형질전환된 담배현탁세포를 통한 hGM-CSF 생산을 위한 배양공정 개발 및 세포배양기 운전

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Process Development and Bioreactor Operation for the Production of hGM-CSF from Transgenic *Nicotiana tabacum* Suspension Cells

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Introduction

Media pH has been recognized as one of the most important process parameters in cell culture. Especially, extracellular pH is regarded as a key factor in the mammalian cell culture for the production of diagnostic and therapeutic proteins. In many documents regarding mammalian cells culture, effect of culture pH on cellular function and properties including cell proliferation, cell differentiation, and nutrient consumption, was reported (McDowell and Papoutsakis, 1998). Also, it has been shown that culture pH affects protein production and quality (Borys et al., 1994). The concentration of hydrogen ions in the medium changes during the development of the culture. This is due to uptake of compounds required as essential nutrients or as buffer components. Meanwhile, the media pH also indicates sugar uptake in plant cell cultures. Generally, the sugar uptake by plant cells on lag period leads to an acidification of media and the pH increases again up to nearly 6.0 during exponential growth. Several reports have been reported the effect of pH on the growth of plant suspension cells but those applications were limited to the production of secondary metabolites (Endress, 1994).

Recently, plant cells have focused increasing attention as an alternative expression system for production of foreign proteins. However, few reports regarding the bioreactor operations of plant cell cultures for the production of foreign proteins and studies on scale-up were not documented. In this study, the effect of pH on the stability of hGM-CSF, as a model system, and physiological changes of plant cells such as secretion of synthesized hGM-CSF were investigated. Then, pH control strategies were applied to bioreactor cultivations. Additionally, effects of DO and aeration rate on the transgenic *N. tabacum* cell growth and production of hGM-CSF were examined in a bioreactor.

Materials and Methods

Transgenic *Nicotiana tabacum* L. cv Havana SR cells transformed with *Agrobacterium tumefacience* for expressing human granulocyte-macrophage colony-stimulating factor (hGM-CSF) was kindly donated by Prof. Yang (Chonbuk National University, Korea). Cell suspensions were cultured in a modified MS medium

containing 30 g/L of sucrose, 0.2 mg/L of 2,4-D, and 0.02 mg/L of kinetin. For bioreactor experiments, a 5-L stirred tank bioreactor (Kobiotech Co., Incheon, Korea) with 2 L working volume was used for perfusion culture. Mixing was achieved by 4-bladed hollowed-paddle impeller with 80 rpm of initial agitation rate. According to cell growth, agitation rate was gradually increased up to 250 rpm. Aeration rate and temperature were maintained at 0.2 vvm and 25℃, respectively.

For the estimation of intracellular hGM-CSF content, 0.2 g of fresh cell was mixed with an extraction buffer (0.8 mL) containing 0.15 M NaCl, 0.25% NP-40, 50 mM Tris/HCl (pH 7.4) and the mixture was warmed for 30 min at room temperature. The cell suspension was sonicated on ice using Vibra Cell VCX 400 (Sonics & Materials Inc., USA) at 20% of amplitude for 90 sec with 8:2 (on:off) pulse and, then, cell extract was centrifuged (12,000 g, 15 min) at 4℃. The supernatant was taken and kept on ice until the estimation of intracellular hGM-CSF content.

Extracellular and intracellular hGM-CSF was quantified by using an ELISA kit according to the manufacturers instructions (PharMingen Inc., USA).

Results and Discussion

Among various environmental factors, pH can affect the secretion of foreign proteins. However, it is difficult to evaluate the effect during cell culture periods because protein synthesis, protein secretion and protein degradation are involved. Therefore, short-term changes within a few hours of hGM-CSF concentration in buffer solutions pre-adjusted to different pH were monitored to minimize the variation resulted from the protein synthesis and degradation. As shown in Figure 2, changes in buffer pH apparently affected the hGM-CSF secretion within relatively short period. It was found that higher pH enhanced the hGM-CSF secretion. At pH 6.5, 2.1 μg/L of hGM-CSF was detected, which was 4.5-fold increase compared to 0.47 μg/L at pH 4.5.

In order to investigate the effect of pH, *N. tabacum* suspension cells were cultivated in a 5-L bioreactor with or without pH control. It was found that, without pH control, pH initially adjusted to 5.8 prior to autoclaving rapidly dropped to 4.0~4.5 after inoclulation within 1 day. Then the level of pH gradually increased up to 6.0 according to cell growth. Figure 3 shows the profiles of cell growth during batch culture in a 5-L bioreactor at a different pH. The cell growth at pH 5.5 showed most similar with that without pH control. The maximum cell density (15.8 g/L) could be obtained on day 10. Although the values of cell density and growth rate at pH 5.5 were not varied but cell-browning in color was observed. At pH 6.0, the cell growth was severely inhibited and cell-browning was more pronounced. Also, at acidic conditions (pH 4.5 and 5.0), the cell growth was limited but cell browning was not observed. It was thought that these differences might be originated from the hindrance of sugar uptake at low pH and unfavorable ion uptake at high pH.

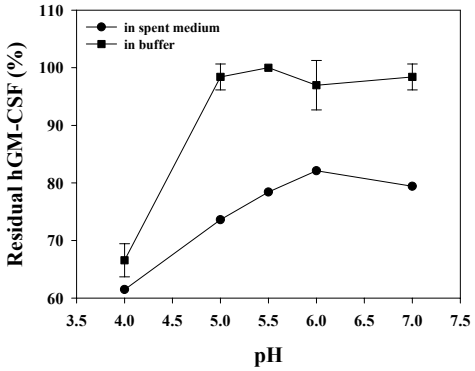

Figure 1. Stability test of hGM-CSF under different pH conditions in buffer and in conditioned media.

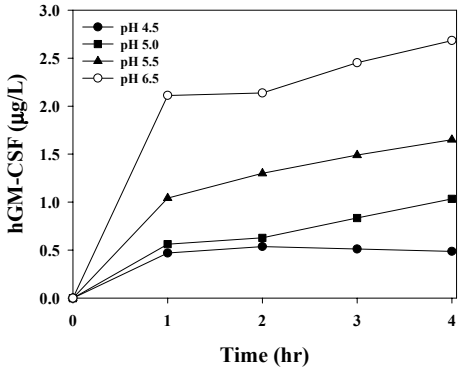

Figure 2. Short-term changes of hGM-CSF concentrations in buffer solution by various pH conditions.

In terms of the production of hGM-CSF, the effect of pH control was more pronounced (Figure 4). Compared to the control culture without pH control, when the pH was controlled at 4.5 and 5.0, the production of hGM-CSF was not significantly influenced even though the cell growth was inhibited. In the control culture, the production of hGM-CSF followed the typical patterns of the production of extracellular foreign proteins. It was observed that the maximum hGM-CSF in media reached about 10 μg/L on day 4 or 6. Meanwhile, with pH control at 4.5 and 5.0, the maximum values were 10.08, 12.15 μg/L on day 4, respectively. In contrast, cultures with pH control to 5.5 and 6.0 significantly enhanced the production of hGM-CSF. When pH was maintained at 5.5, 79.66 μg/L of extracellular hGM-CSF could be obtained on day 6, which was 8-fold increase compared to the control culture. With pH control at 6.0, the production of hGM-CSF was also stimulated up to 58.46 μg/L on day 8 although the cell growth was poor. A noticeable fact was that a sudden decrease in extracellular hGM-CSF levels after the peak was apparently reduced at pH 6.0.

Effects of DO on the growth of *N. tabacum* cells and the production of hGM-CSF were also investigated. To maintain a constant DO level, aeration was solely controlled at a fixed rate. For a 4-days of culture, 5.190, 6.157, and 6.231 g/L of cell density could be obtained at DO 20, 50, and 80%, respectively. It was investigated that growth index and average specific growth rate did not increase above DO 50%. However, growth index and specific growth rate of culture with DO 50% were respectively increased to 1.251 and 0.203 day^{-1} from 0.842 and 0.153 day^{-1} obtained in DO 20%. Based on this result, it is thought that DO 50% is enough to support the cell growth and a decrease in DO level below 50% may reduce the cell growth.

On the other hand, DO also influenced on the production of hGM-CSF and its secretion. The production of extracellular hGM-CSF was enhanced by an increase in DO. When DO was controlled at 80%, 8.716 μg/L of hGM-CSF could be obtained on day 4, which was 2.5-fold higher than that of DO 20%. Not only the extracellular hGM-CSF, but the production of intracellular hGM-CSF was stimulated at higher DO levels, also. The culture with DO 80% gave the highest results and the specific- and the volumetric-term of intracellular hGM-CSF were 4.30 μg/gDCW and 26.78 μg/L,

respectively. It was observed that a higher DO level was effective for the secretion of hGM-CSF. At DO 80%, any detectable evidence for cell lysis could be observed. Nevertheless, the fraction of extracellular hGM-CSF was increased to 24.552% at DO 80% from 14.122% at DO 20%.

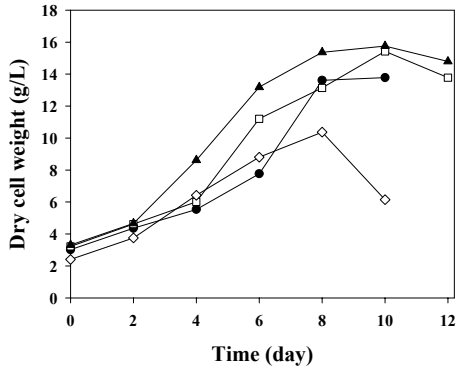

Figure 3. Time course changes of *N. tabacum* cell growth at different pH: ●, pH 4.5; □, pH 5.0; ▲, pH 5.5; ◇, pH 6.0.

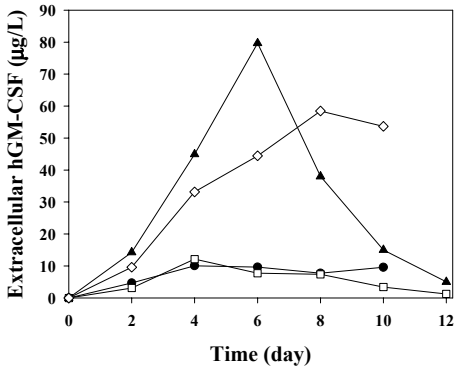

Figure 4. Effect of pH on the production of hGM-CSF: ●, pH 4.5; □, pH 5.0; ▲, pH 5.5; ◇, pH 6.0.

Conclusions

Similar to the production of secondary metabolites, pH, DO, and aeration rate are also key factors affecting the plant cell growth and the production of foreign proteins in bioreactor operation for plant cells. Media pH influenced the stability of hGM-CSF and its secretion. It was found that the maintenance of pH at 5.5 was effective for the cell growth, which was identical to that of control, and the production of hGM-CSF. In terms of DO, a higher DO gave positive effects on the cell growth and the production of hGM-CSF and the secretion of hGM-CSF was facilitated by an increased DO. In addition, the aeration rate was found to have an important effect on the production of hGM-CSF. Although the cell growth in a lag period was delayed by excess aeration rate, but the production of hGM-CSF was pronouncedly stimulated. Consequently, the information obtained from this study will contribute to the reproducibility in scale-up and the process control.

References

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