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Use of Moving Aeration Membrane Bioreactor for the Efficient Production of Tissue Type Plasminogen Activator in Serum Free Medium

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A moving aeration-membrane (MAM) bioreactor was employed for the production of 2 μg/mL of tissue type Plasminogen Activator (tPA) in serum free medium from normal human fibroblast cells. This system could maintain high cell density for long periods of steady state conditions in perfusion cultivation. Under normal operating condition, shear stress was as low as 0.65 dynes/cm^2 at the agitation speed of 80 rpm. Even though cell density gradually decreased with increasing agitation speed, tPA production increased linearly with increasing shear stress within a moderate range. This culture system allowed production of 2 μg tPA/mL while maintaining a high cell denisty of 1.0×10^7 viable cells/mL.

Key words: moving aeration-membrane bioreator, tissue plasminogen activator, normal human cell line

INTORDUCTION

There have been many approaches to develop cell culture systems to grow mammalian cells to a high concentration for the efficient production of pharmaceutically active proteins. One critical criterion in designing such a production system is to overcome the oxygen limitation problem while applying low shear stress to the bioreactor [1]. To effectively supply oxygen into the system, membrane aeration methods are often used. The membranes (usually silicone tubing) are fixed in the reactor, and is thus called as a static membrane aeration system.

However, this static membrane aeration has some limitations in maintaining high density cell cultures such as dissolved oxygen concentration gradients within the reactor, the requirement for a relatively high agitation speed, and the existence of non-homogeneous regions due to the membranes. A partially moving aeration type bioreactor has been introduced to resolve this kind of problems [2]; however, it also has limitations in scaling-up the culture system.

In this report, a novel bioreactor, moving aeration-membrane (MAM) reactor, where a whole membrane frame is agitated, is introduced and characterized to eliminate oxygen limitation at very low shear stress with an aim to enhance the productivity of tPA. Intensive studies have been carried out to produce tPA from various normal and recombinant cell lines [3-5] since its pharmaceutical activity has been proved by many clinical trials [6]. Many kinds of perfusion processes have been employed to economically produce tPA using serum containing or serum-free media [3, 7-9].

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MATERIALS AND METHODS

Cell line and culture condition

A normal human fibroblast cell line (CCD-112 CoN) established from 18 week old female fetus was maintained by DMEM/F12 (1:1) mixtures (pH 7.1) with 5% FBS (GIBCO, USA) in a 75 T-flask. When the cell density reached 1×10^4 viable cell/mL the cells were inoculated with 4 g/L of microcarriers (Cytodex III) into a MAM bioreactor shown in Fig. 1. Serum-free (SF) medium was fed into the reactor when the system started perfusing the medium into the reactor. The SF medium contained DMEM basal medium enriched with 10 mg/L of human transferrin, bovine insulin and selinite mixture, 10 KIU/mL aprotinin (Sigma, USA for all reagents) and 2 μg/L of *Centella asiatica* powder [10]. Cell density was measured every day by the nuclei count method [11]. The concentration of tPA was quantitatively measured by both ELISA kit (Imubind, USA) and fibrin agar plate [12]. The enzymatic activity of tPA was also measured by using a chromogenic substrate, S-2288 to confirm the specific activity of produced tPA [13].

Reactor configuration and perfusion cultivations

Fig. 1 shows the diagram of MAM bioreactor (working volume, 1.5 L). The whole membrane frame (8 in Fig. 1) is agitated by attaching to the shaft (2 in Fig. 1) and a top driven motor. Air is introduced to the reactor at the top holes (4 in Fig. 1) through a 0.2 μm filter (13 in Fig. 1) and the input air is exchanged (9 in Fig. 1) in the medium by silicone tubing (I.D. 1.8 mm, wall thickness 0.4

mm). The gas is exhausted through the bottom holes on the top of the vessel (7 in Fig. 1). The shaft and the housing of the head plate is connected by two double sealing compact bearings to prevent diffusion into and out of the reactor (15 in Fig. 1). The pH, D.O. and temperature were controlled at 7.1, 40% of air saturation and 37°C, respectively. Fresh medium was fed into the system by a peristaltic pump controlled by a level probe. The conditioned medium was also perfused out through a decantor by a peristaltic pump (silimar to Celligen system, NBS, USA), containing 80 m and 15 μm pore size two filter screens at the top of the vessel. The concentrations of glucose and lactate in the media were measured by a glucose-lactate analyzer (YSI, USA). Glutamine and ammonia concentrations were estimated by using Wako kit (Wako, Japan) and HPLC (Waters, USA). Oxygen uptake rate was calculated from the mass balance of oxygen concentrations between inlet and outlet gas streams using an on-line gas chromatography (Varian, USA).

Mass transfer coefficient (k_la) was calculated by the following equation by using the data obtained from a gas chromatography.

$$(dC_l/dt)=k_la(C^*-C_l)-rX \quad (1)$$

where C_l represents the dissolved oxygen concentration (mole/L), C is the dissolved oxygen concentration in the liquid phase at equilibrium with the oxygen concentration in the gas phase (mole/L), and kla is the volumetric oxygen transfer coefficient (h^{-1}). It was assumed that rX, oxygen consumption rate of the cell (mole/L/h), remained constant during the measurement [14].

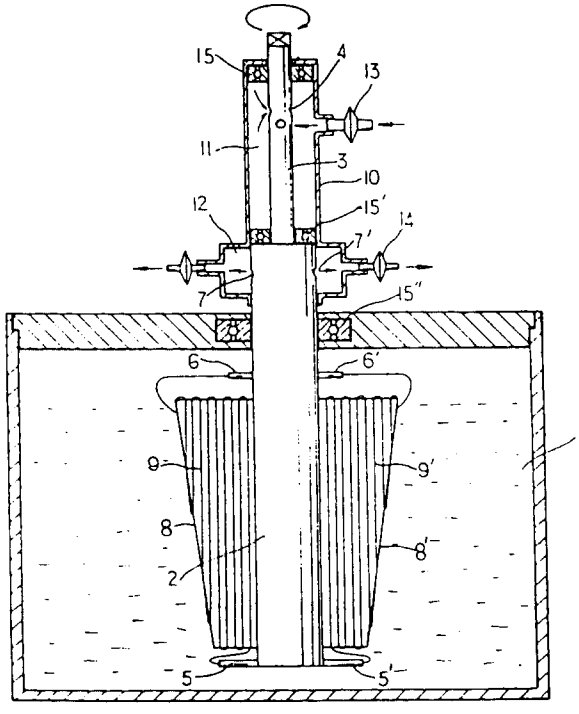

Fig. 1. A diagram of top driven MAM bioreactor.
1. reactor vessel 2. main shaft, 3. center-empty shaft, 4. inlet air, 5. connector to inlet tubing, 6. connector to outlet tubing, 7. exhaust gas, 8. frame for holding tubing, 9. membrane tubing, 10. casing for the shaft, 11. space for inlet air, 12. space for exhast gas, 13. and, 14. 0.22 m filter, 15. compact bearing set

The shear stress was calculated by the following equation [15].

$$\tau=T/2\pi R^2L \quad (2)$$

where τ is the shear stress (dynes/cm^2), T is the torques applied to agitate the shaft (dynes), and R and L are the radius and height of the vessel (cm), respectively.

RESULTS AND DISCUSSION

Fig. 2 shows the results of cultivating human fibroblast cells in the MAM bioreactor. Maximum cell density of 1.0×10^7 viable cell/mL was maintained after 30 days of perfusion cultivation. This is a relatively high cell concentration for human fibroblast cells grown in SF medium, compared to $1\text{-}6.8\times10^6$ viable cell/mL obtained by other culture techniques [3, 5, 16, 17]. 2×10^{-7} μg/cell of maximum specific tPA production (300 IU/mL based on 150,000 IU/mg of specific tPA activity) was obtained at the perfusion rate of 0.88 mL/min for 20 days. This is significantly higher than the previously reported values: specific tPA production of $0.1\text{-}1\times10^{-7}$ μg/cell from normal human cells [18, 19] even though tPA production is partially affected by medium formulation [20]. Figs. 3 and 4 illustrates the kinetics of utilization and production in a novel MAM bioreactor during perfusion cultivation of fibroblast cells. The system reached the steady state after 35 days of cultivation since the residual glucose and glu-

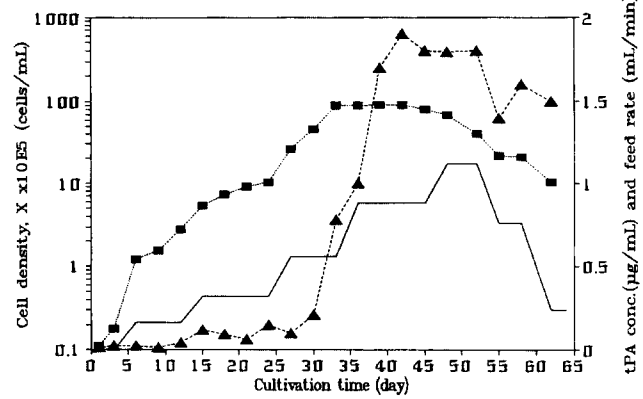

Fig. 2. Cell growth and tPA production during the perfusion cultivation of human fibroblast cells.
-■ -: cell density, -▲-: tPA conc., —: perfusion rate

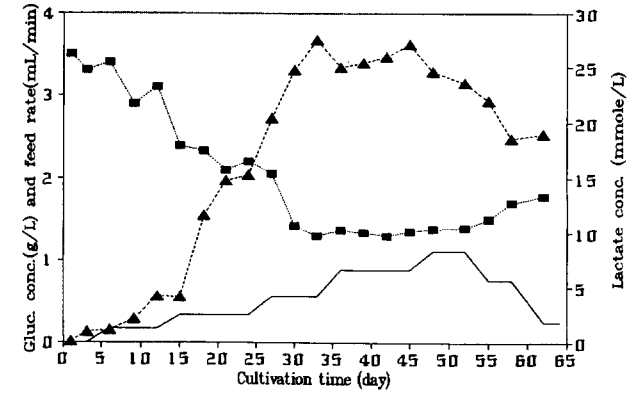

Fig. 3. Glucose consumption and lactate production during the perfusion cultivation of human fibroblast cells.
-■ -: glucose conc. -▲-: lactate conc. —: perfusion rate

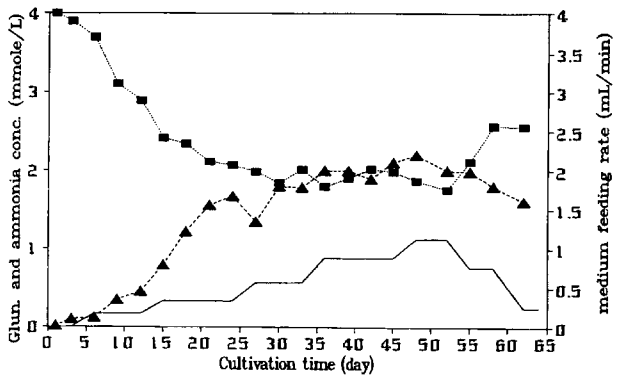

Fig. 4. Glutamine consumption adn ammonia production during the perfusion cultivation of human fibroblast cells.
-■ -: glutamine conc. -▲-: ammonia conc. —: perfusion rate

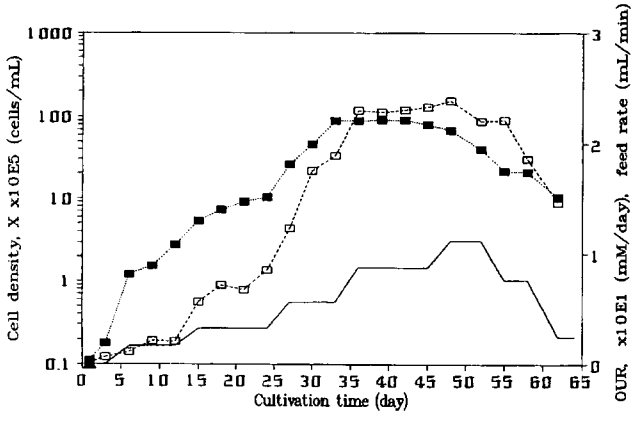

Fig. 5. Cell growth and oxygen uptake rate during the perfusion cultivation of human fibroblast cells.
-■ -: cell density, —: perfusion rate, -□ -: oxygen uptake rate

tamine concentrations remained relatively constant after this peroid except during the last period of cultivation. It could also be confirmed by that the cell concentration was maintained relatively constant during this period.

The average ratio of glucose consumption to lactate production was estimated to be 2.6 under the steady state, which is higher than 1.0-2.0 observed in traditional cell growth. The ratio of glutamine to ammonia was about 1.0. It should be pointed out that the production of lactate sharply decreased when the cell density dropped during the last period of the cultivation. In contrast, ammonia production was not much affected even though the glutamine consumption was significantly reduced in this peroid.

Fig. 5 shows the relationship between cell density and oxygen consumption rate in perfusion cultivation. The cell growth seems to be correlated to the oxygen consumption rate. However, the tPA production seems to be much correlated to specific substrate comsumption rate as shown in Fig. 6. It can be seen that the MAM bioreactor can transfer oxygen molecules into the media more efficiently than other culture processes since the system maintains relatively high cell density and oxygen uptake rate [21, 22]. The specific oxygen uptake rate continuously increased while the cell density decreased in the latter period of the cultivation, possibly due to the screen clogging by the cells.

Fig. 7 shows the effect of agitation speed on mass

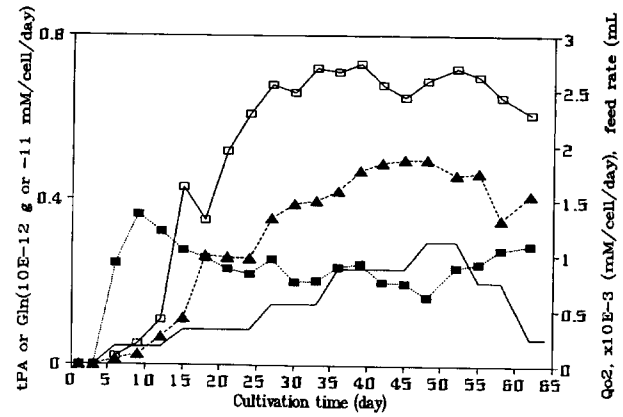

Fig. 6. Specific consumption rates of glucose and oxygen and tPA production rate during the perfusion cultivation of human fibroblast cells.
-■ -: Sp. O2 consum. rate, -▲-: Sp. tPA prod. rate, -□ -: Sp. gln. consum. rate, —: Feed rate (mL/min)

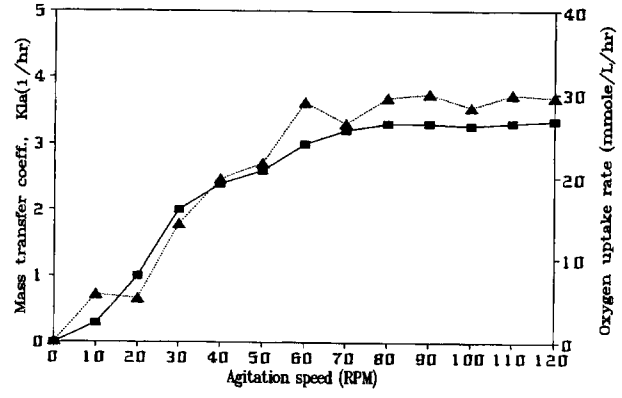

Fig. 7. The effect of agitation speed on mass transfer and oxygen uptake rate at the aeration rate of 200 mL/h for the growth of human fibroblast cells.
-■ -: Mass trans coeff. -▲-: OTR

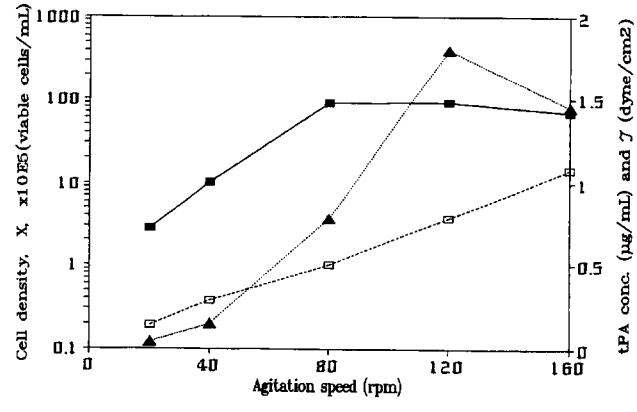

Fig. 8. The effect of agitation speed on the maximum cell growth and tPA production. The correlation between the agitation speed and shear stress is also shown.
-■ -: cell density, -▲-: tPA conc, —: shear stress

transfer coefficient and oxygen uptake rate for the growth of human fibroblast cells. It should be noted that relatively high mass transfer coefficient and oxygen consumption rate were observed at the slow agitation speed of 30-40 rpm. At 80 rpm the maximum agitation speed of this system, the kla and oxygne uptake rate of 3.3 h^{-1} and 29.5 mmole/L-h, respectively were obtained [23, 24]. It is also confirmed that high oxygen up-

take rate could be mainatained under high mass transfer conditions.

Fig. 8 shows the effect of shear stress on cell growth and tPA production in the MAM bioreactor as a function of agitation speed. Overall shear stress calculated from Eq (2) was relatively low in the MAM bioreactor compared with other types of bioreactors, mainly because of low agitation speed. High agitation speed (above 80 rpm) seemes to inhibit the cell growth, but tPA production could be enhanced at moderate shear range (80-120 rpm).

CONCLUSION

A novel moving aeration-membrane (MAM) bioreactor in which the whole membrane aeration system is rotated by being attached to the main shaft, is developed for the efficient production of tPA. tPA production was enhanced by effectively supplying oxygen while generating low shear stress. This culture system yielded ca. 2 μg of tPA/mL at the cell density of 1.0×10^7 viable cells/mL. High cell density could be maintained in the MAM bioreactor due to the high values of oxygen uptake rate and mass transfer coefficient. tPA production could be enhanced at higher shear stress up to 0.8 dynes/cm^2.

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