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Effect of Gelatin on the Stability of Heavy Chain Monoclonal Antibody Production from Plant Suspension Cultures

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Abstract The heavy chain monoclonal antibody (HC MAb) was produced in suspension cultures of genetically modified *Nicotiana tabacum*. The HC MAb secreted to the medium was unstable due to unfavorable interactions in the plant cell medium. The addition of gelatin (5 g/l) stabilized the extracellular HC MAb and increased its production 10-fold. A kinetic model was developed describing the interaction between the secreted protein and the stabilizer. The model accounted for the inactivation of the protein by simple aggregation and general instability. It was assumed that the secreted protein and the stabilizer form a stable complex. Culturing the cells semi-continuously could further increase the productivity of HC MAb.

Key words: Monoclonal antibody, plant suspension cultures, gelatin, protein stabilization, kinetic model

The production of recombinant proteins in cultured transgenic plant cells offers some advantages over mammalian or *Baculovirus* systems. The media used to cultivate plant cells are well defined and inexpensive. Contamination by bacteria or fungi is easily monitored and plant cell cultures do not produce potent pathogens to humans. Plant cells have the capacity for posttranslational modifications including glycosylation.

Magnuson *et al.* [8] reported the production of a heavy chain monoclonal antibody (HC MAb) from genetically modified plant suspension cultures. The amount of functional HC MAb quickly decreased after secretion into the culture medium, which could be prevented partially by the addition of stabilizing agents such as polyvinylpyrrolidone [6, 8] and dimethyl sulfoxide [13].

In this paper, we report the effect of gelatin on the production of HC MAb from genetically engineered tobacco suspension cultures. A kinetic model was developed to describe the mechanism of the denaturation and the subsequent stabilization process. It was also demonstrated that HC MAb productivity could be increased by culturing the cells semi-continuously.

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Materials and Methods

Plant Cell Lines and Culture Conditions

Nicotiana tabacum (NT-1) cells were genetically modified to carry the heavy chain monoclonal antibody gene specific for *p*-azophenylarsonate [8]. The gene was placed downstream of the CaMV 35S promoter and terminated by the T-DNA transcript 7 gene terminator. The vector also included the neomycin phosphotransferase gene to encode kanamycin resistance [8].

The production cell line, 70γ, was cultivated in a suspension media containing a 4.3 g/l Murishige and Skoog (MS) salt mixture [10] supplemented with 30 g/l sucrose, 0.18 g/l KH_2PO_4, 0.1 g/l inositol, 1.0 mg/l thiamine hydrochloride, and 0.2 mg/l 2.4-dichlorophenoxyacetic acid (2.4-D). The pH was adjusted with 1 N KOH to 5.8 prior to autoclaving at 121°C for 15 min. The antibiotic selection in the media consisted of filtered 50 mg/l kanamycin and 100 mg/l cefataxime.

The suspension cells were cultivated at 29°C in 250-ml Erlenmeyer flasks containing 60 ml of the media on an orbital gyratory shaker at 150 rpm. Batch cultures were initiated by adding a 5% (v/v) inoculum of seven-day-old cells.

The cell concentrations were determined by using the method of Mills and Lee [9].

Gelatin (JT Baker, Phillipsburg, NJ, U.S.A.) was added to the media after the pH was adjusted and dissolved during the autoclaving step.

ELISA Analysis

The concentration of HC MAb was determined by an enzyme-linked immunosorbent assay (ELISA) using affinity

purified goat antibody to mouse IgG (Organon Teknika Co., Durham, NC, U.S.A.), alkaline phosphatase-labeled goat anti-mouse IgG that is heavy chain specific (Southern Biotechnology Associates, Birmingham, AL. U.S.A.), and *p*-nitrophenyl phosphate (Sigma, St. Louis, MO, U.S.A.) [8].

Semi-Continuous Cultures

Three types of semi-continuous cultures were tested with gelatin-fortified media (5 g/l). All of the cultures were allowed to grow for four days before semi-continuous operation was initiated by replacing part of the culture broth or supernatant with fresh medium every 12 h. In the first trial, 20 ml (about one third of the total cell suspension) of the culture broth (both supernatant and cells) was removed and replaced by the same amount of fresh medium. In the second trial, 20 ml of supernatant (without any cells) was removed (leaving all cells in the flask) and replenished with fresh media. In the third trial, various amount of culture broth were removed to reduce the wet cell concentration to 150 g/l (to mimic the turbidostat).

Stabilization Model Development

The secreted foreign proteins may be deactivated, which is possibly due to aggregation, insolubilization, and denaturation. Proteolysis was ruled out as a potential inactivation mechanism because Western blot analysis did not show any degradation products [8].

The model consisted of two forms of protein inactivation. The first is the aggregation of two molecules to form an inactive aggregate (PP). The inactivation by aggregation can be prevented by the competitive action of a stabilizer (S). The second is the inactivation of the foreign protein by all other inactivation mechanisms. This model can be described as:

$$P+P \xrightarrow{k_A} PP \tag{1}$$

$$P+S \underset{k_{S_2}}{\overset{k_{S_1}}{\rightleftharpoons}} PS \tag{2}$$

$$P \xrightarrow{k_I} I \tag{3}$$

where only the free protein (P) and the protein-stabilizer complex (PS) are biologically functional.

The model can be represented by three differential equations:

$$\frac{dC_P}{dt} = -k_A C_P^2 - k_I C_P - k_{S_1} C_P C_S + k_{S_2} C_{PS} \tag{4}$$

$$\frac{dC_{PS}}{dt} = k_{S_1} C_P C_S - k_{S_2} C_{PS} \tag{5}$$

$$\frac{dC_S}{dt} = -k_{S_1} C_P C_S + k_{S_2} C_{PS} \tag{6}$$

where C_P, C_S, and C_{PS} are the concentration of protein, stabilizer, and the stabilized complex, respectively. Since all of the concentration terms in the above equations should be in molar units, we used approximate molecular weights for HC MAb (55 kDa) and gelatin (350 kDa) to convert mass units into the molar unit [8, 14].

The model was simulated using the Simusolv™ numerical parameter optimization package (Dow Chemical Company, Midland, MI, U.S.A.), which utilized a modified generalized reduced gradient technique [7] as well as a direct search method [11]. Model parameters were chosen and evaluated based on the standard error between model estimates and experimental data, and the standard deviation of each parameter. An initial estimate for each parameter was given and then changed in a stepwise fashion to minimize the difference between the experimental value and the model value.

Results and Discussion

Stability of the HC MAb Produced during Batch Cultures

Figure 1 shows the cell growth and intracellular and extracellular HC MAb production during a batch culture of the 70γ cell line. The cell growth and intracellular HC MAb concentration followed similar trends during the exponential growth phase, which indicated that HC MAb production is growth related. However, after reaching the maximum 150 μg/l level at the end of the growth phase, the intracellular HC MAb concentration was decreased to 40 μg/l during the stationary period, possibly due to the secretion and the degradation of HC MAb.

However, the extracellular HC MAb concentration remained low (about 7 μg/l) throughout the culture period. The low level of extracellular concentration may be due to the instability of HC MAb in plant cell media, which lack the protein stabilizing agents commonly present in mammalian cell media containing blood serum or its substitutes.

To determine if the extracellular HC MAb was adhering to the culture flask walls, the 70γ cells were cultured in a

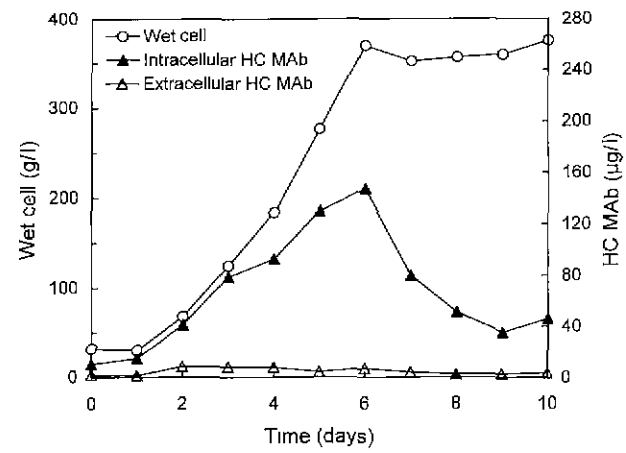

Fig. 1. Cell growth and extracellular and intracellular HC MAb production during a batch culture.

silicone-coated glass flask (Sigmacote, Sigma, St. Louis, MO, U.S.A.) because silicone is known to be resistant to protein binding. However, the silicone treatment did not increase the production level of HC MAb (data not shown). A batch culture grown in polypropylene flasks yielded similar results. Therefore, it was concluded that the low level of the extracellular concentration of HC MAb was not due to its adsorption on the glass surfaces.

Stability of IgG in Growth Media

In order to determine the extent of HC MAb instability in the culture media, an immunoglobulin (mouse IgG, purified from serum; Sigma, St. Louis, MO, U.S.A.) was added to fresh MS media with no cells in it, a culture of four-day-old NT-1 cells, and a culture of four-day-old 70γ cells. The stock solution of IgG was diluted with water and then sterile-filtered to maintain culture integrity. Enough IgG was added to the flask to bring the initial concentration of IgG up to 80 μg/l. Using whole molecules does not guarantee that the heavy chain alone will be stable, but it does supply data on the stability of the whole molecule. From what is known about antibodies, the whole molecule in its native state is more stable than its separate parts (heavy and light chains).

As shown in Fig. 2, the mouse IgG was quickly lost in all the growing cultures, declining to low levels within two days of the addition of IgG. In the MS media flask, without any cells, it was only slightly detectable even on the day of addition. There are known differences between fresh MS media and the media present in growing cultures that could cause the loss of IgG [2]. The four-day-old flasks with growing cells would have much lower concentrations of nutrients than are initially present in the MS media. However, as the plant cells grow, they may release some molecules that may slow the rapid loss of IgG observed in the MS-media-only flask. It could be that the salt concentration is too low, or the total protein concentration is not high enough, to maintain stable HC MAb. Both have been found to be important factors for maintaining stable antibodies in dilute solutions [2].

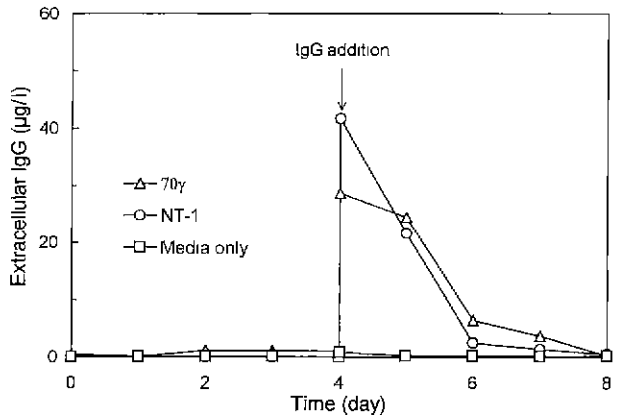

Fig. 2. Addition of whole chain IgG to culture media (80 μg/l) containing no cells, NT-1 cells, or 70γ cells after four days of batch cultivation.

The initial loss of IgG from 80 μg/l to about 40 μg/l could possibly be due to the deactivation that occurs as soon as IgG contacts the media, or to the filtration of the solution. With the large size of IgG molecules (160,000 MW and approximately 0.03 microns in diameter), it is very possible that part of the IgG formed a gel on the 0.2 micron filter, hence lowering the initial concentration of IgG. This could account for the value of 40 μg/l seen in the flasks on the day of addition.

Effect of Gelatin Addition

In order to stabilize the secreted HC MAb in the medium, various amounts of gelatin were added to the culture medium. Figure 3 shows the concentration of HC MAb during the batch cultures. The gelatin addition increased the extracellular HC MAb concentration markedly. When 5 g/l of gelatin was added, the maximum concentration reached 82 μg/l, which is more than a ten-fold increase over the control. The further increase of the gelatin concentration did not improve productivity. A decrease to 1 g/l of gelatin resulted in a good stabilization effect until about 4 days of culture, and then gradually lost its effectiveness. The addition of gelatin did not affect the intracellular production of HC MAb significantly.

Gelatin, being a long and free-moving molecule, has the ability to associate with other molecules in solution. Gelatin

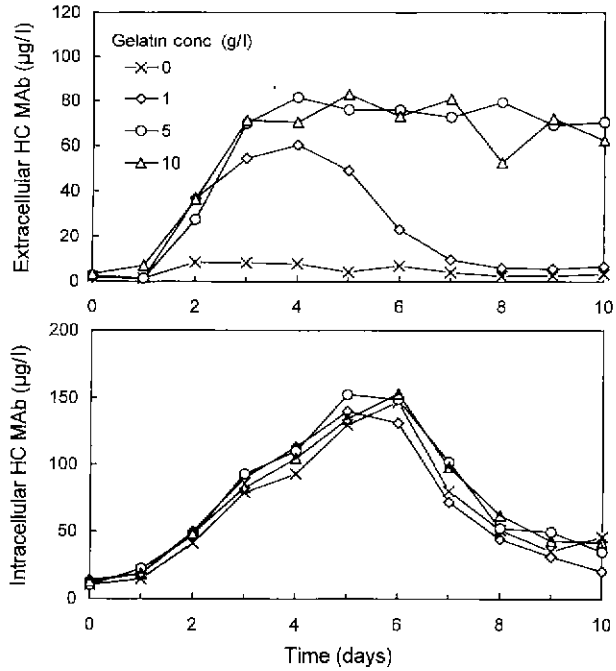

Fig. 3. Effect of gelatin on the production of HC MAb during batch cultures.

molecules are very flexible due to their high concentration of glycine, 33% [12]. They are also multivalent, which allows them to associate with various proteins in solution. It has been reported that gelatin molecules (up to 60) form bundles in a solution [5]. The bundles are loosely intertwined, allowing other molecules to enter them, and thus acting somewhat as a chaperone. The name chaperone is usually reserved for native intracellular molecules that aid in the folding of large globular proteins [1, 4]. In this case, however, we are using gelatin to aid in the stabilization of the HC MAb.

However, the addition of gelatin could not completely prevent the loss of produced HC MAb in the cell because the extracellular concentration was not further increased, even though the intracellular level dropped sharply after 6 days of culture (Fig. 3). This may be partly because of the loss of the stabilizing property of gelatin and partly because of the degradation of the intracellular HC MAb by the action of various metabolites present in the cells.

The cell growth rate was unaffected by the presence of gelatin except when the gelatin concentration was 15 g/l (data not shown). The high concentration of gelatin resulted in a slight increase in the wet cell concentration, approximately 50 g/l higher than the control flasks, which could be a result of the plant cells metabolizing a portion of the gelatin. Therefore, the optimum amount of gelatin addition was considered to be about 5 g/l.

Kinetic Model for HC MAb Stability in Plant Culture Media

In order to understand the mechanism of the deactivation of HC MAb in plant cell media, *Baculovirus*-expressed HC Mab [3] was added to fresh MS media and the change of the active concentration was measured by ELISA assays. As shown in Fig. 4, the experimental data indicated two different inactivation phenomena.

First, there was a rapid and immediate inactivation (45% loss) in the first few minutes after the addition of the HC MAb, followed by a much slower inactivation process.

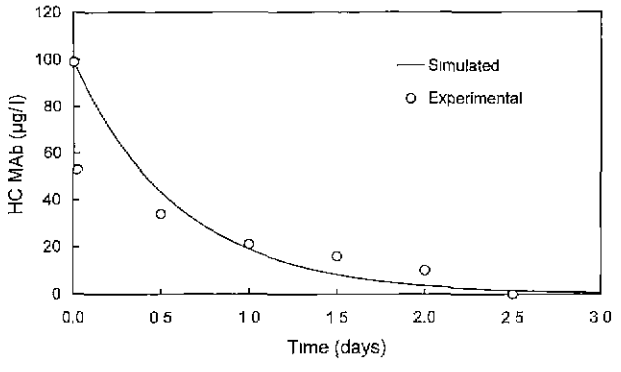

Fig. 4. Change of HC MAb concentration in MS medium without gelatin.

The open circles are the experimental data and the line is simulated results with k_A=1.12 $\mu M^{-1}day^{-1}$ and k_I=1.65 day^{-1}.

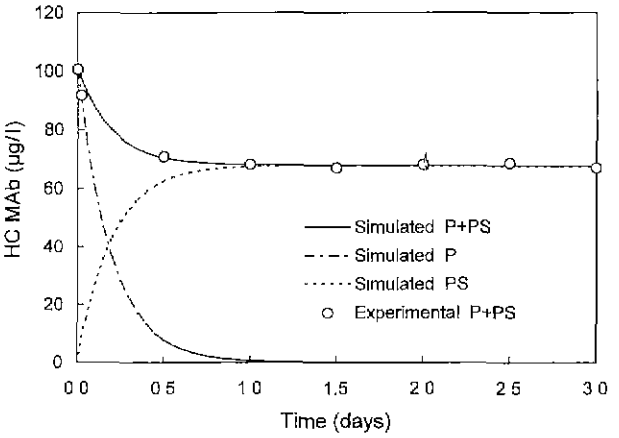

Fig. 5. Change of HC MAb concentration in MS medium with gelatin (5 g/l).

The open circle is the experimental data for the total active HC MAb (P and PS) and the lines are simulated results with k_A=1.12 $mM^{-1}day^{-1}$, k_I=1.64 day^{-1}, k_{S_1}=0.24 $mM^{-1}day^{-1}$, and k_{S_2}=0.0065 day^{-1}.

After one day, 80% of the HC MAb was lost, and after two-and-a-half days all the protein was inactivated.

When the stabilizer was not present, the deactivation model was expressed with Equations (1) and (3), which have only two parameters, k_A and k_I. Several initial guesses were used to verify the convergence of the optimization program. The line in Fig. 4 is the simulation result with optimized parameter values: k_A=1.12 $\mu M^{-1}day^{-1}$ and k_I=1.65 day^{-1}. The model agreed well with the experimental data except for the initial rapid inactivation phenomena, which was difficult to model due to the unknown nature of the sudden inactivation.

When the stabilizer (gelatin 5 g/l) was added, the HC MAb stabilized and only 9% of initial value was lost during the first 30 min after mixing with the plant cell medium (Fig. 5). After one day, only 30% of HC MAb was lost compared to 80% with the stabilizer.

Figure 5 also shows a simulation with 5 g/l of gelatin with the open circles representing experimental data and the lines representing the model. It took about one day for all free HC MAbs (P) to form a complex with the stabilizer (S), which was stable. The simulation results agreed well with the experimental data, showing a 97.4% variation explained (100% variation explained represents a perfect model fit, analogous to a linear coefficient of multiple determination r^2 value of 1.0). The optimal kinetic parameter values for k_{S_1} and k_{S_2} were 0.24 μM^{-1} day^{-1} and 6.5×10^{-3} day^{-1}, respectively.

Effect of Gelatin on the Stability of Whole Mouse IgG

Since the whole IgG with both heavy and light chains is known to be more stable than single-chain antibody such as HC MAb, we tested whether whole IgG is also susceptible to instability in a plant cell medium by adding 100 μg/l of whole mouse IgG (purified from serum, Sigma,

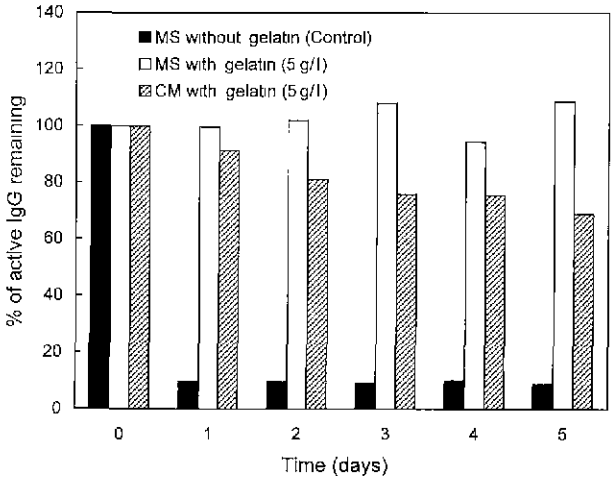

Fig. 6. Effect of gelatin on the stability of whole chain IgG in MS medium and in conditioned medium (CM).

St. Louis, MO, U.S.A.) into MS media and monitoring the activity of IgG. As shown in Fig. 6, about 90% of the IgG was lost within one day in the plant cell medium (control). When gelatin was added (5 g/l) to the medium, the IgG remained active during the entire 5-day period, thus showing the stabilizing effect of gelatin. Since fresh MS media cannot represent the actual condition during a batch culture, we also tested the stability of the IgG in a conditioned medium (CM). It consisted of extracellular fluid that had been separated from non-transformed cells (normal NT1 cells) after 4 days of growth. In this case, about 20% was lost over the 5-day period. This 20% decrease of the IgG in CM suggests the presence of some inactivating compounds produced from plant cells.

Semi-Continuous Production of HC MAb

The long-term goals of this project are to produce immunoglobulin or biomolecules on a large scale. Semi-continuous culture is an attractive method for HC MAb production, as it can prolong the culture in the exponential growth phase, where the production of HC MAb is maximal.

Figure 7 shows the change of wet cell concentration during the semi-continuous cultures. In the case of the supernatant replacement, the cell concentration increased until it reached the maximum level, as in the case of the batch cultures. When both cells and supernatant (cell suspension) were replaced, the growth rate of the cells was not fast enough to keep up with the cell loss and the cell concentration decreased gradually, which showed that the optimum level of the replacement fraction should be less than 33%.

Figure 8 shows the effects of semi-continuous culture on HC MAb production. The extracellular levels of HC MAb were maintained at constant levels with the continuous introduction of the new medium, allowing the periodic harvest of produced HC MAb in the cells and extracellular medium.

The intracellular concentrations followed a trend similar to that of the wet cell concentrations. The intracellular level in the "supernatant-only replacement" flask showed the highest HC MAb concentration. The continual inflow of fresh medium provided an environment for the continual production of HC MAb. However, this mode of operation cannot be continued indefinitely. The overpopulation of cells will make long-term semi-continuous operation very difficult. The optimum operation mode may consist of the batch operation for the first 4 days, the supernatant-only replacement for the next 3 days, and cell suspension replacement for the rest of the run.

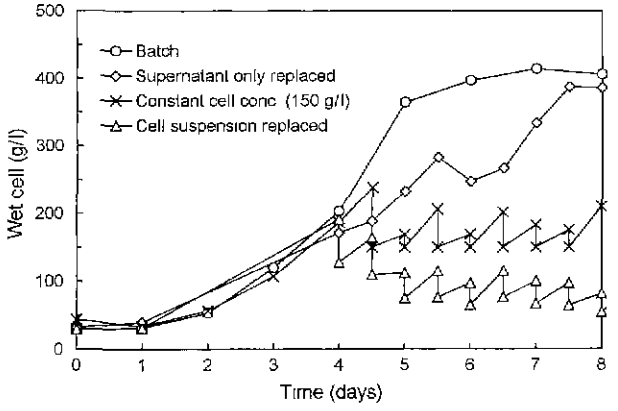

Fig. 7. Wet cell concentrations during various semi-continuous cultures in MS media with 5 g/l gelatin.

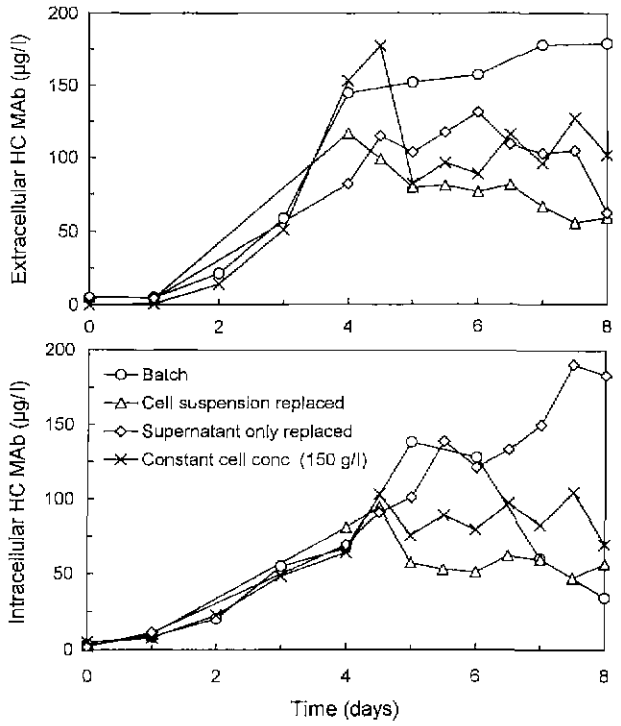

Fig. 8. HC MAb concentrations during various semi-continuous cultures in MS media with 5 g/l gelatin.

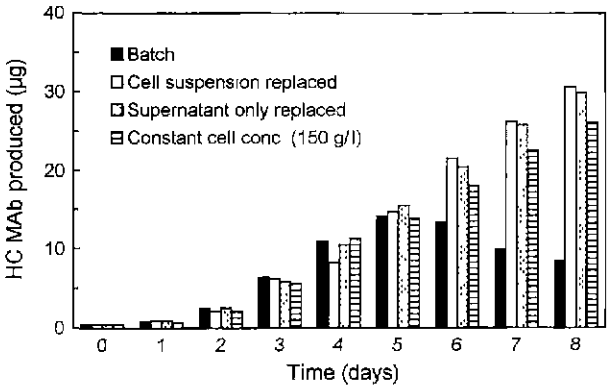

Fig. 9. Total amount of HC MAb produced (µg) by semi-continuous cultures (60 ml) during 8 days of running.

Figure 9 shows the overall production of HC MAb in the semi-continuous cultures. The totals include the HC MAb that is contained within the 60 ml of the flask, both intracellular and extracellular. Additionally, for the semi-continuous trials, all of the HC MAbs harvested were also added to the totals. The "supernatant-only replacement" flask yielded a total of 31 µg after eight days of cultivation in a shaker flask, compared to the maximum 14 µg produced by a batch culture. The total amount can be further increased with the continued operation of the semi-continuous cultures.

As far as the productivity goes, the batch system produced 46 µg/l · day based on the 5-day cultures. The semi-continuous trials, "supernatant-only replaced", "cell suspension replaced", and "constant cell concentration" had productivity levels of 64, 62, and 54 µg/l · day, respectively. Therefore, maintaining the same reactor volume, although changing the operating method, will help to optimize the production. The productivity of the batch culture cannot be directly compared to the semi-continuous runs because batch cultures require down times that will reduce the overall productivity.

Another factor to consider in deciding on a mode of operations is the extracellular fraction of the total HC MAb produced. HC MAb in the extracellular medium is easier for the purification process than HC MAb contained in the cells, because the latter requires a complete cell rupture to release the product.

Acknowledgments

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References

1. Craig, E. A. 1993. Chaperones: Helpers along the pathway to protein folding. *Science* **260:** 1902–1903.
2. Harlow, E. and D. Lane. 1988. *Antibodies: A Laboratory Manual*, pp. 283–318. Cold Spring Harbor Laboratory, New York, New York, U.S.A.
3. Hasemann, C. and J. Capra. 1990. High level production of a functional immunoglobulin heterodimer in a *baculovirus* expression system. *Proc. Nat. Acad. Sci. USA* **87:** 3942–3946.
4. Hayes, S. A. and J. F. Dice. 1996. Roles of molecular chaperones in protein degradation. *J. Cell Biol.* **132:** 255–258.
5. Kawashmi, N., H. Christenson, and B. Niham. 1990. Measurement of the interaction between absorbed polyelectrolytes: Gelatin on mica surfaces. *J. Phys. Chem.* **94:** 4611–4617.
6. 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.
7. Lasdon, L. S., A. D. Waren, A. Jain, and M. Ratner. 1978. Design and testing of a GRG code in liquid culture. *Plant Physiol.* **82:** 353–368.
8. Magnuson, N. S., P. M. Linzmaier, J. 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. *J. Protein Exp. Purification* **7:** 220–228.
9. 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.
10. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth bioassays with tobacco tissue cultures. *Physiol. Plant* **15:** 473–497.
11. Nelder, J. A. and R. Mead. 1965. A simplex method for functional minimization. *Comp. J.* **7:** 308–313.
12. Veis, A. 1964. *The Macromolecular Chemistry of Gelatin*, pp. 1–44. Academic Press, New York, New York, U.S.A.
13. Wahl, M. F. and J. M. Lee. 1995. Effects of dimethyl sulfoxide on heavy chain monoclonal antibody production from plant cell culture. *Biotechnol. Lett.* **17:** 463–468.
14. Wu, C. 1993. Simultaneous calibration of size exclusion chromatography and dynamic light scattering for the characterization of gelatin. *Macromolecules* **26:** 5423–5426.