Biotechnol. Bioprocess Eng. **1999**, 4, 259-263

Effect of Culture Conditions on Monoclonal Antibody Production from Genetically Modified Tobacco Suspension Cultures

Feng Liu and James M. Lee*

Department of Chemical Engineering, Washington State University, Pullman, WA 99164-2710, U.S.A.

Oxygen supply and inoculum age were found to affect the production of the heavy chain monoclonal antibody (HC MAb) from genetically modified tobacco suspension cultures. The increase of oxygen supply increased both cell growth and HC MAb production. Furthermore, the increased aeration and mixing improved the production of HC MAb based on the unit amount of cells or total soluble proteins. This indicated that the increased aeration improved the production and secretion of HC MAb more than other cell components. HC MAb production and cell growth also improved when batch cultures were inoculated with actively dividing cells (5-day old) rather than the fully-grown cells (7- or 10-day old cells) that are commonly used for subcultures. The addition of glutamine to the medium also improved cell growth and HC MAb production.

Key words: monoclonal antibody, tobacco suspension cultures, oxygen supply, inoculum age, glutamine addition

INTRODUCTION

There has been a continued interest in producing functional monoclonal antibodies from intact transgenic plants [1-7] or plant cells [8-10]. There are several advantages to using plant cells rather than mammalian tissue for the production of large quantities of secreted proteins. Plant cell media are mainly composed of simple sugars and salts and are much less expensive than complex mammalian media. It is therefore much easier and more economical to purify secreted foreign proteins from plant cell media than from complex mammalian cell media. Additionally, owing to their rigid exterior walls, plant cells are more resistant than mammalian cells to the shear forces involved in large-scale suspension cultures. Furthermore, plant cell-derived transgenic proteins are likely to be safer for human use than those derived from mammalian cells, since plant cell contaminants and viruses are not pathogenic to humans. Plant cell system is able to process the post-translational modification whereas prokaryotic system cannot.

Magnuson *et al.* [10] reported the production of a heavy chain monoclonal antibody (HC MAb) from genetically modified plant suspension cultures. The produced HC MAb was secreted to the medium. However, the secreted protein was not stable due to degradation in the plant cell medium, which could be partially prevented with the addition of polyvinylpyrrolidone (PVP) [11].

In this paper, we examine the effects of various culture conditions such as oxygen supply, inoculum age, and glutamine addition on the production of HC MAb.

* Corresponding author
Tel: +1-509-335-5252 Fax: +1-509-335-4806
e-mail: jmlee@wsu.edu

MATERIALS AND METHODS

Plant Cell Line and Growth Medium

The plant cell line used in this research was a genetically modified tobacco cell, transformed *Nicotiana tabacum* 70γ [10]. The transformed tobacco cell line carried the gene for a heavy chain antibody specific to *p*-azophenylarsonate. This plant cell line also carried the kanamycin antibiotic resistance gene, which enabled the positive maintenance of the genetically transformed cell line and the prevention of bacterial contamination.

The growth medium contained 4.3 g/L Murashige and Skoog (MS) mixture of inorganic nutrients [12] supplemented with 30 g/L sucrose, 0.18 g/L KH_2PO_4, 0.1 g/L inositol, 1.0 mg/L thiamin hydrochloride, and 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The pH of this solution was adjusted to 5.8 with 1.0 N KOH, prior to autoclaving. Prior to inoculation, cefotaxime and kanamycin were filter sterilized and added to the established medium to achieve the concentrations of 100 mg/L and 50 mg/L, respectively.

Batch Cultures

Shake-flask experiments were performed in 250-mL Erlenmeyer flasks containing 60 mL MS medium (unless otherwise stated) on a gyratory orbital platform shaker operating at 120 rpm at 30°C. Batch cultures were initiated with 5% (v/v) inoculum of 7-day-old cell suspension.

A 5-L stirred tank bioreactor (Bioflo III, New Brunswick Scientific, New Brunswick, NJ) with a working volume of 3.5 L was used for fermenter batch studies. The fermenter was equipped with a 6-blade disk impeller (7.6 cm in diameter and 14.0 cm in width) developed by Hooker *et al.* [13]. The impel-

ler was positioned 3 cm above the bottom of the vessel. The aeration was supplied by a 4-hole ring sparger located directly below the impeller. The batch culture initiated with 5% (v/v) inoculum of 7-day-old cells. The impeller rotation speed was set at 150 rpm. The aeration rates were 0.1, 0.3, 0.6, or 0.9 vvm (volume of air supply per unit volume of liquid per minute).

The cell concentrations were determined using the method developed by Mills and Lee [14].

All experiments were repeated at least twice to confirm the trends of the effects for various operating conditions tested. The error bars were not included in figures because number of experiments were too few to establish good error bars. Since a complete batch culture takes about 8 to 10 days, multiple repeats to establish valid error bars were not practical.

Stabilization of Extracellular HC MAb

For all of the experiments in this study, filter sterilized PVP was added to the cultures to make the final concentration of 0.75 g/L as suggested by LaCount *et al.* [11]. For both the shake flask and the stirred tank fermenter studies, PVP was added aseptically at the time of inoculation.

Oxygen Mass Transfer Coefficient k_La

The dynamic gassing out method [15] was used in measuring the initial k_La values by following the rate of resaturation of the deoxygenated medium. The medium in the fermenter or 250-mL Erlenmeyer flasks were deoxygenated to the level of 0.3 mg O_2/L by sparging the medium with nitrogen. Immediately after the deoxygenation, the air was introduced by sparging the air in the fermenter, or by shaking the aluminum foil covered flasks on a shaker table with the rotational speed of 120 rpm. During this time, the amount of dissolved oxygen in the medium was measured and recorded continuously by a polarographic oxygen probe (YSI Model 58, YSI, Inc., Yellow Springs, OH).

The oxygen balance during the initial stage of oxygenation in the absence cell respiration yields

$$-\frac{dC_{O_2}}{dt} = k_La(C_{O_2} - C_{O_2}^*) \quad (1)$$

where $C_{O_2}^*$ is the equilibrium concentration of oxygen. The integration of Equation (1) between time t_0 and t yields

$$\ln\left[\frac{C_{O_2}^* - C_{O_2}(t_0)}{C_{O_2}^* - C_{O_2}(t)}\right] = k_La(t - t_0) \quad (2)$$

Therefore, k_La can be estimated by the slope of $\ln\left[\frac{C_{O_2}^* - C_{O_2}(t_0)}{C_{O_2}^* - C_{O_2}(t)}\right]$ versus $(t\text{-}t_0)$ plot.

ELISA and Protein Assays

The concentration of HC MAb was determined by an enzyme-linked immunosorbent assay (ELISA) using the affinity purified goat antibody to mouse IgG (Organon Teknika Co., Durham, NC), alkaline phosphatase-labeled goat anti-mouse IgG that is heavy chain-specific (Southern Biotechnology Associates, Inc., Birmingham, AL), and *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) [10].

The total soluble protein was determined by the Bradford Reagent (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a protein standard.

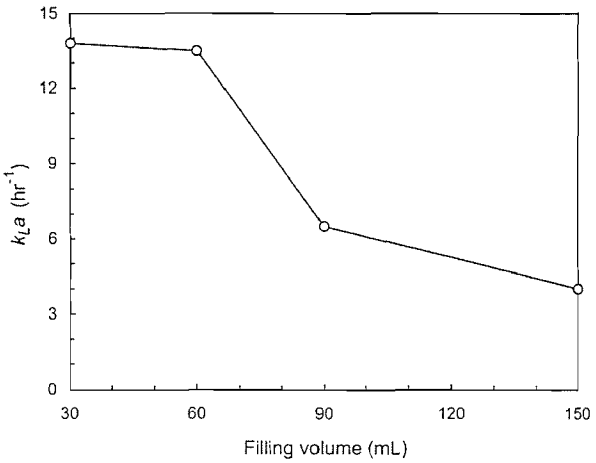

Fig. 1. Effect of the filling (medium) volume of a 250-mL Erlenmeyer flask on the oxygen mass transfer coefficient (k_La).

RESULTS AND DISCUSSION

Effect of Oxygen Supply

Genetically modified tobacco cells (70γ) were cultured in 250-mL Erlenmeyer shake flasks with different medium filling volumes. The decrease in medium volume not only increases the surface area per unit volume, but also enhances the mixing intensity. As shown in Fig. 1, the oxygen mass transfer coefficient (k_La) increased from 4 to 14 hr^{-1} as the filling volume decreased from 150 to 30 mL.

Fig. 2 shows the change of cell concentration and the intracellular and extracellular HC MAb concentrations during batch cultures with different filling volumes. When the filling volume was reduced to 60 mL, the cell growth improved. Similarly, the production of intracellular and extracellular HC MAb improved with the increased aeration. The major reason for the increase of HC MAb production was the increased cell growth. However, no further improvements in cell growth and HC MAb production were observed when the medium volume was further decreased to 30 mL. It may be because the decrease of the filling volume to 30 mL does not increase the k_La value significantly (see Fig. 1) and also because the decrease increases the mixing intensity that affects the cell growth and the foreign protein stability adversely.

The increase of HC MAb production was even more than that expected from the better cell growth. As

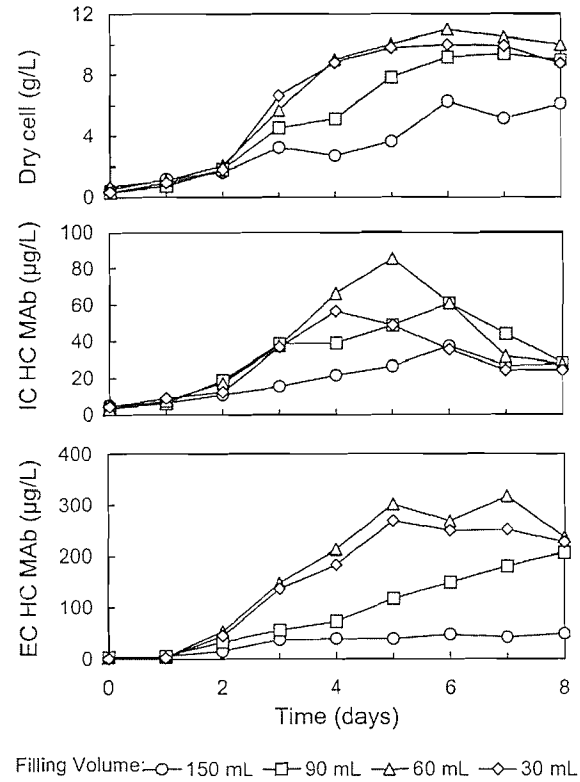

Fig. 2. Effect of the filling volume of a 250-mL Erlenmeyer flask on cell growth and HC MAb production.

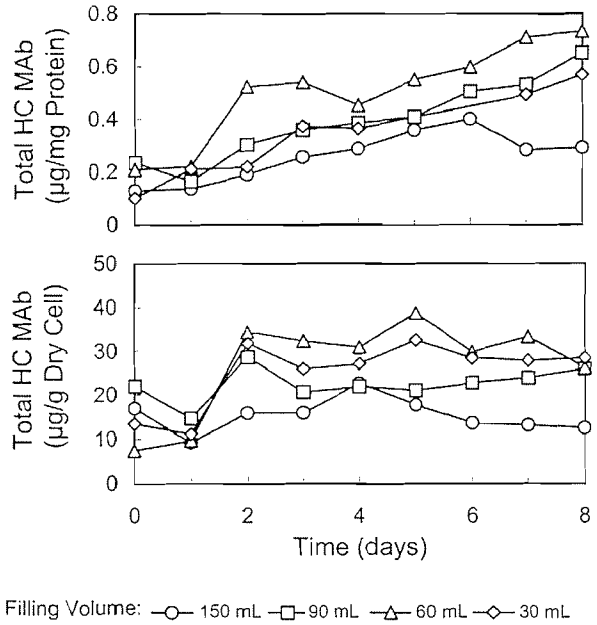

Fig. 3. Effect of the filling volume of a 250-mL Erlenmeyer flask on the production of total HC MAb based on the total protein and the dry cell weight.

shown in Fig. 3, the production of HC MAb based on the unit amount of total protein or dry cell also improved when the filling volume was decreased up to 60 mL. This indicated that the increased aeration improved the production and secretion of HC MAb more than other cell components. These results are

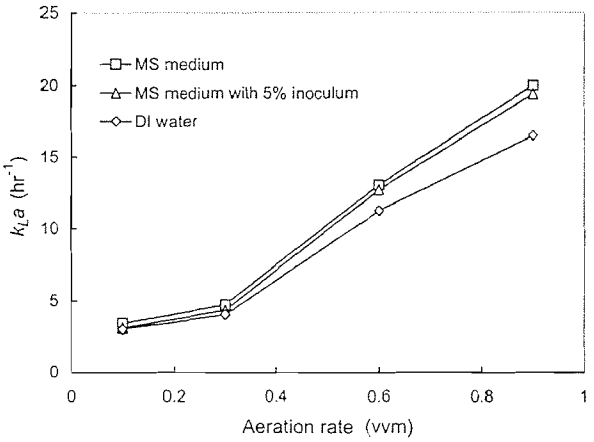

Fig. 4. Effect of aeration rate on the oxygen mass transfer coefficient (k_La) in a 5-L stirred tank fermenter (working volume 3.5 L).

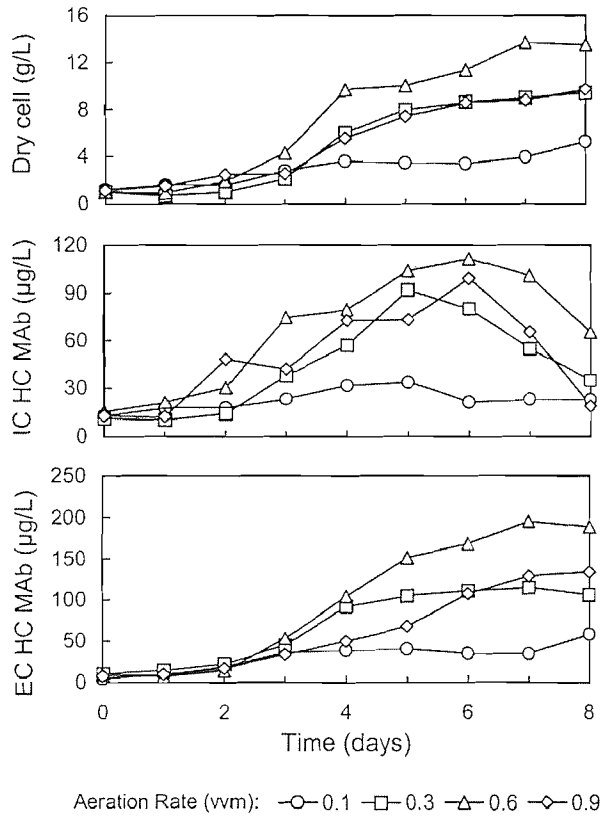

Fig. 5. Effect of aeration rate on cell growth and HC MAb production in a 5-L stirred fermenter (working volume 3.5 L).

in agreement with the results of Gao and Lee [16] for the production from β-glucuronidase (GUS) from genetically modified plant suspension culture.

To further investigate the effect of oxygen supply, the genetically modified tobacco cells were cultured in a 5-L stirred tank fermenter with a working volume of 3.5 L. The effect of aeration rate on the oxygen mass transfer coefficient (k_La) is shown in Fig. 4, which shows a wider range of k_La (3 to 19 hr^{-1}) than that obtained from the shaker flasks (see Fig. 1). The oxygen transfer in the MS medium with or without cells was slightly better than that in deionized water.

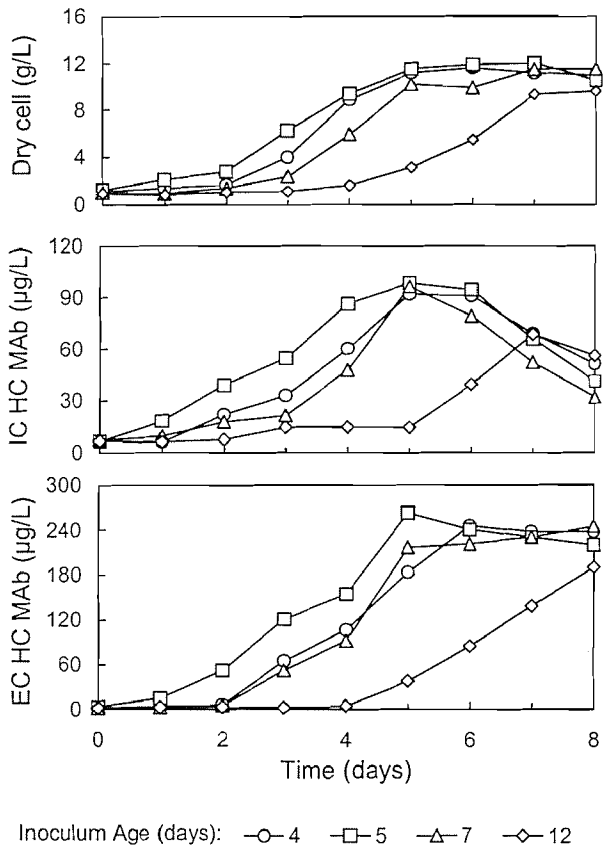

Fig. 6. Effect of inoculum age on cell growth and HC MAb production in shaker flasks.

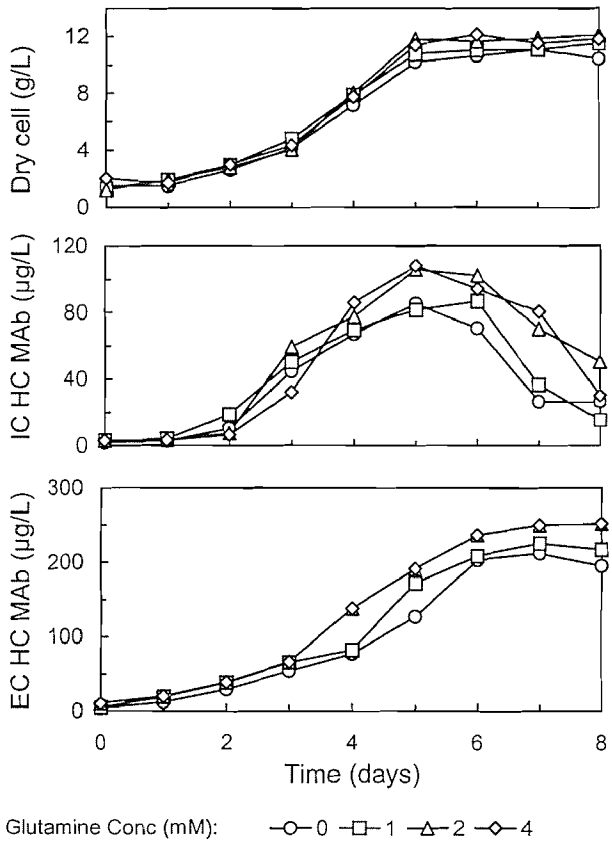

Fig. 7. Effect of glutamine on cell growth and HC MAb production in shaker flasks.

This may be because the presence of various salts in the medium affects the oxygen solubility.

Fig. 5 shows cell growth and HC MAb production in the stirred tank fermenter. As in the case of the shaker cultures, the increase in aeration rate (up to 0.6 vvm) increased cell growth and HC MAb production. This result shows that the adequate aeration is very important to plant cell cultures even though the oxygen demand of plant cells is not as high as that of aerobic organisms. However, when the aeration rate was increased further to 0.9 vvm, cell growth and the HC MAb production decreased, which was due to excessive foaming at the empty space at the top of the fermenter. Cells carried up by the rising bubble and trapped in the foam layers die due to lack of nutrition. Additionally, Hegarty *et al.* [17] indicated the importance of volatile compounds, such as carbon dioxide, for cell growth.

A comparison of Fig. 2. and Fig. 5 shows that the maximum extracellular HC MAb production in the stirred fermenter was lower than that in the shaker flasks. This is due to the fact that the high shear environment (by agitation and aeration) of the stirred fermenter affects the stability of extracellular HC MAb adversely, which can be improved by the optimization of operating conditions and stabilizers. Another potential cause for the decrease is variability of the foreign gene expression level as the cell line goes through numerous subcultures, which will be discussed later when two batch runs with the same culture conditions were compared in the next section.

Effect of Inoculum Age

As described in the "Materials and Methods" section, we inoculated the medium with 7-day-old cells to start batch cultures. However, Fig. 3 shows that the HC MAb production per unit cell weight reached its maximum value after only two days of cultures. This indicates that HC MAb production is most active in the early part of the exponential growth phase. This motivated us to study the effect of inoculum age on cell growth and HC MAb production.

As shown in Fig. 6, the batch cultures with the 5-day old inoculum grew the fastest among tested. As the age of the inoculum increased, the growth rate decreased. This was expected because the length of the lag phase increases with the increased differences of the stock culture (where the inoculum was taken) and the fresh culture medium [18]. When the 12-day-old inoculum was used, active growth was delayed for almost 4 days. This shows the importance of using young healthy cells less than 7 days old. The reduction of the inoculum age to 4 days did not further improve the growth. The production of HC MAb followed a trend similar to that of the cell growth by showing the highest value with a 5-day old inoculum. Therefore, subculturing every 5 days rather than every 7 days can provide maximum production of HC MAb in batch cultures.

The comparison of the EC HC MAb production levels of the cultures with the same condition in Fig. 2 and Fig. 6 (filling volume of 60 mL, inoculation age of 7 days) showed that the maximum EC HC MAb

concentration was decreased about 13% from 300 μg/L (Fig. 2) to 260 μg/L (Fig. 6). This is because the inoculation age experiment (Fig. 6) was performed about 6 months later than the experiments for Fig. 2, during which time the cell line was subcultured every 7 days. Though the foreign gene expression in plant cell system is very stable because foreign DNAs are integrated into nuclear chromosome, there is some variation of the production levels as a cell line goes through numerous subculturing [19].

Effect of Glutamine

Since the production of antibodies is growth related, the modification of cell growth can have a great effect on the level of production. Glutamine is known for enhancing plant cell growth rate [20]. Also, in the hybridoma technique, glutamine has been adopted to improve antibody secretion [21]. In order to examine the role of glutamine in genetically modified tobacco cell culture, batch shake flask cultures were initiated with various glutamine concentrations.

Fig. 7 shows cell growth and HC MAb production in the presence of glutamine. The addition of glutamine did not affect cell growth significantly, though some improvement was noticed. However, both intracellular and extracellular HC MAb productions increased with the increasing glutamine concentrations. When the glutamine concentration was 2 mM, the intracellular and extracellular HC MAb were 20% and 10% higher than the maximum concentration of the control, respectively. Further increases in glutamine concentration did not result in improved HC MAb levels. The increased production of HC MAb was likely due to a prolonged production period in a glutamine-enriched environment. The improvement of secretion was another possibility for the increased extracellular HC MAb [21].

Acknowledgments This research was supported by the National Science Foundation (BCS-9308407 and BES-9808021).

REFERENCES

[1] Hiatt, A., R. Cafferkey, and K. Bowdish (1989) Production of antibodies in transgenic plants. *Nature* 342: 76-78.
[2] Düring, K., S. Hippe, F. Kreuzaler, and J. Schell (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. *Plant Mol. Biol.* 15: 282-293.
[3] Benvenuto, E., R. J. Ordás, R. Tavazza, G. Ancora, S. Biocca, A. Cattaneo, and P. Galeffi (1991) Phytoantibodies: a general vector for the expression of immunoglobulin domains in transgenic plants. *Plant Mol. Biol.* 17: 865-874.
[4] Owen, M., A. Gandecha, B. Cockburn, and G. Whitelam (1992) Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. *Bio/Technol.* 10: 790-794.
[5] Tavladoraki, P., E. Benvenuto, S. Trinca, D. De Martinis, A. Cattaneo, and P. Galeffi (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* 366: 469-472.
[6] Ma, J. K.-C., A. Hiatt, M. Hein, N. D. Vine, F. Wang, P. Stabila, C. van Dolleweerd, K. Mostov, and T. Lehner (1995) Generation and assembly of secretory antibodies in plants. *Science* 268: 716-719.
[7] Schouten, A., J. Roosien, F. A. van Engelen, G. A. M. de Jong, A. W. M. Borst-Vrenssen, J. Zilverentant, D. Bosch, W. J. Stiekema, F. J. Gommers, A. Schots, and J. Bakker, (1996) The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol. Biol.* 30: 781-793.
[8] Hein, M. B., Y. Tang, D. A. McLeod, K. D. Janda, and A. Hiatt (1991) Evaluation of immunoglobulins from plant cells. *Biotechnol. Prog.* 7: 455-461.
[9] De Neve, M., M. De Loose, A. Jacobs, H. Van Houdt, B. Kaluza, U. Weidle, M. Van Montagu, and A. Depicker (1993) Assembly of an antibody and its derived antibody fragment *Nicotiana* and *Arabidopsis*. *Transgenic Res.* 2: 227-237.
[10] Magnuson, N. S., P. M. Linzmaier, J. W. Gao, R. Reeves, G. An, and J. M. Lee (1996) Enhanced recovery of a secreted mammalian protein from suspension culture of genetically modified tobacco cells. *Protein Exp. Pur.* 7: 220-228.
[11] LaCount, W., G. An, and J. M. Lee (1997) The effect of polyvinylpyrrolidone (PVP) on the heavy chain monoclonal antibody production from plant suspension cultures. *Biotechnol. Lett.* 19: 93-96.
[12] Murashige T. and F. Skoog (1962) A revised medium for rapid growth bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
[13] Hooker, B. S., J. M. Lee, and G. An (1990) Cultivation of plant cells in a stirred vessel: effect of impeller design. *Biotechnol. Bioeng.* 35: 296-304.
[14] Mills D. and J. M. Lee (1996) A simple, accurate method for determining wet and dry weight concentrations of plant cell suspension cultures. *Plant Cell Rep.* 15: 634-636.
[15] Taguchi, H. and A. E. Humphrey (1966) Dynamic measurement of volumetric oxygen transfer coefficient in fermentation systems. *J. Ferm. Technol.* 44: 881-889.
[16] Gao, J. and J. M. Lee (1992) Effect of oxygen supply on the suspension culture of genetically modified tobacco cells. *Biotechnol. Prog.* 8: 285-290.
[17] Hegarty, P. K., N. J. Smart, A. H. Scragg, and M. W. Fowler (1986) The aeration of *Catharanthus roseus* L. G. Don suspension cultures in airlift bioreactors: the inhibitory effect at high aeration rates on culture growth. *J. Exp. Bot.* 37: 1911-1920.
[18] Lee, J. M. (1992) *Biochemical Engineering*, p. 141 Prentice Hall Inc., Englewood Cliffs, NJ.
[19] Gao, J., J. M. Lee, and G. An (1991) The stability of foreign protein production in genetically modified tobacco cells, *Plant Cell Rep.* 10: 533-536.
[20] Tate, J. L. and G. F. Payne (1991) Plant cell growth under different levels of oxygen and carbon dioxide, *Plant Cell Rep.* 10: 22-25.
[21] Flickinger, M. C., N. K. Goebel, T. Bibila, and S. Boyce-Jacino (1991) Evidence for posttranscriptional stimulation of monoclonal antibody secretion by L-glutamine during slow hybridoma growth. *J. Biotechnol.* 22: 201-206.