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Development of High Density Mammalian Cell Culture System for the Production of Tissue-Type Plasminogen Activator

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Abstract A high cell density culture system for the anchorage dependent CHO cells was developed based on the combination of *in situ* removal of ammonium ion and microcarrier culture system, and semi-fed-batch feeding of glucose and glutamine was employed to the developed culture system. The glass bead was selected as an optimum microcarrier in terms of cell growth. An ammonium ion selective zeolite, Phillipsite-Gismondine, was packed in a dialysis membrane and equipped on the agitator of spinner reactor for *in situ* removal of ammonium ion. The semi-fed-batch operation was employed to the novel culture system for the high density cell culture, and the results showed the cell growth was improved by 32% and tPA productivity by 250%.

Keywords: CHO cells, tPA, zeolite, *in situ* ammonium ion removal, microcarrier, semi-fed-batch

INTRODUCTION

Tissue-type plasminogen activator (tPA) is a glycoprotein with a molecular weight of 56 to 83 kDa and consists of single chain that is converted into two chains during fibrinolysis [1]. It activates plasminogen into plasmin, and this activation rate is remarkably increased in the presence of fibrin clot [2-4] Tissue-type plasminogen activator is free of immune side effects and has short half-life These properties make tPA an excellent thrombolytic agent for medical use Nowadays tPA is commercially produced by using recombinant CHO cells. But market share of tPA is relatively low because selling price of tPA is higher than that of streptokinase or urokinase. Therefore, development of high density culture systems and searching for an optimum operational strategy for high tPA productivity is essential to reduce production cost.

The nutrient depletion and cellular waste product accumulation, which are two major factors limiting cell growth and protein production, should be overcome in order to achieve high density culture. Glucose and glutamine are the major carbon and energy sources for mammalian cells and easily depleted during culture. Semi-fed batch feeding of glucose and glutamine is usually practiced to make up their depletions.

Ammonium ion and lactate are the major inhibitory waste products in mammalian cell cultures. In animal cell culture, glutamine is essential amino acid used as a source of energy and precursors of other amino acids, antibodies, nucleotides, purine or pyrimidine [5-10]. The accumulation of ammonium ion in animal cell culture media is inevitable because ammonium ion is excreted as a byproduct of glutamine metabolism [11]. Ammonium ion is also generated in cell culture medium during storage by spontaneous decomposition of glutamine to pyrrolidone carboxylic acid. Ammonium ion has been shown to inhibit cell growth and product formation. The inhibitory effects have been observed at the ammonium ion concentration of 0.5 mM in mouse L cell [12], 1 mM in 3T3 cell and BHK cell [13], 2-3 mM in mouse hybridoma cell [14], and 4 mM in MDCK cell [15,16]. Ammonium ion has been also reported to inhibit virus production such as influenza virus, New Castle disease virus, Columbia SK virus [17-19]. Therefore, it is important to reduce inhibitory effects of ammonium ion in order to enhance cell growth and product formation.

There have been two ways of reducing the accumulation of ammonium ion. One approach was the reduction of production rate of ammonium ion and the other was to remove ammonium ion continuously from the system as it is formed during cultivation of cells (*in situ* removal). Ammonium ion production rate could be reduced by decreasing glutamine concentration in the medium [20] or by culturing in glutamine-free medium after adaptation [21-23]. However, the stimulation of cell growth was not achieved through these trials although ammonium ion production rate was significantly reduced. Recently, a biological approach was reported by Bebbington *et al.* [24]. Myeloma cells were transfected with a glutamine synthetase (GS) containing vector and the transfectants were shown to grow in glutamine-free medium due to the formation of gluta-

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mine from ammonium ion and glutamate by glutamine synthetase. An ammonium ion resistant cells were also tried by adapting cells to high concentration of ammonium ion [25].

In situ removal of ammonium ion has been attempted by several groups. Ito *et al.* [26] tried to apply synthetic zeolite ZCP-50 to the human hybridoma culture in serum free media. Jeong and Wang applied another synthetic zeolite Phillipsite-Gismondine, which has a high selectivity and high capacity of ammonium ion adsorption, to the serum supplemented culture of mouse hybridoma cells and demonstrated the enhancement in both cell growth and antibody production [27]. They also immobilized zeolite into calcium alginate bead or dialysis membrane in order to resolve the problem of powder form and applied to the culture of hybridoma cells [28,29] and TGF-β1 producing CHO cells [30,31]. Matsumura *et al.* [32] also integrated zeolite packed bed into perfusion culture of hybridoma cells and resulted in the significant enhancement of both growth and antibody production of cells. Another approach utilizing electrophoretic mechanism was tried by Chang *et al.* to remove ammonium ion and lactate *in situ* from suspension hybridoma culture in order to enhance cell growth and productivity [33].

In the present work, a high cell density culture system for the anchorage dependent CHO cells was developed based on the combination of *in situ* removal of ammonium ion and microcarrier culture system, and semi-fed-batch feeding of glucose and glutamine was employed to the developed culture system The integration of semi-fed-batch operation and novel culture system developed in this work was intended to overcome both nutrient limitation and inhibitory ammonium ion accumulation The performance of semi-fed-batch operation of the novel culture system was evaluated in terms of cell growth, viability, tPA productivity, and degree of reduction of ammonium ion concentration.

MATERIALS AND METHODS

Cell and Culture Media

Anchorage dependent CHO cell producing tPA (ATCC CRL 9606) was used as a model system. For cell culture, basal medium of DMEM/F-12 (Sigma, 1:1 v/v) was supplemented with 10% FBS (GibcoBRL), 2 5 g/L sodium bicarbonate (Sigma), 10 mM HEPES (Sigma), 10,000 units/L penicillin (Sigma), 100 mg/L streptomycin (Sigma), 6.5 g/L glucose (Sigma), 4 mM glutamine (Sigma). Medium for cell culture was sterilized by membrane filtration (pore size, 0.22 μm)

Cell Line Maintenance and Microcarrier Culture

The anchorage dependent CHO cells were maintained in T-flask (25 cm^2, Corning). Cells were inoculated at 5×10^5 cells/mL and maintained at 37°C in a humidified CO_2 incubator (5% CO_2). For microcarrier culture, anchorage dependent CHO cells were immobilized on the several microcarriers, such as glass beads (Sigma), gelatin-coated microcarrier (Sigma), Biosilon (Nunc), and Cytodex 3 (Sigma). Microcarrier culture was performed at the microcarrier concentration of 10 g/L and inoculation level of 3×10^5 cells/mL. Microcarrier culture was maintained in the spinner reactor located in a humidified CO_2 incubator (5% CO_2).

Analysis of Cell Concentration, Viability, Ammonium Ion Concentration and tPA Concentration

Cells were detached from the sampled microcarriers by treating with trypsin solution (200 unit/mL, 1 mM EDTA, Sigma) and cell concentration was measured by hemocytometer *The cell viability was measured by* trypan blue exclusion method Ammonium ion concentration was measured by ammonium ion electrode (Orion). The 200 mL of pH adjusting ISA solution (Orion) was added to 5 mL sample and the electromotive force was measured in mV unit. Calibration curve of standard ammonium chloride solution was prepared, and mV unit of sample was converted into mM unit [34] For the measurement of tPA concentration, a chromogenic enzymatic method was used [35] Samples containing tPA were incubated with plasminogen (Sigma) and chromogenic peptide (S-2251, D-Val-Leu-Lys-*p*-nitroanilide, Sigma) for one hour, and the absorbance *of generated para-nitroanilide was measured by* using ELISA Reader (Bio-RAD) at 405 nm.

A High Density Culture System and Semi-fed-batch Operation

Phillipsite-Gismondine IW-85 synthetic zeolite (UOP) was used for the *in situ* removal of ammonium ion by adsorption [26-32]. Park *et al.* already developed various types of immobilized adsorbent and applied to anchorage dependent CHO cells for tPA production [30]. They selected membrane type immobilized adsorbent as an optimum form and successfully applied to anchorage independent CHO cells for TGF-β1 production [31]. Therefore, the membrane type immobilized adsorbent was explored to apply to the microcarrier culture of

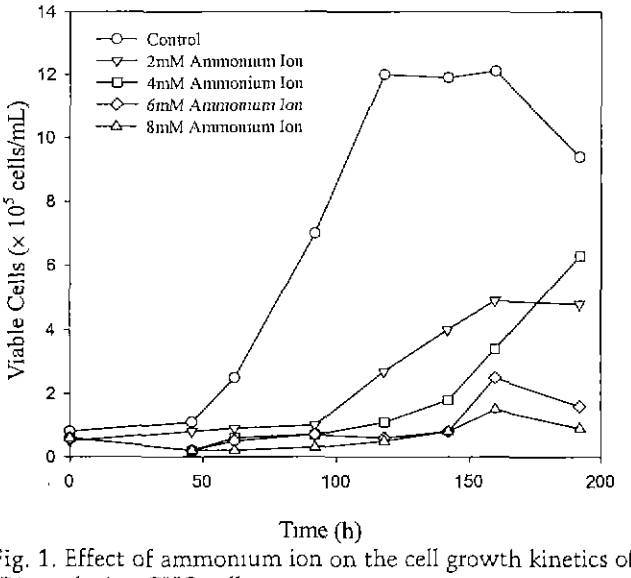

Fig. 1. Effect of ammonium ion on the cell growth kinetics of tPA producing CHO cells.

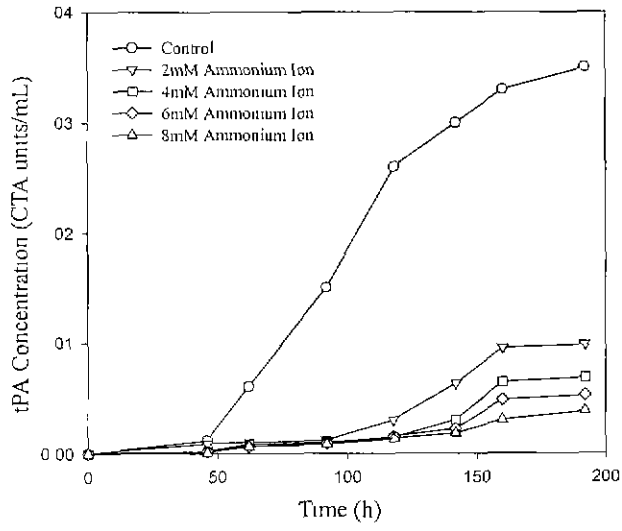

Fig. 2. Effect of ammonium ion on the tPA productivity of CHO cells.

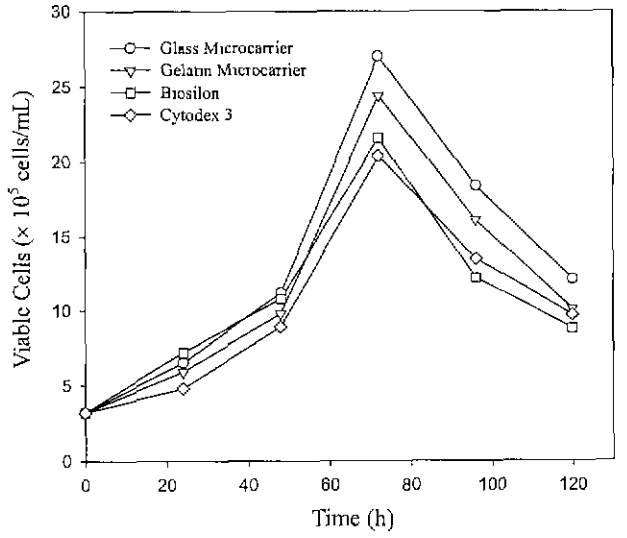

Fig. 3. Comparison of cell growth kinetics of tPA producing CHO cells cultured on several types of microcarriers

anchorage dependent CHO cells for high density culture system in the present work. The membrane type immobilized adsorbent could be easily prepared by packing Phillipsite-Gismondine zeolite powder into dialysis membrane. For the pretreatment of dialysis membrane, the membrane was flushed with water for 3-4 h, treated with 0.3% (w/v) sodium sulfide solution at 80°C for one hour, acidified with sulfuric acid (0.2%), and washed to remove residual acid. Then, a high density culture system was assembled by attaching the packed membrane to the impeller of spinner reactor *In situ* removal of ammonium ion was started after 48 h by replacing the normal impeller into the membrane attached impeller. At the same time, semi-fed batch was also operated. For semi-fed batch, 1.5 g/L/day of glucose and 300 mg/10^6 cells/day of glutamine were added to the system

RESULTS AND DISCUSSION

Effect of Ammonium Ion on Cell Growth and tPA Productivity

Fig 1 shows the effect of initial ammonium ion concentration on the live cell growth kinetics of CHO cells. The ammonium ion inhibited cell growth by retarding initial cell growth rate and reducing maximum attainable cell density Fig. 2 shows the effect of initial ammonium ion concentration on the tPA productivity of CHO cells. It could be observed that the inhibition pattern of tPA production was almost similar to that of cell growth. Therefore, it is believed that the inhibitory effects of ammonium ion on tPA production is due to mainly growth inhibitory effects of ammonium ion. Several mechanisms have been proposed to explain the inhibitory effect of ammonium ion. However, the following three mechanisms are generally accepted because of the existence of sufficient experimental evidences. The first mechanism involves the uptake of weak base NH_3 into lysosomes causing increase in intralysosomal pH [36]. Second mechanism involves the uptake of weak acid NH_4^+ into the cytoplasm causing cytoplasmic acidification [36]. Recently, Ryll *et al* reported that UPD-GNAc (sum of uridine diphosphate-N-acetylglucosamine and uridine diphosphate-N-acetylgalactoseamine) mediates the growth inhibitory action of ammonium ion [37]. Ammonium ion is incorporated into fructose-6-phosphate to synthesize glucosamine-6-phosphate which is a direct precursor of the UDP-GNAc. No matter which mechanism involves ammonium ion toxicity of animal cells, *in situ* removal of ammonium ion from cell culture media by immobilized adsorbents is a promising strategy to obtain high cell density and high tPA productivity because ammonium ion obviously inhibits both cell growth and tPA productivity as shown in Fig 1 and Fig. 2.

Establishment of High Density Culture System and Fed-batch Operation

The membrane type immobilized adsorbent was explored to apply to microcarrier culture of anchorage dependent CHO cells for the establishment high density culture system through *in situ* removal of ammonium ion in the present work. The optimum microcarrier was selected prior to the development of novel system because microcarrier culture is basic culture system for the large scale application of anchorage dependent animal cells. For the selection of optimal microcarrier, CHO cells were immobilized on several microcarriers, such as glass bead, gelatin-coated microcarrier, Biosilon, Cytodex 3, and cultured in spinner reactors with microcarrier concentration of 10 g/L. Fig 3 shows the cell growth kinetics of tPA producing cells cultured on several types of microcarriers The glass bead was selected as an optimum microcarrier and used for following experiments because the cells on glass beads showed best cell growth among the microcarriers tested.

Then the ammonium ion removal efficiency of mem-

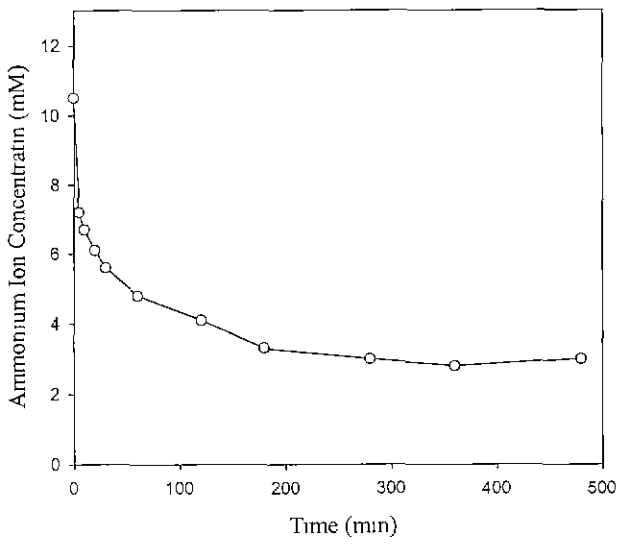

Fig. 4 Removal efficiency of ammonium ion by membrane type adsorbent attached in the spinner flask (agitation speed, 60 rpm).

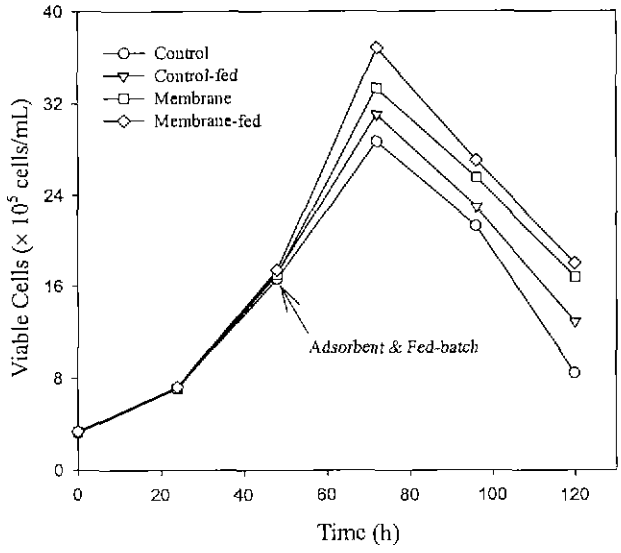

Fig. 5. Cell growth kinetics of tPA producing CHO cells cultured in 100 mL spinner flask with various operation modes.

brane type immobilized adsorbent was examined before application to microcarrier culture system. Two grams of Phillipsite-Gismondine zeolite powder was packed in dialysis membrane and the membrane was attached on the agitator of spinner by string. The 100 mL of 10 mM ammonium ion solution was then added to the spinner and ammonium ion concentration was measured according to time. Fig. 4 shows the removal kinetics of ammonium ion by membrane type adsorbent attached on the agitator of spinner reactor. It shows relatively quick equilibrium in three hours and removal efficiency of 70% It is therefore concluded that membrane type adsorbent could be successfully applied to *in situ* removal of ammonium ion in order to keep ammonium ion concentration below toxic level. The time required to reach equilibrium was also relatively short as compared with culture period of one week.

Since the nutrient depletion and cellular waste product accumulation are two major factors limiting cell growth and protein production, both *in situ* removal of ammonium ion and semi-fed-batch feeding of glucose and glutamine were finally integrated together in this study in order to significantly enhance the tPA productivity through high density cell culture. By periodically providing inexpensive key nutrients such as glucose and glutamine into the microcarrier culture system with in situ removal of ammonium ion, a significant increase of cell growth and tPA productivity could be obtained without further providing expensive serum components

To explore the applicability of proposed system, four spinner reactors were run. Fig 5 shows the live cell growth kinetics of various modes of operations. 'Control' is just a regular batch microcarrier culture using glass bead. 'Membrane' is a batch microcarrier system with *in situ* removal of ammonium ion. *In situ* removal of ammonium ion was started after 48 h by replacing the normal impeller of spinner reactor with the membrane attached impeller, because Park *et al.* [30] suggested that the optimum addition time for membrane type adsorbent was 48 h after inoculation. 'Control-fed' is the system operated in batch mode at exponential phase and afterwards in semi-fed-batch of glucose and glutamine feeding to make up for consumed nutrients Semi-fed-batch operation was also started 48 h after inoculation. 'Membrane-fed' is the combined system of 'Membrane' and 'Control-fed' This system is the integrated culture method of semi-fed-batch operation and novel culture system with *in situ* removal of ammonium ion and microcarrier culture. Cell growth of combined system showed about 32% increase of maximum attainable cell density as compared with simple batch operation. This result demonstrated the improved performance of combined system. Also Fig 5 shows that the cell growth of culture with adsorbent or semi-fed-batch culture was better than simple batch culture Therefore it is concluded that either removal of ammonium ion or supply of depleted nutrient is necessary to enhance cell growth, and the combination of these two approaches could maximize the cell growth. It is noticeable that the cell growth of culture with adsorbent was better than that of semi-fed-batch culture. This means that removal of ammonium ion was more effective than supplement of depleted nutrients. This was because ammonium ion accumulation became more serious in case of semi-fed-batch culture due to increase of glutamine concentration by continuous feeding. The high accumulation of ammonium ion in case of culture with only semi-fed-batch can be confirmed by Fig. 6. This high concentration of ammonium ion inhibited cell growth and stimulated cell death in case of semi-fed-batch culture. This means that fed-batch culture alone could not support further cell growth because of high ammonium ion accumulation caused by continuous feeding of glutamine. Consequently fed-batch mode should be incorporated with any means of ammonium ion removal method in order to get high cell density.

Fig. 6 shows ammonium ion concentration change in

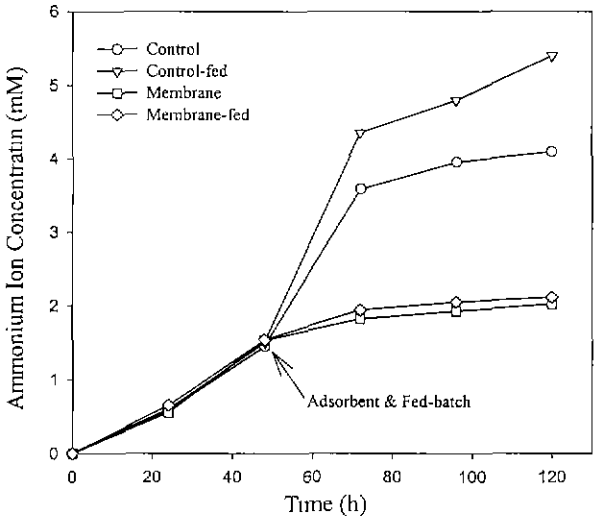

Fig 6 Ammonium ion production kinetics of CHO cells cultured in 100 mL spinner flask with various operation modes.

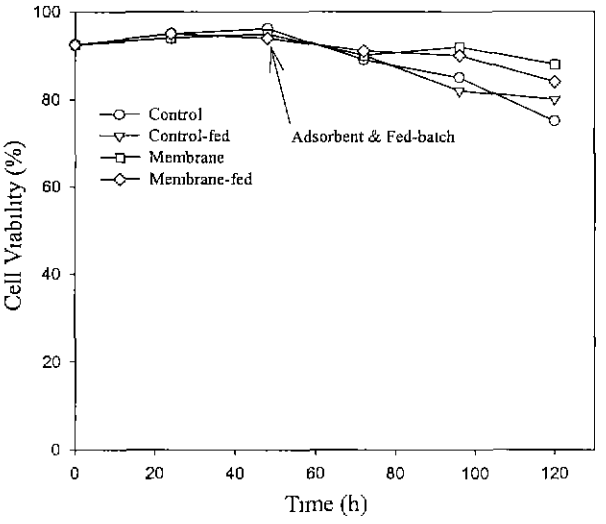

Fig 7. Cell viability change of tPA producing CHO cells cultured in 100 mL spinner flask with various operation modes.

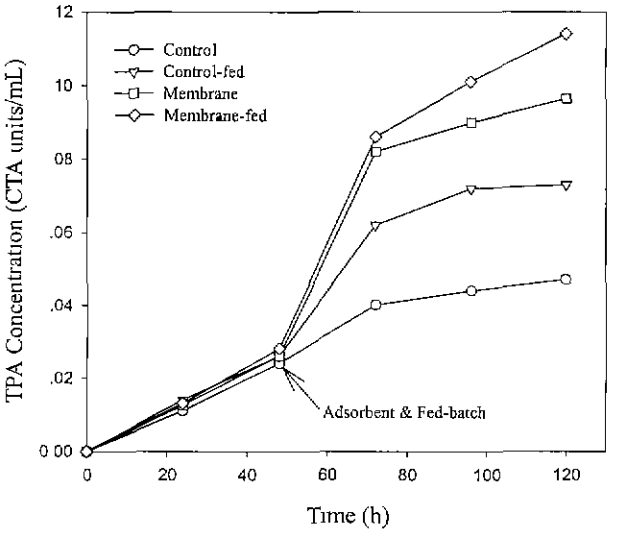

Fig. 8. tPA production kinetics of CHO cells cultured in 100 mL spinner flask with various operation modes.

the same run. It shows that ammonium ion concentrations of culture with adsorbent and combined system were kept even lower than simple batch culture. This represents that *in situ* removal of ammonium ion was successfully accomplished by membrane type immobilized adsorbent As mentioned before, the accumulation of ammonium ion was most serious in case of semi-fed-batch culture due to the addition of glutamine. It is well known that high glutamine concentration causes increase of ammonium ion production rate as well as glutamine consumption rate [38]. Therefore, ammonium ion concentration was continuously increased in the semi-fed-batch culture due to supply of glutamine, so cell growth could not continue unless ammonium ion was removed from the system.

Fig 7 shows cell viability changes in the same run. Both systems with adsorbent ('Membrane' and 'Membrane-fed') showed higher viabilities than those of two systems without adsorbent ('Control' and 'Control-fed'). This was because ammonium ion concentrations of systems with adsorbent were much lower than those of two systems without adsorbent as shown in Fig. 6 due to *in situ* removal of ammonium ion by immobilized adsorbent. This means that high accumulation of ammonium ion not only inhibits cell growth but also accelerates cell death. In other words, ammonium ion is a major factor to reduce cell viability. Consequently, it is necessary to remove ammonium ion for enhancing cell viability as well as cell density.

Fig. 8 shows the tPA production kinetics in the same run. The combined system showed 250% increase in tPA production as compared with simple batch operation. Simply combining additional feeding of small amount of glucose and glutamine with a small pack of immobilized adsorbent resulted in 250% increase of valuable tPA, which was impossible to be accomplished in a simple batch operation no matter how much serum and other nutrients were provided into the system. It is noticeable that tPA productivity was enhanced in the combined system much more intensively than cell growth. Like live cell growth kinetics, the tPA productivity of culture with adsorbent was better than that of semi-fed-batch culture. This means that removal of ammonium ion was more effective in enhancing tPA productivity than supplement of depleted nutrients. In other words, tPA productivity was more sensitive to ammonium ion accumulation than nutrients depletion. Therefore, tPA production could not be stimulated by only feeding additional glucose and glutamine unless ammonium ion was removed from the system. Eventually it is concluded the combined system of semi-fed-batch and removal of ammonium ion could stimulate not only cell growth but also tPA production.

CONCLUSION

Ammonium ion showed inhibitory effect on both cell

growth and tPA productivity. The application of membrane type immobilized adsorbent was successful in reducing ammonium ion concentration in culture media. A novel culture system combining *in situ* removal of ammonium ion in microcarrier culture system and a semi-fed-batch operation was developed and explored as a high cell density culture system for high tPA productivity. It showed more than 32% increase in maximum cell density and 250% increase in final tPA concentration as compared with batch microcarrier culture system. The proposed system is economical because it can accomplish considerably high cell density and high tPA productivity with employing inexpensive adsorbents, glucose, and glutamine.

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