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Dialysis cultures with immobilized hybridoma cells for effective production of monoclonal antibodies

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Abstract

An industrial scale reactor concept for continuous cultivation of immobilized animal cells (e.g. hybridoma cells) in a radial-flow fixed bed is presented, where low molecular weight metabolites are removed via dialysis membrane and high molecular products (e.g. monoclonal antibodies) are enriched. In a new “nutrient-split” feeding strategy concentrated medium is fed directly to the fixed bed unit, whereas a buffer solution is used as dialysis fluid. This feeding strategy was investigated in a laboratory scale reactor with hybridoma cells for production of monoclonal antibodies. A steady state monoclonal antibody concentration of 478 mg l^{-1} was reached, appr. 15 times more compared to the concentration reached in chemostat cultures with suspended cells. Glucose and glutamine were used up to 98%. The experiments were described successfully with a kinetic model for immobilized growing cells. Conclusions were drawn for scale-up and design of the large scale system.

Abbreviations: c_{Glc} – glucose concentration, mmol l^{-1} ; c_{Gln} – glutamine concentration, mmol l^{-1} ; c_{Amm} – ammonia concentration, mmol l^{-1} ; c_{Lac} – lactate concentration, mmol l^{-1} ; c_{MAb} – MAb concentration, mg l^{-1} ; D – dilution rate, d^{-1} ; D_i – dilution rate in the inner chamber of the membrane dialysis reactor, d^{-1} ; D_0 – dilution rate in the outer chamber of the membrane dialysis reactor, d^{-1} ; $q_{\text{FB,Glc}}^*$ – volume specific glucose uptake rate related to the fixed bed volume, $\text{mmol l}_{\text{FB}}^{-1} \text{ h}^{-1}$; $q_{\text{FB,Gln}}^*$ – volume specific glutamine uptake rate related to the fixed bed volume, $\text{mmol l}_{\text{FB}}^{-1} \text{ h}^{-1}$.

Introduction

Fixed bed reactors with immobilized cells enable cultivation of high volume specific cell concentrations under low shear conditions (Looby *et al.*, 1990; Ong *et al.*, 1994; Pörtner *et al.*, 1995). If a radial-flow geometry is applied, oxygen is not limited along the radius and high fixed bed units can be build (Kurosawa *et al.*, 1991; Matsumura *et al.*, 1993). High volume specific concentrations of cells require high perfusion rates to supply nutrients and to remove toxic metabolites. Consequently the product concentration (e.g. monoclonal antibodies) remains low even at high volume specific production rates. The product concentration in the harvest stream can only be increased, if devices for

product retention (e.g. dialysis techniques) are installed (Linardos *et al.*, 1992; Bohmann *et al.*, 1992, 1995).

In Figure 1 a reactor concept for the cultivation of immobilized cells is shown, which offers a high potential for scale up. The cultivation unit consists of radial-flow fixed bed elements and is coupled with an aeration unit, a dialyzer module and a medium reservoir (compare Bohmann *et al.*, 1995). Low molecular metabolites (ammonia, lactate) are removed over the membrane into a dialysis fluid. Because high molecular components can not pass through the membrane, products (e.g. monoclonal antibodies) are accumulated. Bohmann *et al.* (1995) investigated this concept on a laboratory scale in a membrane dialysis reactor with integrated radial-flow fixed bed (Figure 2). In

Figure 1. Scheme of a fixed bed reactor system coupled with a dialyzer module, an aeration unit and a medium reservoir for large-scale cultivation of immobilized animal cells. In the fixed bed an element of the reactor is indicated, which was investigated in the membrane dialysis reactor (compare Figure 2).

Figure 2. Membrane dialysis bioreactor for laboratory scale with integrated radial-flow fixed bed (working volume of the inner (culture) chamber: 1.2 l, working volume of the outer (dialysis) chamber: 4.9 l, fixed bed volume: 0.6 l).

this reactor the radial-flow fixed bed containing porous carriers for cell immobilization has the same geometry as the fixed element indicated in Figure 1. Therefore data obtained with this reactor are representative of the large-scale situation.

The performance of the reactor system was demonstrated in long term fermentations with hybridoma cells up to 75 days (Bohmann *et al.*, 1995). The highest MAb concentration (368.1 mg l^{-1}) could be obtained at dilution rates of 0.2 d^{-1} in the inner chamber, which is ten times higher compared to chemostat cultures with suspended cells. In these studies low molecular nutrients were supplied over the membrane. Thus nutrient supply and removal of metabolites were coupled. The efficiency of medium use was low, because a high concentration difference of nutrients over the membrane had to be maintained for sufficient supply. To solve this drawback a novel "nutrient-split" feeding strategy (compare Figure 1 and 2) was developed, where concentrated medium is fed directly to the fixed bed unit, whereas a buffer solution is used as dialysis fluid (Lüdemann *et al.*, 1995).

In this report experiments with the "nutrient-split" feeding strategy will be discussed and described with a kinetic model for immobilized growing hybridoma

cells. The efficiency will be compared to other reactor systems and conclusions for design of the large scale concept will be drawn.

Materials and methods

Cell line and standard medium

A mouse-mouse hybridoma cell line producing monoclonal antibodies (MAb) against penicillin-G-amidase was cultivated in medium, which consisted basically of a 1:1 mixture of Iscove's MDM and Ham's F12 supplemented with 2 mmol l⁻¹ L-glutamine and 2 g l⁻¹ NaHCO₃, 0.01% (w/v) PEG and 50 mmol l⁻¹ ethanolamine. To complete the medium either 3% (w/v) horse serum (HS) (referred to as *HS-medium* in the following text) or an iron-rich supplement as a 100 x concentrated mixture (referred to as *IR-medium* in the following text) was added. The formulation described in a previous paper (Franěk and Dolníková, 1991) was modified to yield final concentrations of 200 µmol l⁻¹ ferric citrate and 100 µmol l⁻¹ ethanolamine. Hydrocortisone was omitted. For use in bioreactors 100 mg l⁻¹ streptomycin and 100,000 IU l⁻¹ penicillin were further added to the media.

"Nutrient-split" medium

The standard medium was split into a nutrient concentrate (10 x) and a buffer solution containing all inorganic components. In respect to the fermenter geometry (see Figure 2) the buffer was concentrated 1.24 x to obtain 1 x salt concentration in the whole fermenter. To prevent precipitation of the medium concentrate (purchased by BioConcept, Umkirch, Germany) the pH had been adjusted to rather basic conditions, which enhances glutamine decomposition. Therefore glutamine was fed as an extra solution (50 x concentrated). Both nutrient concentrate and buffer solution were supplemented with the iron-rich protein-free supplement.

Analysis

Glucose and lactate were measured by a glucose analyser (YSI 2700-D, purchased by Fa. Schlag, Bergisch-Gladbach, Germany). Ammonia and L-glutamine were determined enzymatically, monoclonal antibodies by a mouse-IgG-ELISA (all kits from Boehringer, Mannheim, Germany). Cells were counted

Table 1. Cultivation of hybridoma cells in the membrane dialysis bioreactor with integrated radial-flow fixed bed with supply of nutrients over the membrane (experimental data from Bohmann *et al.*, 1995). Comparison of experimental and calculated values for the inner chamber at a dilution rate of 1.22 d⁻¹ in the outer chamber and 0.2 d⁻¹ in the inner chamber

Component	Measured	Calculated
glucose [mmol l ⁻¹]	6.94	6.44
glutamine [mmol l ⁻¹]	2.10	1.78
lactate [mmol l ⁻¹]	16.90	15.94
ammonia [mmol l ⁻¹]	1.0	1.02
MAb [mg l ⁻¹]	368	366

using a haemocytometer. Cell viability was determined by trypan blue exclusion.

Fixed bed cultures

Basic data on the metabolism of immobilized cells were determined in an axial flow fixed bed (volume 50 ml and 176 ml, respectively), connected with 750-ml reservoir vessel for aeration and medium exchange (Bohmann *et al.*, 1992). The medium in the reservoir vessel could be changed in a semi-batch mode or continuously. Dissolved oxygen was controlled in the reservoir vessel and at the outlet of the fixed bed. Porous SIRAN® carriers (Schott, Mainz, Germany, for specific data compare Looby *et al.*, 1990) with diameter 3-5 mm were selected out of a number of carriers (Ong. *et al.*, 1994; Pörtner *et al.*, 1992).

For dialysis cultures a membrane dialysis reactor was used (Figure 2) (Bohmann *et al.*, 1992, 1995). The reactor consists of two chambers (outer chamber 4.9 l, inner chamber 1.2 l) separated by a dialysis membrane (cuprophane, Enka, Germany) with a cut-off of 10 000 Dalton (for specific data see Bohmann *et al.*, 1995). In the inner chamber a radial-flow fixed bed (volume 0.6 l) containing the porous carriers for cell immobilization is introduced. In contrast to the concept shown in Figure 1 in this reactor aeration was performed in the outer chamber (medium reservoir) due to practical reasons and oxygen had to permeate through the membrane. It could be confirmed during the experiment that the membrane area was sufficient to supply enough oxygen to the inner chamber.

The mass balance equations for the different types of reactors were discussed extensively by Bohmann *et al.* (1995).

Table 2. Comparison of different reactor systems. Data from: (1) Lüdemann *et al.*, 1996, (2) Pörtner *et al.*, 1996, (3) Bohmann *et al.*, 1992, (4) Pörtner and Märkl, 1995, (5) Bohmann *et al.*, 1995, (6) Lüdemann *et al.*, 1995

Reactor system	Suspension			Fixed bed perfusion (4)	Membrane dialysis reactor with integrated fixed bed	
	batch (1)	fed-batch (2)	chemostat (3)		supply of nutrients over the membran (5)	“nutrient-split” feeding strategy (6)
dilution rate [d ⁻¹]	–	adapted	0.67	0.26	D _i : 0.2 D ₀ : 0.6	D _i : 0.1 D ₀ : 0.12
cell conc. [cells ml ⁻¹]	2*10 ⁶	3.2* 10 ⁶	2.4* 10 ⁶	–	–	–
MAB-conc. [mg l ⁻¹]	35	54	35	65	383	400
MAB-yield [mg l ⁻¹ d ⁻¹]	8.4	12.7	26.4	16.9	76.6	40
medium demand [l g _{MAB} ⁻¹]	28	18.5	23.5	15.4	35.4	15.5

Results

Kinetic model for immobilized growing cells

The immobilized cells are not accessible to direct sampling, counting and analysis. Modeling of the cell metabolism has therefore to be based on volume specific substrate uptake and metabolic production rates. The experiments were performed in HS-medium as well as in IR-medium. The data for the following model were obtained from steady state conditions, where a saturation of the fixed bed with cells can be expected. Up to now the growth of cells within the fixed during growth phase can not be described.

At high substrate concentrations (glucose above 4 mmol l⁻¹, glutamine above 1.5 mmol l⁻¹) the volume specific glucose uptake rate $q^*_{FB,Glc}$ (mmol l_{FB}⁻¹ h⁻¹) was expressed as a function of the glucose concentration c_{Glc} (mmol l⁻¹),

$$q^*_{FB,Glc} = 0.801 + 0.419 * c_{Glc} \quad (1)$$

the volume specific glutamine uptake rate $q^*_{FB,Gln}$ (mmol l_{FB}⁻¹ h⁻¹) as a function of the glutamine con-

centration c_{Gln} (mmol l⁻¹):

$$q^*_{GB,Gln} = 0.12 + 0.27 * c_{Gln} \quad (2)$$

At low substrate concentration (below 4 mmol l⁻¹ glucose and/or 1.5 mmol l⁻¹ glutamine) the metabolism is dominated by glutamine limitation, similar to suspended cells (Pörtner *et al.*, 1996). The volume specific glucose (equation 3) and glutamine uptake rates (equation 4), respectively, could be expressed as a function of the glutamine concentration:

$$q^*_{FB,Glc} = 4.9 * \frac{c_{Gln}}{c_{Gln} + 0.55} \quad (3)$$

$$q^*_{FB,Gln} = 0.6 * \frac{c_{Gln}}{c_{Gln} + 0.2} \quad (4)$$

The yield of lactate from glucose was 1.9 mol mol⁻¹ at glucose concentrations higher than 4 mmol l⁻¹ and 1.4 mmol l⁻¹ below this concentration. The yield of ammonia from glutamine was 0.9 mol mol⁻¹. The antibody productivity was not constant and varied between appr. 3.5 and 6.1 mg l_{FB}⁻¹ h⁻¹. The reason for this is not yet clear.

Figure 3. Experiment in the membrane dialysis bioreactor with integrated radial-flow fixed bed with supply of nutrients over the membrane (from Bohmann *et al.*, 1995, HS-medium). Time course of the dilution rates D and the glucose concentrations c_{Glc} in the inner and outer chamber, respectively and the MAb-concentration c_{MAb} in the inner chamber. Calculations for the steady-states were made at a later time with the kinetic model obtained with a small-scale fixed bed.

Experiments in the membrane dialysis bioreactor with integrated radial-flow fixed bed

The kinetic model for the metabolism of immobilized cells was applied to the data available for the membrane dialysis reactor. In Figure 3 an experiment performed by Bohmann *et al.* (1995) is shown. As already mentioned in the introduction, low molecular nutrients were supplied over the membrane in this case and thus nutrient supply and removal of metabolites were coupled. At a dilution rate of 0.2 in the inner chamber a MAb concentration 368 mg l^{-1} could be obtained. Experimental data and calculations are in good agreement, as shown in Table 1 for a dilution rate of 1.22 d^{-1} in the outer chamber and 0.2 d^{-1} in the inner chamber.

Figure 3 underlines the problem bound up with the strategy of feeding low molecular substrates over

Figure 4. Experiment in the membrane dialysis bioreactor with integrated radial-flow fixed bed with "nutrient-split" feeding strategy (Lüdemann *et al.*, 1995, IR-medium). Time course of the concentrations of glucose c_{Glc} , lactate c_{Lac} , glutamine c_{Gln} , ammonia c_{Amm} and monoclonal antibodies c_{MAb} in the inner chamber. Calculations for the steady-states were made at a later time with the kinetic model obtained with a small-scale fixed bed. 1: start of feeding nutrient concentrate, protein-free (10x), dilution rate $D_1 = 0.12 \text{ d}^{-1}$. 2: start of feeding dialysis fluid (buffer), dilution rate $D_0 = 0.15 \text{ d}^{-1}$.

the membrane. The glucose concentration in the outer chamber had to be approx. 6 mmol l^{-1} higher than in the inner chamber due to the transport resistance of the membrane. The usage of glucose was 57% and of glutamine only 26%.

This problem can be overcome by using the "nutrient-split" feeding strategy. Figure 4 shows the time course of an experiment reported by Lüdemann *et al.* (1995). The experiment was started with both chambers containing complete medium (protein-free IR-medium). After 70 h of batch fermentation continuous perfusion of the inner chamber with 10 x concentrated nutrient solution was started (dilution rate $D = 0.12 \text{ d}^{-1}$). To keep the ammonia concentration below the toxic level (5.96 mmol l^{-1} at pH 7.1, Lüdemann *et al.*, 1994), perfusion in the outer chamber with dia-

lysis fluid was started at 148 h ($D = 0.15 \text{ d}^{-1}$). Steady state levels for glucose, glutamine, lactate and ammonia concentrations were reached after 600 h. Until the experiment was finished after 850 h cultivation time appr. 400 mg l^{-1} MAb were accumulated. Glucose and glutamine were used up to 98%. The steady state values are described by the kinetic model rather well.

Considerations on the potential of the “nutrient-split” feeding strategy

A comparison of different reactor systems and process strategies underline the potential of dialysis cultures run with the “nutrient split” feeding strategy (Table 2). The antibody concentrations obtained with dialysis cultures in the membrane dialysis reactor are up to 15 times higher compared to conventional suspension cultures. The volume specific yield is as well considerably higher. For dialysis cultures the supply of nutrients over the membrane has some advantages in respect to the volume specific yield, which can be overcome by further increasing the nutrient concentration in the nutrient solution in case of the “nutrient-split” feeding strategy.

The rather good agreement between model calculation and experiment allows some further theoretical considerations on the potential of the “nutrient-split” feeding strategy applied to fixed bed reactors coupled with dialysis. Process simulations were performed for the geometry of the membrane dialysis reactor with integrated radial-flow fixed bed, as the ratio of fixed bed volume, volume of inner (culture) chamber, membrane area and volume of the outer (dialysis) chamber can be used as well for the large scale system shown in Figure 1. The initial glutamine concentration was 35 mmol l^{-1} in the concentrated feed. The dilution rate of dialysis fluid in the outer chamber was adjusted to keep the ammonia concentration in the inner chamber at 5.4 mmol l^{-1} , which is below the toxic level of 5.96 mmol l^{-1} at pH 7.1 (Lüdemann *et al.*, 1994). Figure 5 shows the concentration of monoclonal antibody c_{MAb} and the total medium demand as a function of the dilution rate D_i of the inner chamber. As expected the MAb concentration increases and the medium demand decreases with decreasing dilution rate. As in this case the volume specific antibody productivity was constant and did not depend on the substrate concentration, the volume specific yield is independent of the dilution rate ($40 \text{ mg l}^{-1} \text{ d}^{-1}$).

With respect to process efficiency the lowest dilution rate possible should be used. But with decreasing

Figure 5. Simulation for the geometry of the membrane dialysis reactor with integrated fixed bed run with “nutrient-split” feeding strategy. Concentration of monoclonal antibodies c_{MAb} in the inner chamber and total medium demand versus dilution rate in the inner chamber D_i . The calculated curves represent steady state values. The dilution rate in the outer chamber was adjusted to keep the ammonia concentration at 5.4 mmol l^{-1} . Circle and square are measured values from Lüdemann *et al.* (1995).

dilution rate the time required to reach a steady state increases. At a dilution rate of 0.05 d^{-1} it takes 20 days for a total medium exchange in the inner (culture) chamber. At these long residence times proteolytic effects on the product quality can not be excluded (Schaefer *et al.*, 1987). Furthermore the substrate concentration drops to very low values at very low dilution rates and instability of the fixed bed culture becomes likely. Therefore it is recommended to keep the dilution rates in the inner (culture chamber) at 0.1 d^{-1} .

Discussion

The concept of fixed bed cultures coupled with dialysis proved to be very effective for production of biologicals with animal cells, even with non-adherent hybridoma cells. The yield can be maximized, if a “nutrient-split” feeding strategy for supply of nutrients and removal of low molecular weight metabolites is applied. The kinetic model, which was obtained from small scale fixed bed reactors, was successfully applied to experiments performed in the membrane dialysis reactor with integrated radial-flow fixed bed. The radial-flow fixed bed in this reactor has the same geometry as the fixed bed elements of the large-scale version indicated in Figure 1. Therefore design and scale up of the indus-

trial scale reactor system can be done with basic data on carrier type and metabolism of the immobilized cells obtained in small scale reactor systems and data on the properties of the dialysis membrane.

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