Re-use of spent cell culture medium in pilot scale and rapid preparative purification with membrane chromatography

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Abstract

Based on experiments in bench scale, a recycling of spent cell culture medium was performed in a 100-l pilot scale bioreactor. The cell cultivation has been done as a repeated batch procedure after the initial batch in the following four repeated batches spent medium from the previous batch was partially re-used. After microfiltration and ultrafiltration a part of the filtrate was mixed with a concentrate of amino acids and glucose, sterile filtered and subsequently filled back into the bioreactor. Up to 65% of the harvested cell- and product-free spent medium was re-used in each repeated batch. This procedure results in a saving of pure and waste water volume and saving of supplemented proteins as transferrin, insulin and lipoproteins and, therefore, also in a reduction of the production costs.

A strongly acidic membrane ion exchanger was evaluated for the ability to purify the monoclonal antibodies from the pilot scale cultivation. Within minutes, gram quantities of product could be purified in a high flux system, especially developed for this purpose, achieving purities of 80%. The capacity of the acidic membrane ion exchanger was found in former investigations to be 1 mg cm^{-2} with recoveries up to 96%. Final purification was carried out by gel column filtration.

Key words: Monoclonal antibody; Economy; Large scale; Medium; Membrane ion exchanger; Re-use; Waste water minimizing

1. Introduction

During the first period of cell culture used for industrial processes attempts have already been made to minimize the costs for medium. Mizrahi and Avihoo (1977) tried to re-use serum containing spent cell culture medium after supplementation of the amino acids consumed. They re-used 100% of the spent medium, but the medium could only be recycled once because cell growth was negatively influenced. Therefore, this procedure was not introduced into industrial production processes. Interpreting the growth inhibition reported by Mizrahi and Avihoo caused by an accumulation of toxic substances, Kempken et al. (1991) proposed a partial medium recycling; this system was called, medium cycling bioreactor

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(MCB). In a 2-1 scale reactor with serum-free medium, it could be shown that it is possible to continue to re-use the spent medium as long as the percentage of re-used medium was less than 75% of the total volume per recycling step (Munzert, 1992).

In cell culture processes the re-use of medium allows the production costs to drop because the volume of medium is less than for conventional processes. To avoid inhibitory effects caused by an accumulation of toxic metabolites in the spent medium the re-used part of the medium was diluted with fresh medium.

This strategy is schematically depicted in Fig. 1. The liquid stream out of the bioreactor was substituted by an equal volumetric stream consisting of a supplemented medium concentrate. Using this strategy, possibly accumulated inhibitors may be diluted down to non harmful concentrations. In contrast to small-scale, where continuous processes are very common, for the large-scale experiments described in this paper a repeated batch process in 110-l working volume has been used. The flow scheme of the apparatus employed for this process is given in Fig. 2.

Cells were separated by a semicontinuous centrifuge which was linked to the bioreactor in a way such that it was possible to disconnect it from the bioreactor for cell harvest without interrup-
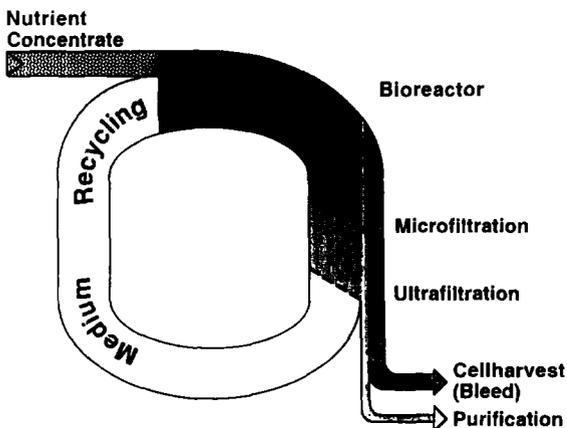
Medium Recycling Process

Fig. 1 Liquid flow scheme of the recycling process. The output streams for cell harvest and purification are substituted by a fresh medium nutrient concentrate.
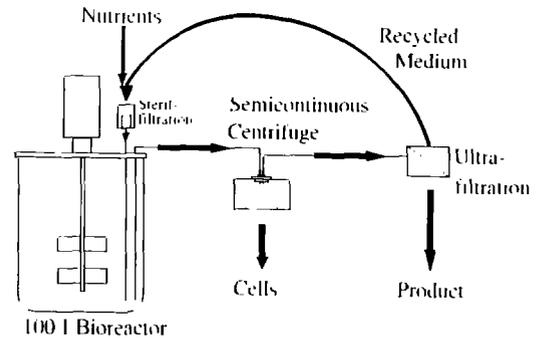

Fig. 2. Flow scheme of the experimental setup. The cells were separated in a semicontinuous centrifuge. The product was separated from the cell free medium by ultrafiltration. The UF permeate was supplemented with nutrients. This volume was then sterilfiltered directly back into the bioreactor.

tion of the cultivation process. The product, a monoclonal antibody (mAb), was separated from the cell-free supernatant stream of the centrifuge by crossflow ultrafiltration. The filtrate of the UF was recycled batchwise.

For the purification and preparation of proteins, peptides and enzymes, ion exchange is a common technique. However, using classical soft gels or porous particle media as a matrix results in a limitation arising from the restriction of mass transport in these diffusion-dominated systems. A different approach is the attachment of functional groups to the inner surfaces of synthetic microporous membranes. The main advantage of using membranes as a matrix is the fact that the mass transport is convective and, therefore, avoids the aforementioned limitation of classical matrices. Other interesting features in addition to the high flux rate include: high adsorption rates and binding velocities; no problems arising from channeling, bed shift or bed collapse; the ease of scale up and the comfort of using a prepacked stabilized matrix.

2. Materials and methods

2.1. Process procedure

After inoculating and filling the bioreactor, the first batch was performed. When cells reached

stationary phase the batch was terminated by pumping the reaction mixture out of the bioreactor. A small amount was left in the bioreactor as inoculum for the following repeated batch. The spent medium was passed through a centrifuge for cell separation and the supernatant was given to a crossflow ultrafiltration system, where the product was removed. Then the UF filtrate was analyzed for its amino acid and glucose contents. The lacking amounts of amino acids and glucose were calculated and added as concentrate which was finally mixed with the UF filtrate. For the preparation of the concentrate, amino acids and glucose were dissolved in fresh medium. The complete volume was sterile filtered and fed back into the bioreactor. During the subsequent cell growth period the centrifuge was disconnected, cells were harvested from the rotor, which was then cleaned, autoclaved and relinked to the system for the cell harvest within the next repeated batch.

At the beginning of the experiment the ultrafiltration unit was initially steam sterilized inline. It was not cleaned or sterilized between the recycling steps.

2.2. Materials

The bioreactor was a BIOSTAT 100 l from B. Braun (Biotech International, Melsungen, Germany). This bioreactor was equipped with a fixed silicone tube for bubble-free aeration and a marine-type impeller for mixing. During cultivation, temperature was set at 37°C, pH to 7.10 and the stirrer speed to 30 rpm. The dissolved oxygen concentration was controlled at 40% air saturation.

Cell separation was performed with a Biofuge 17 RS with an autoclavable titan rotor 8575 (both Heraeus, Osterode/Harz, Germany). The crossflow system for ultrafiltration was a Sartocon II system (Sartorius AG, Göttingen, Germany) equipped with a single Ultrasart module (filter material: Polysulfon, filtration area: 0.7 m², cut off: 100 kDa). Large media volumes were mixed in a stirrer-equipped stainless steal buffer tank (B. Braun Biotech International, Melsungen, Germany).

The cell line used in this experiment was a mouse-mouse hybridoma secreting a monoclonal antibody (mAb) type IgG_1, kindly provided by KabiPharmacia (Sweden). The medium was sterile filtered with a filtration system consisting of two filters in line with 0.45 μm and 0.2 μm pore size, respectively (Sartorius AG, Göttingen, Germany). This system was sterilized inline by steam. The filter integrity was checked by a forward flow test using the Sartocheck II device (Sartorius AG, Göttingen, Germany) before and after each filtration step.

Two samples per day were taken from the bioreactor and analyzed as follows: Living and dead cell numbers were microscopically analyzed after Trypan blue exclusion; the free amino acids in the samples were analyzed with HPLC (System 400, Kontron, Eching, Germany) using the OPA method (Büntemeyer, 1988); antibody concentration was analyzed using a kinetic sandwich ELISA. For fast antibody quantification a new analyzing system based on an affinity column (Abicap, Abion, Benediktbeuern, Germany) has also been used.

To separate the cells from the reaction mixture it was passed through the centrifuge with a flow rate of 300 ml min^{-1}. In the first recycling step, the cell separation was performed at a rotor speed of 6500 rpm (3543 $\times g$). The resulting clearance rate was 97%. In the following steps the rotor speed was set to 9000 rpm (6792 $\times g$) but no increase of clearance had been observed.

The purification of the antibodies was performed with Sartobind membrane ion exchanger prototypes, which were kindly supplied by Sartorius AG (Göttingen, Germany). The strongly acidic membrane ion exchanger (MIEX) with a pore size of 0.45 μm and a membrane thickness of 170–190 μm was folded into the module. The area of the module was 1400 cm². The membrane is autoclavable and stable over a range of pH 2–13. The maximum operation pressure was 4 bar.

In order to bind proteins to the ion exchanger, the conductivity of the supernatant had to be reduced from 13 mS cm^{-1} (normal cell culture medium) to 1.3–1.8 mS cm^{-1} (Fig. 3). In addition, the supernatant was first concentrated by
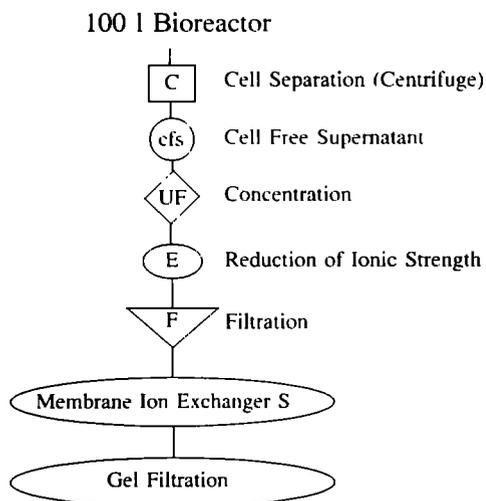
100 l Bioreactor


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        |
      [ C ]      Cell Separation (Centrifuge)
        |
      ( cfs )    Cell Free Supernatant
        |
      <UF>       Concentration
        |
      ( E )      Reduction of Ionic Strength
        |
      \ F /      Filtration
        |
   ( Membrane Ion Exchanger S )
        |
   (   Gel Filtration   )
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Fig. 3. Purification scheme of a batch of the 100-l cultivation. After cell separation the supernatant was concentrated by ultrafiltration. The reduction of ionic strength was performed by electrodialysis. After a filtration step the supernatant was purified by membrane chromatography. Final purification was carried out by gel filtration.

ultrafiltration. To achieve this reduction in conductivity, an electrodialysis system (StanTech GmbH, Geesthacht, Germany) was used. After adjusting the pH to 5.8 and a filtration step (0.2 μm), the supernatant was passed through the S-type membrane ion exchanger using the high flux system (HFS; Fig. 4). The product was eluted from the membrane by a shift in salt concentration.

For the acidic ion exchanger the following buffers were used: buffer A: 10 mM Na-phosphate + 10 mM NaCl pH 5.8; buffer B: 10 mM Na-phosphate + 250 mM NaCl pH 5.8.

For cleaning and regeneration 0.2 M NaOH (60° C, 20 min) was used. The membranes could be cleaned without loss of binding capacity and were regenerated by washing with buffer A.

Final purification was carried out using a preparative gel filtration column (Superdex 200 PG, Pharmacia Biosystems, Freiburg, Germany) with 600 ml gel volume. A 20 mM phosphate buffer containing 150 mM NaCl was used. The flow rate was set to 6 ml min^{-1}.
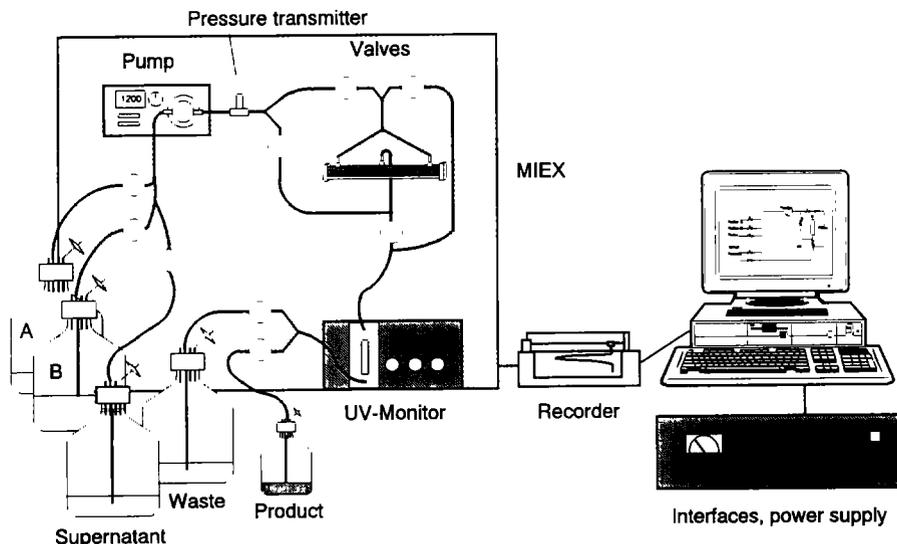

Fig. 4. High flux system consisting of a gear wheel pump, a UV monitor, a pressure transmitter and special valves. A personal computer was equipped with interfaces for A, D, D, A and TTL signal transfer. Specially developed software offers the opportunity to control the valves and to monitor the UV and pressure signals, thus enabling complete automatization. The system was developed for optimal use of the preparative membrane ion exchanger units and allows flow rates up to 1200 ml min^{-1} and a maximum pressure of 1.2 bar.

2.3. Medium preparation

The cell line was grown in a serum-free basal medium based on a 1:1 mixture of DMEM/Ham's F 12 supplemented with 50 μM ethanolamine, 2 mM sodium pyruvate and the following proteins: 10 mg l^{-1} bovine insulin (Sigma, Deisenhofen, Germany) 10 mg l^{-1} human transferrin (Behringwerke AG. Marburg, Germany) Fe-saturated, 0.5 g l^{-1} bovine serum albumin (Serva, Heidelberg, Germany) complexed with 2 mg oleic acid (Serva) and 1.0 ml l^{-1} Pentex Excyte I (Bayer Diagnostics, München, Germany; Büntemeyer et al., 1991). To avoid nutrient limitation at high cell densities for the initial batch this medium was additionally supplemented with several amino acids and 11 mM glucose (Table 1).

At the end of every repeated batch, a culture medium sample was taken and analyzed for amino acid and glucose concentrations. The results were used for the calculation of the amounts of amino acids and glucose to be added to the fresh medium to substitute these consumed nutrients. After adding this amount at the beginning of the following repeated batch, the concentrations in the reactor were again nearly the same as at the beginning of the previous one. In contrast to this procedure, the amount of proteins to be added to every repeated step was not calculated from its consumption. It was just added into the fresh medium concentrate at concentrations according to the figures mentioned above.

3. Results

The experiment was divided in an initial batch and four repeated batch cultivations. After the first batch, the four repeated batches were performed with recycled medium, they are called recycling processes. Each recycling process was started with an inoculum taken from the previous batch. The added volume consisted of two parts, a part of fresh medium concentrate and a part of recycled medium. For the calculation of the percentage of the recycled medium, summarized in Table 2, the recycled medium was regarded as the sum of the UF filtrate volume and the remaining medium from the previous batch.

The inoculum for the first batch culture was produced with a 2-l perfusion culture. The starting volume in the 100 l reactor was 35 l. After 1 d, the bioreactor was filled up to 55 l and after 2 d to 110 l. The cell density was then 3.2×10^5 viable cells per ml. The whole time course of the overall process is depicted in Fig. 5.

The initial batch was finished after 5.2 d of cultivation and reached a cell number of 2.6×10^6 viable cells per ml and runs at 112 l constant working volume. The first repeated batch was performed with 63% of spent medium. The viable cell number decreased at 1.3×10^6 viable cells per ml, not caused by a limitation of amino acids or glucose. As can been seen from Fig. 6, the supply of amino acids was sufficient at this time of the cultivation. This batch was finished after a

Table 1
Amino acid and glucose concentrations in the basal medium (DMEM/Ham's F12), the supplements and the optimized medium for high cell density cultures

Substrate	Basal medium (μM)	Amount of added supplements (μM)	Optimized medium for high cell densities (μM)
His	150.2	143	293.2
Gln	2469.6	6566	9035.6
Gly	249.7	–	249.7
Arg	697.7	332	1029.7
Tyr	313.7	220.7	534.4
Trp	44.2	146.7	190.9
Met	115.5	268.1	383.6
Val	449.3	511.9	961.2
Phe	214.7	90.8	305.5
Ile	414.4	457.3	871.7
Leu	449.4	457.3	906.7
Lys	497.4	246.3	743.7
Asn	49.96	–	49.96
Ala	50.5	–	50.5
Asp	50.3	50.3	100.6
Cys–Cys	203.1	208	411.1
Glu	49.96	–	49.96
Pro	150.0	–	150.0
Ser	249.8	–	249.8
Thr	448.9	419.8	868.7
Glucose	18149.3	11098.8	29248.1

Table 2
Composition of the reactor working volume

Culture mode	Inoculum (l)	Recycled UF filtrate (l)	Fresh medium (l)	Reactor working volume (l)	Recycled spent medium (%)
1 batch	55	–	55	110	–
1 recycling	16	55	41	112	63
2 recycling	29	42	38	109	65
3 recycling	31	44	41	116	65
4 recycling	26	39	51	116	56

Table 3
Apparent specific growth rates and specific antibody production rates for the initial batch and the recycling processes

Culture mode	Apparent spec. growth rate μ_{max} (d^{-1})	Specific antibody production rate q_p (μg per 10^6 viable cells per d)	
batch	0.90	min: 15	max: 29.3
1 recycling	0.99	min 8.4	max: 31
2 recycling	0.65	min: 18.3	max: 84.5
3 recycling	0.76	min: 13	max: 21.6
4 recycling	0.83	min: 13.4	max: 19.8

period of 3.5 to 8.7 d of overall cultivation time at a cell number of 7.5×10^5 viable cells per ml. The second recycling process was run at a total volume of 109 l and started with 1.0×10^5 viable cells per ml now containing 65% of recycled medium. This batch was finished after 12.7 d overall cultivation time with a viable cell number of 8.4×10^5 ml^{-1}. For the third recycling step also 65% spent medium was recycled. However, this process was started with 2.2×10^5 viable cells per ml and run at 116 l total volume. After 2.6 d batch time the bioreactor was harvested; the cell number was then 1.4×10^6 viable cells per ml. In the fourth recycling process the amount of medium was decreased to 56%. This batch was

started with 2.45×10^5 viable cells per ml. The experiment was finished after 19.7 d overall cultivation time. The viable cells reached a density of 1.7×10^6 per ml. Finally, the total reactor volume was harvested. In this process a total amount of 21 g mAb was produced.

From the monoclonal antibody and cell concentration data shown in Fig. 5, the integral specific production rates for the monoclonal antibody have been calculated according to the following formula:

$$q_p(t) = \frac{c_p(t) - c_p(t = 0)}{\int_0^t x(t)\, dt} \qquad (1)$$
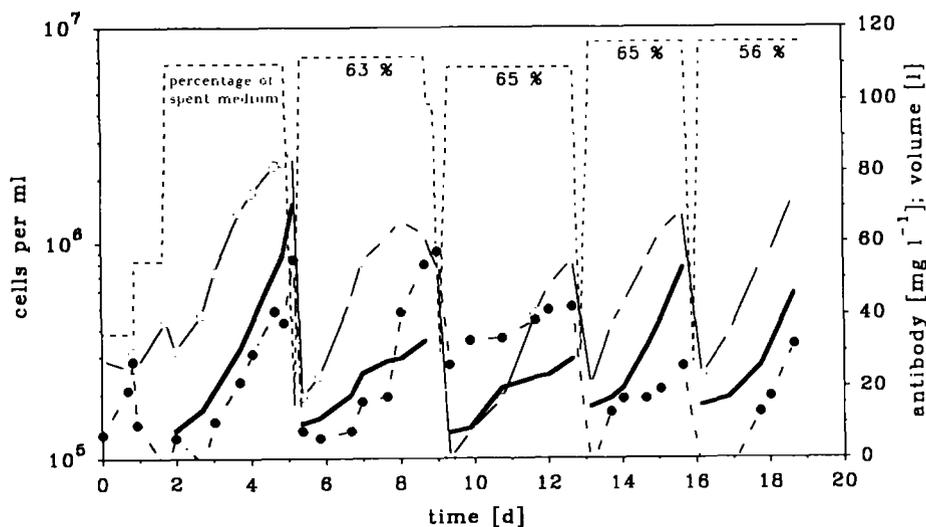

Fig. 5 The time course of the overall cultivation. Shown are viable and dead cell numbers, the product concentrations in the bioreactor and the working volume. For each recycling process the percentage of the spent medium (inoculum and recycled medium) is indicated. ○, viable cells; ●, dead cells; ——, antibody; ------, volume.
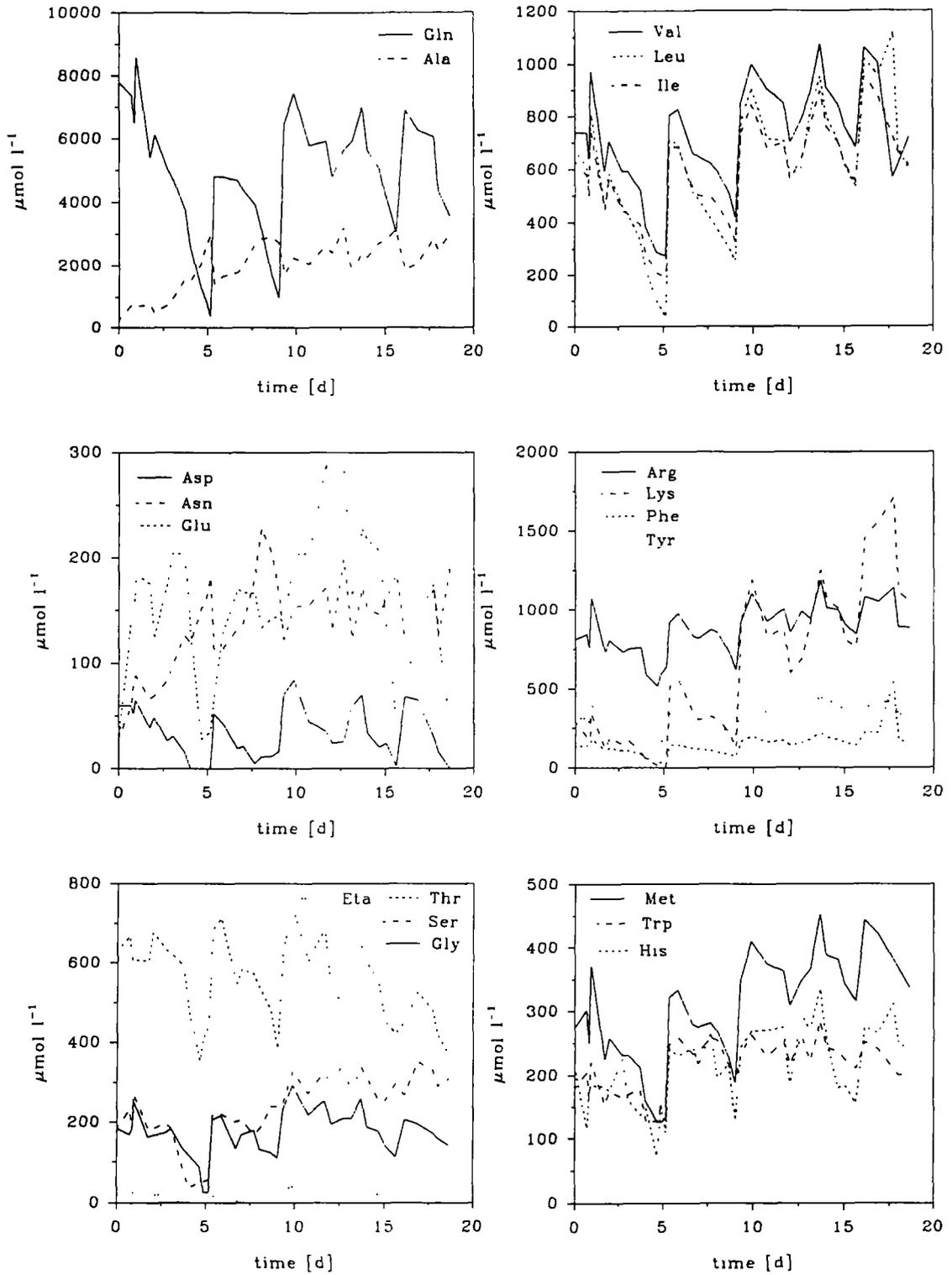
Fig. 6. Concentration of amino acids in the bioreactor. By adding the fresh medium nutrient concentrate the consumed nutrients were refreshed at the beginning of each recycling process.

1. batch   1 recycling   2 recycling   3 recycling   4 recycling
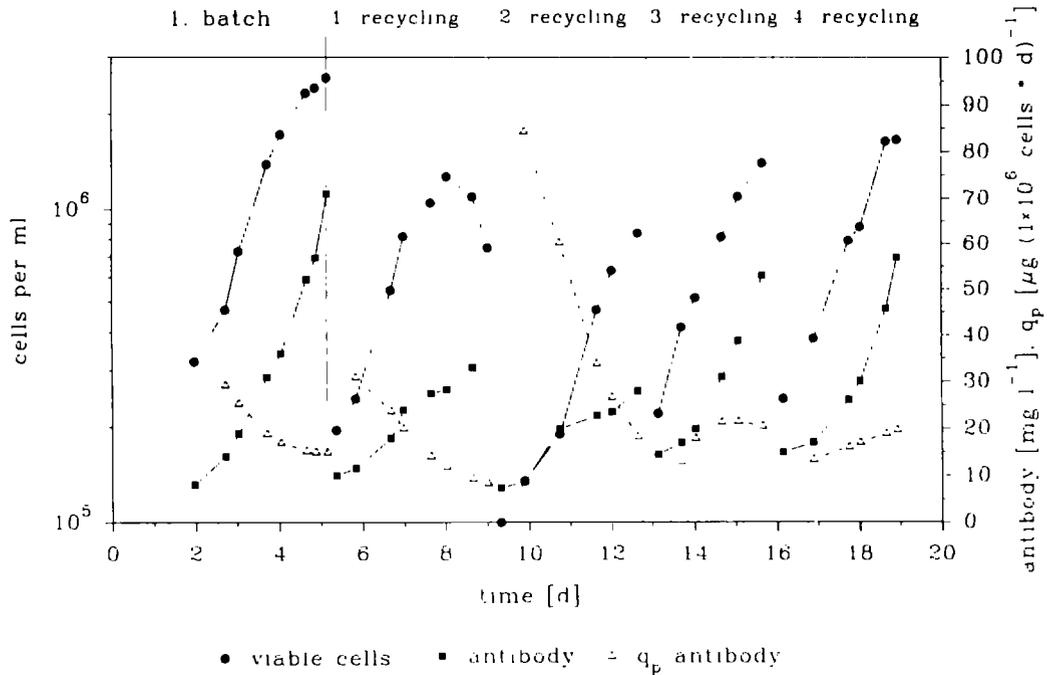

● viable cells   ■ antibody   ▵ q_p antibody

Fig. 7. Integral specific production rates calculated by formula (1) during the five batch processes calculated by integration of a cubic regression curve fit. ●, viable cells; ■, antibody; ▵, q_p antibody.

The results of the calculations are shown in Fig. 7. The specific rates (q_p) could not be observed as constant during the batch process. In the initial batch and the following two repeated batches there was a decrease, where in contrast in the last two repeated batches we found an increase. The minima and maxima values are given in Table 3 where the apparent specific growth rates are also shown.

To characterize the efficiency of the recycling processes the ratio of antibody produced to the amount of fresh medium used have to be re-

garded. The overall yield per batch $Y_{mAb\ vol}$ (mg mAb per litre fresh medium) is calculated as: [(final mAb concentration) − (initial mAb concentration)](batch volume)/(fresh medium volume):

$$Y_{mAb\ vol} = (c_{final} - c_{start}) \frac{V_{batch}}{V_{fresh}}$$ (2)

The results of this calculations are listed in Table 4 and given in Fig. 8 with cumulative amounts of antibody.

Table 4
Antibody concentrations (mAb) and antibody yield $Y_{mAb\ vol}$ (mAb produced per litre fresh medium) for the initial batch and the four recycling processes

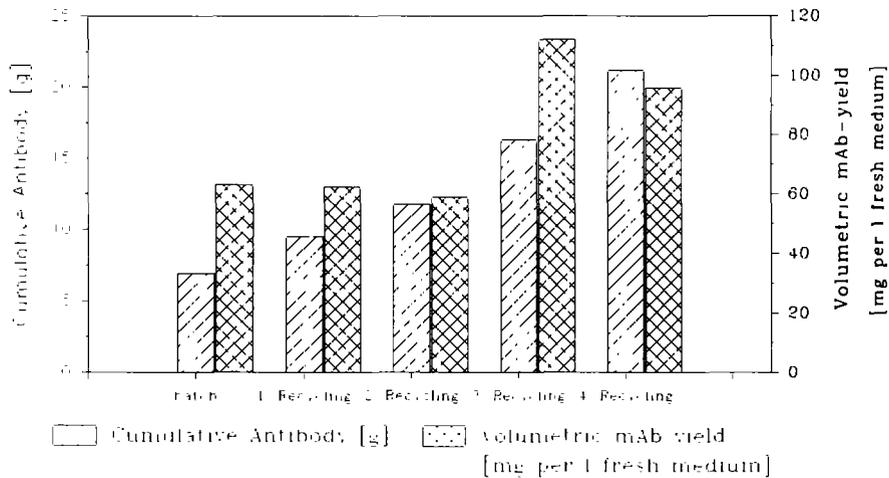| Culture mode	Initial mAb concentration (mg l⁻¹)	Final mAb concentration (mg l⁻¹)	Batch volume (l)	Fresh medium volume (l)	mAb produced per l fresh medium (mg l⁻¹)
batch	8	71	110	0	63
1 recycling	10	33	112	41	62.8
2 recycling	7.4	28	109	38	59
3 recycling	14.5	53.8	116	41	111.2
4 recycling	14.9	57	116	51	95.8

Fig. 8. Total amount of produced mAb (product yield), calculated yield coefficient.

In the batch process a yield of 63 mg mAb per l fresh medium was achieved. In the first and second recycling process the yield dropped under this level, but recovered in the third and fourth recycling process.

It can be seen from Fig. 5 and Table 4 that the cells may have adapted to the recycled medium. In the first and second recycling process the maximum viable cell density reached 1.3×10^6 viable cells per ml which is less than the viable cells of 2.6×10^6 per ml in the initial batch. The product yield dropped under a lower level (62.8 mg l^{-1} fresh medium) compared to the initial batch. However, in the following two recycling steps the viable cell density recovered and reached 1.7×10^6 viable cells per ml. Also the product yield increases up to 111.2 mg mAb per l fresh medium.

Regarding the cost of medium: For the initial batch process the medium costs have been calculated to 12.30 DM l^{-1} (Kempken et al., 1991). From Fig. 9 it can been seen that by starting of the recycling process the medium costs for the total process decreased to a level between 4.20 DM l^{-1} and 4.94 DM l^{-1}.

The preparative membrane ion exchanger using the high flux system was used for rapid purification of one batch of the 100-l bioreactor. In four repeated runs one batch was purified using flow rates of 0.85-1.06 l min^{-1}. These high flow rates resulted in a very high throughput of 3.9 g h^{-1}. The binding rate was about 94% and the recovery rates in excess of 82% (Table 5). It was possible to purify the whole batch without cleaning the module. Former investigation showed that one preparative module could bind up to

Table 5
Membrane chromatography with Sartobind S

No.	IgG (mg)	Volume (l)	IgG bound (%)	Flow rate (l min^{-1})	Recovery (%)	Concentration factor	Process time (h)	Throughput (g h^{-1})
1	456	1.4	91	0.85	90	3.1	0.11	4.2
2	489	1.5	91	0.85	84	4.0	0.11	4.3
3	391	1.2	98	0.85	75	3.0	0.11	3.6
4	267	1.1	97	1.06	80	4.4	0.08	3

Within minutes gram quantities of product could be purified in the HFS. The concentration factor was calculated by dividing the IgG concentration in the elution by the IgG concentration in the supernatant. The binding rate was about 94% and the recovery rates in excess of 82%. The throughput was 3.9 g h^{-1}.
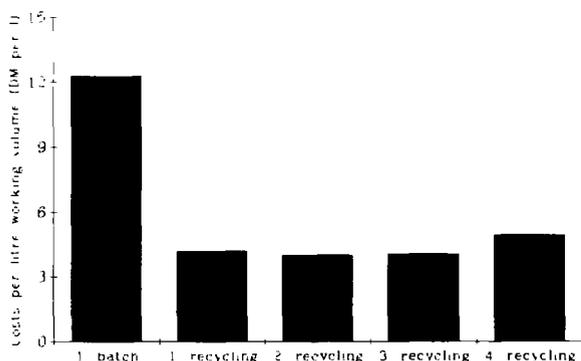
Fig. 9. Medium costs per litre working volume for the initial batch and the following recycling processes

910 mg mAbs without any loss in recovery (Lütkemeyer et al., 1991) (greater amounts were not determined). The capacity of the membrane ion exchanger of 1 mg IgG per cm^2 (equivalent to 50 mg IgG per ml bed volume), as determined with a single filter sheet (12.56 cm^2 effective surface), is very high compared to filter materials of other suppliers.

Up to a flux rate of 571 l m^{-2} h^{-1} (maximum achieved by the system) the binding of the mAbs

on the MIEX was better than 90%. The concentration factor achieved by the MIEX is still somewhat unsatisfactory in comparison with a common ion exchanger, but optimization of the ion exchanger unit towards smaller elution volumes could change that. Up to now one MIEX unit was used 25 times without loss in binding capacity, recovery and flow rate. The main advantage of the MIEX in comparison to ion exchanger based on gel matrices is the much higher throughput (> 13 times).

Final purification of the mAbs was carried out using a preparative gel filtration column. The elutions from the MIEX were applied directly, without prior concentration, to the gel filtration column and resulted in two distinct peaks. The second peak contained the purified mAbs (Fig. 10).

4. Conclusions

The experiment shows that it is possible to recycle up to 65% of spent medium, the specific
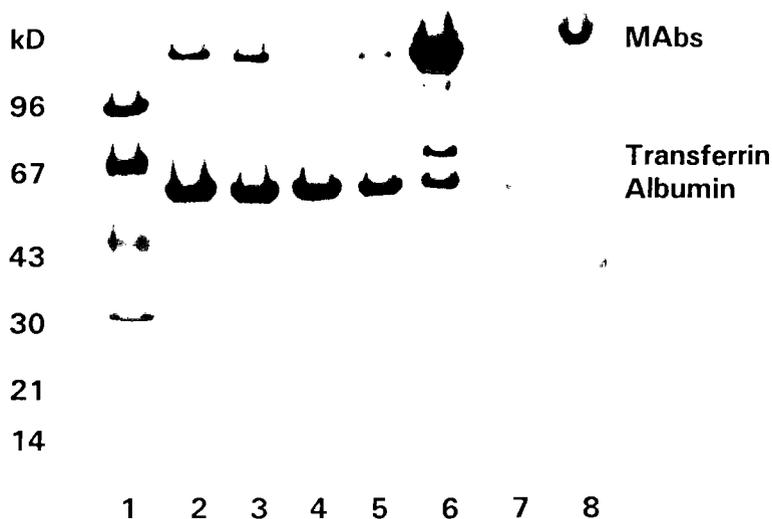

Fig. 10. SDS polyacrylamide gradient gel (8–25%) using a modified silver stain under unreduced conditions. Isolation of mAbs with strong acidic membrane ion exchanger and gel filtration. Lane 1, marker 14–96 kDa (reduced); lane 2, supernatant; lane 3, supernatant after salt reduction; lane 4, unbound proteins (MIEX); lane 5, first wash (MIEX); lane 6, concentrated IgG (MIEX); lane 7, gel filtration 1 peak; lane 8, gel filtration 2 peak, purified mAbs.

antibody production pattern was only influenced when recycling started. The first recycling process shows that it is important to stop the batch at the exact point of no limitation. In the total process it was only necessary to use 281 l of fresh medium instead of 563 l which would have to be used in a conventional repeated batch procedure. For the spent medium which was only supplemented with amino acids and glucose, 52% of the total medium costs were cut.

By medium recycling, 112 mg mAb per l fresh medium could be obtained, compared to 63 mg mAb per l fresh medium in the initial batch.

Compared to the batch culture, the specific productivity decreased in the first recycling process but recovered in the second and reached its peak value in the third. Therefore, an adaptation of the cells to the recycling is obvious.

Membrane ion exchangers are versatile tools for the concentration and purification of mAbs. The binding capacity and the recovery are similar to gel matrices. Using the high flux system very high throughput of the MIEX was demonstrated as the main advantage, which is easy to handle and can be used repeatedly.

Acknowledgements

Abbreviations and notations

c_p	product concentration (mg l^{-1})
q_p	specific production rate (μg per 10^6 viable cells per d)
t	time (d)
V	volume (l)
x	viable cell concentration (cells per ml)
$Y_{mAb, vol}$	mAb yield (mg product per l)

DM	Deutsche Mark
dt	time interval
kDa	kilo Dalton
M	mol l^{-1}
mAb	monoclonal antibody
rpm	rounds per min

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