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Enhancement of Oxygen Transfer in Animal Cell Culture by Using a Perfluorocarbon as an Oxygen Carrier

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과불소 탄소화물을 산소전달매체로서 사용하여 동물세포 배양의 산소전달 증진

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ABSTRACT

In order to increase the oxygen transfer in a bioreactor for animal cell culture, a perfluorocarbon, Flutec^{R} ppll was used in a modified Celligen ^{TM}system. Also, the effects of ppl1 on the hybridoma cell growth and on the production of monoclonal antibody were investigated. There was no harmful effect of ppl1 on the cell growth and on the production of monoclonal antibody.

INTRODUCTION

Recently, large scale cultivation of animal cells has drawn great attention, because large quantities of pharmaceutical and health-related products, such as vaccines, growth hormones, tissue plasminogen activator(TPA), interferons, interleukins and monoclonal antibodies, are in great demand(1). And, it is very important to maintain high cell density in animal cell bioreactors in order to increase the yield and the productivity of products, which results in the reduction of serum cost, downstream processing cost, and hence the reduction of the overall cost(2).

The order of cell densities in dense culture systems is in the range is 10^{7}-10^{8} cells / ml(3). But, the normal cell density in a suspension cell culture is in the order of 10^{6} cells / ml. Since, in the high cell density systems, the cell concentration increases 10-100 times higher than in the low cell density systems, there can be severe oxygen limitations and higher waste accumulations such as CO_2, lactic acid and ammonium ion. These problems are the barriers to the scale-up of animal cell culture. The most critical barrier to the scale-up of the animal cell culture is the supply of an adequate oxygen into the bioreactor(4). Many oxygenation methods were tested to overcome the problem of oxygen limitation, such as cage aeration, air lift, silicone tubing, surface aeration, circulation system, and artificial red cells. But, any of these methods cannot simultaneously satisfy the requirements of good mixing, low flow shear, no foaming, and an adequate oxygen transfer rate at high cell density(4).

Therefore, it is very importnt to find a new method of oxygenation for the large scale animal cell cultures without foaming and high shear or surface tension force, which damages cells.

The perfluorocarbons(PFCs) has proved to be biologically inert, and have no toxicity(5). Emulsion of PFCs have been successfully been used in "blood-transfusions" to several different animals and, recently, to humans(6). In addition to potential applications of emulsified PFCs in mammalian systems, PFCs have been used for microbial and animal cell cultures as oxygen carriers(7-11).

And, Brink and Tramper(12) reported that the PFCs, FC-40 and FC-70 caused almost no inactivations of Mycobacterium cells. Adlercreutz and Mattiasson(13)

also reported that the PFC, FC-72 and the surfactant, pluronic F-68 did not affect the viability of alginate-immobilized G. oxydans cells for 12 days. But, Chandler et al.(6) found that the growth of E. coli and S. cerevisiae cells was inhibited by a commercial Fluosol-DA and an emulsion made of perfluorodecalin with the poloxamer surfactant pluronic F-68. Also, cytotoxic effects of Fluosol-DA and similar PFCs emulsions have been observed using mammalian cells in vitro(14-15). The toxicity of these emulsions is thought to be partly caused by the surfactants.

Here, an alternative oxygenation method by using a perfluorocarbon (Flutec^R pp11) is presented. At first, capabilities of oxygen transfer were compared between a Flutec^R-bioreactor and a Celligen^{TM} bioreactor (New Brunswick Sci., NJ). Next, the effects of Flutec^R pp11 on a hybridoma cell growth and monoclonal antibody(IgG2a) production were investigated in a T-flask and a spinner flask. And, finally a new design of Flutec-Celligen bioreactor is recommended based on the previous experimental results.

MATERIALS AND METHODS

Cell line

The mouse-mouse hybridoma cell line(#824) used in this study was given by New Brunswick Sci. Co.. The monoclonal antibody produced by this cell line is the mouse immunoglobulin, IgG2a.

Culture media

The cells were maintained in DMEM(1.337 g/l, Dulbecco's modified Eagle medium, Irvine Sci., santa Ana, CA) containing 4.5 g/l of glucose, supplemented with NCTC 135(0.94 g/l, GIBCO Lab, Grand Island, NY), oxaloacetate(1 ppm), insulin(75.5 μg/l), $NaHCO_3$(3.7 g/l), pluronic(0.1 g/l), primaton RL(2.5 g/l), mercaptoethanol(3.5 ppm), penicillin(100 U/ml), streptomycin(100 μg/ml) and 5%(v/v) calf bovine serum, and medium was filtered through a 0.2 μm membrane.

PBS (phosphate-buffered saline)

Before inoculation, the reactor should be autoclaved with PBS to keep pH & DO probes sterile. PBS includes NaCl(8 g/l), KCl(0.2 g/l), Na_2HPO_4(1 g/l) and KH_2PO_4(0.2 g/l), and the pH of PBS was adjusted to 7.2 with 3N HCl.

Perfluorocarbon

The perfluorocarbon used was perfluoromethyldecalin, whose common brand name is Flutec^R pp11. This chemical was obtained from ISC Chemicals Ltd., Avonmouth, England. The properties of pp11 are almost same as those of pp9(Damiano and Wang, 1985) except the boiling point(B.P. of pp11=215℃).

Radial immunodiffusion(RID) assay

The mouse immunoglobulin, IgG2a was analyzed by using a RID kit(ICN ImmunoBiologicals, Lisle, IL). Three standard samples were used to make a calibration curve. Each sample of 5 μl centrifuged broth was injected twice into each hole of the RID plate, which contains a monospecific antiserum in agarose, over a period of 30 min. After 50 hours at room temperature, the ring size of each sample was measured, and the actual concentration of antibody was rad from the calibration curve.

Viable cell count

Cell numbers were determined in culture medium using the trypan blue exclusion method as the following. A 0.2 ml sample of culture medium was mixed with 0.2 ml trypan blue stain (0.4%). Small amount of this mixture was injected into a hemacytometer. An inversion microscope was used to count viable cells(transparent) and dead cells(blue color).

Cell propagation

Cells were cultivated as follows: 1) in 25 and 75cm^2 T-flasks as stationary cultures; 2) in 250 ml spinner flasks (Bellco Glass, Vineland, NJ) containing 100 ml medium stirred at 60 rpm; 3) in roller bottles containing 250 ml medium. The culture medium was inoculated to contain about $3x10^5$ cells/ml and incubated at 37°C. T-flasks were put in the 5% CO_2 incubator. Spinner flasks and roller bottles were inoculated, gassed with a mixture of 5% CO_2 in air and incubated at 37°C.

K_La measurement

The overall oxygen transfer coefficient, K_La was measured by the static gassing-out method(16). The pH of

medium was adjusted to 7.2 with CO_2 gas before starting the measurements. And, the temperature of medium was always controlled as 37°C.

Cell Culture Systems

Celligen™ bioreactor

Animal cells in culture are shear-sensitive. Thus, a special low shear agitation system is required to minimize the physical damage to the cells, New Brunswick Sci. Co.(Edison, NJ) developed a new concept of bioreactor which employs cell lift impellers and a cage aeration(Fig. 1). Liquid mixing was gently accomplished by rotating a hollow tube impeller that has three discharge arms. Centrifugal force of these arms pulls the liquid up through the hollow tube so as to make a very gentle mixing. And, aeration was done in the 200 mesh screen through a ring sparger. This cage aeration is very useful for the microcarrier cell culture, because microcarriers are separated from the aeration environment by the screen. But, suspension cells can penetrate into the screen and can contact with the gas bubbles. So, the cage aeration is not useful for the suspension cell culture except the advantage that foam can be captured in the foam elimination chamber at the top of the impeller, which is cocated above the liquid surface of the medium. And, a controller was designed to combine DO and pH control through the regulation of the air, O_2, N_2 gas mixture introduced into the reactor. Details of the Celligen™ bioreactor were described in theliterature(17).

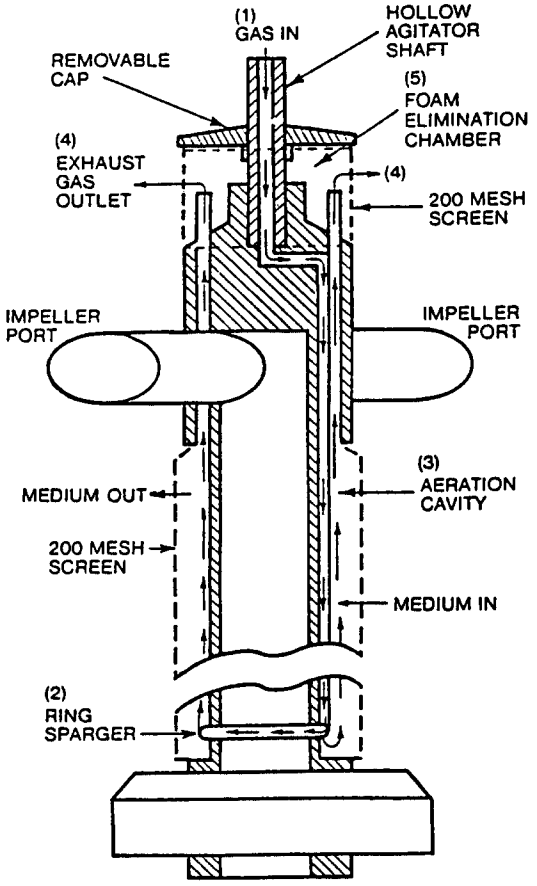

Fig. 1. Schematic diagram of the Celligen™ bioreactor.

Flutec-Celligen bioreactor

A 1.2 L Celligen™ bioreactor was modified to increase the oxygen transfer rate by using a perfluorocatbon, Flutec® ppll as a gas carrier instead of using the direct sparging of gas into the reactor. The screen cage and the ring sparger were removed, and a slit was made on the cell lift impeller in order to allow medium to circulate through the slit and the hollow tube of the impeller(Fig. 2).

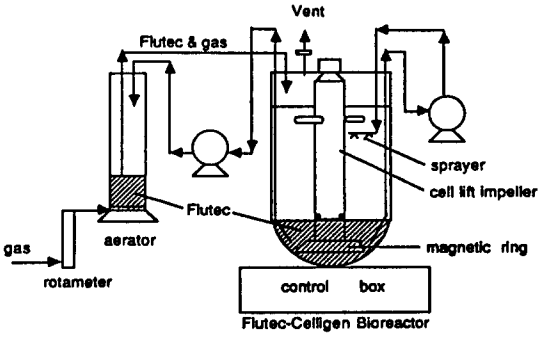

Fig. 2. Schematic diagram of the Flutec-Celligen bioreactor.

A bar sprayer of 10 syringe needles was mounted just below the top surface of the medium. A peristaltic pump was used to spray the ppll through the sprayer. The ppll droplets settled down to the bottom of the reactor, where the ppll droplets colesced and form a separate layer from the medium. Another pump circulated the

ppll from the reactor to a separate aerator, where the ppll was saturated with the mixed gas of air, O_2, CO_2 and $N3_2$, and returned to the reactor by the gas pressure. The returning ppll and gas hit the wall of the reactor, and separated from each other. The ppll fell down into the reactor, and the gas went up into the head space of the reactor and finally was vented out. The pH and DO of the medium were controlled as the same way as the Celligen™ bioreactor system except that the mixed gas was sparged into the ppll in the separate aerator through a glass-sintered sparger.

RESULTS AND DISCUSSION

Increase of K_L in the Flutec-Celligen bioreactor

The hybridoma cell density in the bioreactor can be increased up to 2.6X10^7 cells/ml, which is 10 times higher than that in the normal batch system, by using a cell-recycle perfusion system(18). For this system with high cell density, the oxygen transfer rate may be limiting because of high oxygen requirement. It is clear that the K_La value cannot be increased to meet the oxygen requirement under these circumstances even with the oxgenenriched air used in the Celligen™. In order to increase the K_La value for the high-cell-density system, a Flutec-Celligen bioreactor was considered. In this bioreactor, ppll was recirculated via a separate aerator into the CelligenTM.

In Fig. 3 & 4, the comparisons of K_La values between Celligen™ and Flutec-Celligen bioreactors are shown for different impeller speeds and air flow rates. The K_La values of Flutec-Celligen bioreactor are 2-5 times higher than those of the Celligen™, even though the flow rates of ppll are small(recirculation rate=60 ml/min and spraying rate=42 ml/min). If ppll flow rates higher than these minimum value are used, the K_La value can be higher than 10 hr^{-1} for the flutec-Celligen bioreactor operated at 50 rpm of impeller speed and 0.5 L/min of air flow rate.

above 0.7 L/min of air flow rate, the K_La's of the Celligen™ system dropped, and stayed almost constant as 1 hr^{-1} above 1 L/min air flow rate(Fig. 4). This phenomenon was caused by the fact that the liquid level inside the screen-cage aerator decreased at high air flow rates to a level close to the ring sparger. Since high

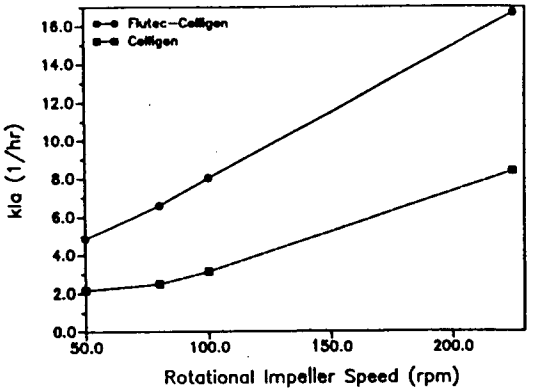

Fig. 3. Comparison of k_La values between 1.2 L Celligen™ and Flutec-Celligen at different impeller speeds, 37°C, 0.5 L/min air flow, 60 ml/min ppll recycle rate, and 42 ml/min ppll spray rate with 5% CBS medium.

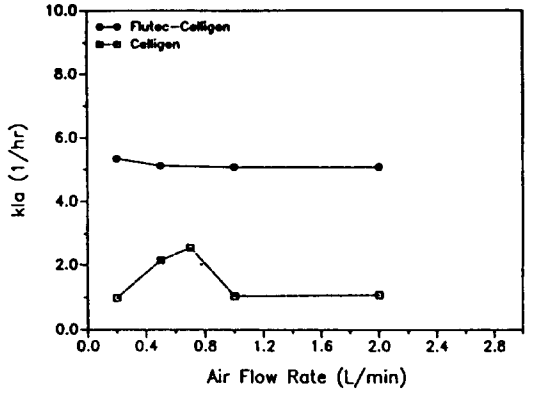

Fig. 4. Comparison of k_La values between 1.2 L Celligen™ and Flutec-Celligen at different air flow rates, 37°C, 50 rpm, 60 ml/min ppll recycle rate, and 42 ml/min ppll spray rate.

protein concentration of the serum decreased the surface tension of the medium, it is possible that this would cause the partial blockage of the screen of the foam elimination chamber so as to make high back-pressure inside the cage. And, the air flow rate in the aerator does not affect K_La of the Flutec-Celligen bioreactor-(Fig. 4). This means that ppll can be saturated easily

with a small amount of air, and this saturation step is not the rate-limiting step for oxygen transfer.

At a cell density of 10^6 cells / ml, hybridoma cells in the culture consume oxygen at a rate of 0.2 mmol / L / hr. In order to meet this demand the K_La value needed to maintain a DO level of 50% air saturation (0.1 mmol / l) in the medium is 2 hr^{-1}, if we consider that the oxygen solubility in the water at 37°C is 0.2 mmol / L, and that the air is supplied into the reactor. Since the cell-recycle perfusion system can get the cell density in the order of 10^7 cells / ml or higher, the K_La value needed to maintain a 50% airsaturation DO in the medium is $20hr^{-1}$ or higher. If the oxygen-enriched air that contains 50% O_2 is used, the required K_La can be reduced to 8 hr^{-1}, which is still too high to be obtained by the Celligen™ bioreactor with a gentle mixing environment. On the other hand, the K_La value of the Flutec-Celligen system can be increased beyond 10 hr^{-1} not by using high impeller speeds and high direct air sparging into the medium, but by increasing ppl1 flow rates which dos not create a severe environment for the cells. Therefore, the Flutec-Celligen bioreactor system has a great potential for cultivating animal cells and other fragile cells to a high cell density without cell damage.

Effects of Flutec® pp11 on hybridoma cell growth and monoclonal antibody production

The mouse-mouse hybridoma cell line(#824) was grown in the T-flasks and the spinner-flasks with / without the ppl1 in order to investigate the effects of the ppl1 on the cell growth and the monoclonal antibody(IgG2a) production.

The cell passages were done in the T-flasks that contained 15ml medium and 5 ml ppl1, over one month. No decrease of cell viability was observed. The initial cell growth in the T-flask with ppl1(25 vol%) appeared to be faster than that in the T-flask without ppl1.(Fig. 5) This phenomenon can be attributed to an extra oxygen supply to the cells in the T-flask with ppl1.

Fig. 6 & 7 show the cell growth and the monoclonal antibody production in a 250ml spinner-flask the contains 100ml medium with / without 25 ml ppl1. In the spinner-flask with ppl1, the magnetic bar located just above the interface of the ppl1 and the medium in order to renew the liquid-liquid contact-interface. The agitation

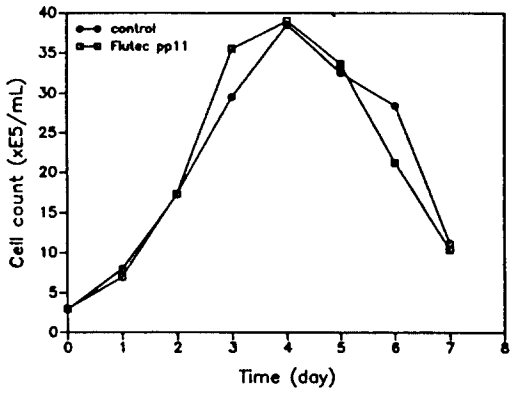

Fig. 5. Comparison of hybridoma cell growth in 15 mL T-flask between control and pp11 system which has 5 mL pp11 at the bottom of the T-flask at 37°C and 5% CO_2 environment.

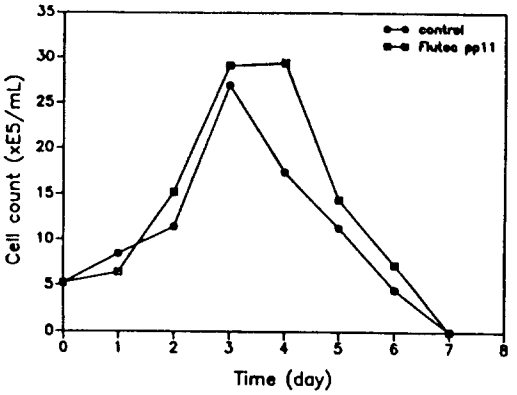

Fig. 6. Comparison of hybridoma cell growth in 100 mL spinner flask between control and pp11 run.

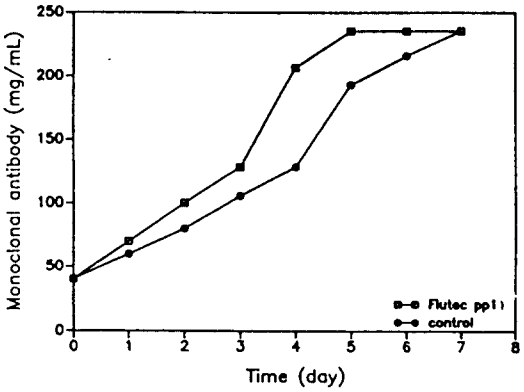

Fig. 7. Comparison of monoclonal antibody production in 100 mL spinner flask between control and pp11 run.

speed of the magnetic bar was 60 rpm. The initial cell growth and antibody production rates are faster in the ppl1 spinner-flask system than in the control system. The maximum antibody concentration(235 mg/L) was achieved 2 days earlier in the ppl1 system than in the control system. But, the final antibody concentrations were same for the both systems, because all essential nutrients were depleted finally in both systems. Therefore, we can conclude that the Flutec^R ppl1 used here does not have any harmful effect to the hybridoma cells, and that in can increase the rate of oxygen supply to the cells.

Problems and recommendations in the performance of the Flutec-Celligen bioreactor

Several batch runs were tried to culture the hybridoma cells in the 1.2 L Flutec-Celligen bioreactor with a ppl1 flow rate of 25 ml/min, an impeller speed of 40 rpm and a total gas flow rate of 0.5 L/min. The seed culture from roller bottles was inoculated into the sterile medium in the Flutec-Celligen bioreactor that contained 180 ml ppl1 in the bottom of the reactor. The total broth volume was 1.2 L and the initial cell density was $5x10^5$ cells/ml. And, the separate aerator contained 110 ml ppl1. The flow rates of O_2, CO_2 and air were controlled individually by the interactive Celligen^{TM} controller in order to keep the DO and the pH of the medium to be 60% and 7.2, respectively. The control of DO, pH and temperature in this reactor was accomplished very well. But, there occurred a problem of cell aggregation in the region around the interface of the medium and the ppl1. This phenomenon was also observed in the roller bottle that contained the ppl1. The cells aggregated to make clumps at the interface of the ppl1 and the medium, while the roller bottle was rotating. But, there was no difference in cell viability due to clumping. In the Flutec-Celligen reactor, the magnetic ring submerged in the ppl1 phase rotates, which may be a possible reason for the cell aggregation. The common reason for the cell aggregation in both systems may be the rotation energy, which is transferred into the interface and become the energy needed for aggregating the cells at the interface. On the other hand, the spinner-flask did not have the aggregation problem, because there was no moving part in the ppl1 phase, and the interface was always renewed by rotating the magnetic bar in the broth phase.

Based on these experiences, some modifications for the Flutec-Celligen bioreactor are suggested as follows; 1) there should be no moving part in the ppl1 phase; 2) the interface of the two phases should be renewed with a smooth stirrer(marine or paddle impeller) which should be located in the broth phase; 3) emulsions of ppl1 and medium components should be avoided, or eliminated by using a phase-separator; and 4) the spraying of ppl1 should be gentle to reduce the hydrodynamic shear stress which create emulsions; so a shower or ring sprayer is recommended instead of a nozzle sprayer.

요 약

동물세포배양을 위한 생물반응기내의 산소전달을 증가시키기 위하여 과불소탄소화물의 한 종류인 Flutec^R ppl1을 수정된 Celligen^{TM} 생물반응기내에서 사용하였다. 또한, ppl1이 하이브리도마 세포성장과 모노클로날 항체 생산에 미치는 영향을 조사하였으나 ppl1의 나쁜 영향을 찾아볼 수 없었다.

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