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Continuous Stable Production of von Willebrand Factor Monoclonal Antibody in Spin Filter Bioreactor with Bleeding Technology

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Abstract The characteristics of two different modes of perfusion culture, intermittent and continuous bleedings, were investigated by culturing the hybridoma cells producing von Willebrand Factor (vWF) monoclonal antibody (McAb) in a 15 L bioreactor without clogging the filter. Both culture methods exhibited similar profiles of cell density and metabolite concentrations during the culture period at the cell concentration of around 1×10^7 cells/mL. When the perfusion rate was increased, the intermittent bleeding culture showed problems of ammonia accumulation and decrease of cell viability. The continuous bleeding culture exhibited higher physiological activity than that of the intermittent bleeding culture in terms of nutrient consumption and metabolite production kinetics. But the analysis of specific oxygen consumption rate showed that the specific oxygen consumption rate of intermittent bleeding culture was similar to that of exponential growth phase. The continuous bleeding culture showed higher specific vWF McAb productivity and cumulative production than those of the intermittent bleeding culture. Finally we proved the possibility of long-term operation of continuous bleeding culture and produced approximately 40 g of vWF McAb in a 15 L bioreactor after one-month operation.

Keywords. cell culture, hybridoma, bleeding mode, perfusion, spin filter, monoclonal antibody

INTRODUCTION

Many industrial cultures of hybridoma are performed in batch or fed-batch system [1,2]. In those systems, the reactor size must be large enough to produce large amount of protein. Perfusion culture has the advantages of high cell density and small reactor size required [3]. There have been many reports on perfusion strategies such as spin filter [4-7], centrifuge [8], internal and external membrane filter [9-13], decanting column [14], and packed bed [15,16]. But the perfusion culture requires a complicate operating system, so it is difficult to control the process and maintain long-term operation and is prone to contamination. Recently, the process control of perfusion culture has been studied extensively and it is possible to culture the hybridoma at high cell density with this method [17-19]. Also spin filter system has been studied intensively and found that it is easy to scale-up to thousands of liters. In spite of well-known technology, spin filter system has a problem of clogging filter. Bleeding of cells is a useful strategy to maintain high viability of the cells and to prevent the problem of filter clogging [20,21]. The optimization of bleeding is complicate because it is affected by many parameters such as growth rate, death rate, perfusion rate, and product titer. In this study, we cultured the hybridoma cells producing vWF McAb in a 15-L bioreactor with two different bleeding modes of perfusion culture, intermittent and continuous bleedings. Both blee-ding modes maintained the cell density of around 10^7 cells/mL at 1st steady state and the perfusion rate was increased to achieve higher cell density. To investigate the physiological state, metabolite profiles and kinetics of each culture were monitored. Finally, we evaluated the possibility of long-term perfusion culture with bleeding technique and the specific productivity with cumulative production.

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MATERIALS AND METHODS

Cell Line and Culture Medium

The hybridoma cell line vR8 was obtained by cell fusion following the previous method [22]. The cell line produces IgG2a McAb against human vWF that binds the factor VIII in plasma. The serum-free medium [23], which consists of 1:1:1 mixture of DME (Sigma), F12 (Sigma), and RPMI 1640 (JRH), was used for the culture. The supplements were glucose (1.73 g/L), glutamine (0.44 g/L), insulin (5 mg/L), transferrin (5 mg/L), sodium selenite (5 μg/L), ethanolamine (20 μM), and pluronic F68 (0.1%). All of the reagents were cell culture grades. HEPES (15 mM) and $NaHCO_3$ (2 μg/L) were used for buffering

Bioreactor Operation

A 15-L bioreactor (BIOSTAT® ED, B.BRAUN, Germany) was used to perform high cell density perfusion culture. Two large pitch-blade impellers were used for mixing and 15 μm spin filter was installed as a cell retention device. Oxygenation was accomplished through the sparger with pure oxygen and was maintained at 40% of saturation with air at 1 atm by using a PID controller. Carbon dioxide and air were supplied by surface aeration to control the pH of the culture media. The reactor was operated at 37°C, 80 rpm. For the perfusion culture, the harvest was removed from the inner space of spin filter by pumping out with peristaltic pump. Continuous bleeding was accomplished by pipe installed between spin filter and culture vessel. Aseptic sampling device was used to take samples and to harvest culture broth calculated from sampling for intermittent bleeding.

Analytical Methods

Cell density and viability were determined using hemocytometer by trypan blue dye-exclusion method twice a day. Samples were centrifuged to measure the concentrations of residual nutrients and metabolites and stored at -20°C for the determination of McAb concentration. Concentrations of glucose, glutamine, and lactate were measured using Biochemistry Analyzer (Model 2700, YSI, U S.A.). Ammonium ion concentration was measured by ammonia probe (Model 95-12, Orion Research Inc , U.S A.) The vWF McAb concentration was determined by enzyme-linked immunosorbent assay (ELISA) on the basis of reference antibody, which was purified by protein-A affinity chromatography and Q-sheparose ion exchange chromatography, and the concentration of reference antibody determined by measuring the O.D at 280 nm The specific oxygen consumption rate was calculated off-line once a day by measuring the decrease of dissolved oxygen from 90% to 40% without any nutrient and gas supply. The metabolite kinetics was calculated by the following equation [17]; specific nutrient consumption rate is,

$$\frac{48(C_1-C_2)/(t_2-t_1)+2\{C_0-(C_1+C_2)/2\} \times R_P}{(x_1+x_2)/10^3}$$

Where, C_0 is the initial chemical concentration of fresh media and C_1 is the concentration of chemical at time t_1 and C_2 is the concentration of chemical at time t_2 and x_1 is the viable cell concentration at time t_1 and x_2 is the viable cell concentration at time t_2 and R_P is the average perfusion rate from t_1 to t_2.

For the metabolite production rate, same equation was used except exchanging C_1 and C_2 each other.

RESULTS AND DISCUSSION

Cell Growth in Two Different Bleeding Modes

The hybridoma vR8 cell was employed as a model system to investigate the possibility of long-term high-density perfusion culture in a 15-L bioreactor. In a simple perfusion culture, cell density increased more than 3 × 10^7 cells/mL but the filter clogging made further operation impossible. To solve this clogging problem, we tested two different bleeding systems, continuous and intermittent modes The concept of bleeding is to remove cells that are in excess to certain density. The growth profile of perfusion culture with intermittent bleeding is shown in Fig. 1(a). According to the profile, cell density increased to 1.3 × 10^7 cells/mL at culture time of 96 hours. At that time, the excess cells more than 9 × 10^6 cells/mL were drained out by aseptic sampling device. Then fresh medium was supplied to the reactor over 2 h. After the medium supply, sampling was performed again to measure the cell density and the concentrations of glucose, glutamine, lactate, ammonia, and vWF McAb. Sampling was performed three times a day; before bleeding, after filling fresh medium, and 8 h from bleeding. The average cell growth between bleedings was increased by 80% and cell viability was increased by more than 95%; these are comparable to those in exponential growth phase. After 230 h-stationary phase from bleeding, perfusion rate was increased up to 4 v/v/d (perfusion volume/reactor working volume/day) to observe culture characteristics at higher density. The expecting cell density after bleeding was around 1.4 × 10^7 cells/mL. As the cell density increased, average cell growth between bleedings decreased from 80% to 45% and viability was also decreased to less than 90%. This indicates the maintenance of culture more than 2.0 × 10^7 cells/mL is not favorable in this system. The profile of perfusion culture with continuous bleeding is shown in Fig 1(b) Two peristaltic pumps were used to maintain cell density around 1 × 10^7 cells/mL, one is for perfusion and the other is for bleeding. Perfusion rate was controlled to 3.5 v/v/d for 168 hours after cell density reached to 1 × 10^7 cells/mL and then increased to 4 v/v/d. Bleeding rate was about 0.4 v/v/d throughout the culture. The cell density was maintained at 1 x 10^7 cells/ml in the period of 1st stabilization and then increased to 1.2 × 10^7 cells/mL as the perfusion rate was increased The viability was maintained higher than 95% in whole operation period These results showed the possibility of culturing hybridoma cells for an extended period using continuous bleeding mode.

Metabolites Analysis in Different Two Bleeding Modes

One of the obstacles in high cell density culture is toxic effect of by-products accumulated in culture medium such as lactate, ammonia, and other unknown metabolites during the culture. To observe these toxic effects in two different bleeding cultures, metabolite concentrations were measured at the time course According to Fig. 2(a), lactate concentrations were less than 1.3 g/L in both modes, which is within a safe range. The concentrations of ammonia were in the range of 2.5-5 mM at the perfusion rate of 3.5 v/v/d in both cultures. But this value increased gradually when the perfusion rate increased up to 4 v/v/d in intermittent bleeding culture. This result shows that the perfusion rate should be higher than 4 v/v/d above a cell den-

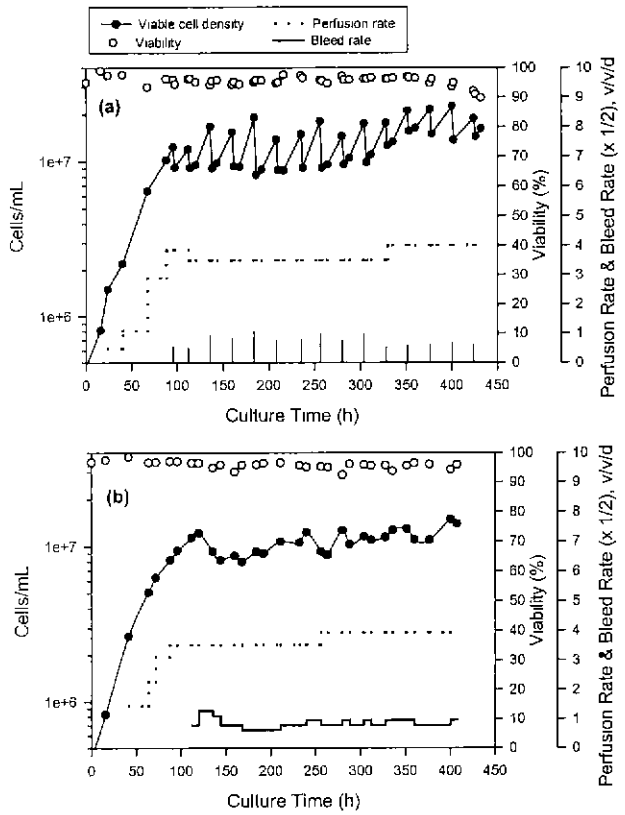

Fig. 1. The growth profiles of vR8 hybridoma perfusion culture in a 15-L spin filter bioreactor; (a) represents the intermittent bleeding mode and (b) represents the continuous bleeding mode

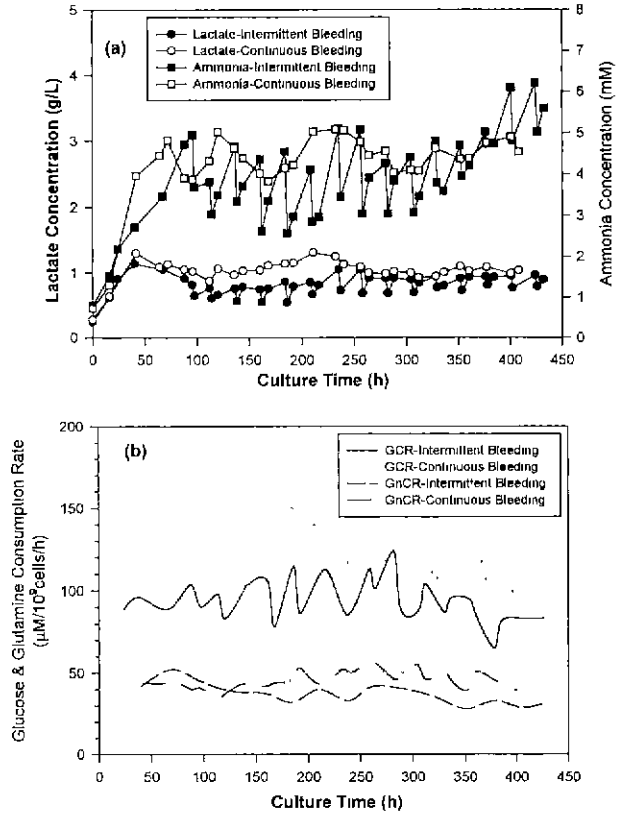

Fig. 2 Time courses of metabolite concentration (a) and nutrients consumption rate (b) in two different bleeding modes, intermittent and continuous. GCR: glucose consumption rate, GnCR glutamine consumption rate

sity of 2×10^7 cells/mL because this vR8 hybridoma cell line has inhibitory effect on the growth at higher than 7 mM of ammonia (data not shown). On the other hand, the concentration of ammonia was maintained less than 5 mM throughout the culture in continuous bleeding mode. Toxic materials did not cause any problems in continuous bleeding culture. Glucose and glutamine are main energy sources of animal cells along with amino acids and the changes of their consumption rates are shown in Fig 2(b). For glucose and glutamine consumption rates, continuous bleeding culture showed 28% and 31% higher values than those of intermittent bleeding culture, respectively. The metabolic parameters in two different bleeding cultures are summarized in Table 1. The glucose consumption rates, around 90 μmole/10^9 cells/h, in both bleeding cultures were comparable to other results [24]. For lactate and ammonia production rates, continuous bleeding culture showed 70% and 40% higher values than those of intermittent bleeding culture, respectively. This metabolite analysis suggests that the physiological activity of the culture is higher in the continuous bleeding culture than in the intermittent bleeding culture. On the other hand, for the ratio of lactate production rate to glucose consumption rate (LPR/GCR) and ammonia production rate to glutamine consumption rate (APR/GnCR), the continuous bleeding culture showed 33%, 7.5% higher values than those of the intermittent bleeding culture, respectively. This indicates that the continuous bleeding culture was not an efficient system in terms of nutrient metabolism as compared to the intermittent bleeding culture.

Table 1. Comparison of metabolic parameters in two different bleeding modes

Parameters	Intermittent Bleeding	Continuous Bleeding
GCR (μM/10^9cells/h)	96	123
LPR (μM/10^9cells/h)	104	177
GnCR (μM/10^9cells/h)	35.4	46.3
APR (μM/10^9cells/h)	47.1	66.0
OCR (μM/10^9cells/h)	199	161
LPR/GCR	1.08	1.44
APR/GnCR	1.33	1.43
OCR/GCR	2.07	1.31
Specific Productivity (μg/10^6cells/d)	13.1	18.2
Volumetric Productivity (mg/L/d)	138	195

Specific Oxygen Consumption Rate in Two Different Bleeding Modes

The time course change of oxygen consumption is

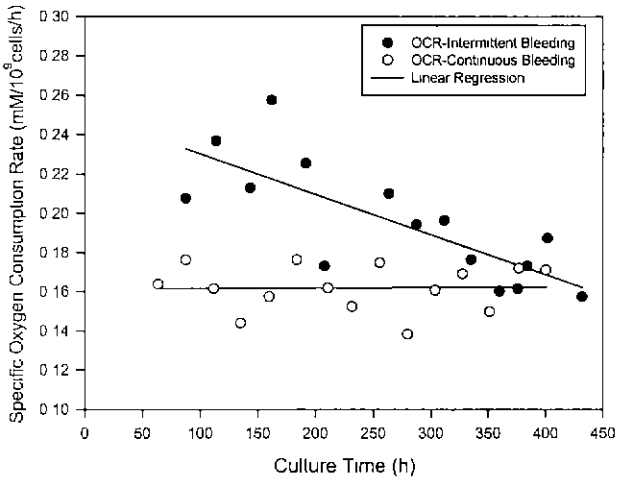

Fig 3 Specific oxygen consumption rate in perfusion culture with two different bleeding modes. Straight lines represent the linear regression of each bleeding mode.

shown in Fig. 3 in two different bleeding modes. In the intermittent bleeding culture, the average specific consumption rate was 0.199 mM/10^9 cells/h with a tendency of decrease. This declining trend suggests that the physiological state of cells in the intermittent bleeding culture was getting worse along with the culture time. But the specific oxygen consumption rate in the intermittent bleeding culture was higher than in the continuous bleeding culture because cells were exposed to the favorable environment (growth-like phase) temporarily in a intermittent bleeding culture due to supplying fresh medium at one time. For the continuous bleeding culture, the specific oxygen consumption rate showed almost constant values throughout the culture with an average value of 0.161 mM/10^{9}cells/h. This result agrees with the result of metabolite analysis in Fig. 2, which suggests that the continuous bleeding culture could be maintained in a steady state. We calculated the ratio of oxygen consumption rate to glucose consumption rate (OCR/GCR) to compare the efficiency of glucose consumption in both bleeding cultures. As shown in Table 1, OCR/GCR value of the intermittent bleeding culture was 58% higher than that of the continuous bleeding culture. This means that the intermittent bleeding culture was more efficient in using glucose as an energy source than the continuous bleeding culture.

vWF Monoclonal Antibody Production in Two Different Bleeding Modes

The vWF McAb in culture supernatant was measured by ELISA method The specific productivity was calculated from antibody titer and cell density in two different bleeding cultures. The results are shown in Fig. 4(a). The average specific productivity in continuous bleeding culture was 18 2 μg/10^{6}cells/d, which is 17% higher than that of intermittent bleeding culture. In continuous bleeding culture, the specific productivity of early growth phase was lower than that of steady state phase.

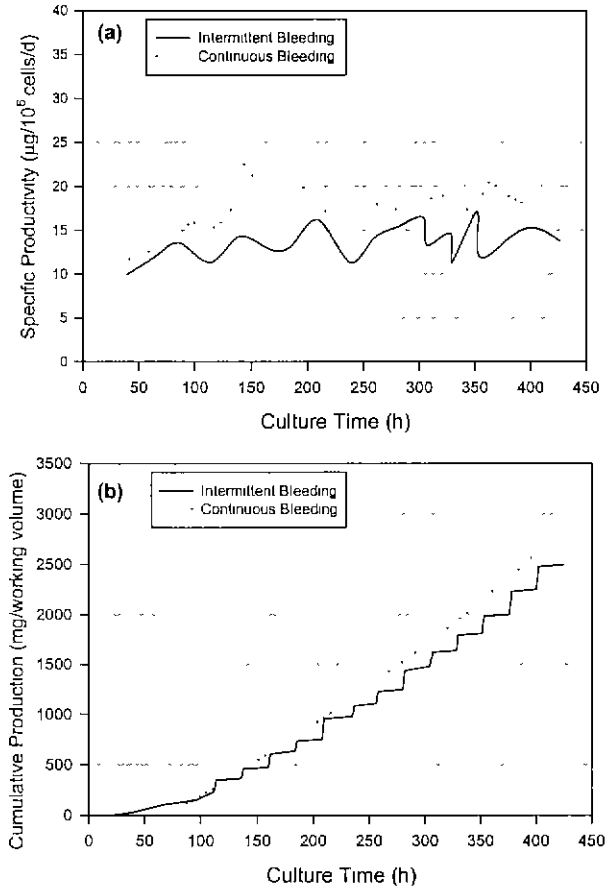

Fig 4 The time course profiles of specific productivity (a) and cumulative production (b) in two different bleeding modes. Cumulative production is the sum of antibody produced at that time and divided by working volume (liter).

This result suggests that this cells produce antibody more in stationary phase than in exponential growth phase. The same result can be observed in intermittent bleeding culture. In that culture, the specific productivity was almost uniform in both growth and bleeding phases because of growth-like characteristics in bleeding phase (Fig 1(a)). The cumulative antibody production from each culture was represented in Fig. 4(b). To compare precisely, antibody titer was measured before and after bleeding and the harvest drained was pooled with perfusate in intermittent bleeding culture. As shown in Fig 4(b), there was no difference between two cultures in spite of higher specific productivity in continuous bleeding culture because higher density was achieved in intermittent bleeding culture.

Stable Production of Monoclonal Antibody With Continuous Bleeding

High-density perfusion culture was performed with continuous bleeding to test possibility of long-term operation. As shown in Fig. 5, the cell density reached to 1 × 10^7 cells/mL from the inoculum density of 7 × 10^5 cells/mL after 96 h operation. From this time, perfusion rate was set to 3.3 v/v/d to the end and the bleeding

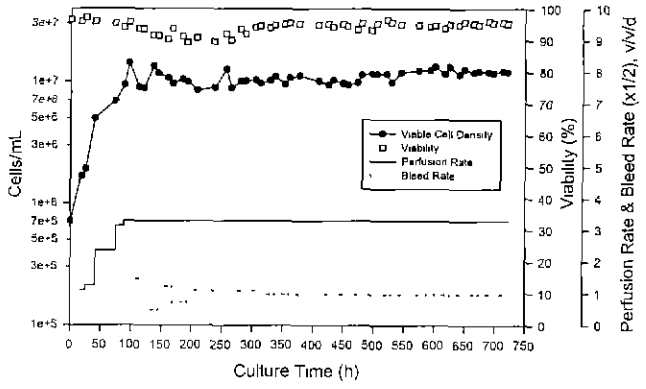

Fig. 5. The growth profiles of vR8 hybridoma perfusion culture in a 15-L spin filter bioreactor The perfusion rate was fixed and bleeding was performed in continuous mode. After adjustment period bleed rate was also fixed.

rate was controlled to maintain steady state for 200 hrs. Thereafter the culture was maintained steady state for 400 h with fixed perfusion and bleeding rates and the viability of cell was maintained higher than 95% in this phase. For the nutrients and metabolites concentrations, all of these profiles were observed to be very stable throughout the culture

CONCLUSION

The use of spin filter as a cell retention device in high-density long-term perfusion culture has a problem of filter clogging One of the methods to solve this problem is a bleedings technology. In this study, we tested two different types of perfusion culture, intermittent and continuous bleedings, in a 15-L bioreactor. To compare the characteristics of these two culture modes, cell density, residual nutrient concentration, metabolite concentration, oxygen consumption and product titer were measured at the time course. And with these measurements, we calculated the kinetics terms; specific nutrients consumption rates, specific metabolites production rates, specific oxygen consumption rates and specific McAb production rate According to these analyses, intermittent bleeding culture has similar characteristics of exponential growth phase, in which higher cell growth rate and more efficient consumption of nutrients and oxygen were observed, compare to continuous bleeding culture On the contrary, specific McAb production rate and cumulative McAb were higher in continuous bleeding culture than in intermittent bleeding culture. Moreover continuous bleeding culture showed stable production of McAb without adverse effects of by-products and clogging filter. As a result, we could produce approximately 40 g of vWF McAb using this continuous bleeding culture method in a 15-L bioreactor for one-month operation.

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