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The Super-Spinner: A low cost animal cell culture bioreactor for the CO_2 incubator

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Abstract

The production of small quantities of monoclonal antibodies and recombinant proteins was carried out using a new low cost production system, the Super Spinner. Into a 1 l standard Duran® flask a membrane stirrer equipped with a polypropylene hollow fiber membrane was installed to improve the oxygen supply by bubble-free aeration. The aeration was facilitated by using the CO_2 conditioned incubator gas, which was pumped through the membrane stirrer via a small membrane pump. The maximal oxygen transfer rate (OTR_{max}) of the Super Spinner was detected. For this purpose one spinner flask was equipped with an oxygen electrode. The OTR_{max} was measured by the dynamic method. The ratio of membrane length to culture volume was adapted corresponding to the oxygen uptake rate of the cells according to the desired cell density. A balanced nutrient supply resulted in an optimal formation and yield of products.

Introduction

Small quantities of monoclonal antibodies have been traditionally produced in ascites cultures *in vivo*. Following the new animal protection law this procedure is now prohibited in Germany. Therefore, there was a need to develop new *in vitro* cultivation systems, which are inexpensive, easy to handle and efficient. Conventional spinner systems can only partly fulfil this objective. In recent years several approaches have been tested and some systems have been established and are commercially available. The "Glass Mouse" (Haardt and Falkenberg, 1988) and the TecnoMouse (Tecnomara Integra Bioscience) are both high cell density systems. The cells are located in dialysis membrane compartments and supplied with air saturated culture medium. The advantages of such systems are the high cell concentration and high product accumulation. Disadvantages are the impossibility to observe the cells and the risk of product decomposition by enzymatic processes. A similar system is the Milstein/Pannell Production System (Pannell *et al.*, 1992) (distributed by Techne, England). The cells are also located in three dialysis membrane compartments, but there the cells are kept in suspension by a gently oscillating bubble within the dialysis tube, though estimation of the cell number is possible. Oxygenation is carried out by bubbling gas manually into the outer medium reservoir. Another system is the Biott spinner (Able K K, Japan), which is equipped with a coil of a porous

Teflon tube for aeration. This system allows the cell numbers to be counted during the cultivation and it is suitable for suspension as well as microcarrier cultures (Shirokaze *et al.*, 1993). This system is equipped with pH and oxygen electrodes. The values are controlled by a bioreactor controller. Therefore, this system is more expensive than spinner flasks.

This paper describes the development and investigations of an alternative batch system for suspension cultures with an internal membrane stirrer for aeration (Lehmann *et al.*, 1992; Heidemann *et al.*, 1993). It is a scale down version of the membrane aerated bioreactor described by Lehmann *et al.*, 1987.

Materials and methods

The Super Spinner

The so-called Super Spinner system consists of a 1 l Duran flask (Schott, FRG) equipped with a tumbling membrane stirrer moving a polypropylene hollow fiber (Microdyn, FRG) through the medium to improve the oxygen supply. This small device is placed into a CO_2 Incubator. The CO_2 conditioned incubator gas (5% CO_2, 95% air) was used for aeration. A small membrane pump (Whisper 500, Japan) was connected via a sterile filter with the membrane. To avoid bubble formation the head space of the Super Spinner flask was connected to the gas inlet tube, generating a head space pressure equivalent to the gas pressure in the membrane inlet gas stream. The degree of aeration can be seen

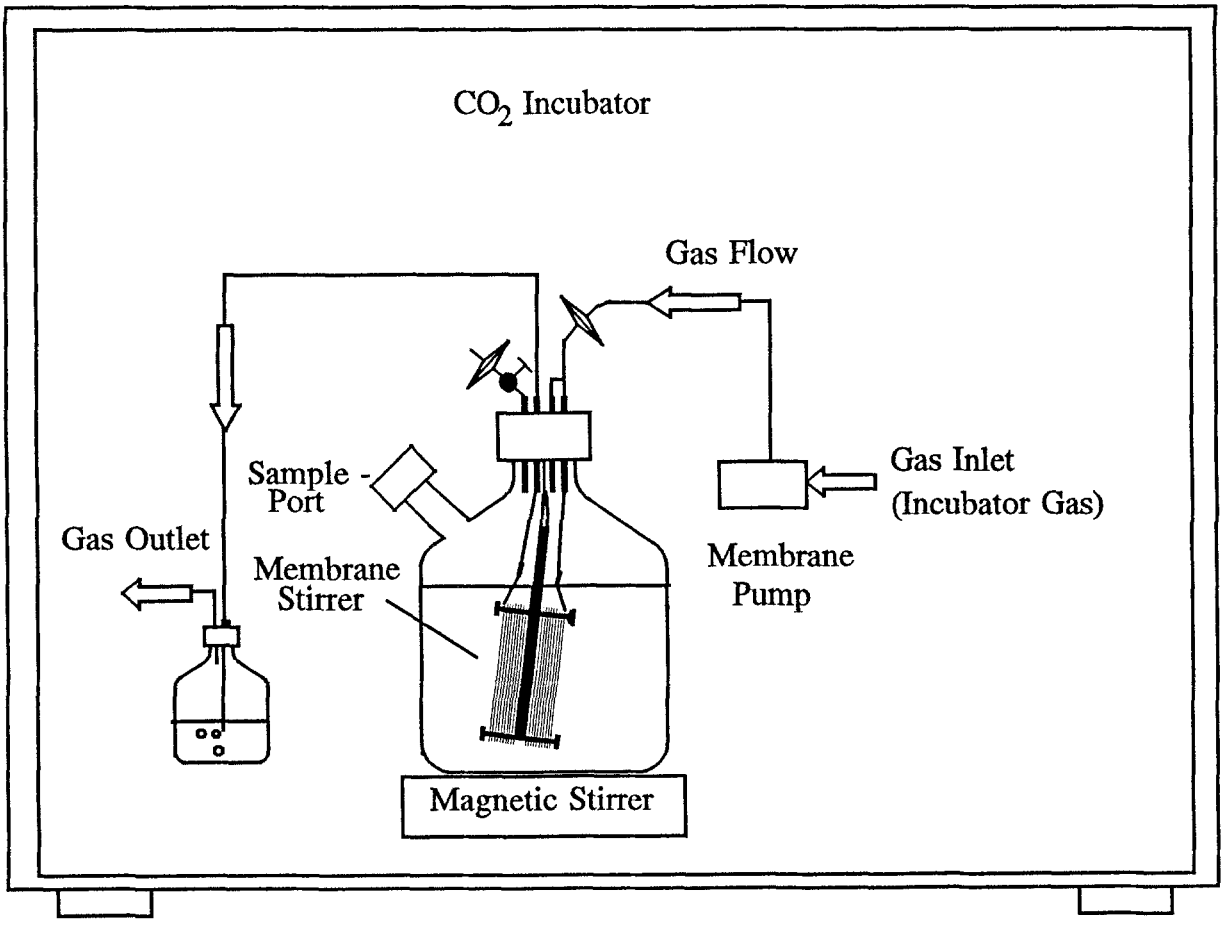

Fig. 1. Schematic diagram of the Super Spinner with membrane stirrer and membrane pump as used in a CO_2 incubator.

from the bubble frequency appearing in a flask containing pure water, connected to the outlet of the membrane tube. The system can be put on common magnetic spinner stirring stations. The working volume can be varied from 300 to 1000 ml. The Super Spinner is shown in Fig. 1. A legal protection for registered designs (No. G 92 15 153.1, B. Braun International, FRG) for the system is given.

Oxygen transfer rate of the super spinner

For the optimal use of the Super Spinner it was necessary to determine the maximal oxygen transfer rate (OTR_{max}). For that purpose once a spinner flask was equipped with an oxygen electrode and the OTR_{max} was estimated using the "dynamic method". The measurements were carried out in pure water. The oxygen electrode (Ingold, FRG) was connected to an amplifier and the data were recorded for later calculations.

Maximum OTR numbers of the Super Spinner aerated with air are given in Table 1a and 1b.

Three different membrane lengths (1, 1.5 and 2 meter) were used at a volume of either 700 ml or 900 ml. The membrane pump was set to flow rates of 28 l/h (position low, data in Table 1a) or 55 l/h (position high, data in Table 1b), respectively. The temperature was 20°C and the stirring speed was 35 rpm. The OTR measurements were carried out using the following formula:

$$OTR = k_L \cdot a \cdot (c^* - c_L)$$

where

OTR = oxygen transfer rate (mg/l/h)

Table 1a. Oxygen transfer rates (OTR) and $k_L \cdot a$ values of the Super Spinner. Gas flow: 28 l/h

Membrane length (m)	700 ml volume	
	$k_L \cdot a$ (h^{-1})	OTR_{max}* (mg/l·h)
1	1.8	15.8
1.5	2.4	21.0
2	3.3	29.5

*At maximum driving force (c_L = zero) and at 20°C.

$k_L \cdot a$ = volumetric mass transfer coefficient (h^{-1})

c^* = maximum oxygen concentration in the liquid (mg/l)

c_L = momentary oxygen concentration in the liquid (mg/l)

A linear correlation between membrane length and maximum oxygen transfer rate was indicated, where as increasing the pump speed to maximum power resulted only in an increase of about 4%. The $k_L \cdot a$ value of 0.4 d^{-1} for head space aeration proves that high cell densities cannot be obtained if volumes of 700 ml or more are used. The results correspond with data obtained in bioreactor systems (Lehmann *et al.*, 1988).

Additional experiments showed that the polypropylene membrane can be used several times without decrease in oxygen transfer rate after sterilization.

Cell lines and medium

Two different recombinant CHO cell lines and one hybridoma cell line were used in this investigation.

— mouse-mouse hybridoma BSC 24 (cell lines

Table 1b. Oxygen transfer rates (OTR) and $k_L \cdot a$ values of the Super Spinner. Gas flow: 55 l/h

Membrane length (m)	700 ml volume		900 ml volume	
	$k_L \cdot a$ (h^{-1})	OTR_{max}* (mg/l·h)	$k_L \cdot a$ (h^{-1})	OTR_{max}* (mg/l·h)
1	1.9	16.4	1.3	11.5
1.5	2.4	21.5	1.9	16.6
2	3.5	30.6	2.8	24.8
Head space aeration	0.4	3.8	—	—

*At maximum driving force (c_L = zero) and at 20°C.

supplied from Kabi Pharmacia AB, Stockholm, Sweden), producing a monoclonal antibody of IgG_1 type;
- recombinant CHO cell line TF 70 R (cell lines supplied by Kabi Pharmacia AB, Stockholm, Sweden), producing human active tissue plasminogen activator (t-PA);
- recombinant CHO cell line SS3 A2 (cell line supplied by Behring Werke AG, Marburg, FRG) (Zettlmeissl *et al.*, 1887), producing human active antithrombin III (AT III).

For all cell lines as described previously a serum free medium (DMEM: Ham's F12) containing insulin and transferrin was used (Jäger *et al.*, 1988). For some cultivations albumin and ExCyte I (Lipoprotein, Bayer Diagnostics, FRG) were also added.

The medium was supplemented with additional amino acids and glucose to avoid limitations during early periods of the cultivation processes. This supply of nutrients based on the OTR resulted in a good formation and yield of the products (Büntemeyer *et al.*, 1991).

Analytical methods

The cell number was counted microscopically by trypan blue exclusion, total CHO cell number by crystal violet staining. Glucose and lactate were analyzed using the YSI 2700 analyzer (Yellow Springs Instruments, USA). The free amino acids for the medium optimization were analyzed with an automated reversed phase HPLC system DS 450 (Kontron GmbH, FRG) with precolumn derivatization using the OPA method (Büntemeyer, 1988). Antibody and recombinant protein concentration in

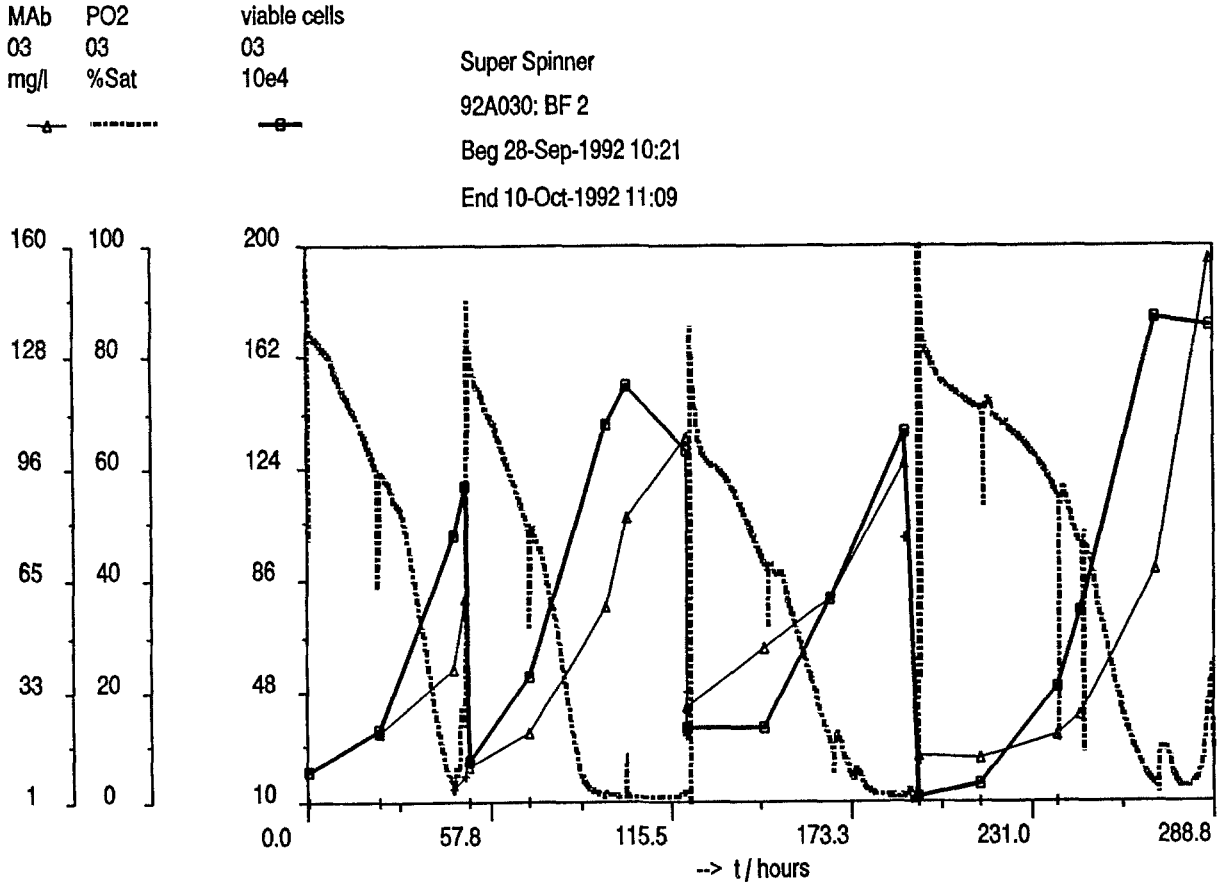

Fig. 2. Original history plot from the Micro MFCS. Repeated batch cultivation with the hybridoma BSC 24 is shown. The first three batches were performed with 1.5 m, last batch with 2 m membrane. Cell and antibody concentration (MAb) reached the maximum in the last batch.

the supernatant were analyzed using a kinetic sandwich ELISA method and the EL 311 autoreader (Tecnomara GmbH, FRG).

Results and discussion

Mammalian cell cultivations in the Super Spinner System

This section describes the cultivation experiments with this new system. The aim was to demonstrate that the Super Spinner is a suitable tool for the cultivation of several suspended mammalian cell lines.

Experiments with the mouse-mouse hybridoma BSC 24

This cell line was cultivated in a series of two repeated batch cultures. In the first set of batch cultures (Fig. 2) the optimal ratio membrane length to culture volume was determined under sterile conditions. To perform this the oxygen concentration was measured on-line and recorded with the Micro Multi Fermenter Control System (Micro MFCS, B. Braun International, FRG). The oxygen electrode was connected to a Digital Control Unit (DCU, B. Braun International, FRG) which was controlled by the Micro MFCS. In the first three batches the membrane length was 1.5 m and the culture volume 700 ml. In the first batch the viable cell number reached 1.2×10^6 cells/ml. Some amino acids were totally consumed, especially glutamine (data not shown). After the second batch the medium was supplemented with several amino acids and glucose. The viable cell number increased to 1.5×10^6 cells/ml. The oxygen concentration decreased to zero at this time, so the cell growth was limited. For the last batch the membrane length was increased up to two meters. Therefore, the cells for the inoculum were transferred into another flask. Then the Super Spinner was equipped with the new membrane, autoclaved and inoculated with the stored cells again. Figure 3 shows the cultivation of this final (fourth) batch in detail. Total cell concentration reached 2×10^6

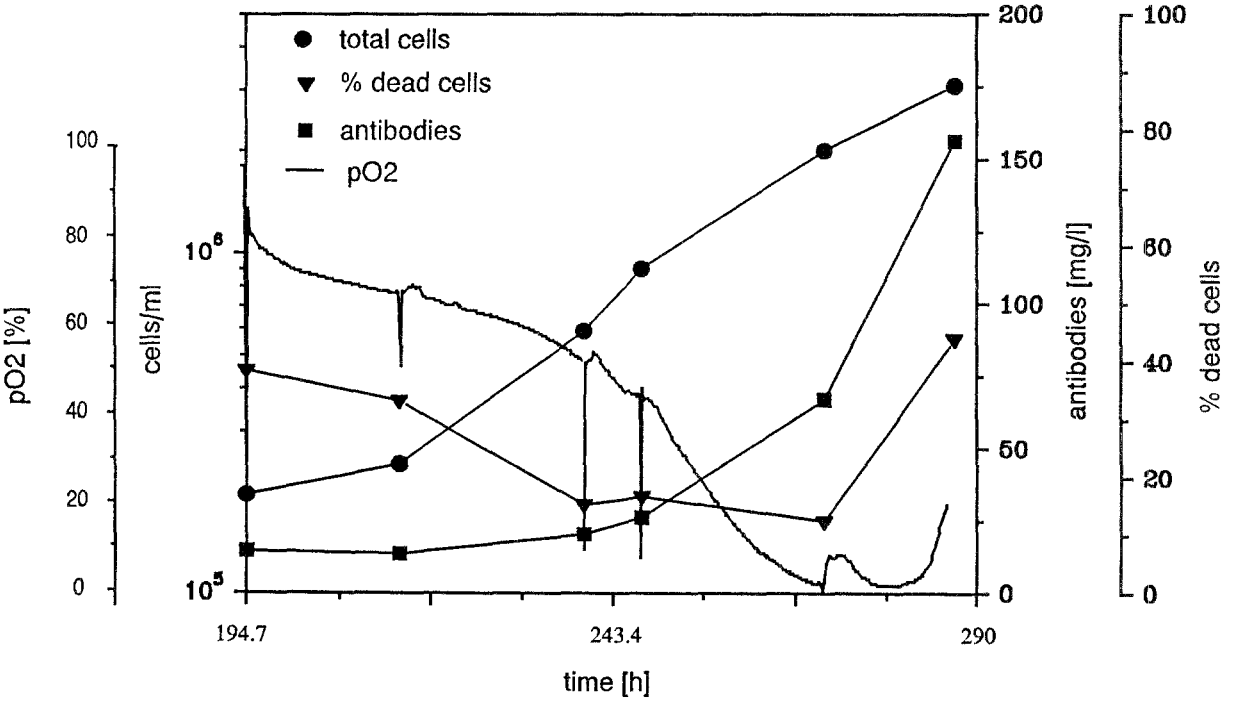

Fig. 3. Detailed plot of the last batch with the hybridoma BSC 24 from Fig. 2. Antibody concentration, total cells, % dead cells and dissolved oxygen concentration (pO_2, in % air saturation) are shown. PO_2 level decreased during the cultivation, and increased at the end after 3.5 days (due to amino acid depletion).

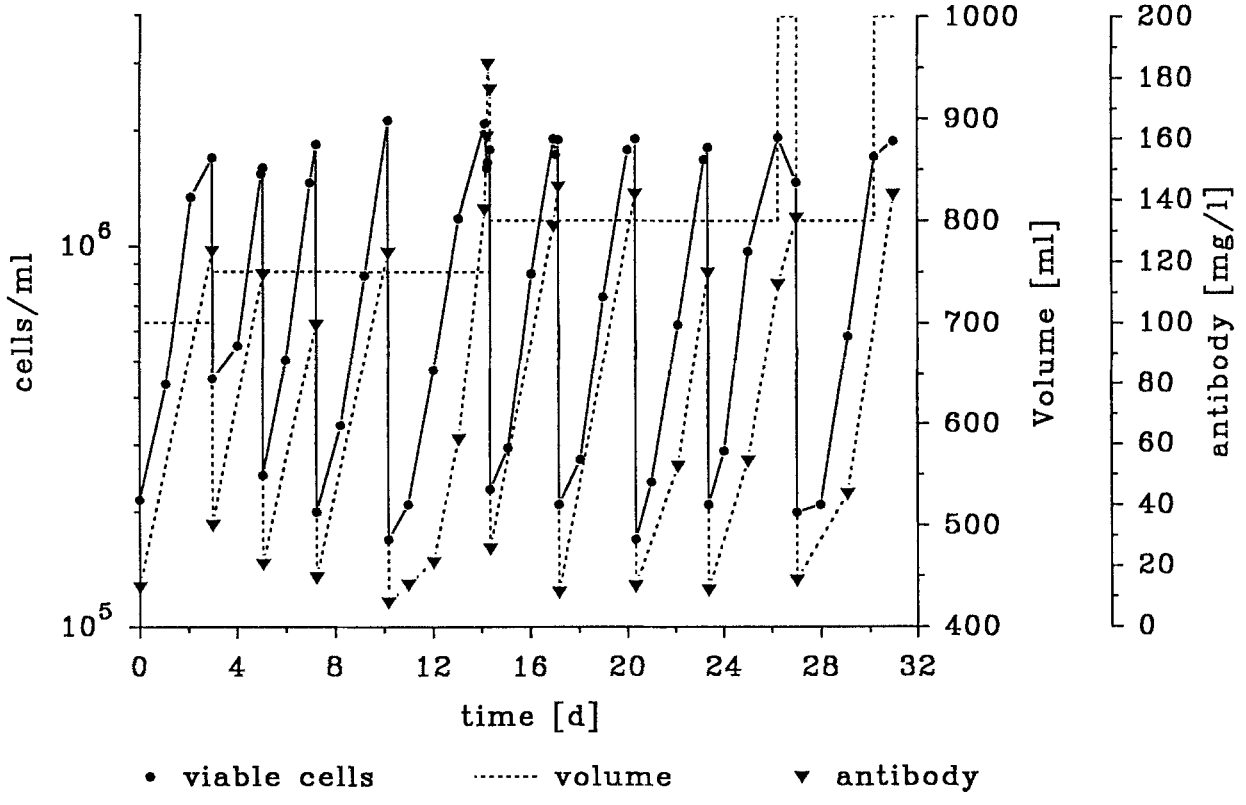

Fig. 4. Production of monoclonal antibodies using the Super Spinner in repeated batch mode with the hybridoma BSC 24. Viable cell concentration, antibody concentration and culture volume are shown.

cells/ml, final antibody concentration was 160 mg/l (specific productivity: ~50 μg/(10^6 cells·d)). Dissolved oxygen concentration decreased to zero but increased due to amino acid depletion at the end of the cultivation. Several amino acids were totally exhausted when the maximum OTR was reached, resulting in a good product yield per unit medium consumed.

The time course from the second series of repeated batch cultivations is shown in Fig. 4. During a cultivation period of 32 days altogether 970 mg antibody (about 100 mg per batch) was produced. Every batch was terminated by harvesting ca. 80% of the culture broth. The remaining part was used as inoculum for the following batch. The series of cultivations started with a working volume of 700 ml in the first batch, increased up to 750 ml for the next four batches and 800 ml for the last five batches. Additionally, the volume was increased up to 1000 ml in the last two batches one day before harvesting. This procedure resulted in a high accumulation of monoclonal antibodies (~140 mg per batch) during the stationary growth phase, which is typical for the antibody production with hybridomas described previously (Merten *et al.*, 1985; Musielski *et al.*, 1993).

The process dynamics of monoclonal antibody production may follow growth-dissociated pattern (Dalili *et al.*, 1990; Bushell *et al.*, 1993). The maximum specific antibody production rate was found during exponential phase and correlated with the highest specific growth rate (Merten *et al.*, 1991; Riese *et al.*, 1993).

Figure 5 shows a SDS PAGE of the product obtained from the cultivation. No decomposition of the antibody was observed. After the fourth batch the medium did not contain human serum albumin (HSA) and therefore it was washed out in the

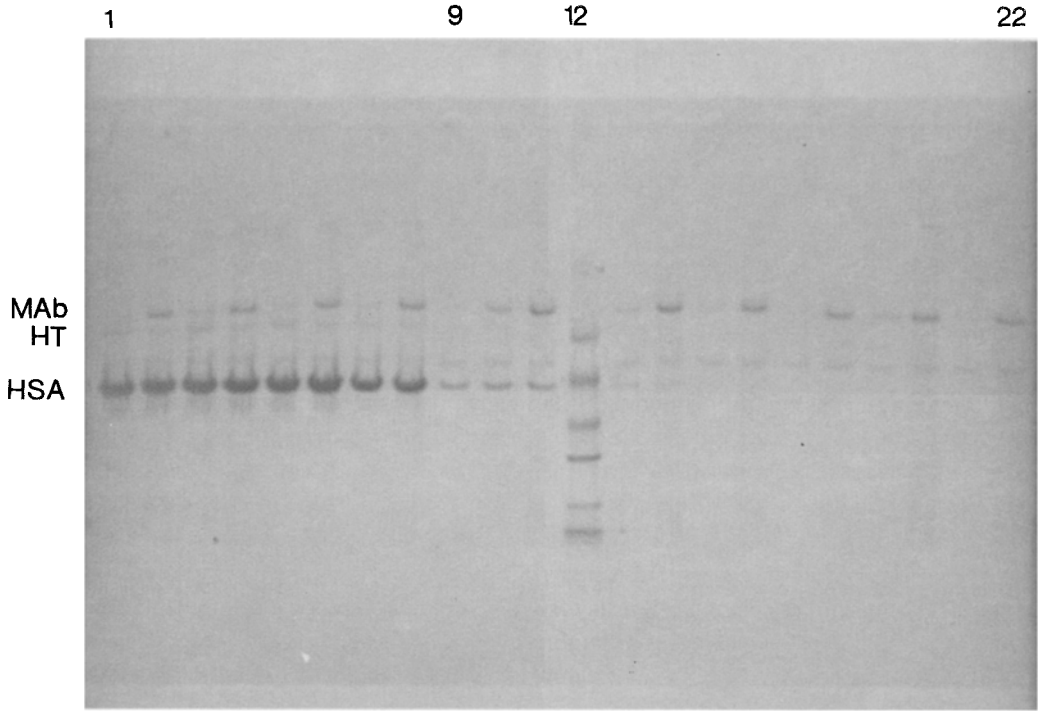

Fig. 5. Unreduced SDS-PAGE of cell culture supernatant of the repeated batch cultivation from Fig. 4. Initial and last samples (before harvesting) of every batch were used (lane 1 — lane 11 and lane 13 — lane 22, lane 12 marker). Human serum albumin (HSA) was only used in the first three batches (lane 1 — lane 8), it was washed out in the following batches. Antibody and human transferrin (HT) patterns were constant during the process, no decomposition was observed.

following batches as can be seen from lane nine on in Fig. 5. Growth and antibody productivity was not affected.

Experiments with the recombinant CHO cell line TF 70 R

The recombinant CHO cell line TF 70 R producing human t-PA was cultivated in a repeated batch mode (Fig. 6). This cultivation was performed to compare head space aeration and membrane aeration. Therefore, the first batch was carried out with head space aeration only and the second batch with membrane and head space aeration. In the first batch viable cell concentration reached 5.5×10^5 cells/ml using a culture volume of 780 ml. In the second batch the starting culture volume was 800 ml, increased to 900 ml after 7 days and finally to 1000 ml after 10 days (fed batch procedure). At this volume the cell density was nearly constantly at 2×10^6 viable cells/ml. The t-PA concentration reached 3.5 mg/l (total yield 3.5 mg) compared to 2 mg/l (total yield 1.6 mg) in the head space aerated batch. This experiment shows the positive effect of membrane aeration. It was found possible to reach high cell densities at maximum volume in this spinner flask.

Experiments with the recombinant CHO cell line SS3 A2

The recombinant CHO cell line SS3 A2, producing active human AT III, was cultivated. A comparison between head space (dotted lines) and membrane aeration (solid lines) was carried out. The time course is shown in Fig. 7. Employing the membrane stirrer resulted in a four-times higher viable cell density (2×10^6 cells/ml to 5×10^5 cells/ml) at the same culture volume of 650 ml. The product yield increased from 3.9 to 11.7 mg.

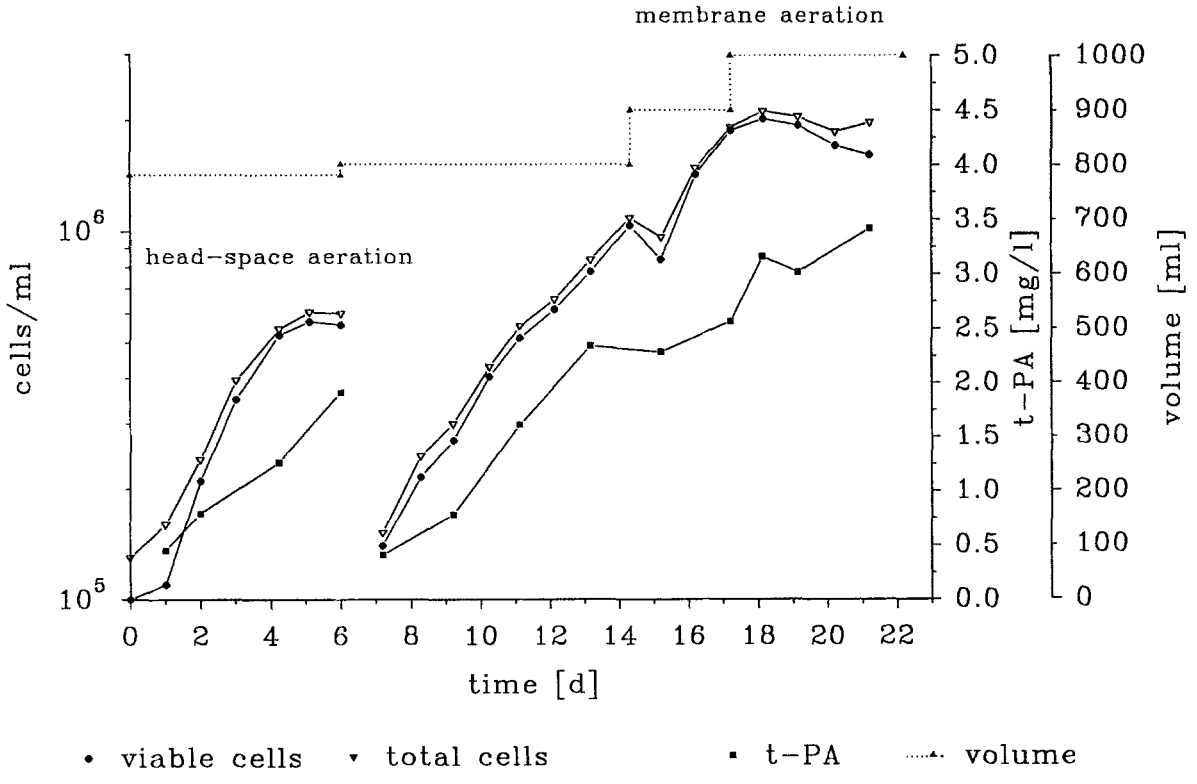

Fig. 6. Repeated batch fermentation with the recombinant CHO cell line TF 70 R. First batch was performed with head space aeration, second fed batch with normal membrane aeration, possible viable cell number increased 4 times, product yield ~2.5 times compared to head space aeration.

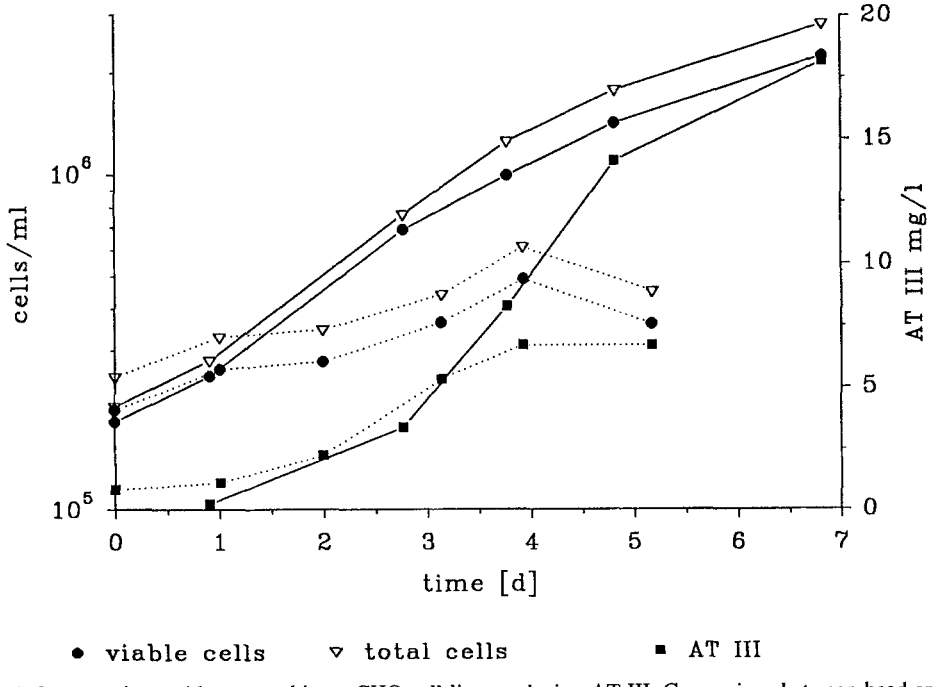

Fig. 7. Batch fermentations with a recombinant CHO cell line producing AT III. Comparison between head space (dotted lines) and membrane aeration is shown, cell number and AT III yield increased up to 4 times with membrane aeration.

Conclusion

The Super Spinner is a new small scale, inexpensive cultivation system for suspended mammalian cells constructed for the use in a CO_2 incubator, and for easy handling. By using the CO_2 conditioned incubator gas the pH value can be kept nearly constant over the whole cultivation time. Optimizing the nutrient concentrations in the culture medium and using the correct membrane length according to the OUR of the cells resulted in a better yield of products. Two meters of membrane are necessary for cells with high metabolic rates such as hybridomas. For CHO cells membrane lengths between 1 and 2~m are possible. The maximum volume of the spinner flask is one liter. It is a suitable tool for different cell lines and the initial experiments show also the possible effect for microcarrier cultures.

If only small quantities of monoclonal antibodies are required, this system offers a very good alternative to ascites cultures.

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