28 Membrane-stirrer-reactor for bubble free aeration and perfusion

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Introduction

The advantages of perfusion systems for the cultivation of mammalian and human cells in vitro are well known. With a perfusion system higher cell densities than in batch or fed-batch systems can be obtained. Therefore, higher product concentrations and productivities are possible /2/.

Up to now there are several different perfusion systems commercially available. One group of these systems is constructed for homogeneously stirred cultures with continuous cell retaining (New Brunswick, Bilthoven). These systems almost use fast rotating spin filters mounted on the stirrer shaft. The spin filters are made of microporous stainless steel or synthetic material with a pore size of 5 μm or greater /2, 3, 4/. Another construction is a satellite filter vessel for the reprocessing of the cells /5/. In all cases aeration is done by sparging air and/or oxygen into the culture /2, 3, 5/ or by using membrane aeration with a silicone tube /6/. The possibility of monitoring cell growth and behavior is one advantage of these systems. One great disadvantage is the clogging of the spin filter systems after about 1 - 2 weeks /2/. The other group of perfusion systems demonstrates the inhomogeneous type of high density cell culture like hollow fibre reactors /7/, dense cell reactors or reactors with immobilized cells /8/. In these systems the medium is circulated fast through the cell containing matrix. The advantages of these systems are very high cell densities up to $5 \cdot 10$ cells/ml and high product concentrations. The disadvantages are uncontrolled cells and high perfusion rates.

In this paper a new homogeneously stirred reactor with bubble free aeration and perfusion system is described. The core of this sy-

stem is a stirrer on which two hollow fibre membranes are mounted. The first, a hydrophobic membrane, is used for bubble free aeration described earlier /1/. It is connected to a gas supply system creating the necessary gas mixture depending on pH and DO. The second, a hydrophilic membrane, is used for supplying the culture with nutrients and for removing the spent medium and waste products. This membrane is connected on one side via a feeding pump to the medium reservoir and on the other side via a harvest pump to a harvest container. Feeding and harvesting pump are running alternatively. The advantage of this construction is the possibility to avoid clogging by back flushing the filtration system with fresh medium. The pore size of this membrane is 0.3 μm. Therefore, only very small particles can clog the pores. With the whole system several cell lines have been successfully cultivated. The system has been scaled up without any problems to a volume of 20 litre until now.

Material and Methods

Cell propagation system:
The cells are cultivated in a homogeneously stirred fermentor. The core of the system is a membrane stirrer. On a glass support in case of a 1 litre fermentor or on a teflon support in a 20 litre fermentor two separate hollow fibre membranes out of polypropylene (Accurel®, ENKA, Wuppertal) are mounted. The membranes have a specification shown in Table 1. The hydrophobic membrane used for bubble free aeration of the culture is connected via a sterile filter to a gas supply unit. This unit creates a gas mixture of carbon dioxide, oxygen, nitrogen and air depending on pH and DO of the culture. At the outlet of the aeration membrane a sterile trap is connected. With this aeration system it is possible to supply the culture at almost any time of the fermentation period with the optimal gas mixture. At the beginning of a fermentation process, when cell densities are low, the culture can be supplied with CO_2, N_2 and air to perform the optimal culture conditions. Later on, when cell densities are high, the culture can be additionally supplied with oxygen. In addition, the aeration membrane can be used to remove carbondioxide produced by the cells. Specific oxygen transfer rates in dependence of gaflow and stirrer speed obtained in a 23 litre cell culture fermentor with 2.3 m hydrophobic membrane per litre culture are shown in Fig. 1. The second

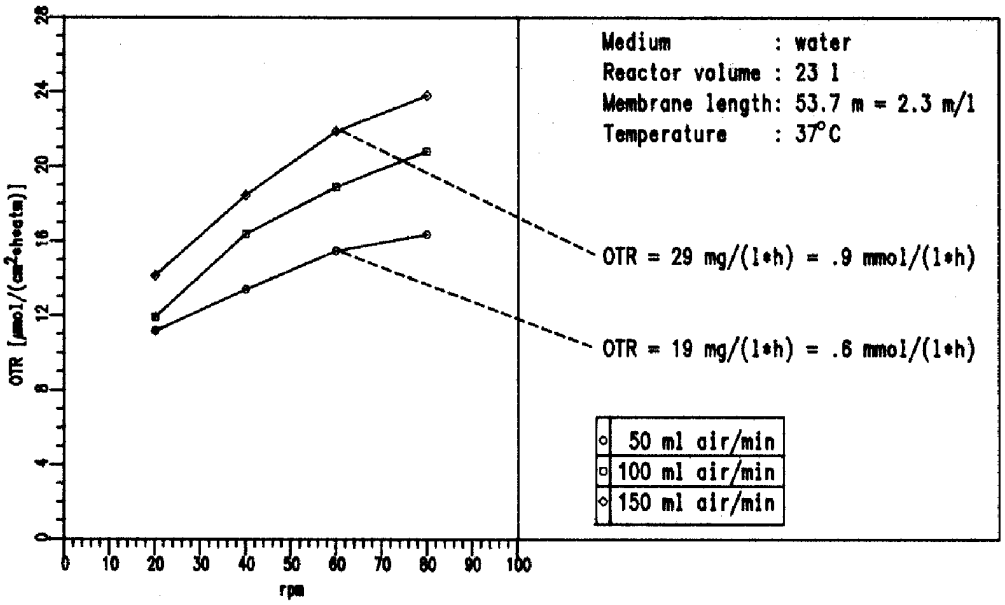

Fig.1. Oxygen transfer rates at a membrane stirrer for a 23 litre fermentor

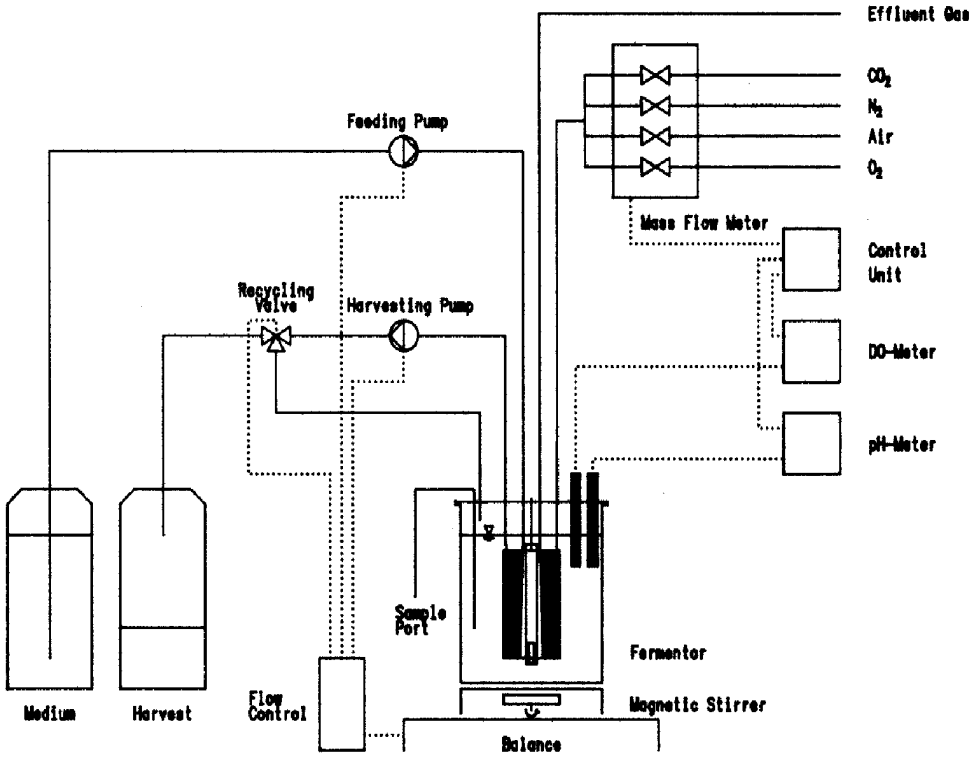

Fig.2. Scheme of the fermentor system with bubble free aeration and perfusion

membrane mounted on the support consists of the same material as shown in Table 1. Before mounting it is hydrophilized with ethanol for 1 hour and washed with pure water to remove the solvent. With this membrane it is possible to carry out continuous microfiltration. The whole system is schematically shown in Figure 2.

Perfusion mode:
The perfusion system is constructed as shown in Fig. 3. The pumps are connected at each side of the hollow fibre membrane. The liquid level of the fermentor is controlled by a level or weight control.
The perfusion process starts with filling the fermentor using the feeding pump. At that time the harvest pump stops and closes the outlet of the hydrophilic membrane. Therefore, the medium pumped by the feeding pump into the fermentor has to pass the membrane. When the maximum level is reached, the feeding pump is stopped and the harvest pump is started. In this case the feeding pump closes the inlet of the membrane. The medium removed from the fermentor by the harvest pump has to pass the membrane. When the minimum level is reached, the harvest pump is stopped and the feeding pump is started to fill the fermentor again. This backflushing procedure makes it possible to prevent an early clogging of the membrane. Each pumping cycle, feeding and harvesting, lasts about 15 to 20 minutes. The perfusion rate is controlled by setting the pump rates of the two pumps. With the recycling valve in the harvesting stream it is possible to recycle fresh medium which is in the membrane tube at the end of each feeding cycle.

Fermentation strategy:
The cells are seeded out in the fermentor at viable cell densities of about 2 to $4 \cdot 10^5$ cells/ml. In a batch mode the cells were cultivated to viable cell densities of about $1 \cdot 10^6$ cells/ml. At that state the perfusion mode is started with a small perfusion rate. Depending on cell density the perfusion rate has to be changed to higher values. For process guide values easily measurable components of the feeding medium or of the waste medium are possible like glucose, special amino acids, lactate or else. Which guide value is chosen depends on each cell line.

Cells and culture media:
With this cultivation system several different cell lines were

Table 1.
Membrane Data of the Accurel^{R} hollow fibre (ENKA, Wuppertal)

d_e = 2.6 mm, d_i = 1.8 mm		
Pore volume	:	75.0 %
Medium pore size	:	0.3 µm
Burst pressure	:	6.5 bar
Implosion pressure	:	1.5 bar
Specific surface, outside	:	81.7 cm^2/ m
Specific surface, inside	:	56.5 cm^2/ m
Specific volume, outside	:	5.3 ml / m
Specific volume, inside	:	2.5 ml / m

cultivated. One group of cells was antibody producing in suspension cultured cell lines such as mouse-mouse-hybridomas, rat-mouse-hybridomas and EBV-transformed human B-lymphocytes. The other group of cells was in suspension cultivated adherent cells such as Hela, BJAB and genetically manipulated BHK 21 cells for different purposes. The media used for these cultivations based on commercially available compositions like DMEM (Dulbellco's modified Eagle Medium), IMEM (Iscove's modified Eagle Medium), HAM F12 and RPMI 1640. They were often used serum free with some additional supplements or in the other cases with fetal calf serum up to 10 % (v/v).

Cell count:
Viable and dead cells were estimated microscopically using trypan blue exclusion.

Results and Discussion

Depending on cell line and cultivation purpose viable cell densities up to $2 \cdot 10^7$ cells/ml with perfusion rates of D = 1.2-1.5 d^{-1} could be obtained.

Fig. 3 shows a time course of a cultivation of EBV-transformed B-lymphocytes over a period of about 2 months in a 1.2 litre scale. For aeration a 2.4 m hydrophobic membrane was mounted on the stirrer support in addition to a 2 m hydrophilic membrane corresponding to a specific length of about 2 m hydrophobic membrane and 1.7 m hydrophilic membrane per litre culture, respectively. At the beginning the cells were cultivated in batch-mode to a cell density of about $1 \cdot 10^6$ cells/ml in a period of 5 days. The perfusion was started with an initial rate of D = 0.45 d^{-1} for one week. The cells reached viable densities of about $3 \cdot 10^6$ cells/ml. In the next 7 days the perfusion rate was set to D = 0.9 d^{-1}. In this case a viable cell density of about $7 \cdot 10^6$ cells/ml could be obtained. In order to measure cell specific utilisation rates of glucose and amino acids and production rates of waste products the perfusion rate was set down to D = 0.45 d^{-1} for a period of 2 weeks. In the last 3 weeks the perfusion rate was set to several values up to D = 1.3 d^{-1}. It could be shown that the viable cell density is strongly correlated with the perfusion rate. However,

at the last month of the fermentation period the concentrations of glucose and some amino acids in the fermetor were nearly zero. Over the complete fermentation period only one double membrane stirrer unit was used. In order to prevent an early clogging of the hydrophilic membrane, maximum flow rates of about 0.7 litre medium per meter membrane and day were performed in addition to the back flushing procedure.

In Fig. 4 a time course of a 3 week cultivation of EBV-transformed B-lymphocytes in a 1.2 litre scale is shown. The membrane length was the same as in Fig. 3. After a one day batch phase the perfusion was set with increasing cell density up to a maximum value of $D = 1.3\ d^{-1}$. At 3 times (at day 9, 12 and 14) cells were harvested for other purposes. Except the first 2 days the cells grew with constant growth rate. After this fermentation period of 3 weeks the filter unit was still at full flow efficiency.

Fig. 5 shows a time course of a 16 days cultivation period in a 23 litre cell culture fermentor with the same cell line used in the figures above. In this case 2.3 m hydrophobic and 0.9 m hydrophilic membrane per litre culture were built on to the support, respectively. The fermentation strategy was the same as described above. The cells were cultivated in batch mode to a viable cell density of about $1 \cdot 10^6$ cells/ml (day 4). At that time the perfusion is increased with increasing cell density. In this case a viable cell density of about $1 \cdot 10^7$ cells/ml could be obtained with a perfusion rate of $D = 0.95\ d^{-1}$ corresponding to 20 litre medium per day.

Summary

The results shown above demonstrate that the cultivation system described here is a valuable tool for mammalian cell culture. Bubble-free aeration with microporous moving membranes is an excellent possibility for culture aeration.
With cell free medium exchange through a hydrophilic membrane high viable cell densities of about $2 \cdot 10^7$ cells/ml can be obtained. The back flushing procedure is necessary to prolong membrane stability to more than 2 months. A clogged membrane can be cleaned by treating with pure water and biochemical or soft chemical reagents like pepsine/HCl, H_3PO_4, $NaHCO_3$. To our experience the membrane

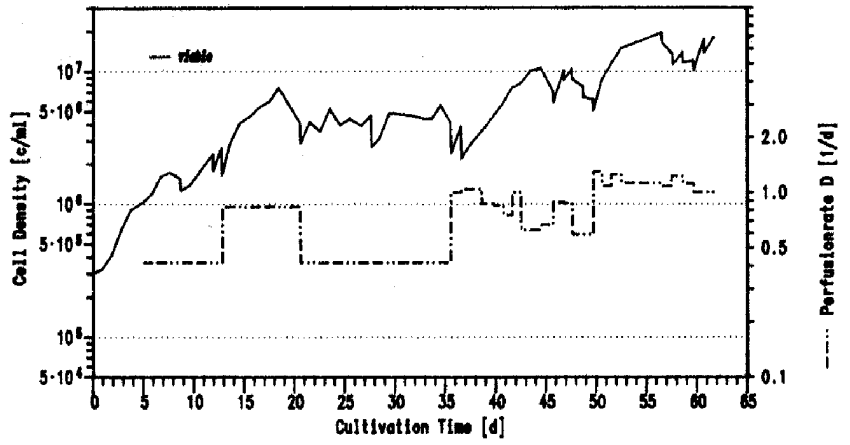

Fig.3. Continuous cultivation of EBV - transformed B-Lymphocytes in a 1.2 litre fermentor with 2.4 m hydrophobic membrane and 2 m hydrophilic membrane

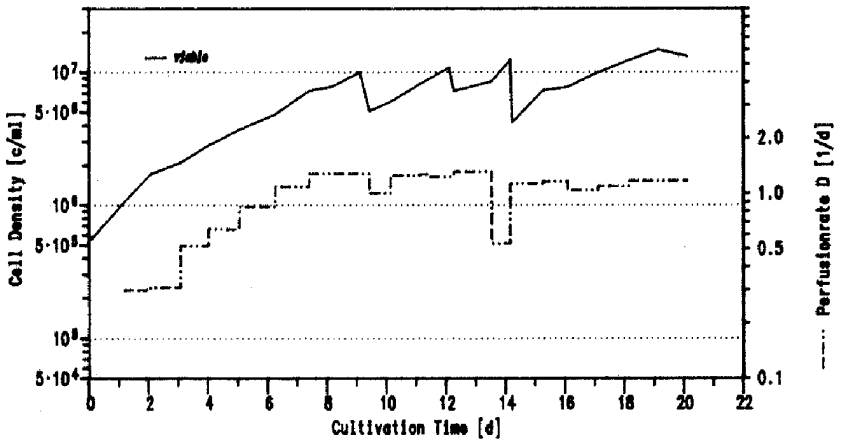

Fig.4. Continuous cultivation of EBV-transformed B - Lymphocytes in a 1.2 litre fermentor (same conditions as in Fig.3.)

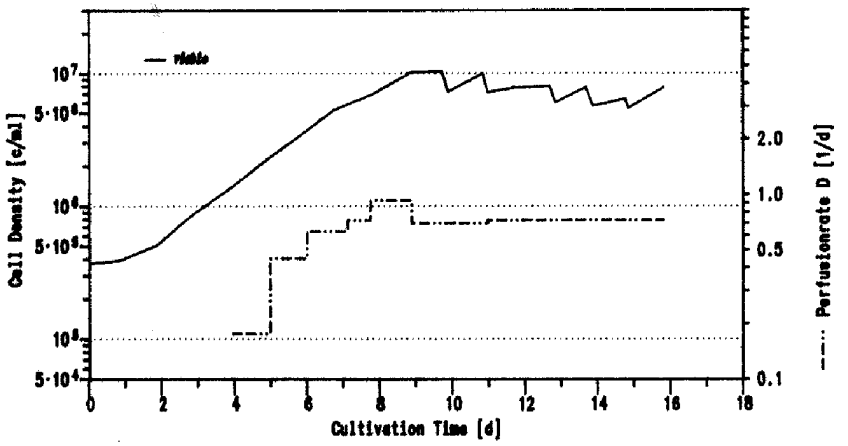

Fig.5. Continuous cultivation of EBV-transformed B - Lymphocytes in a 23 litre fermentor with 2.3 m hydrophobic membrane and 0.9 m hydrophilic membrane per litre, respectively.

should not filtrate more than one litre culture broth per meter membrane and day to guarantee a stable behavior for weeks.

References

/1/ Lehmann, J.; Piehl, G.W.; Schulz, R.
Develop. biol. Standard. 66, 227 (1987).

/2/ Reuveny, S.; Velez, D.; Miller, L.; Macmillan, J.D.
J. Immunol. Methods 86, 61 (1986).

/3/ Van Wezel, A.L.; van der Velden-de Groot, C.A.M.; de Haan, H.H.; van den Heuvel, N.; Schasfoort, R.
Develop. biol. Standard. 60, 229 (1985).

/4/ Himmelfarb, P.; Thayer, P.S.; Martin, H.E.
Science 164, 555 (1969).

/5/ Tolbert, W.R.; Feder, J. in: Annual Reports on Fermentation Processes, Vol. 6, eds. G.T. Tsao, M.C. Flicker and R.K. Finn (Academic Press, New York), p 35.

/6/ Kuhlmann, W. Develop. biol. Standard. 66, 263 (1987).

/7/ Schönherr, O.T.; van Gelder, P.T.J.A., van hees, P.J.; van Os, A.M.J.M.; Roelofs, H.W.M.
Develop. biol. Standard. 66, 211 (1987).

/8/ Littlefield, S.G.; Gilligan, K.J.; Jarvis, A.P.
Hybridoma 3, # 1, 75 (1984).

Discussion

S. REUVENY — Can you specify which amino acid was limiting?

BUNTEMEYER — Serine, tryptophan and methionine.

H. KATINGER — Can I confirm that you used an EBV transformed lymphocyte?

BUNTEMEYER — Yes that is correct

M. KAYLANPUR — How do you sterlise your membranes, and how many times can you re-use them?

BUNTEMEYER — By steam in the fermenter and they are used 5-10 times.