Sparging-shear sensitivity of animal cells

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Sparging-shear sensitivity of animal cells

Proefschrift

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Stellingen

behorende bij het proefschrift 'Sparging-shear sensitivity of animal cells'.
Leo van der Pol

1. De samenstelling van het kweekmedium is ten onrechte veronachtzaamd door veel auteurs van publicaties over de gevoeligheid van dierlijke cellen voor afschuifkrachten (dit proefschrift).

2. Het gebrek aan een gestandaardiseerde methode voor het meten van de gevoeligheid van dierlijke cellen voor afschuifkrachten maakt een zinvol vergelijk van gegevens en het trekken van algemene conclusies vrijwel onmogelijk (D.W. Murhammer, C.F. Goochee 1990. Biotechnol.Prog. 6, 391-397, dit proefschrift).

3. In het onderzoek naar de gevoeligheid van dierlijke cellen voor afschuifkrachten wordt 'verklaring' regelmatig verward met 'verband' (publicaties over shear van M. Hülscher *et al.* en van J. Wu *et al.*).

4. De beweringen dat celschade en verlies van dierlijke cellen plaats vinden in de schuimlaag zijn onvoldoende onderbouwd (S. Zhang *et al.* 1992 J. of Biotechnol. 25, 289-306).

5. Het beschermende effect van serum en albumine tegen afschuifkrachten is niet af te leiden uit simpele groeiexperimenten met dierlijke cellen (S.O. Ozturk, B.O. Palsson 1991. J. of Biotechnol. 18, 13-28; C.B. Elias *et al.* 1995. Chem.Eng.Sc. 50, 2431-2440).

6. Het belang van 'serendipity' wordt geïllustreerd door geneesmiddelen die voor hun doel ongeschikt, maar in hun bijwerking zeer succesvol bleken.

7. Balkaniseren lijkt rechtstreeks afgeleid van kannibaliseren.

8. Het einde van 'eindeloos voor de wind' is doorgaans lagerwal.

9. Veel relatienetwerken werken beter dan computernetwerken.

10. Men moet 'een carrière' niet verwarren met 'een leven'.

11. Het is opmerkelijk dat veel Nederlanders hun trouw aan leveranciers beloond willen zien in de vorm van doorgaans waardeloze spaarzegels.

12. In de maakbare samenleving wordt het woord 'zorg' steeds vaker verward met 'zelfredzaamheid'.

13. Na Schiphol in de zee en de Betuwelijn in de grond, zal de Zuiderzeelijn wel in de lucht blijven hangen.

14. Onthaaste spoed is meestal goed.

15. Er is nog hoop voor mensen die 'hun millenium niet hebben'.

Wageningen, 4 december 1998

Aan mijn ouders

Voorwoord

Het is niet te hopen dat mijn capaciteit voor het plannen van projecten wordt afgelezen aan het project 'promoveren'. Het is verbazingwekkend hoe lang ik mezelf en mijn omgeving in de waan kon laten dat het proefschrift binnen een bepaalde tijd zou zijn afgerond. Het is te danken aan mijn promotor Hans Tramper dat ik de kans kreeg om wetenschappelijke artikelen te gaan schrijven die later gebundeld konden worden tot dit proefschrift. Er zat af en toe meer dan fysieke afstand tussen Wageningen en Groningen, met name tijdens het schrijven van het laatste artikel. Toch was Hans altijd een constante factor die mij steun en vertrouwen gaf. Kees de Gooijer was voor mij een lichtend voorbeeld hoe werk, gezinsleven en promoveren gecombineerd kunnen worden.

Het inhoudelijke werk is uitgevoerd bij het oude Bio-Intermediair in de binnenstad van Groningen door de studenten Marcel Thalen, Gerben Zijlstra, Geert van der Wouw, Wilfried Bakker, Dick Bonarius, Iedo Beeksma en Irene Paaijens van de Landbouwuniversiteit Wageningen. Het leek wel of er in Wageningen alleen maar kanjers studeerden. Het doet mij deugd dat allen na een aangename en succesvolle stagetijd goed terecht zijn gekomen, al hadden Gerben, Irene en Iedo nog enige nascholing bij Bio-Intermediair nodig.

Dank ben ik verschuldigd aan de oprichters en eerste directeuren van Bio-Intermediair, Kees van der Graaf en Jos van Weperen, die mij in de gelegenheid hebben gesteld een deel van de werktijd aan de R&D activiteiten van dit proefschrift te besteden. Zij hadden een onmiskenbare inbreng in de bruisende pioniersfeer die het bedrijf kenmerkte. Er wordt nu wel eens meewarig teruggekeken op die turbulente beginperiode, maar ik hoop dat dit proefschrift een bewijs is dat er ook kwaliteit werd geleverd. Als verdere katalysatoren uit die tijd wil ik noemen Klaas Riepma en Koos Koops die tijdens vele lunchbesprekingen en borreluurtjes een bijdrage leverden aan messcherpe analyses van het onderzoek, het bedrijf en de toestand in de wereld. Van de vele goede collega's van het grotere Bio-Intermediair aan de Zuiderweg wil ik speciaal het PPD-team danken (Carina, Esther, Monique, Romi, Marion, Saskia, Gerben, Maurice en Jurjen).

De Landbouwuniversiteit Wageningen en DSM-Biologics (voorheen Gist-brocades/ Bio-Intermediair), met name de afdeling Marketing en Sales featuring Peter Ketelaar, bedank ik voor de geldelijke steun voor het maken van dit proefschrift.

Voor het relativeren van allerhande zaken tijdens kaarten, risken en andere spelactiviteiten inherent aan bier drinken moet ik danken de biologievrienden van het eerste

uur (of eigenlijk eerste jaar) Harm Geerligs, Klaas Vrieling, Kees Talsma, Anne van der Velde en 'last but not least' Menno Engelkes.
Mijn vader beschouw ik als de stimulerende kracht om aan een universitaire studie te beginnen en mijn moeder als de leverancier van het doorzettingsvermogen om het ook af te ronden.
Marjolein was gedurende de jaren dat er veel vrije tijd aan het schrijven van dit proefschrift werd besteed een onmisbare steun. Eva en Idsard: wat kon ik er soms doorheen zitten aan het eind van de dag en dan toch weer energie putten uit een uurtje ongecompliceerd spelen. Ik hoop dat na de promotie vrije tijd ook weer echt 'vrij in te delen tijd' zal zijn.

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1. General introduction

Summary

Animal cells are increasingly used for the production of high-value proteins like monoclonal antibodies, primarily because these cells produce the correctly processed protein and the protein product is secreted into the culture medium. Homogeneous culture in stirred tank reactors is the prefered fermentation technique for large-scale production of biopharmaceuticals with animal cells; sparging is the prefered method of oxygen supply. Direct sparging of these cultures can be applied if the culture medium contains a protective agent like Pluronic F68.
The most efficient use of culture media and reactor volume and time is associated with systems that maintain a high cell density at low growth rate. This requires reduction of the cell death rate to a minimum. A better understanding of shear phenomena as a result of sparging is necessary to be able to control the cell death rate. A generic approach of research is chosen here to contribute effectively to the fragmented knowledge of shear sensitivity of hybridoma cells in sparged cultures.

Introduction

The application of animal cells for the production of biologicals is growing. Originaly the large-scale culture of animal cells was mainly applied for vaccin production (1970's) using adherent cells on microcarriers. During the 1980's the market for monoclonal antibodies from hybridoma suspension cells developed. The main advantages of animal cells for the production of high-added-value biologicals are that these cells produce a correctly folded protein containing the correct additional groups and that this mature protein product is secreted into the culture medium. The correct post-translational modifications of the product are of importance for the quality of the product. The secretion of protein product makes the purification of product from animal-cell fermentations far more simple than for instance the purification of product in inclusion bodies from bacteria.
The correct processing involves all post-translational activities in the ER and Golgi system (figure 1, ref.18), like protein folding, creation of disulfide bonds and addition of sugar groups (glycosylation) or other groups (such as phosphate, adenylate, palmitate). This holds especially for protein products with larger molecular weight, having in mind clinical applications for which small differences can create differences in bioactivity, clearance from blood and immunogenicity.
The traditionally mentioned disadvantages of animal-cell culture include slow and variable growth, low and unstable productivity, and shear sensitivity. Overall the use

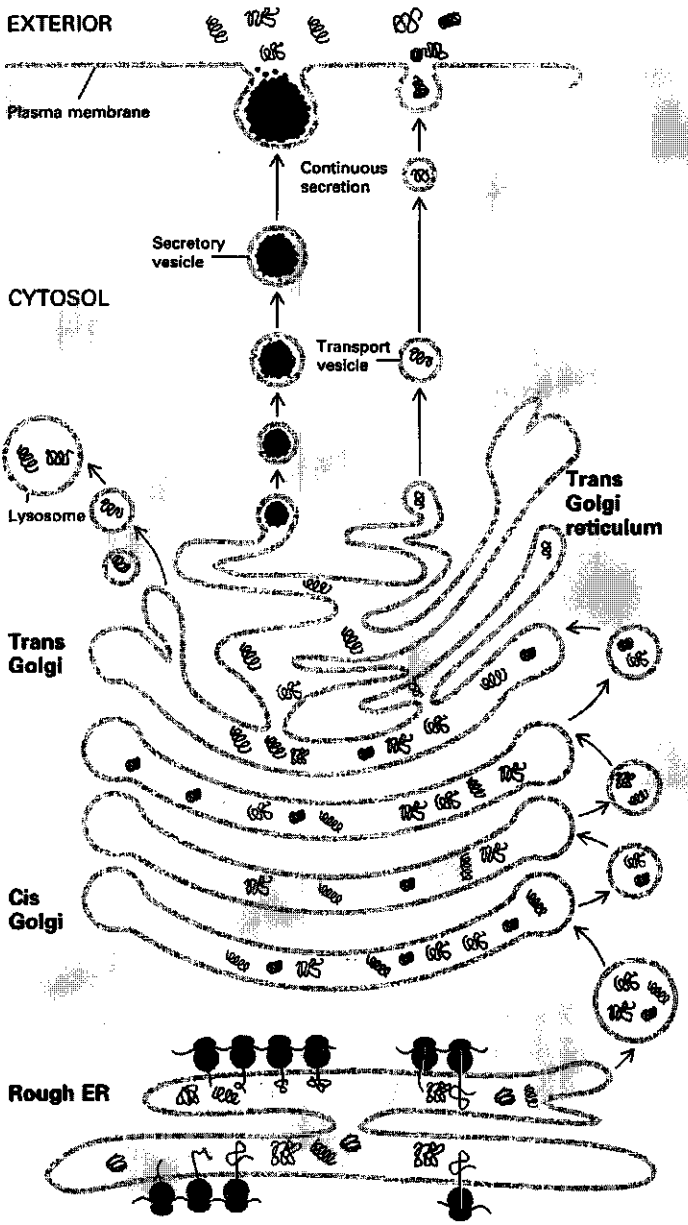

Figure 1 The maturation of secretory proteins during the transport in the endoplasmatic reticulum and Golgi system (adapted from ref.18).

of animal cells for large-scale protein production seemed to involve inherent inconsistencies preventing it to reach a state of robustness that is needed for a reliable production process. These disadvantages - especially the shear sensitivity of animal cells - urged many research groups to look for alternative reactor types that reduce or prevent some of the disadvantages. Examples of such systems are hollow fibre, fluidized bed, encapsulation (cell immobilization), celligen cell lift (mild mixing), membrane aeration, caged aeration, perfluorocarbons (alternative oxygenation systems).
For the culture of animal cells *in vitro* the general approach is to copy the conditions in the body, that is physiological values for temperature, pH, DO and other parameters. For the culture medium body fluid was the target, including serum as component that presents a natural fluid environment. In tissue, cells are packed as stationary adherent cells in a density of at least 10^9 viable cells per mL. In tissue the capilairy system of the blood circulation combined with the flexible activity of different cell types is capable of maintaining this tissue culture cell density in a healthy state within certain gradient ranges of homeostasis. Bioreactor systems like the hollow fibre are attempting to mimic these tissue conditions. In practice this approach often results in the development of larger gradients than allowed, leading to the accumulation of dead cells.
In general the gradient (non-homogeneous) bioreactor systems have one or more of the following disadvantages :

1) limited scaleability
2) non homogeneous : accurate cell count is impossible; no adequate data on cell growth and viability; difficult process control
3) lack of simplicity, creating increased risk of errors

Therefore in the biopharmaceutical industry the use of conventional stirred-tank reactors is the general cell-culture method. For most products the required annual amounts for the market are in the range of 10-1000 g purified protein product. For cell lines producing 5-25 pg/cell/day, this implies the use of homogeneous stirred-tank reactors of 500-5000 L for a batch process or 50-500 L working volume for continuous perfusion fermenters. A continuous fermenter produce several working volumes of harvest per week while a batch reactor will generate one volume per week and the mean cell density in continuous perfusion fermenters is higher compared to batch reactors.
The general production scheme for a biopharmaceutical protein [eg. MAb] by animal cells is shown in figure 2. Starting point for a production is a vial from the Manufacturers Working Cell Bank (MWCB). This bank usually consists of 200-400 vials and is generated from the Master Cell Bank (MCB), the original back-up cell bank of around 100 vials of the production cell line. Both MCB and MWCB are extensively tested as required by the different regulatory authorities. The vial of the MWCB is expanded in a preculture phase to generate sufficient cell material for the

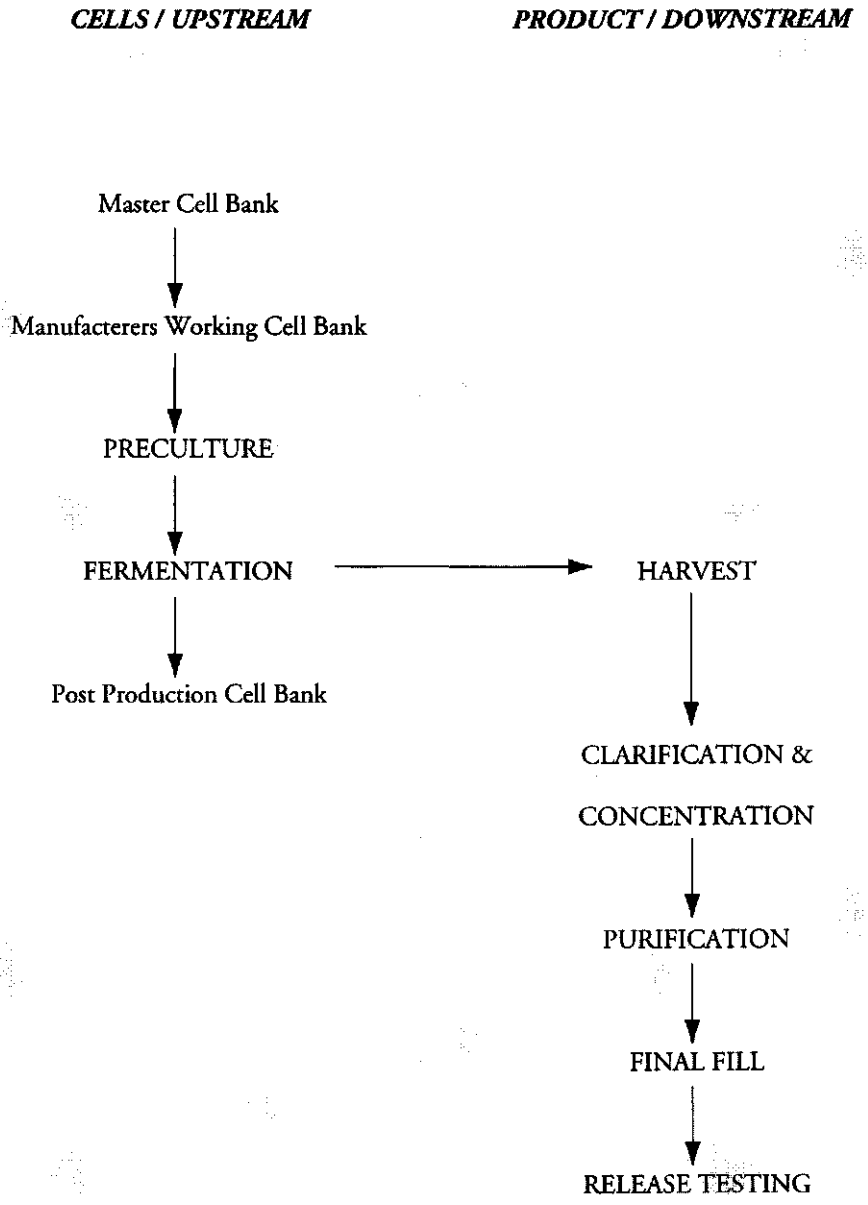

Figure 2 General scheme for the production of clinical grade monoclonal antibody.

inoculation of a bioreactor. The fermentation that is executed after the growth phase in the reactor can be either batch, fed batch or continuous perfusion. A fraction of the cells at the end of the production are used to generate a Post-Production Cell Bank (PPCB) to be able to test the stability of the cells from start to end of the production process.
The first step of the Down-Stream Processing (DSP) usually consists of the removal of cells and cell debris by depth filtration followed by a tangential flow filtration. The resulting concentrate (or retentate) is the starting material for the purification using several column steps. Besides obtaining the correct amount of product in a certain concentration and purity, the biopharmaceutical end product has to meet a set of specifications as listed in the Bill of Testing (BOT). This includes maximum concentrations for toxic/waste materials like DNA and column resins. Also in the BOT specifications are given for the production process like :

a) cell stability as measured by determining several parameters of cells originating from MCB, MWCB, and PPCB.
b) product stability as measured by determining several parameters of the product in the early, mid, and late phase of the fermentation process.
c) efficiency of the DSP process to remove virusses.

Hybridoma cells

Hybridoma cells produce antibodies that specifically bind a target sequence (epitope) of another large-molecular-weight molecule (the antigen). This property of selective non-covalent binding of a target makes these antibodies very useful for showing the presence of the antigen (analytical techniques; diagnostic applications), purification of the antigen (immunoaffinity chromatography), or therapeutic applications, where the antibodies are used to locate diseased cells in patients.
Hybridoma cells are fusion cells of a myeloma cancer cell that is capable of infinite growth and a b-lymphocyte that produces a specific type of antibody (figure 3, ref.18). The generation of a hybridoma fusion cell producing monoclonal antibody (MAb) was first succesfully described for mouse lymphocytes by Köhler and Milstein in 1975 (Nobel prize in 1984, ref.5). Known antibody types are IgG, IgM, IgA, IgE, and IgD; from IgG at least four subtypes exist. The antibodies used for commercial production are predominantly of the IgG type. This IgG antibody consists of two heavy chains and two light chains, linked together to create a 150 kD protein with two identical antigen binding regions (figure 4, ref.18).
The myeloma parent cell is deficient in an enzyme involved in nucleotide synthesis and can not grow in media without precursors. By selection on production of antibody and growth in culture medium without precursors the suitable hybridoma fusion cells can be selected. During a further selection process the hybridoma producing the

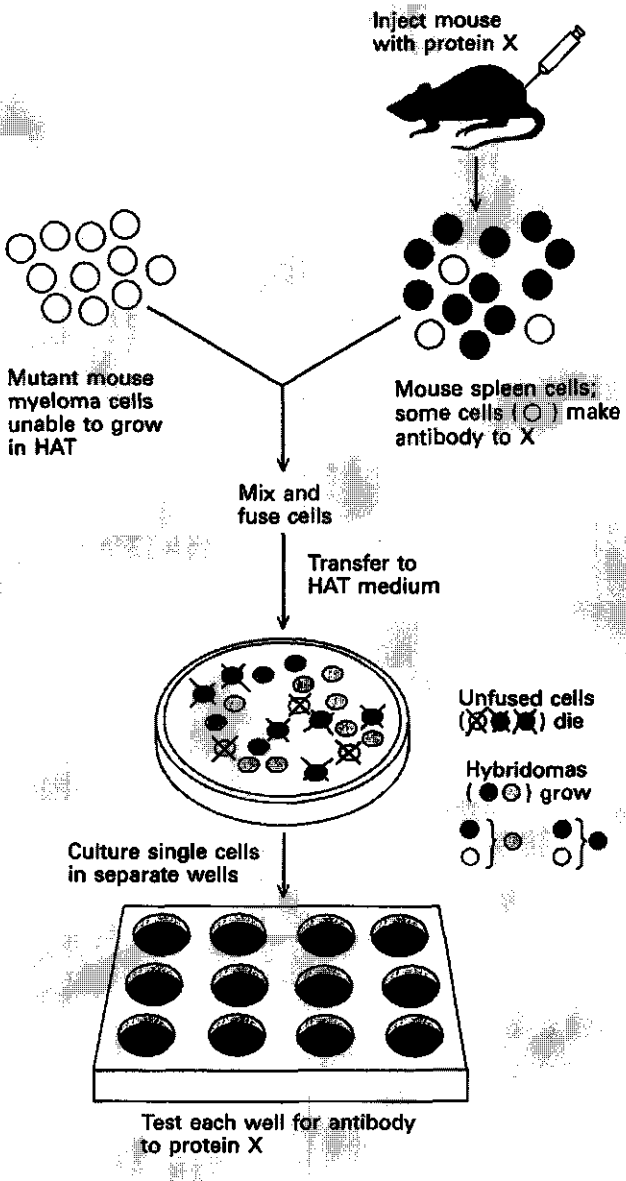

Figure 3 The procedure for the selection of a monoclonal-antibody-producing hybidoma cell line (adapted from ref.18).

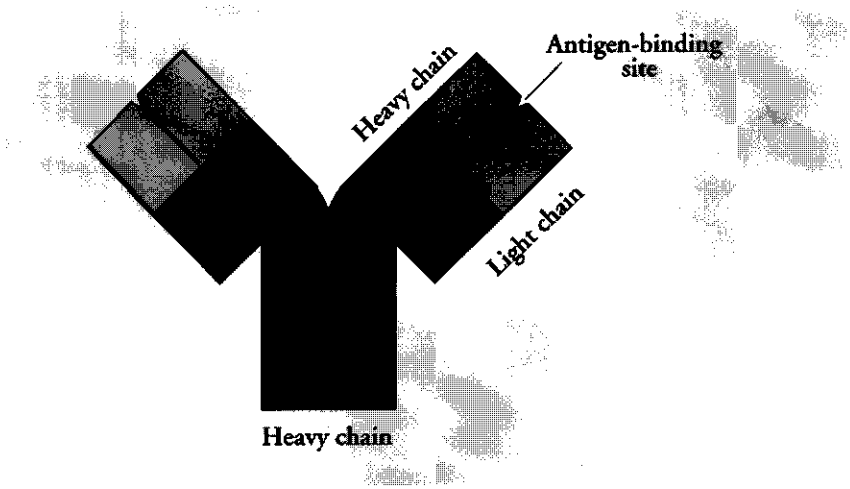

Figure 4 The structure of an IgG antibody (adapted from ref.18).

antibody with the right growth properties and the required product specifications can be obtained. This selection process is done by a dilution technique that selects for clones originating from a single cell producing one specific antibody.
Hybridoma cells are usually growing as single suspension cells, showing the transformed background of the myeloma parent. Transformed cells (cancer cells) are capable of growing in suspension, because they contain two mutations for as well immortality as for loss of contact inhibition.

Growth and death

Hybridoma cells cultured at physiological conditions will produce a batch growth curve similar to the generalized bacterial growth curve but with a few differences. Animal cells will show a shorter exponential phase, because the high inoculum density will allow a limited number of generations before reaching the maximum viable-cell concentration. A real stationary phase will be absent and the death rate will be relatively high. Reason for this is that even under optimal growth conditions the viability is rarely 100%, indicating that some cells are dying. The programmed, genetically controlled death is called "apoptosis", and can be discriminated from accidental/ sudden death or "necrosis". The reduction in growth rate after the exponential phase is in parallel with an increasing death rate, creating a maximum in viable cell number for a short period of time.
Dependent on the cell line and the used culture medium the maximum growth rate

will be in the range of 0.5 to 1.0 d^{-1}, corresponding to doubling times of 17 to 34 h. The minimum inoculum density applied is generally in the range of 10^4 to 10^5 viable cells per mL while the maximum viable cell number generally is close to 2 $x10^6$/mL, if oxygen is not limiting, with an average viability during exponential growth of 80-98%.

Antibody production

A murine hybridoma produces MAb in the range of 5-25 pg/cell/d. Human hybridoma's produce 10 times less and human/murine heterohybridoma's have a specific productivity in between these two types. For a "wild type" myeloma cell the reported maximum specific productivity is 72 pg/cell/d (10). It is one of the challenges of developments in expression systems to create recombinant myeloma's with high specific productivity. For murine hybridoma cells in a culture flask reaching a maximum viable cell density of 10^6/mL after 4 days the antibody concentration in the supernatant will be 10-50 mg/L. After 6-7 days the antibody yield will have doubled with antibody generated by "stationary" cells and released from dying cells.
The production of antibody by hybridoma cells is in general not strictly coupled to the growth rate. This enables the use of continuous perfusion culture systems that maintain a concentrated viable cell mass at low growth rate and relatively high antibody production rate. Continuous perfusion creates a "near stationary phase" at minimal growth rate, requiring a further reduced death rate.

Animal suspension cells and shear

Animal cells are generally considered as relatively sensitive to shear forces compared to bacteria and yeasts cells because of their relative large size (8 - 16 μm) and lack of a cell wall. Whether damage by shear forces will actually occur during a culture of animal cells is dependent on the energy input, the reactor dimensions, the composition of the culture medium and the cells themselves.
For the culture of animal cells in a homogeneous system mixing is required to maintain a homogeneous cell suspension and to provide effective transfer of nutrients to all cells. Mixing in reactors for animal-cell culture is generated by rotating impellers (stirred-tank reactors) or by sparging (bubble column, air-lift loop reactor). Mixing can generate shear forces, resulting from differences in liquid flow pattern (17). For animal suspension cells the differences in liquid flow that matter are the flow differences over a distance in the range of the cell size (diameter).

Sparging and shear

Sparging is the most efficient way of providing oxygen to a high-density cell culture. Though animal cells have a relatively low specific oxygen consumption, the low solubility of oxygen in water or culture medium is causing oxygen to be a key parameter for upscaling. Above a working volume of 5L it is often difficult to reach high cell densities with only aeration via the headspace of the reactor. However, sparging can be detrimental for animal cells (1, 6, 11, 15). The cell death rate shows a positive correlation with the gas flow rate (1, 15) and a negative correlation with the suspension volume in the bubble column (2, 16). The size of the bubbles used for sparging showed a variable correlation with cell death : small bubbles (1.6 - 0.2 mm) caused a greater detrimental effect (1); no difference in cell death rate was found in the range of bubble size from 2 to 5 mm (16).

The results of the group of Tramper confirmed the killing-volume hypothesis for insect cells in a bubble column in TNM-FH medium. The killing-volume hypothesis postulates the existence of a constant killing volume around each air bubble during its life time. The first-order rate constant k_d of cell death caused by sparging is correlated with the gas flow F, the air-bubble volume d_b^3, the suspension volume V, and the killing volume V_k, according to :

$$k_d = \frac{6 F V_k}{\pi^2 d_b^3 V}$$

The cell killing event is associated with the disengagement of bubbles at the suspension surface (2, 4) or with the formation of bubbles at the nozzle (4, 9). The rising of bubbles was identified as harmless for the cells.

Additives like FCS and Pluronic F68 can provide concentration-dependent protection against sparging (1, 2, 6-9, 12). In serum-free culture medium the addition of BSA provides a concentration-dependent protection for hybridoma cells growing in a continuously sparged air-lift loop reactor (3).

Several mechanisms have been suggested for as well the explanation of cell damage caused by sparging, as for the protective effect of certain components. Two mechanisms of cell damage were proposed by Handa et al.: damage due to rapid oscillations caused by bursting bubbles, and damage due to shearing in draining liquid films in foams. It was proposed that polymers like Pluronic F68 act as cell protective agents by stabilizing foams and thereby reducing film drainage and bubble bursting in the vicinity of the cells.

Pluronic is decreasing the surface tension (8) and it was shown that the hydrophilic-lipophilic balance of Pluronic polyols determines the protective effect (9). Therefore the protective effect of Pluronic was interpreted as a coating of the cell/ media interface and/or the cell/ bubble interface, resulting in reduced cell/bubble interaction (9).

Animal-cell culture media

Culture media for *in vitro* culture of animal cells are complex compared to most chemically defined prokaryotic media. A minimal complete formulation contains near forty different components. Since the start of animal-cell culture the trend has been towards the development of chemically defined serum-free media.
The standard production medium has a viscosity (and density) close to that of water. The surface tension of most basic media formulations are close to the value of water (0.070 N m^{-1}) while the surface tension of most complete serum-containing or serum-free media is lower (around 0.060 N m^{-1}). Harvests of high-density hybridoma fermentations using protein-free media contain the product Mab (50-100 mg/L) and a mixture of host-cell proteins in the range of 50 - 200 mg/L. When serum is used or a serum-free medium containing albumin, than the fermenter harvest will contain 2 - 4 g/L total protein. Products of cells or cell debris can change the surface tension because cells contain many surface-active components that can be actively or passively be released.

This thesis

In general animal cells can be cultured if the cell growth rate is substantially larger than the cell death rate. As it is favourable for production fermentations to culture a high density of cells at low growth rate, the logic consequence is that the death rate has to be further reduced. Causes of cell death include nutrient limitation, presence of toxic components, and shear stress. The study of cell death caused by shear stress due to sparging is the topic of this thesis. The primary variable in the quantification of the shear sensitivity for sparging of the animal suspension cells and the prevention of cell death is the composition of the culture medium.

Thesis contents

This chapter consists of a general introduction, providing an overiew of the status of research on the shear sensitivity of animal cells untill 1990, the start of work on this thesis. In chapters 2 and 3 the effect of a reduction of the serum content of the culture medium is described on the death rate of hybridoma cells in a bubble column. In addition the killing volume hypothesis as developed for insect cells is tested for hybridoma cells.
The effect of antifoam on the shear sensitivity of hybridoma cells in the bubble column is described in chapter 4. In chapters 5 and 6 the effect of addition of respectively polyethylene glycol and dextran on the cell death rate caused by sparging is

monitored in serum-free culture medium. In chapter 7 the significance of developments in the production of biopharmaceuticals with animal cells, like the use of protein-free media and fermentation techniques using high cell densities at reduced growth rate, are discussed in the light of the present kwowledge on shear sensivity of animal cells.

Objectives

As general objective of this thesis can be formulated : to qualify and quantify the effect of media composition on the shear sensitivity of hybridoma cells for sparging.
Additional objectives :

1) verify the killing-volume model for hybridoma cells
2) develop a suitable defined serum-free culture medium
3) test the effect of different additives
4) evaluate the results in a broader perspective

Methodology

Making general conclusions in shear research is difficult because many different cell lines have been tested under different conditions and media types in different shear systems for a different shear-related phenomenon. Since a standardized methodology can reduce this fragmention, a generic approach was chosen, as developed for insect cells at the Wageningen Agricultural University (WAU).
As a result of this generic approach one cell line was chosen for the study of shear sensitivity for sparging in one model sytem, the bubble column (figure 5). If the medium composition was varied, one set of standard conditions was used for the sparging shear-stress experiment. For the verification of the killing-volume model one parameter was changed and other conditions kept constant.
A hybridoma cell line was used because it is one of the animal-cell types that is frequently used for large-scale productions; the product, monoclonal anti-

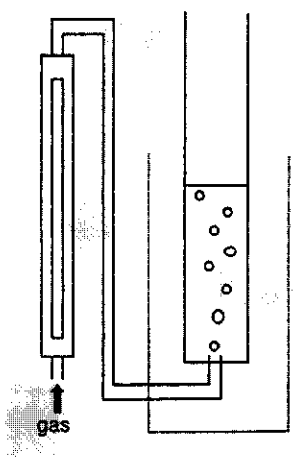

Figure 5 The bubble column used for shear stress experiments. The column is connected to a gas-flow meter and placed in a water jacket.

body (MAb), can be readily quantified and is relatively stable. The cell line used, PFU-83, a murine hybridoma, was well qualified in previous cultures. To generate identical samples for bubble-column experiments, the PFU-83 cells were maintained at steady-state conditions in a chemostat without sparging. This to exclude differences in shear sensitivity caused by a difference in growth rate. The chemostat was not sparged and very mildly agitated so no adaptation of the cells to severe shear-stress conditions could occur. Finally, PFU-83 hybridoma cells are stable cells as measured by their productivity.
The bubble column was chosen as a down-scaled system for sparging shear-stress experiments, that is well suited for testing the different parameters of interest. At the WAU this sytem had been adequately tested for its feasibility. The conditions for the testing of the effect of different media additives were standardized for temperature, volumetric gas flow rate, working volume, height and diameter of the bubble column. A relatively short stress experiment was done (1 to 3 h) to prevent effects of the growth rate interfering the cell death rate.

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2. Effect of serum concentration on production of monoclonal antibodies and on shear sensitivity of a hybridoma

Summary

The effect of serum content of culture medium on the specific production rate of monoclonal antibodies (Mab's) and on shear sensitivity has been studied with hybridoma's, cultured in a continuous stirred tank reactor (CSTR). No decrease in specific Mab-production was found when the serum concentration was reduced from 10 to 2.5%, while steady state cell concentrations were hardly affected as well. In contrast the cell death rate in a bubble column strongly increased when the serum concentration was lowered, which could be ascribed to a reduced physical protective effect by the serum.

Introduction

The use of mammalian cells for the in vitro production of biologicals is increasing. One of the salient examples is the in vitro culture of hybridoma's for the synthesis of monoclonal antibodies (Mab's). Reduction of the percentage of foetal calf serum (FCS) in the culture medium is an important aim in animal cell culture, because of improved economy, easier purification of Mab's and a better defined production process. FCS is a partly undefined and variable mixture of components with a variety of specific and non-specific effects on cells. Even though it seems to be possible to describe a standard serum-free medium, which can support a certain growth of the majority of hybridoma cell lines (1), the use of serum-free medium for large scale production of Mab's is still limited.

The general trends obtained from comparing hybridoma growth and Mab-production in media with (5 or 10%) or without FCS are that the growth rate is equal or less and that the specific Mab-production is comparable or higher in serum-free medium (1,2). However, little research has been done in fermentor systems that allow kinetic analysis with reliable quantification of growth and specific production rate under steady state conditions. One of the non-specific functions of FCS is the physical protective influence it has on the shear sensitivity of cells growing in suspension in aerated cultures (3). Therefore, substituting polymers are often added to animal cells in agitated and aerated cultures on media with low FCS content. Shear sensitivity and oxygen supply generally emerge as ultimate limitations for high-density cultures of suspended animal cells in fermenters, due to the fragility of these cells and the low

solubility of oxygen in aqueous solutions. After receiving little attention in earlier research, the shear sensitivity of animal cells cultured in vitro has received more and more attention in recent years, including studies with hybridoma's (4 - 8).
Agreement exists on the conclusions that animal cells are shear sensitive and that increasing shear forces and longer shear times cause, to a certain level, greater cellular damage. Less clear is the effect of non-lethal shear forces on cell growth and production. Also the extent to which general conclusions can be generated from the studies is limited, due to the lack of similarity in approach and conditions (9). For hybridoma's it has been found that cells in the exponential growth phase are less sensitive to shear than cells in other phases of growth (6,7). No change in growth or production was found for hybridoma's that survived exposure to laminar shear stress (8). In this paper the effects of reducing FCS-content of the medium from 10 to 2.5%, as sole variable of culture conditions, on the specific Mab-production rate and shear sensitivity are reported for hybridoma's, cultured at a fixed dilution rate in a CSTR. The shear sensitivity to air bubbles has been determined in a bubble column. Furthermore the hypothesis derived and experimentally validated for the shear sensitivity of insect cells (11) in a bubble column has been tested on the hybridoma cells with respect to the parameters injected air flow rate and the height of the bubble column. This hypothesis for the death rate of fragile cells in a bioreactor as result of sparging, is based on two assumptions :

1) The loss of viable cells is a first order process ; the mathematical formulation used is

$$\ln C_t/C_o = -K_d\, t \qquad (1)$$

with C_t the viable cell concentration at time = t, C_o the viable cell concentration at t = 0 and K_d = death rate constant

2) Associated with each air-bubble is a hypothetical killing volume X, in which all viable cells are killed :

$$K_d = \frac{24\, F\, X}{\pi^2\, d^3\, D^2\, H} \qquad (2)$$

with F being the volumetric air flow-rate, X the hypothetical killing volume, d the diameter of the air bubble, D the diameter and H the height of bubble column.
During validation of this hypothesis with insect cells in a bubble column (10, 11, 12) it has been demonstrated that the first-order death-rate constant K_d shows indeed a linear correlation with the air low F, the reciprocal height H^{-1} and the reciprocal square diameter D^{-2} of the bubble column. This shows that the hypothetical killing volume X is independent of these physical parameters.

For a bubble diameter d greater than 2 mm it has been found that the K_d is independent of d^{-3}. Therefore, formula (3) can be transformed into :

$$K_d = \frac{4\ F\ X'}{\pi\ D^2\ H} \qquad (3)$$

This by introducing a hypothetical specific killing volume X', which is the ratio of X and the volume of one air bubble. Ongoing research aims at further validation of this hypothesis and at elucidation of the effect of other important process parameters, e.g. dilution rate, growth and Mab-production.

Material and methods

The cell line used in these experiments, PFU 83, is a rat/ mouse hybridoma, producing antibodies directed to corticotropin releasing factor (13). The cells were cultured as a continuous suspension in two separate 1 dm^3 round-bottomed, non- siliconized fermenters with a 0.5 dm^3 working volume and equipped with 0.06 m diameter marine impellers.
The temperature was kept constant at 37°C and the stirrer speed at 60 rpm. The medium consisted of RPMI 1640 (Seromed) with 0.3 g/dm^3 glutamine (Merck) and 2 g/dm^3 NaHCO3, without phenol-red. Just before use 0.1 g/dm^3 streptomycine (Sigma) and 0.3 g/dm^3 glutamine (endconcentration 4 mM) were added. Fermenters were surface-aerated with humidified air containing 5% CO_2, through a 0.2 µm airfilter (Gelman), at a flow of 0.1 dm^3/min. The dilution rate D was 8.33 10^{-6} s^{-1} in all experiments. The % FCS (Sanbio) varied as indicated in the results and discussion section. The pH was not controlled externally, but remained within a range of 7.1 to 7.3. Under the described culture conditions PFU 83 grows as single suspension cells, which show no measurable shear-damage caused by the stirring, nor sedimentate or attach to the glass wall. The limiting factor for growth under these standard conditions is not identified, but regularly measured parameters can be excluded (glucose, glutamin, oxygen).
Product IgG was determined by a quantitative ELISA, with polyclonal Rabbit-anti-Rat antibodies (Dakopatts), screened on a Titertech multiscan(450 nm).
For shear-stress experiments a sample (volume specified in the text) of the above cell-suspensions was taken from the fermenter and transferred to a bubble column with an inner diameter of 0.036 m. On the surface of the suspension 0.02% silicon anti-foam (Baker) was added. The temperature was controlled at 37°C by circulating warm water through a jacket around the bubble column.
During the experiment every 30 minutes a sample from the suspension in the bubble

column was taken and diluted 1:1 with 0.1% Trypan Blue in phosphate buffered saline solution (NPBI). Cell number and viability were determined microscopically using a Fuchs Rosenthal haemacytometer.
As standard set point conditions for a shear-stress experiment in the bubble column were chosen : air flow rate (F) 6 dm^3/h, height (H) 0.08 m and FCS 10%. Under these conditions the time needed (3-4 h) to measure significant cell death is sufficiently short to prevent interference from growth or disturbances from infections. Values of K_d were obtained from the slope of the plot ln (C_t/C_o) versus time t (equation 2).

Results and discussion

Growth and Production

At the chosen, fixed dilution rate of $8.33 \times 10^{-6}\ s^{-1}$, corresponding to a medium flow rate of 0.35 dm^3/day for a 0.5 dm^3 cell culture, a steady state with a sufficiently high cell concentration was obtained, for all serum concentrations tested. Only a slight reduction in viable cell number occurred when the serum content was reduced from 10 to 2,5 % (figure 1). The cell concentration rapidly decreased, however, at 1%

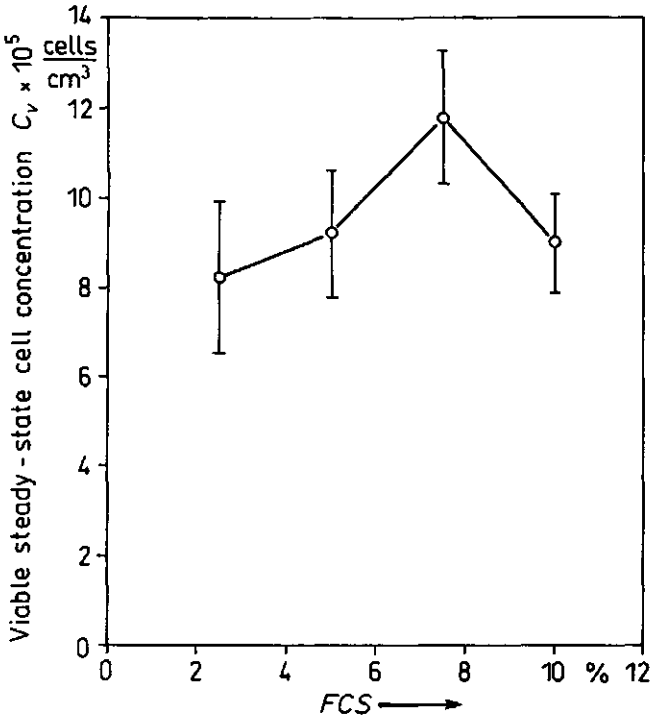

Figure 1
Viable steady-state cell concentration of hybridoma cells in a continuous stirred tank reactor as a function of foetal calf serum concentration. Each point is the average of three different steady states with a minimal length of four days, so the variance is determined for a minimum of 12 values per point.

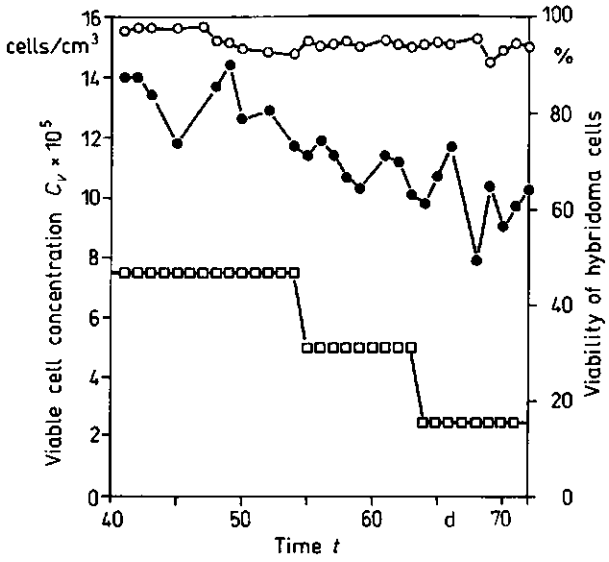

Figure 2 Viable cell concentration (●) and viability (○) of hybridoma cells in a continuous stirred tank reactor during stepwise reduction of foetal calf serum concentration from 7.5 to 2.5 % (□).

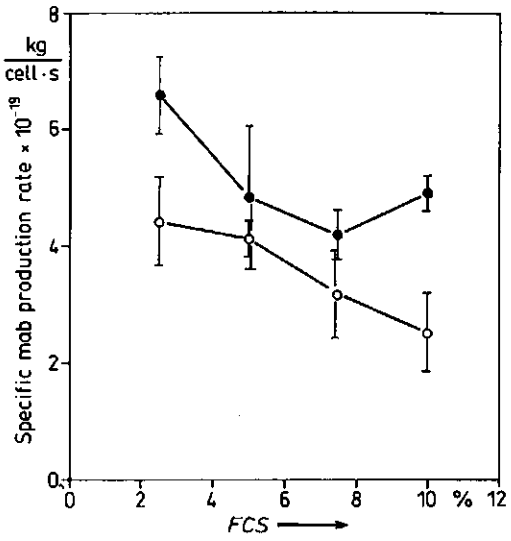

Figure 3 Specific monoclonal antibody production rate of hybridoma cells in a continuous stirred tank reactor as a function of foetal calf serum concentration. One point is the average of double or triple ELISA scores determined for a steady state of minimal 3 days. Two separate runs are shown.

FCS (data not shown), indicating that a serum component becomes growth limiting. Highest steady state cell concentrations were reached at 7.5% FCS, indicating that at a higher serum content some serum factor(s) may become inhibitory for growth.
The viability of the cell culture remained above 90% even at 2.5% FCS, as is illustrated by the daily cell counts of one fermenter-run (figure 2). Dividing the dilution rate by the average viability ratio of 0.93, gives a value for the growth rate of PFU-83 of 8.9×10^{-6} s^{-1}, valid for all experiments.
Average IgG-production of PFU-83 was 4×10^{-19} kg/(s.cel), marking it as a medium class producer (14). Figure 3 shows that the specific Mab-production rate does not decrease as a result of a reduction in serum content. For the shown, different steady-states, obtained for the two fermenter runs, the trend is that the specific Mab-production rate increases slightly with decreasing serum content. This is in aggreement with data in literature, comparing production of Mab's in serum-free or serum containing media (1,2).
Production of Mab's by hybridoma PFU-83 is stable over at least 3 months, since no difference in specific Mab-production rate was found under similar steady state conditions in the CSTR over such a period.

Shear sensitivity

The effect of varying the height of the bubble column results in a linear correlation between death rate constant Kd and the reciprocal height H (figure 4), showing that the hypothetical specific killing volume X' is independent of this parameter. A similar plot can be made for Kd versus the volumetric air flow rate F, resulting in a linear relation (figure 5), meaning X' is independent of this parameter as well.
The model for shear stress sensitivity in a bubble column, such as derived and validated for insect cells is thus also valid for hybridoma's with respect to the parameters H and F. Presently, the effect of the other parameters, i.e. the column and air-bubble diameter, are investigated in conjunction with another important process parameter, i.e. the dilution rate of the continuous culture.
The effect of serum on the air-bubble shear sensitivity of PFU-83 was tested by performing shear experiments using cells, cultured in the CSTR at steady states with different FCS content. To be able to distinguish between physical and biological effects of serum, shear stress experiments in the bubble column were performed twice, once with the same serum content as in the fermenter and once with a serum percentage added up to 10% FCS. Because cell concentrations and viability are hardly affected by the serum reduction to 2.5% (figures 1 and 2) all suspension volumes taken from the fermenter have comparable starting cell numbers and identical growth rate.
As visible in figure 6 the protective physical effect of serum is evident. Adding 2,5% or 5% FCS to a suspension of cells cultured at 7,5% and 5% respectively, completely protects the cells against air bubble shear stress, as compared to cells from a 10 % FCS steady state. This indicates that reducing FCS-content from 10 to 5% has only

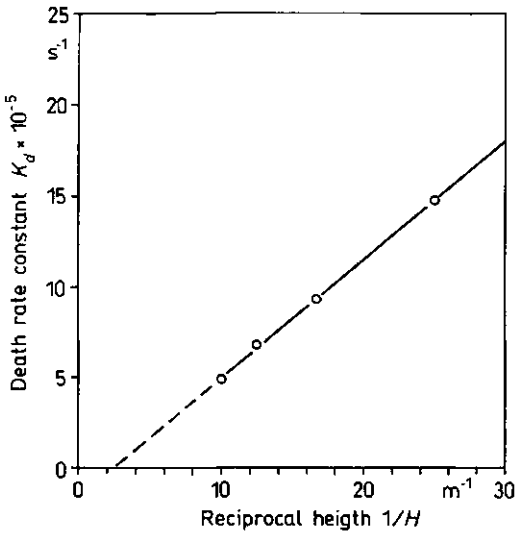

Figure 4 First-order death-rate constant of hybridoma cells in a bubble column as a function of the reciprocal height.

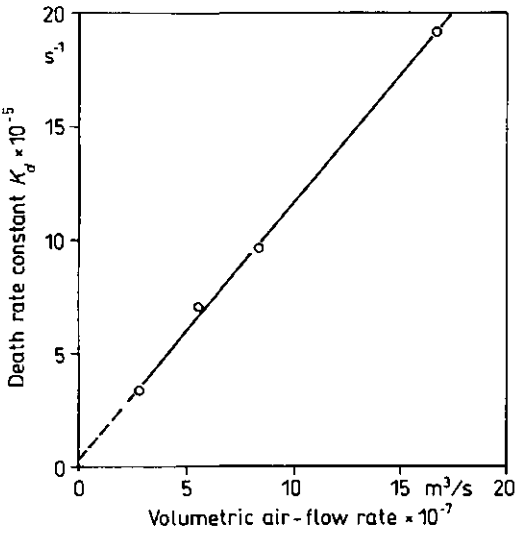

Figure 5 First-order death-rate constant of hybridoma cells in a bubble column as a function of the volumetric air flow rate.

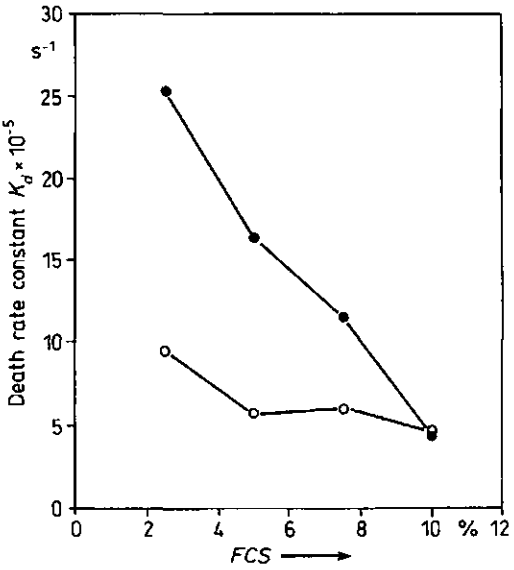

Figure 6 First-order death-rate constant of hybridoma cells in a bubble column as a function of the foetal calf serum concentration, at which the cells were growing in a steady state situation in the continuous stirred tank reactor. Without addition of extra serum (l) and with serum concentration added up to 10% (m) in the bubble column.

a physical protective effect for sparger-aeration. A slight biological effect can be noticed at 2.5% FCS.

Derived from the data in figure 6 and formula (4) values for X' are found to increase from 0.0022 at 10% FCS to 0.0121 at 2.5% FCS. For insect cells an average value for X' was found of 0.004 in medium with 10% FCS and 0.1% methylcellulose, as an average of all experiments in the bubble column (11). This indicates that PFU-83 hybridoma cells are about equally sensitive for aeration stress than the insect cells under comparable circumstances.

Conclusions

Reduction of the serum content of culture-medium from 10 to 2.5% for PFU-83 at a growth rate of 8.9×10^{-6}, has the following effects :

- the steady state cell concentrations and viability are hardly affected at the chosen dilution rate. Because the viability was approximately constant and above 90%, the growth rate was also constant for all experiments. Adding compensating factors for serum to maintain this growth rate only seems usefull at serum concentrations under 2.5%.

- the specific Mab production rate is certainly not decreasing as a result of serum reduction.
- the shear sensitivity of the cells for air bubbles is increasing as a result of the reduction in serum content, because of the loss of the physical protective effect of serum. The hypothetical killing volume X is directly related to FCS-content of media, as can be expected since the killing volume is a factor that contains both cell and media components.

In vitro animal cell culture systems that exert considerable shear stress on the cells, especially met with large scale culture, shear forces will be a key parameter on media with low serum content. It is therefore logic that in cultures with a lower FCS content serum substituting polymers are added whenever possible.

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Nomenclature

K_d	death rate constant	s^{-1}
K_g	growth rate constant	s^{-1}
D	diameter of bubble column	m
d	diameter of air bubble	m
H	height of bubble column	m
X	hypothetical killing volume	m^3
X'	specific hypothetical killing volume	(-)
F	volumetric air flow rate	m^3*s^{-1}
C	number of viable cells	$cells*m^{-3}$
C_o	number of viable cells at t = 0	$cells*m^{-3}$
t	time	s

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3. Effects of low serum concentrations (0 - 2.5%) on growth, production and shear sensitivity of hybridoma cells

Summary

In a stirred culture of hybridoma cells the effects of serum reduction from 2.5 to 0% on growth and monoclonal antibody production have been investigated. The shear sensitivity of cells from the same culture has been tested in a bubble column. Serum reduction does not greatly affect viable-cell concentrations but cell specific monoclonal-antibody production rate shows a decreasing trend. A gradual increase in sensitivity for sparging has been measured in a bubble column at decreasing FCS concentrations. This increase in sensitivity is not the result of a long-term biological effect. Finally, the model describing the death rate of insect cells in bubble columns has now been completely validated for the pertinent hybridoma-cell line.

Introduction

The serum component of medium is known to provide a protective effect against shear for hybridoma cells (4,7,8). Therefore the use of serum-free medium can cause problems under culture conditions involving higher shear forces. Elucidation and quantification of the protective effect of serum is essential for the rational design of animal-cell bioreactors and one of the aims of our studies. In smaller-scale bioreactors the damaging effect of stirring on cells is negligible at stirrer speeds large enough to keep a homogeneous suspension (1,3,10,11), provided no entrainment of bubbles occurs caused by a vortex around the impeller shaft (6). Improving the oxygen supply by sparging with air can similarly cause significant damage to cells (3,4,10,11), even when aeration is limited, unless protective agents like Pluronic are added to the culture medium (3,4,11).

In our work we study the death rate of cells as result of sparging with air in a bubble column, under conditions at which the growth rate of the cells is slow compared to the death rate. In this way physical parameters determining bioreactor design can be optimized with respect to shear stress and oxygen transfer (15). In a previous study (13) the effects of reducing the serum content of culture medium from 10 to 2.5% on cell growth, monoclonal antibody (Mab) production and shear sensitivity have been reported. In addition to this the effects of varying the air-flow rate and the height of the bubble colum n have been shown to correspond to the model that describes the

death rate of hybridoma and insect cells caused in a bubble column as result of sparging (5,12).
In the work presented here the effect of reducing the concentration of foetal calf serum (FCS) from 2.5 to 0% on the growth, the Mab production and the shear sensitivy of hybridoma cells has been studied. Also the effect of varying the diameter of the bubble column, used for shear-stress experiments, on the death rate is described.

Material and methods

The studied cell line PFU 83, is a P3-myeloma derived rat/ mouse hybridoma (14). A sample from the same working cell bank, as used in previous shear experiments, was thawed (13). The cells were grown on either RPMI 1640 (Seromed, Biochrom KG, Berlin, Germany) medium or a 3:1 mixture of Dulbecco's Modified Eagle's and Ham's F12 (DME/F12) medium (both Flow, ICN Biomedicals, Zoetermeer, The Netherlands). The RPMI contained additions to give end concentrations of 0.6 g/dm^3 (4 mM) glutamine, 2 g/dm^3 $NaHCO_3$ and 0.1 g/dm^3 streptomycine (Sigma, St.Louis, USA). The DME/F12 medium was supplemented with glutamine (0.6 g/dm^3), $NaHCO_3$ (3.5 g/dm^3), 2-mercapto-ethanol (3.9 mg/dm^3), transferrin (Sigma, 5 mg/dm^3), selenite (2.5 $\mu g/dm^3$), ethanolamine (0.7 mg/dm^3), ascorbic acid (5 mg/dm^3) and glutathione (reduced, 1 mg/dm^3). All supplements were obtained from Merck (Darmstadt, Germany), unless stated otherwise.
The media contained a varying content of heat-inactivated FCS (CCClab, Sanbio, Uden, The Netherlands) as sole variable during the experiments, as specified in the results and discussion section.
The cells were cultured as a continuous suspension in a 1 dm^3 round-bottomed fermenter with a marine impeller, as described previously (13). The working volume of the cell suspension was 0.5 dm^3 and the dilution rate 8.33 10^{-6} s^{-1} in all experiments. Cell densities and other parameters were determined 4 mean residence times after a change in the serum concentration.
The Mab concentration in the supernatant of the effluent was determined by a quantitative ELISA with polyclonal Rabbit-anti-Rat antibodies (Dakopatts, Glostrup, Denmark), screened on a Titertech multiscan (450 nm). Total protein in cell-free effluent was determined according to Bradford (2). Samples for these assays, from the CSTR, were centrifuged for 30 s in a DADE immofuge and the supernatant frozen at -20°C until analysis.
The stress experiments were executed identically as in the previous study (13), with as standard conditions : 80 cm^3 of cell suspension in a bubble column with an inner diameter of 0.036 m, height H 0.08 m, are sparged with an air-flow rate F of 6 dm^3/h for 2 or 3 h.
Silicon anti-foam (J.T.Baker, Deventer, The Netherlands) was added at 0.002% (v/v) at the beginning of each experiment and extra anti-foam was added when necessary

by tipping with 1:10 diluted anti-foam solution in a Pasteur Pipet, later during the stress experiment to prevent formation of a foam layer.

Results and discussion

Cell growth and Mab production

PFU-83 hybridoma cells were grown in a continuous culture with decreasing serum content to study the effects on growth and production, as indicated in figure 1. The CSTR culture also supplied well-defined and reproducible samples for shear-stress experiments in a bubble column to determine the effect of FCS concentration on the death rate as a result of sparging with air.

On RPMI medium cell concentrations decrease with a serum reduction from 5 to 2.5%. A further reduction in serum content results in a strongly-reduced growth (13). To obtain comparable samples for shear-stress experiments in the low range of serum concentrations

(2.5 to 0%), it was necessary to use a medium that provides good cell growth under serum-free conditions. The enriched DME/F12 combination, described in the material and methods section, was selected as a simple medium that supported sufficient growth.

A change in basic medium on day 18, i.e. substituting RPMI for DME/F12 - both

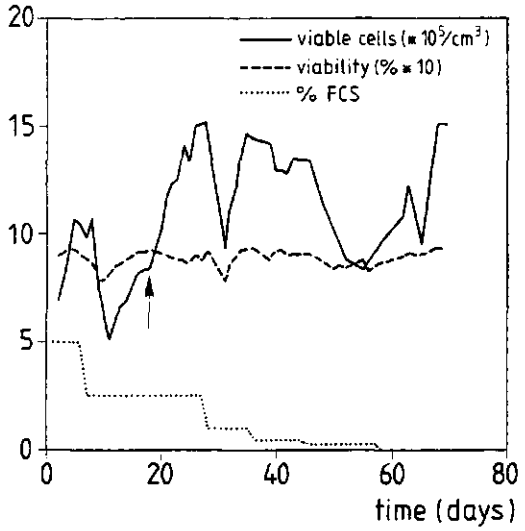

Figure 1 Viable-cell concentration and viability of hybridoma cells in a continuous stirred-tank reactor during step-wise reduction of foetal-calf serum content of the medium from 2.5 to 0 %. The arrow indicates a transfer from RPMI to DME/F12 medium.

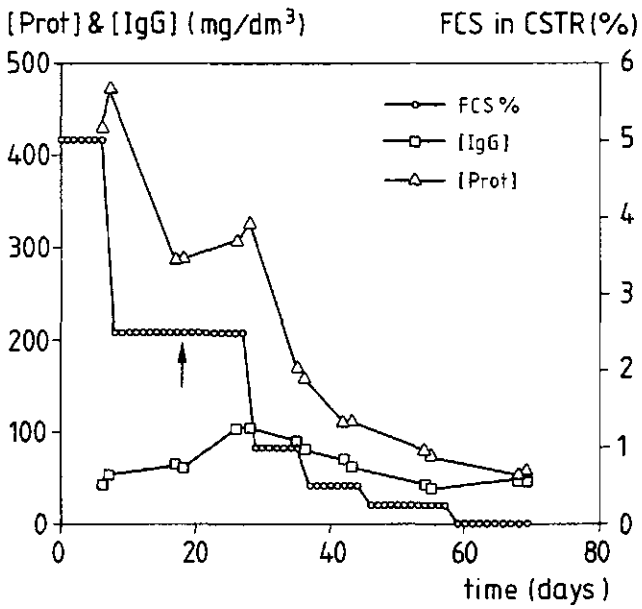

Figure 2 Concentration of total protein and monoclonal antibodies in the cell-free effluent of hybridoma cells in a continuous stirred-tank reactor during step-wise reduction of foetal calf serum content in the medium from 2.5% to 0%. The arrow indicates a transfer from RPMI to DME/F12 medium.

containing 2.5% FCS - results in a considerably-higher cell concentration (figure 1). A reduction in serum content from 2.5 to 0.5% FCS, on DME/F12 medium, causes only a slight reduction in cell density, while viability remains at least at 90%. At 0.25% FCS the cell number decreases, while the viability reaches a minimum value of 85%. The cell concentration restores to higher values on completely serum-free medium (figure 1). The Mab concentration shows an increase, from 60 to 100 mg/dm^3, after the transition from RPMI to DME/F12 medium at 2.5% FCS (figure 2). After the medium switch the product concentration ultimately decreases with the reduction in serum concentration to an equal level as on RPMI with 5% FCS.

Assuming steady-state kinetics, at least four mean residence times after a change in serum concentration, the cell specific MAb production rate (CSMPR) can be estimated [MAb-concentration (kg/dm^3) x Dilution rate (s^{-1}) / Viable cell concentration (number/dm^3). Figure 3 shows the results for the fermenter run described in this section (run A), in addition to the results of previously published runs (B & C, ref. 13). The increasing trend for the CSMPR on RPMI medium with decreasing FCS content from 5% to 2.5% is confirmed by the data of this run.

The transition from RPMI to DME/F12 medium at 2.5% FCS hardly affects the CSMPR. A further reduction of the serum content from 2.5 to 0% causes a decrease

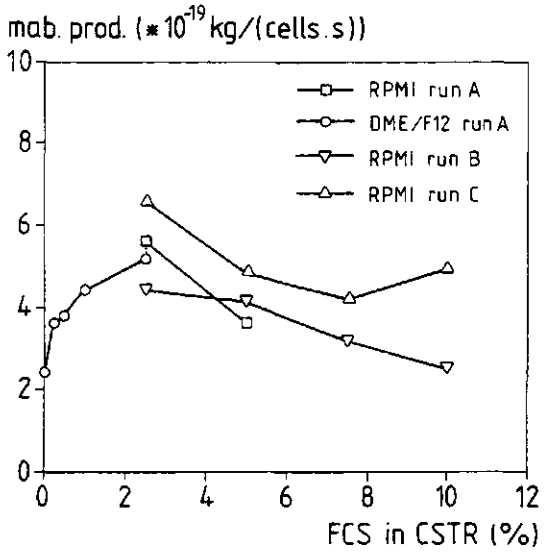

Figure 3 Specific monoclonal antibody production rate of hybridoma cells in a continuous stirred-tank reactor during step-wise reduction of foetal-calf serum content in the medium: from 10% to 2.5% on RPMI, from 2.5% to 0% on DME/F12 medium. Run A is described in this article, the dotted line indicates the transition of basic medium from RPMI to DME/F12 medium. Runs B and C are taken from the previous article (13).

of the CSMPR, indicating that FCS contains factors that stimulate the production of Mab's. Since PFU-83 has proven to be stable in many comparable CSTR runs, non-specific loss of Mab production of this hybridoma cell line is very unlikely. DME/F12 medium is more efficient for Mab production by PFU-83 compared to RPMI medium, mainly because it supports the growth of more viable cells. The total protein content of cell-free supernatant of the effluent decreases concomitant with the reduction of the serum concentration (figure 2). The Mab concentration becomes a considerable fraction of the total protein of the effluent.

Shear sensitivity

Like in the previous study, a shear-stress experiment has been executed in the bubble column at the same serum concentration as in the CSTR as well as at 2.5% FCS, where the difference in concentration is added just before the start of the shear experiment. The reduction in serum concentration from 2.5 to 0% causes a large increase in the death-rate constant k_d (figure 4). However, the increase in shear sensitivity caused by the elimination of serum from the DME/F12 culture medium can be completely nullified by adding extra serum up to 2.5% just before the shear experiment. Thus also at lower serum concentrations FCS has essentially no long-term biological effect,

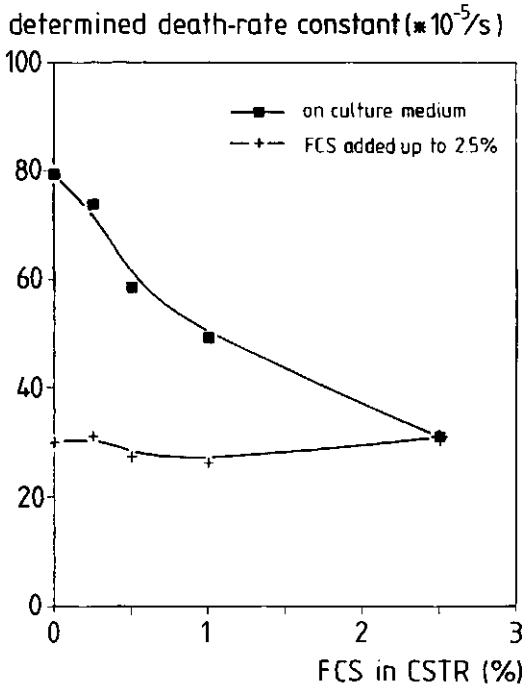

Figure 4 First-order death-rate constant of hybridoma cells in a bubble column as a function of the foetal-calf serum concentration at which the cells were growing in a continuous stirred-tank reactor. Without addition of extra serum, or with serum concentration added up to 2.5% just before the shear experiment in the bubble column.

but rather provides a direct non-specific physical protective effect. This corresponds to the results of Kunas and Papoutsakis (7), who found that FCS provides a short term protective effect against intensive stirring for hybridoma cells in a stirred bioreactor. Recently viscometric studies showed that prolonged exposure to FCS makes hybridoma cells more tolerant to laminar shear, while a short term effect did not occur (9).

For the shear-stress experiments with variable bubble-column diameter D, PFU hybridoma cells were grown in a CSTR on RPMI medium containing 5% FCS. The number of viable cells was constant within a range of 1.2 to 1.5 10^6/ cm^3 with a minimum viability of 90%, which is comparable to previous runs under identical conditions. The concentration of Mab in the effluent did not vary significantly in time. Thus identical samples for shear-stress experiments were obtained.

For insect cells the following equation for the death rate in a bubble column as result of the sparging, has been validated (12) :

$$k_d = \frac{4\,F\,X'}{\pi\,D^2\,H}$$

where X' is the dimensionless specific hypothetical killing volume. Previously (13) we have shown that the death-rate constant kd is proportional to the volumetric air-flow rate F and the reciprocal height H. Figure 5 shows that k_d is also proportional to the reciprocal square diameter D of the bubble column. This means that X' must be independent of these variables and that the hypothetical killing-volume hypothesis derived for insect cells is also valid for the hybridoma cells of this study.

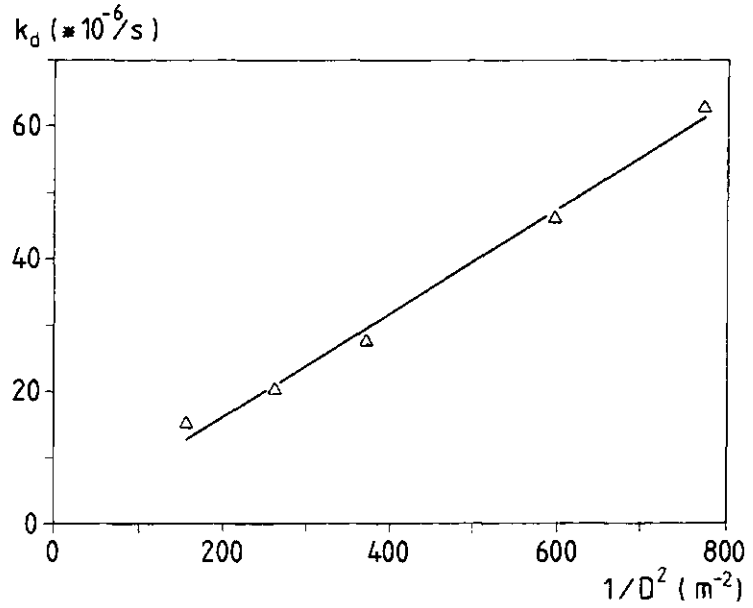

Figure 5 First-order death-rate constant of hybridoma cells in a bubble column as a function of the reciprocal square diameter of the bubble column.

Conclusions

A reduction of the serum content of culture medium from 2.5 to 0% for PFU-83 hybridoma cells in a CSTR at a growth rate of 8.9 x 10^{-6} s^{-1}, has the following effects:

* Viable-cell numbers and viability do not show drastic changes in the selected DME/F12 medium

* The specific Mab production rate decreases to some extent
* The shear-stress sensitivity of the hybridoma cells, measured by determining the death-rate constant k_d in a bubble column, is increasing
* FCS provides a non-specific protective effect against intensive shear stress caused by sparging
* The killing-volume hypothesis derived for insect cells in a bubble column, also applies to the PFU-83 hybridoma cells of this study

Acknowledgements

The authors are indebted to Ms.Wieke Coops and Ms.H.Hoff for technical assistance.

Nomenclature

D	diameter of bubble column	m
F	volumetric air-flow rate	m^3/s
H	height of bubble column	m
k_d	death-rate constant	s^{-1}
X'	specific hypothetical killing volume	(m^3/m^3)

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4. Effect of silicone antifoam on shear sensitivity of hybridoma cells in sparged cultures

Summary

Addition of high concentrations of silicone antifoam (AF) to a suspension of hybridoma cells in a bubble column reduces the death rate when using medium without protective component. In contrast, high AF concentrations can cause detrimental effects in sparged medium with a protective component, such as applied for large-scale animal cell cultures.

Introduction

In the scaling up of in vitro cultures of animal cells great attention has to be given to the shear sensitivity of the cells and as a consequence to the oxygen transfer in the bio-reactor (22). Sparging with air or pure oxygen as a method of oxygen supply is simple and efficient but can cause cell damage. This damage has been attributed to the generation of bubbles in the sparger region (12), the bursting of bubbles at the surface of a suspension (6, 8, 18) or by film drainage in unstable foams (6). Sparging in a protein-containing liquid, like a suspension of animal cells in culture medium, results in foaming. Media used for sparged animal-cell cultures contain protective additives, like fetal calf serum (FCS) and Pluronic F68 (13). These protective components have a foam-stabilizing effect. The formation of a stable foam has been connected with the protective effect against sparging for hybridoma cells growing in a bubble column (6). Another beneficial effect is that foaming liquids usually are non-coalescing, resulting in a larger surface for gas exchange (9,15).
Adverse effects of foam include the loss of cells in the foam (1, 2, 5), reduction of effective reactor volume, and risk of overflow and contamination (15). Also the improvement of oxygen transfer by preventing coalescence can be reduced by the adsorbance of foam-promoting molecules to the interface of bubbles, creating a more rigid interface (9, 15). A certain amount of foaming is acceptable in fermentations but excessive foam formation should be prevented (15). Therefore antifoams (AF), especially silicone emulsions, are widely used in animal-cell culture in bubble columns, air-lift loop reactors and sparged stirred-tank reactors. Drawbacks of the use of AF in cultures of animal cells include toxic effect of certain AF types (1, 11), difficult separation from the product (1, 15) and the possible problem of approval by the authorities for pharmaceutical and food applications (22). The reported toxic effect of certain AF types for different types of animal cells seems to be mainly due to the use of

industrial grade AF emulsions (1, 11), probably indicating the presence of contaminants.
In previous work we have reported the effect of reduction of the serum concentration on the shear sensitivity of hybridoma cells in a bubble column. The first-order death-rate constant k_d was quantified in the presence of a minimum amount of silicone AF (20, 21). One of the aims of our research is to quantify the cell death rate constant k_d under standardized conditions in a bubble column such that the cell death rate is usually much greater than the defined cell growth rate. This is done to evaluate which factors determine cell death caused by sparging without interference of cell growth.
The addition of AF to prevent foam formation in the bubble column causes small fluctuations in AF concentration during a sparging shear-stress experiment, dependent on which protective component is present at a certain concentration in the culture medium. Therefore it becomes necessary to evaluate the effect of different concentrations of AF on the shear sensitivity of hybridoma cells in the bubble column. This also because in a recent paper it has been stated that the foaming properties of culture medium have not been considered adequately and that foaming and cell losses in the foam layer are major problems in stirred and sparged bioreactors (23).
In this study the effect of different concentrations of medical grade silicone AF have been evaluated for hybridoma suspension cells in a bubble column in medium with serum or polyethylene glycol (PEG) as protective component and in serum-free medium without protective component.

Materials and methods

Cell line and medium

The cell line used, PFU-83, is a rat/mouse hybridoma suspension cell which produces monoclonal antibodies against rat/ human corticotropin releasing factor (19).
The culture medium was either RPMI 1640 (Seromed) + 0.6 g/dm^3 (4mM) glutamine + 2 g/dm^3 NaHCO3 + 5% FCS (Sanbio) + 0.1 g/dm^3 streptomycine (Sigma), or 3:1 DMEM (Flow)/Ham's F12 (Seromed) serum-free [DME/F12] medium with 375 µg/dm^3 EDTA (Titriplex III) and further additions as described previously (21). This serum-free medium contains 5 mg/dm^3 transferrin (human, Sigma) as the only protein component. The polyethylene glycol (PEG) polymers PEG 6000 and PEG 20000 were always added in a concentration of 1 g/dm^3, from an autoclaved stock solution of PEG in aqua bidest of 100 g/dm^3. PEG has been been tested previously in sparged animal cell cultures (12).
Components mentioned without the name of the manufacturer have been obtained from Merck.

Culture method and shear stress

The cells were cultured as a continuous suspension in a 1 dm^3 continuous stirred-tank reactor (CSTR) with a working volume of 0.5 dm^3 and a growth rate of 8.5 10^{-6} s^{-1} [growth rate = dilution rate/viability ratio; viability ratio = number of viable cells/total cell number].

The CSTR culture provided defined samples for sparging shear-stress experiments in the bubble column under standard conditions, as described previously (20). The suspension (0.08 dm^3) in the bubble column was sparged with a volumetric air flow rate of 6 dm^3/h, resulting in a gas flow per unit culture volume of 75 h^{-1} [corresponding to 1.25 vvm]. This holds for all shear-stress experiments, unless stated otherwise in the results and discussion section.

Experiments in the bubble column were undertaken to quantify the first-order death rate constant k_d under circumstances that usually cause a much higher death rate than the known growth rate. This means that the standard conditions in the bubble column result in substantial cell death unless a protective component is present in a sufficiently high concentration.

A few seconds before the start of an experiment a certain amount of non-autoclaved 1:10 diluted AF was added. The AF used was medical grade antifoam B silicone emulsion (J.T.Baker) containing polydimethylsiloxan. The generation of a foam layer on the medium surface was prevented by touching the surface with a Pasteur pipet filled with a 1:25 diluted AF (tipping) when necessary. The standard experiment in the bubble column is therefore carried out in the absence of foam. The standard bubble column experiment lasted for 3 hours with sampling every 30 minutes, starting with an initial viable cell concentration above $10^6/cm^3$. Sampling and cell counting was stopped within 3 hours when the number of viable cells dropped below $10^5/cm^3$. When the viable cell number decreased rapidly samples were taken more often.

Cell counting and analysis

Cell numbers were determined under the microscope with a Fuchs-Rosenthal haemacytometer by the trypan-blue exclusion method.

The change in number of viable or dead cells is expressed as a percentage of the total cell concentration (viable and trypan blue stained) at the beginning of the experiment. This was done to make changes in cell numbers comparable to changes in activity of lactate dehydrogenase.

The first-order death-rate constant k_d was determined from the slope of the plot ln $(C_{v,t}/C_{v,0})$ versus time t, with $C_{v,t}$ being the viable-cell concentration at time = t and $C_{v,0}$ the viable- cell concentration at t = 0.

Biological oxygen monitoring

The cell-specific oxygen-consumption rate (CSOCR) of PFU-83 cells was deter-

mined by biological oxygen monitoring with a YSI membrane kit (standard) in a Tamson vessel at 37°C. The 0 and 95% set points for the oxygen electrode were adjusted with nitrogen and air containing 5% CO_2. Suspension samples of 10 cm^3 were taken from the standard 1 dm^3 CSTR culture and transferred rapidly into the Tamson vessel. This takes about one minute. Shortly after adding a certain amount of AF the measuring starts. The CSOCR was calculated by dividing the decrease in dissolved oxygen, the oxygen uptake rate (mol O_2 m^{-3} s^{-1}), by the viable cell number.

Lactate-dehydrogenase (LDH) assay

The LDH assay was executed with cell-free supernatant (2 minutes 500 x g in a DADE Immufuge) with a method based on the Boehringer LDH monotest, similar to Racher et al., 1990 (16). The increase in LDH activity, before and after a shear-stress experiment, is expressed as percentage of the 100% LDH score, which is determined with a 0.1% Triton X-100 (Merck) treated (releasing all LDH) sample of the cell suspension before a bubble column experiment. Cell-free supernatant samples were kept at -20°C for a maximum of two weeks.

Results and discussion

Sparging death rate and antifoam effect

The effect of varying the concentration of silicone AF on the death rate of PFU-83 hybridoma cells was examined in a bubble column in RPMI medium containing 5% FCS and serum-free DME/F12 medium with or without PEG. For PFU-83 cells cultured in RPMI medium with 5% FCS the death-rate constant k_d increases with a higher AF concentration (figure 1). This experiment was repeated with PFU-83 cells cultured in the CSTR in serum-free DME/F12 medium. Just before a bubble column experiment in the bubble column PEG 20000 and a variable concentration of AF were added to the suspension sample. Again enhancing the AF concentration from 0.002 to 0.1% causes a four to five-fold increase in the death-rate constant k_d (figure 1). During these experiments it was occasionally necessary to prevent the formation of foam by tipping with diluted AF emulsion.

In serum-free DME/F12 medium without protective component the sparging in the bubble column causes a high death rate. In order to try to reduce this death rate the volumetric air-flow rate was reduced to 3 dm^3/h. An increase in AF concentration now results in a lower k_d (figure 2). This lower k_d is still substantially higher than the increased k_d values at high AF concentration in medium with a protective component, despite the difference in air-flow rate.

In medium without a protective component and with addition of AF to a low concentration at the beginning of a bubble column experiment, tipping with diluted AF was necessary during the experiment to such a degree that the AF concentration has

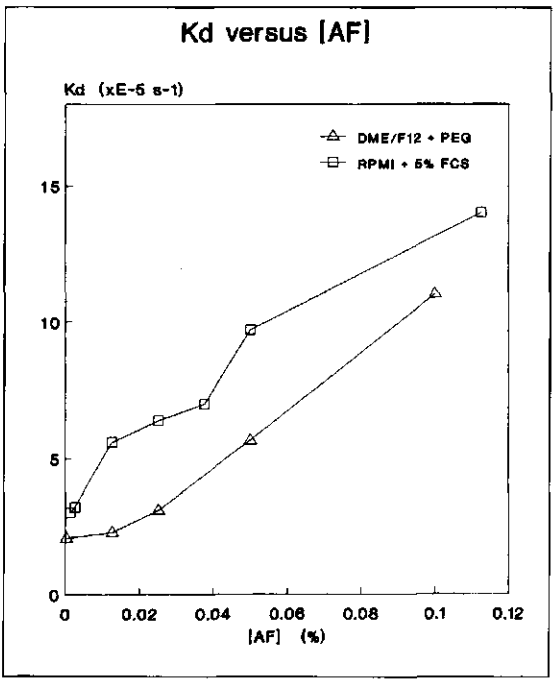


Figure 1 First-order death-rate constant of hybridoma cells in a bubble column at an air flow rate of 6 dm^3/h as a function of the concentration of antifoam, added at the beginning of a bubble column experiment, in culture medium with serum or PEG as protective component.

to be corrected for the extra added 1:25 diluted AF. The corrected end-values are also shown in figure 2. At higher initial AF concentrations the tipping was necessary less often and can be neglected. On the average, stress experiments in medium without protective component lasted about 1.5 hour.

Antifoam in serum-free medium with PEG

Because only medium with a protective component can be applied for the cultivation of animal cells using sparging, the detrimental effect of AF was further examined in DME/F12 medium with PEG 6000 or 20000. Medium supplemented with either PEG 6000 or PEG 20000 resulted in similar k_d values in the standard shear experiments [data not shown].

Bubble column experiments with PEG 6000 or 20000 and a high (0.1%) AF concentration resulted in an increase of trypan-blue stained dead cells (figure 3a and 3b), especially in the beginning. Lysis of cells is indicated by the reduction of the total cell number. The reduction in viable cells before and after a shear-stress experiment cor-

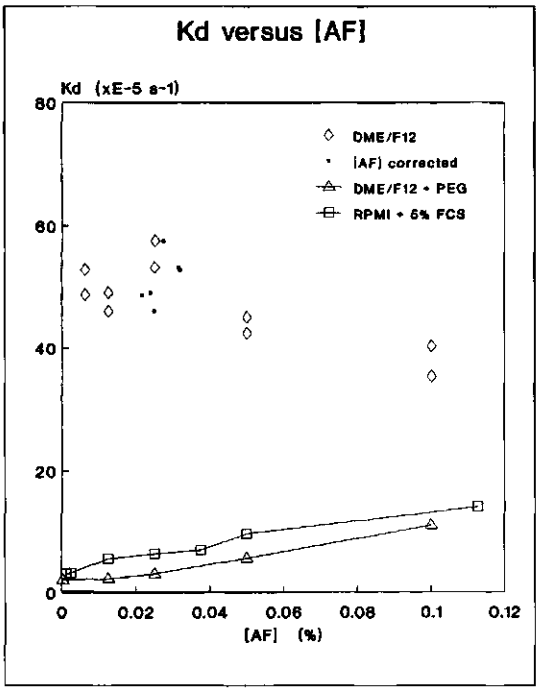


Figure 2 First-order death-rate constant of hybridoma cells in a bubble column at an airflow rate of 3 dm^3/h as a function of the initial concentration of added antifoam in DME/F12 medium without protective component. The values of the death-rate constant for the low antifoam concentrations are also shown after correction for tipping with 1:25 diluted antifoam solution during the bubbling experiment. The results given in figure 1 (obtained at 6 dm^3/h) are included for comparison.

responds well with the increase in relative LDH activity (figure 4). For 0.1% AF the results of duplicate experiments are shown to give an indication of variance.
Addition of 0.1% AF to a control sample of the cell suspension from the CSTR, incubated in culture flasks for 3 to 4 hours, did not cause any change in cell numbers. This shows that AF makes the cells more vulnerable to shear caused by sparging but does not have a short term toxic effect. The addition of 0.1% autoclavated AF emulsion however did have a long term effect because the growth of the PFU-83 cells in culture flasks with this addition was strongly reduced after 3 days in an incubator. This indicates that a high AF concentration does have a cytostatic effect on PFU-83 cells.
The death-rate increasing effect of silicone AF was further investigated by determining the effect of the AF concentration on the consumption of oxygen of PFU-83 cells. The cell-specific oxygen-consumption rate (CSOCR) is decreasing with increasing AF concentration in serum-free DME/F12 medium with PEG (figure 5). A similar

decreasing effect was found using RPMI + 5% FCS and DME/F12 medium without protective component. The reduction is most prominent at the lower AF concentrations; at higher concentrations saturation occurs. A reduction of the oxygen absorption rate [OAR = OUR] caused by addition of 0.1% silicone base compound AF has also been reported for yeast cells (14).
Because AF is a hydrophobic surface-active substance it is likely to exert its effect by changing the properties of either the gas-liquid interface or the cell-medium interface (7). Since the gas-liquid interface is absent during the biological oxygen monitoring the results of figure 5 suggest that the detrimental effect of silicone AF on shear sensitivity of cells might involve an effect of AF on the cell membrane.

Antifoam in medium without protective component
As stated before a medium without protective component cannot be applied for animal cell culturing using sparging. Because adding an increasing AF concentration is causing an opposite effect on cell death in medium without a protective component as in medium with a protective substance, control experiments concerning the effect of silicone AF in DME/F12 medium without protective component, have been exe-

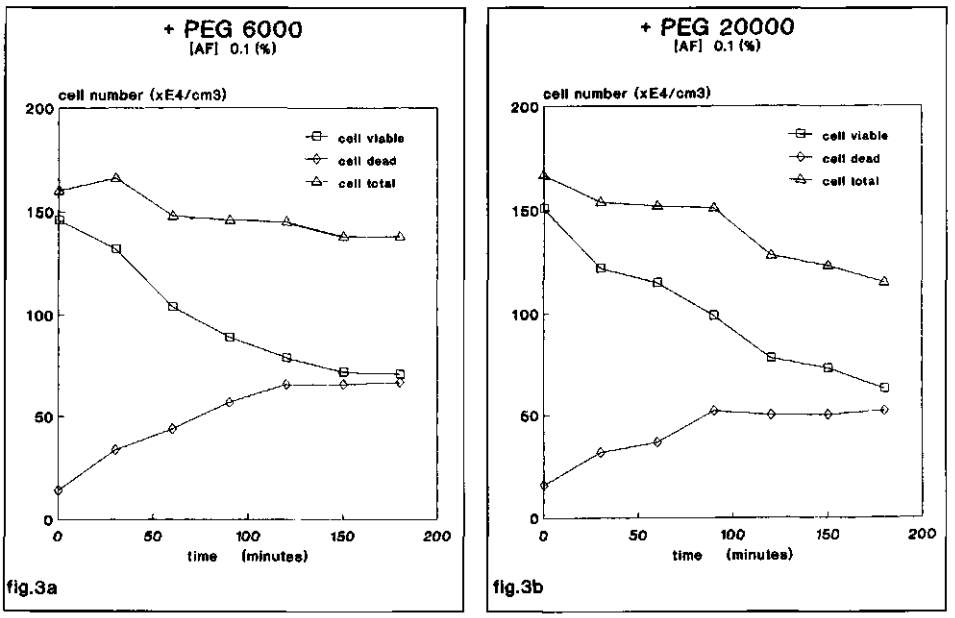


Figure 3a and 3b Change in cell numbers of hybridoma cells in a bubble column during a bubble column experiment in DME/F12 medium with 0.1% antifoam and 1 g/dm^3 PEG 6000 (a) or PEG 20000 (b).

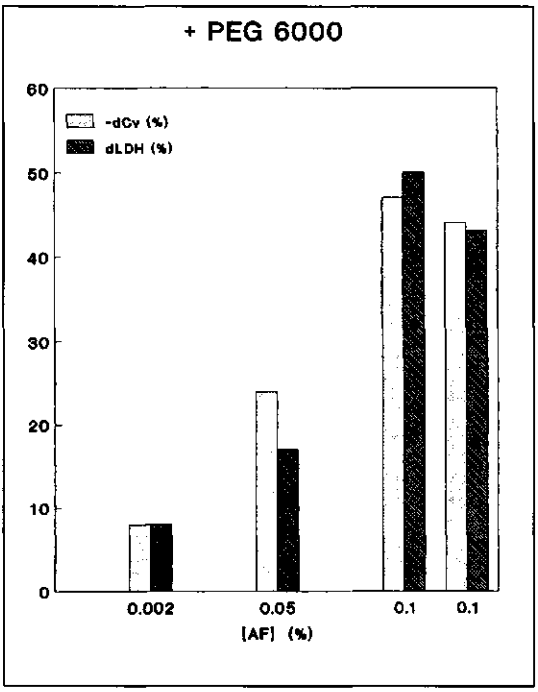


Figure 4
Relative change in viable-cell number (-dCv %) and LDH activity (dLDH %) of hybridoma cells in a bubble column in DME/F12 medium with 1 g/dm^3 PEG 6000 after a bubble column experiment at various antifoam concentrations (data partly used for figure 3a and 3b).

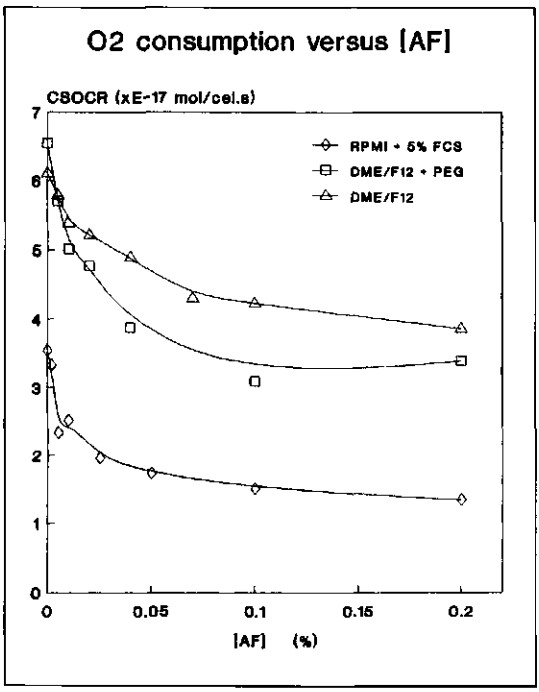
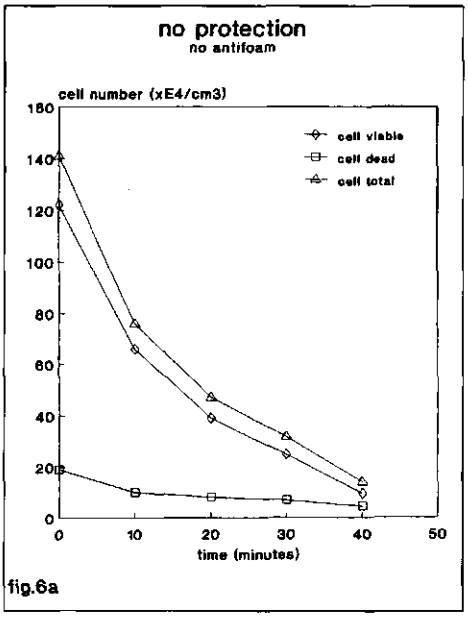


Figure 5
Cell-specific oxygen consumption rate (CSOCR) of hybridoma cells as a function of antifoam concentration.



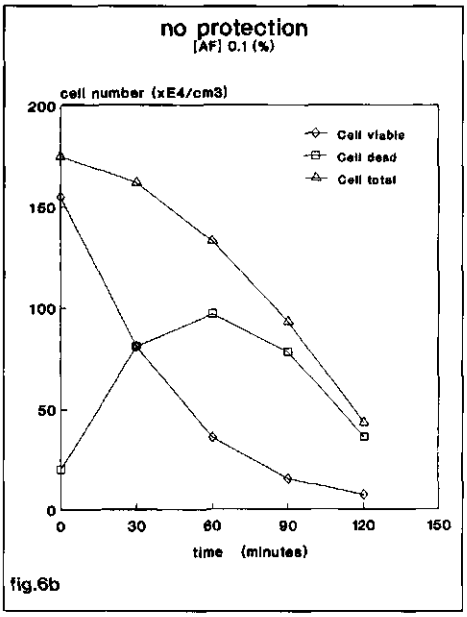


Figure 6a and 6b Cell numbers of hybridoma cells in a bubble column in DME/F12 medium without protective component as a function of time, without (a) or with (b) addition of 0.1% antifoam.

cuted. As mentioned before the standard shear experiment in the bubble column in medium without protective component is difficult to carry out at low AF concentrations, because of the necessary frequent tipping with diluted AF emulsion (figure 2). Therefore a comparison is made between stress experiments completely without AF, allowing foam to develop, or with 0.1% AF, at an air-flow rate of 6 dm^3/h.

Without AF an unstable foam with bubbles of variable size developed from the beginning of the standard shear-stress experiment. This is possibly due to cellular proteins because the DME/F12 medium itself does not generate foaming. The number of viable cells decreased to a value below $10^5/cm^3$ within one hour. Also the total cell number decreased from the beginning of the experiment, indicating cell lysis or loss of cells in the foam (figure 6a). In the small 'deposit' developing at the top of the foam layer, very few dead cells could be detected. The relative increase in LDH activity corresponds with the decrease of the fraction of viable cells (figure 7). In medium without protective component and without AF, cells are thus rapidly lysed in the suspension or in the unstable foam.

The addition of 0.1% AF to a cell suspension in medium without a protective component prevents the development of foam. Comparable to DME/F12 medium with PEG and 0.1% AF (figure 3a and b), first the number of trypan-blue stained dead

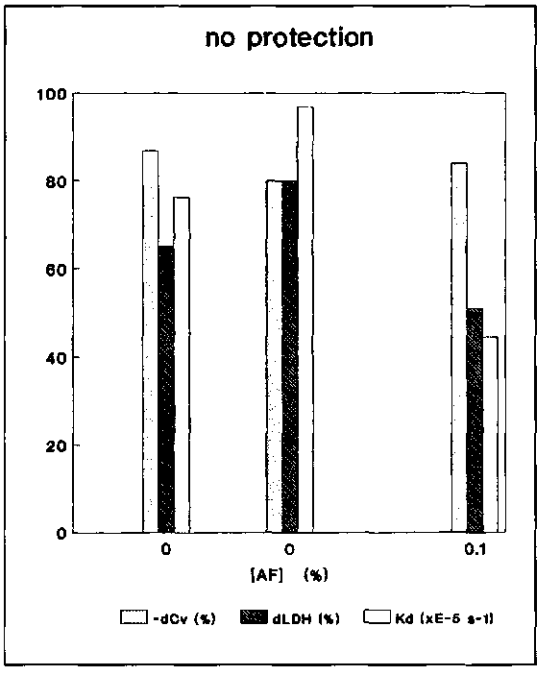


Figure 7 First-order death-rate constant (Kd) and relative change in viable cell number (-dCv %) and LDH activity (dLDH %) of hybridoma cells in a bubble column in DME/F12 medium without protective component, without and with antifoam. The duration of the bubbling experiments are 40 and 45 minutes for duplicate experiments without antifoam and 120 minutes for the single experiment in the presence of 0.1% antifoam (data also partly used for figure 6a and 6b).

cells increases while the total cell number shows a slight reduction (figure 6b). This is followed by lysis of cells as shown by the decrease of the total cell number. The increase in relative LDH activity is less than the relative decrease of the viable cell fraction (figure 7), due to unknown reasons. The death-rate constant k_d in the presence of 0.1% AF is about half of the k_d found without AF (figure 7).

Mechanism of cell death

From the experiments using medium with a protective component and with AF it can be concluded that neither serum nor PEG need the development of a multilayer stable foam to exert their protective effect. Under similar sparging shear-stress conditions but using less defined culture medium, Pluronic F68 protects mouse LS suspension cells in the absence of a foam layer (10). The same finding has also been made for Pluronic F68 with insect cells (11). Therefore it seems unlikely that polymers

like Pluronic and PEG act as cell-protective agents only by stabilizing foams, as suggested as by Handa-Corrigan et al., 1989 (6).
The experiments using DME/F12 medium without protective polymers show that without AF an unstable non-homogeneous foam layer causes rapid lysis of cells. This might be caused by film drainage and bubble break up in the foam. In the presence of 0.1% AF processes associated with the break up of single bubbles at the surface of the cell suspension cause first an increase of intact dead cells and later lysis. This might be due to rapid oscillations after bubble break up (6) or to adherence of cells to bubbles resulting in 'collective' injury after the bubble break up by vertical jetting and ejection of a liquid drop (2, 3). Recently Cherry and Hulle, 1992 (4), suggest that the rupture of thin films accounts for most if not all of the death of insect cells within a sparged bioreactor, using medium with either 0.1% Pluronic F68 or 5% FCS as protective component.
In the DME/F12 medium it can also not be excluded that cells die in the suspension due to rising bubbles because experiments that indicate the bubble rising zone as the relatively harmless have all been done using medium with protective components (6, 17), which can change the gas-liquid interface properties of bubbles. This underlines the importance of a standard set-up of shear-stress experiments using a fully defined medium.

Conclusions

Relatively high concentrations of medical grade silicone AF emulsion cause an increase in cell death during sparging of PFU-83 hybridoma cells. The detrimental effect is not substantial at the low concentrations needed for subtle handling of an existing foam during batch cultivations, using sparging in a medium with a protective component. However the excessive use of AF to prevent development of any foam, or the repeated use of AF during a continuous cultivation might cause higher cell death or reduced growth. The reducing effect of AF on the cell-specific oxygen consumption of PFU-83 cells occurs mainly at the low concentrations.

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5. Polyethylene glycol as protectant against damage caused by sparging for hybridoma suspension cells in a bubble column

Summary

For hybridoma cells cultured in a continuous stirred-tank reactor in a defined serum-free medium the protective effect of polyethylene glycol (PEG) against damage caused by sparging with air has been evaluated in a bubble column.
Varying the molecular weight and concentration of PEG results in a diverse pattern of the death rate caused by sparging. Measuring of the surface tension with the same PEG concentrations and molecular weights results in a similar pattern though without the great variation in values as found for the cell death rates. This indicates the involvement of the gas/ liquid interface in the protective mechanism of PEG.
PEG in the protective range of concentrations and molecular weights did not have a negative effect on the overall oxygen transfer coeficient $k_{O,l}a$.

Introduction

Current developments in the application of in vitro cultures for animal cells in order to produce biologicals are directed towards scale up of culture volumes and the use of chemically defined serum-free medium. For scale up, the important criterium is to supply sufficient oxygen without creating excessively high shear forces, causing cell death (1). Sparging with air or oxygen, the most efficient method of oxygen transfer, can be applied if a protective component is present in the culture medium.
Serum provides some protection against sparging (2-6) but also generates foam. Pluronic F68 is generally used as a protective component to substitute for serum in serum-free medium. This block copolymer of poly(oxyethylene) and poly (oxypropylene), is also added to many serum-containing media because its protective effect against sparging is much greater than that of serum and because including Pluronic usually reduces foaming.
The use of media additives for the protection of suspended animal cells has been reviewed by Papoutsakis (7). The effect of a certain additive varies and seems to be dependent on the type of cell line, the culture medium and the shear-stress circumstances. This is illustrated below by the effects of the polymer poly(oxyethylene) or more commonly, polyethylene glycol (PEG).

Keywords: Animal cells; sparging; shear stress; gas-liquid interface; polyethylene glycol.

Adding 0.1% or 0.2% of PEG (20 kDa) stimulated the growth of several different cell lines in serum-free medium in static culture and in spinner flasks (8). The growth-stimulating effect for mouse/human hetero-hybridomas was less for PEG polymers with smaller molecular weights. In sparged cultures of recombinant CHO cells in medium with 10% FCS 0.004% PEG (2 kDa) had no effect on growth of one cell type whilst causing reduced growth of another type of CHO cell (9).
PEG (10 kDa) protected hybridoma cells from sparging damage during growth in a bubble column (10). For SF9 insect cells in TNM-FH medium with 5% FCS, addition of 0.2% PEG (8 or 2 kDa) provided some but variable protection in sparged and agitated culture, but no protection in an air-lift reactor (11).
Addition of 0.1% (w/v) autoclaved PEG 400 to ATCC CRL-8018 hybridoma cells growing in culture flasks in serum-free medium with 1 g/dm^3 BSA, caused cell lysis (12). Adding the same amount of PEG 1400, 8000 or 15000 resulted in an identical growth curve as in medium without PEG and also provided protection against agitation with bubble entrainment. PEG 8000 did not protect the cells against a laminar-flow shear in a Couette viscometer (13). This summarizes the different effects of PEG on the growth and cell death of animal cells in various media and culture systems.
As for PEG, as with other polymers different effects have been found, resulting in the indication of different damaging events and different protective mechanisms (7, 14). It is unclear whether there is one uniform damaging event caused by sparging and one mechanism for polymers that provide protection or if different explanations are required, depending on the afore-mentioned differences in cell type, culture medium or shear-stress conditions.
In the review of Papoutsakis it was concluded that damage to cells is always caused by shear forces acting on the cells through the surrounding fluid layer, the boundary layer which is always in a state of laminar flow (7). All additives that protect suspended cells from fluid-mechanical injury must either decrease the fragility of the cells or affect the forces on the cells due to their interactions with gas/liquid interfaces (7). Sparging shear damage and protective mechanisms seem thus either cell-medium interface or bubble-medium interface related.
Even though shear stress in liquids is directly related to bulk viscosity, this property of culture medium cannot explain the protective effect of certain polymers against sparging (12, 15, 16, 17). It is also clear that the surface tension can not be the only determining factor in protecting cells against sparging either, though different results have been obtained for this bubble/medium interface related parameter (12, 15, 18, 19).
If one decisive property exists that explains the protective effect of a certain polymer against sparging it can only be found by a standardization of experiments. Therefore in this study the effects of PEG on sparging shear-stress sensitivity was tested for the hybridoma cell line PFU-83, in a fully defined serum-free medium in a bubble col-

umn under standardized conditions and over a time span in which growth is negligible. In this way there is no need to discriminate between a possible growth stimulating effect or a cell death reducing effect of a certain polymer. It only has to be demonstrated that the polymer to be tested has no toxic effects in the course of a sparging shear-stress experiment. PEG has been chosen because it provides good protection against sparging for the selected hybridoma cell line during a standard shear-stress experiment in the bubble column (23) and because it is a polymer of one single monomer available in many different molecular weights.
Because the mechanism by which polymers provide protection against sparging seems interface related the effect of varying PEG molecular weight and concentration on the cell death rate in the bubble column were determined. To test a possible correlation between protective effect and gas/liquid interface properties, the surface tension and the oxygen transfer were measured for the different concentrations and molecular weights of PEG in culture medium. To test a possible corre- lation between protection and cell/liquid interface, hybridoma cells were incubated with PEG to allow the generation of a PEG coating. After this, "PEG-coated" cells were washed in medium without PEG and then subjected to stress in the bubble column without protective polymers.

Material and methods

Cell Line and Maintenance

The cell line used in this study, PFU-83, is a rat/mouse hybridoma, producing an IgG antibody against corticotropin-releasing factor (22). The cells were cultured in a 1 or a 5 dm^3 continuous stirred-tank reactor (CSTR) at a dilution rate of 6.3 to 8.4 $x10^{-6}$ s^{-1}. Both CSTR's were round-bottomed glass vessels and were stirred by a four-blade marine impeller (48 and 61 mm diameter, respectively) at a constant stirring speed of 100 rpm (no vortex). The CSTR's were aerated by leading sterile air with 5% CO_2 through the headspace (5).
The culture medium for all experiments was a serum-free 3:1 DMEM/Ham's F12 medium with 5 mg/dm^3 transferrin as the only protein component (6).

Sparging Shear-Stress Experiments

Shear-stress experiments were carried out in a 36 mm diameter bubble column by sparging an 80 cm^3 suspension sample with air containing 5% CO_2 at a gas flow per unit culture volume of 1.25 vvm (5, 23). Cell samples were taken from the CSTR culture. No antifoam was added. Polyethylene glycol (PEG) was added from an autoclaved 25% (w/v) stock solution. All PEG polymers were obtained from Merck (Darmstadt, Germany).
For the "PEG-coating" experiment 100 cm^3 samples were taken from the 5 dm^3

CSTR and incubated with PEG 6000 for a given period of time (see results and discussion section). After this the cells were washed twice with fresh culture medium, using a MSE Minor S centrifuge [3 minutes, 400x g]. The second time the pellet was resuspended with 80 cm^3 fresh medium and transferred to the bubble column. The control samples were treated with the same wash procedure.

Analytical Methods

Cell numbers and viability were determined using a haemacytometer and the trypan-blue exclusion technique.

The first-order death-rate constant was derived by linear regression from the slope of $\ln C_t/C_0$ (C_t = viable cell number at time t; C_0 = viable cell number at the beginning of the shear-stress experiment; 5).

The concentration of IgG antibody was quantified by using an enzyme-linked immunosorbent assay (ELISA) as described earlier (6).

The static surface tension was determined by the Wilhelmy plate tensiometer method.

Oxygen transfer

The overall oxygen-transfer coefficient $K_{O,l}a$ was determined with a dynamic method (25) in a 2 dm^3 Biolafitte fermenter with a flat-bottomed glass vessel. This standard fermenter was stirred at 250 rpm by a four-blade turbine impeller with a diameter of 45 mm and sparged through eight 0.5-1 mm diameter holes in a steel tube below the impeller. The concentration of dissolved oxygen was monitored using an oxygen sensor with a YSI membrane kit (standard) in DMEM/Ham's F12 culture medium at 37°C. The $K_{O,l}a$ was determined by linear regression of the log version of the exponential part of the saturation curve. The 0% and 100% set points for the oxygen electrode were adjusted with pure nitrogen and oxygen gas. After the calibration of the electrode the saturation curve is determined by introducing pure oxygen at a gas-flow rate of 14.5 dm^3/hour starting at the 0% set point. The measurement was stopped at 80% saturation. The determined response time for the oxygen sensor [8.72 s] was within specifications, indicating that it was smaller than 20% of the maximum $K_{O,l}a$ (s^{-1}).

Results and discussion

Cell line stability

Cultures of PFU-83 hybridoma cells in CSTR's were used as the source of suspension samples for shear experiments during a period of several weeks. Because stirring in the CSTR is very mild and no sparging is applied, the cell population undergoes no possible selection for shear resistance. The viable cell concentration varied in the

range 9.5 to 15.5 x 10^5 cells/cm^3, at the standard fixed dilution rate. The cell-specific MAb production rate [kg $cell^{-1}$ s^{-1}] of PFU-83 cells cultured in a CSTR in serum-free medium showed a good stability. Also repeated sparging shear-stress experiments with cells taken from the CSTR culture at a different period of time showed similar results (data not shown).
Therefore the CSTR culture of PFU-83 in serum-free medium without protective additives against shear, provides well-defined suspension samples for shear-stress experiments in a bubble column.

Protective effect of PEG

PEGs with molecular weights varying from 400 to 35000 Da were tested for their protective effect against sparging for hybridoma cells in a bubble column under standardized conditions. Without any addition of PEG the standard sparging shear stress experiment resulted in a k_d value of 107 x10^{-5} s^{-1}.
The sparging shear-stress experiment was repeated, with the different molecular weight PEGs in the concentration range of 0.005% or 0.25% to 0.2% (table 1). Figure 1 is the result of a three-dimensional fit to the data of table 1. The differences in fitted k_d's for medium without PEG are the result of interpolation from data of a concentration range with a particular molecular weight PEG.

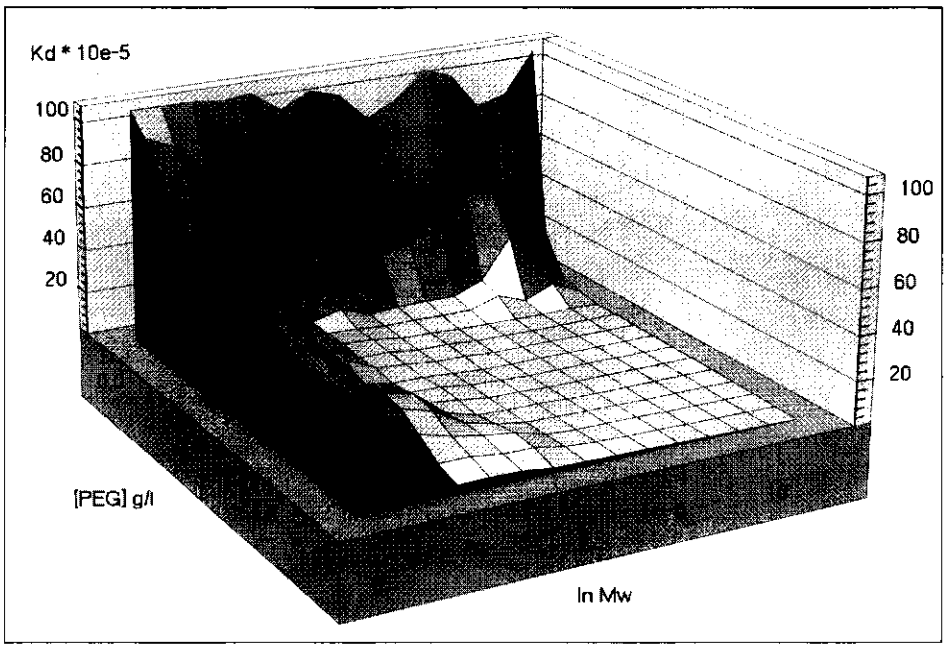

Figure 1 Fitting-program version of the data of Table 1.

Table 1 The first-order death-rate constant k_d ($x10^{-5}$ s^{-1}) of hybridoma cells in a bubble column as a function of the molecular weight and the concentration of added PEG.

PEG Molecular weight (kDa)	Concentration of added PEG (g/dm^3)						
	0.00	**0.05**	**0.10**	**0.25**	**0.50**	**1.00**	**2.00**
400	107.5	ND	ND	92.4	69.7	72.2	47.2
600	107.5	ND	ND	71.9	51.2	58.1	21.8
1000	107.5	ND	ND	40.5	19.0	19.5	3.9
1500	107.5	36.0	ND	8.3	7.0	3.7	4.4
3000	107.5	ND	ND	3.1	3.7	3.0	1.5
6000	107.5	14.2	10.5	6.9	3.1	1.0	1.6
10000	107.	ND	ND	5.9	1.8	1.3	1.5
35000	107.5	36.3	ND	6.4	1.3	1.5	0.5

The trend of figure 1 shows that PEG polymers of a molecular weight greater than 1000 Da provide a good protection against sparging for PFU-83 cells if the polymers are added in a concentration above 0.025% (w/v). These findings correspond with the results found for other hybridoma cells (12). For insect cells however, 0.2% PEG 2000 or 8000 did not offer consistent protection against sparging (11).

The lower values for k_d near the 10^{-6} s^{-1} range are too close to the growth rate of the cells (k_g = 8.5 $x10^{-6}$ s^{-1}) to be accurate. Initially the growth rate of the cells in the bubble column is the same as in the CSTR, because cells in the G_2 phase (which takes several hours) are committed to start the process of cell division. A possible correction for the growth rate would not change figure 1.

Static controls cultures of PFU-83 in T-flasks in serum-free medium with 0.1% PEG 200 showed no inhibition of growth of this hybridoma by a low molecular weight PEG [data not shown].

This is in contrast to the results of Michaels and Papoutsakis (12) for hybridoma cells in serum-free medium with 0.1% PEG 400 in T-flask culture.

Foam

The generation of foam is an important property of fermentations with sparged animal-cell cultures (1, 24). As there was no antifoam added to these sparging shear-stress experiments, foam formation could be evaluated. Addition of PEG 400 resulted in a high [up to 20 cm] unstable foam layer, with bubbles of non-homogeneous size distribution. PEG with a molecular weight larger than 1500 Da generated a small [up to 3 cm] stable foam layer, with homogeneous bubbles of about 6 mm in diameter. When PEG 35000 Da was used, no foam layer developed. The larger molecular weight PEGs reflect the use of PEG as an antifoam agent (9).

Based on the correlation between foam type and the protective effect against sparging, Handa-Corrigan proposed that the generation of a stable foam layer explains the protective mechanism of additives like PEG and Pluronic (3). Since PEG and Pluronic also provide good protection against the damaging events of sparging in the absence of foam this hypothesis can not explain the complete mechanism of protection by these polymers (23, 26). However, because the generation of foam is related to properties of the gas/liquid interface it seems important to mention the development of a specific type of foam.

Surface tension

Changes in surface tension of DMEM/Ham's F12 culture medium caused by addition of the previously tested ranges of molecular weight and concentrations of PEG were measured. The surface tension of DMEM/Ham's F12 medium (69.8 $x10^{-3}$ N/m) was only slightly lower than that of water (72.9 $x10^{-3}$ N/m).

Table 2 shows the values of the surface tension of different molecular weight and concentrations of PEG in DMEM/Ham's F12 culture medium. Figure 2 is the 3-D fit of the data from table 2. The trend is that initial addition of PEG molecules with a molecular weight larger than 1000 Da causes a reduction in the surface tension but that a further increase above 0.01% brings about no further reduction in the surface tension. For PEG 400 more than 0.1% has to be added to the culture medium to bring about a comparable reduction.

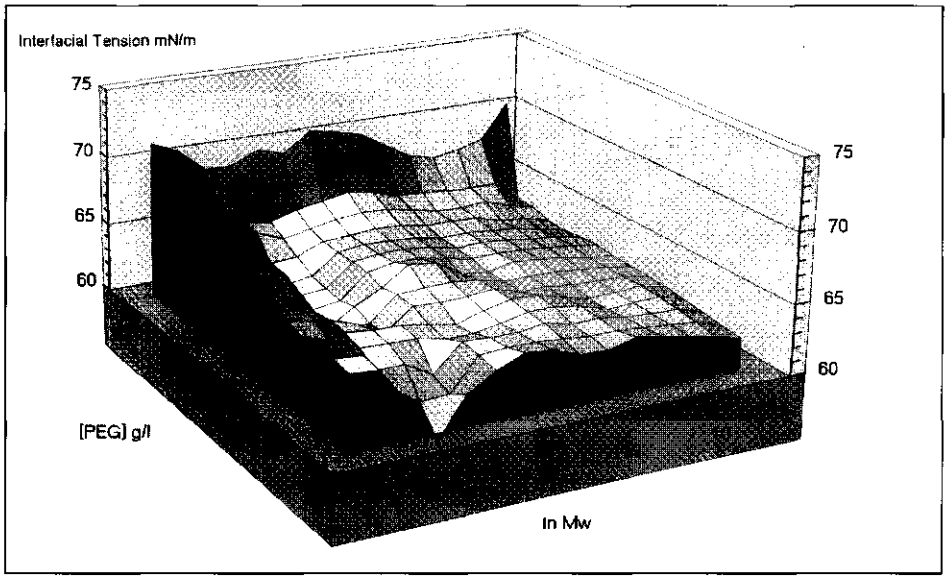

Figure 2 Fitting-program version of the data of Table 2.

Table 2 The surface tension ($x10^{-3}$ N m^{-1}) of DMEM/Ham's F12 culture medium as a function of molecular weight and concentration of added PEG.

PEG Molecular weight (kDa)	Concentration of added PEG (g/dm^3)						
	0.00	**0.05**	**0.10**	**0.25**	**0.50**	**1.00**	**2.00**
400	71.4	72.0	71.8	69.6	69.5	68.3	65.1
600	70.3	68.2	67.4	65.9	67.4	66.7	64.9
1000	68.9	66.7	65.0	65.4	62.0	61.4	59.0
1500	69.6	64.6	64.6	64.1	63.9	63.0	62.6
3000	71.9	66.9	66.9	65.2	64.9	65.5	64.5
6000	70.6	65.7	65.5	65.0	64.1	63.4	63.0
10000	67.4	65.0	64.9	64.3	64.3	64.1	63.9
35000	70.4	63.4	63.5	63.5	62.9	63.1	62.2

Although there is some scatter, it is clear that the shape of the 3-D figure 2 of surface-tension values shows a good similarity with figure 1, the pattern of the k_d values. Because of this similarity in 3-D patterns it is likely that the presence of PEG near the gas/liquid interface is of importance for its protective effect.

The relatively small reductions in surface tension compared to the large reductions in k_d values indicate that the surface tension in itself alone can not explain the protective effect of PEG. This same conclusion is supported more strongly by results with silicone antifoam. Addition of antifoam reduces the surface tension considerably (20, 21) whilst the same concentrations have little effect on the cell death rate caused by sparging (23). In Table 3 it can be seen that the addition of polymers, either proteins or polymers such as PEG and Pluronic cause a reduction in the surface tension of media without surfactants. However, the addition of these polymers to media with a surface tension value below 6 $x10^{-2}$ N m^{-1} has little effect.

Larger molecular weight PEG polymers might induce a decrease in surface tension by multiple adherence to the interface. The extra addition of polymer is unable to bring about a further reduction in surface tension if the interface is already extensively covered. The smaller molecular weight PEGs (< 1000 Da) might be unable to form multiple attachments to the interface and being removed more readily. Therefore, whilst the same weight per volume PEG 400 contains many more molecules than for PEG 35.000 the effect on the surface tension is smaller. The way in which the different molecular weight PEGs are interacting with the gas/liquid interface may have different effects on boundary layer mobility. Michaels concludes that static properties like bulk viscosity and static surface tension are less likely to provide information on protection against sparging but that dynamic properties like dynamic surface tension, surface shear viscosity and dilatational surface viscosity have to be deter-

Table 3 Comparison of values of the surface tension ($x10^{-2}$ N m^{-1}) for different media formulations from published data.

Reference	water	basal medium	complete medium	medium + protein	medium + polymer
12	7.0	7.0 [D/R]		5.3	4.7-5.4
19	7.28	7.47 [R]			6.2-6.45
15	7	7 [R]		5	4
18		[I/H]	5.7	5.4	
This work	7.29	[D/H	]6.98		6.2-6.5

Basal media are standard basic media formulations [D = DMEM; H = Ham's F12; I = IMDM; R = RPMI] or combinations thereof. Complete media contain serum-replacing components but no protective additives against sparging.

mined. The first measurement of these dynamic properties resulted in the conclusion that different protection mechanisms may exist for different shear protectant additives (30).

Oxygen transfer

It is of paramount importance that polymers that provide protection against sparging do not reduce oxygen transfer if they are to be applied in animal-cell fermentations. This is because scale up of these fermentations requires an increase of oxygen transfer without an unacceptable increase in cell death due to sparging. If coverage of the interface with polymers shields the cells from sparging damage but also reduces the oxygen transfer there is not necessarily an overall benefit. For this reason and because the different molecular weight PEGs caused differences in surface tension, another gas/liquid interface related parameter, the overall oxygen transfer coefficient $K_{O,l}a$, was determined.

The effect on $K_{O,l}a$ of adding different molecular weight and concentrations of PEG polymers to the standard culture medium is shown in table 4. In the standardized bioreactor with DMEM/Ham's F12 medium none of the PEG polymers had a significant negative effect on the $K_{O,l}a$. For the larger molecular-weight PEGs (>1000 Da) adding 0.2% even had a slight beneficial effect on oxygen transfer. Pluronic F68 (8400 Da), which was also tested in this experiment, caused a slight reduction of the $K_{O,l}a$.

The effect of polymers such as Pluronic F68 or serum proteins on the oxygen transfer seems to depend on agitation rate and gas-flow rate (27), but also on bubble size and the presence of other surface active additives (28). None of these parameters were changed in the experimental set up for the $k_{O,l}a$ measurements with PEG.

Table 4 The overall-oxygen transfer coefficient $k_{O,l}A$ ($x10^{-3}$ s^{-1}) of 1 dm^3 DMEM/ Ham's F12 culture medium in a 2 dm^3 stirred and sparged tank as a function of molecular weight and concentration of added PEG.

PEG Molecular weight (kDa)	Concentration of added PEG (g/dm^3)			
	0.00	**0.10**	**0.50**	**2.00**
400	3.8	2.8	3.1	3.1
1000	3.4	2.7	3.5	5.3
1500	3.6	3.3	4.4	5.5
3000	4.1	3.7	4.8	5.2
6000	3.4	3.1	4.3	5.9
35000	4.5	3.8	4.1	5.7
Pluronic	3.5	2.1	2.2	2.0

Cell/medium interface

Besides the gas/liquid interface the cell/liquid interface may also be of importance for the protective effect against sparging damage (2, 11, 19, 26, 29). The protective effect of PEG (M_w > 1000 Da) against sparging is a rapid process. If a large polymer binds to an interface with several parts of the molecule, the desorption is generally a very slow process. The often mentioned "coating" of cells does suggest rather a multiple-attachment adsorbtion than some loose interaction. Based on the assumption that PFU-83 cells could be "coated" with PEG 6000 it was tested if "PEG-coated" cells would be better protected against sparging in a standard shear-stress experiment in medium without PEG than control cells that were not incubated in a concentrated PEG solution.

The control experiment was carried out with cells which were not incubated with PEG 6000 but were washed twice in fresh medium and stressed in the bubble column with a minimum amount of PEG 6000. This minimum amount of PEG indicates the maximum concentration of PEG that might be present in the bubble column after the washing procedure for cells that have been incubated with 1 or 3% PEG. A conservative estimate was used for the dilution factor (2500x) accomplished by the washing procedure. This is of importance since 0.05% PEG 6000 already causes a reduction of 87% of the death rate constant k_d determined without PEG (table 1).

Incubating PFU-83 cells for 1 minute in culture medium with 1% PEG 6000 and sparging them in the bubble column in medium without a protective component resulted in a k_d value of 69 $x10^{-5}$ s^{-1}. A prolonged incubation of 3 hours in a culture flask at 37°C in an incubator with 3% PEG caused an even higher k_d of 86 $x10^{-5}$ s^{-1}. The control experiments with minimal amounts of added PEG 6000 (0.003% and 0.0003%) gave k_d values of 10.4 and 56 $x10^{-5}$ s^{-1} respectively. Therefore the coating

experiments indicate that incubating with PEG 6000 does not provide a protective effect compared to the control values. Because the coating experiments did not result in extra protection no further attempt was made to discriminate between a possible coating protection and that from the PEG remaining after dilution by washing. The actual dilution factor of the washing procedure would be close to 10000x and therefore the remaining PEG concentration after incubating with 1% or 3% PEG 6000 and the washing procedure will be around 0.0003% and 0.0001%.
This experiment indicates that cell-associated PEG provides no protection against sparging or that the coating layer of PEG polymer is not as tightly associated with the membrane as to remain intact after the washing procedure with fresh medium.

Conclusions

PEG polymers with a molecular weight > 1000 Da provide good protection against sparging for PFU-83 hybridoma cells in defined serum-free medium in a bubble column. This is in agreement with the results of Michaels (12).
The pattern of k_d values obtained with different molecular weight and concentrations of PEG is similar to the pattern found for surface tension for the same molecular weight and concentrations of PEG. This indicates that the presence of PEG in the gas/liquid interface is of importance for its protective effect.
PEG in the measured range of molecular weight and concentration did not have a negative effect on the oxygen transfer under standard conditions in culture medium.
Incubating cells with PEG 6000 and stressing the cells after a rapid washing procedure in the bubble column in medium without PEG did not provide an extra protective effect.

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Nomenclature

BSA	Bovine Serum Albumin	
D	dilution rate	(s^{-1})
FCS	Fetal Calf Serum	
IgG	immuno globulin type G	

not shown). During some experiments dextran caused such excessive foaming that a considerable fraction of the suspension volume is lost. Therefore silicone antifoam was added to keep constant suspension volume in the bubble culomn during all experiments. The low concentrations of added antifoam used to suppress foam formation have a negligible effect on the measured death rate in the bubble column (20).

Viscosity and Surface tension

The apparent viscosity of the DMEM/Ham's F12 culture medium with varying concentrations of dextran was determined in a Haake rotovisco RV 20. The dynamic viscosity was measured in a Ubbelohde capillary viscometer. Both viscosity measurements were carried out at 37°C.The static surface tension of culture medium containing dextran was determined with the Wilhelmy plate method, using a Wägenzelle HBM tensiometer at 37°C.

Oxygen transfer

The overall oxygen transfer coefficient $k_{O,l}A$ of culture medium containing dextran was determined with the dynamic method (22). A 1.5 L flat-bottomed Applikon reactor with a 6 bladed turbine impeller was used. Measurements were done by sparging 14 L h^{-1} gas through the 1 L liquid volume at 350 rpm and 37°C. The concentration of dissolved oxygen was measured with an Ital-BOM oxygen sensor with a YSI standard membrane. The sensor was calibrated with nitrogen gas at 0% and oxygen gas at 100%. The oxygen transfer was recorded starting at 0% and stopped at 80% saturation. The $k_{O,l}A$ was obtained by linear regression of the log of the saturation curve.

Results and discussion

Cell culturing

Samples for sparging shear-stress experiments in a bubble column were taken from a continuous culture of PFU-83 cells in a CSTR. After the initial growth phase the cell numbers stabilized at a value near 12.5×10^5 mL^{-1} with an average viability of 90%, at a constant dilution rate of 7.4×10^{-6} s^{-1}. During the period of time this fermenter was used for shear-stress experiments the specific MAb production rate was determined and shown to be constant at a level of 5×10^{-17} g $cell^{-1}$ s^{-1}. The specific productivity is used as the parameter for general stability of the cell line. The cells were cultured under very mild agitation conditions (no sparging, 100 rpm stirrer speed generating no vortex), hence no selection for shear resistance could occur during this continuous culture. Together this shows that the continuous culture of PFU-83 cells in a CSTR provided controlled samples for sparging shear stress experiments of a stable hybridoma cell line.

Sparging shear stress

The effect of various concentrations of different molecular weight dextrans was tested for PFU-83 hybridoma cells in a standardized sparging shear-stress experiment in the bubble column. Figure 1 shows the first-order death-rate constant k_d ($x10^{-5}$ s^{-1}) determined in culture medium containing the various combinations of added dextran. The exact data are also given in Table 1. The 40 kDa dextran had no significant effect on the death rate of PFU-83 in the bubble column. The larger molecular weight dextrans showed an increasing protective effect at concentrations of 10% or more. Addition of 2000 kDa dextran, the largest molecular weight tested, gave a 83% reduction of k_d at the highest concentration of 20%. Little difference was found between dextran 222 kDa and dextran 500 kDa. This can be explained by the different distribution of molecular weights around the given average values for different suppliers (information provided by Pharmacia and Fluka) of these types of dextran.

It can be concluded from figure 1 that dextrans with a molecular weight greater than 40 kDa provide substantial protection against sparging at concentrations of 10% or more. The addition of dextran often caused excessive foaming. Therefore antifoam had to be added to prevent overflowing of the bubble column and substantial reduction of the suspension volume during the sparging shear-stress experiment. The

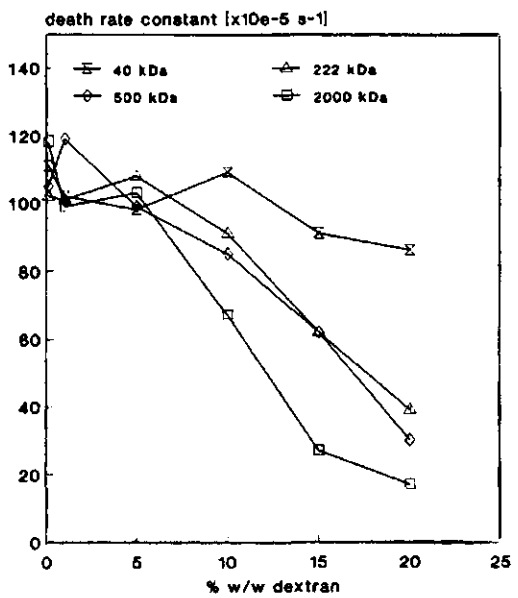

Figure 1 First-order death-rate constant of hybridoma cells in a bubble column as a function of the molecular weight and the concentration of added dextran.

Table 1 The death-rate constant k_d of hybridoma cells in a bubble column, the viscosity and the surface tension of culture medium as a function of molecular weight and concentration of added dextran.

Dextran, molecular weight [kDa]	Added dextran % w/wk_d	k_d x10^{-5} s^{-1}	viscosity mPa x s	surface tension
	0	98	0.72	64
40	0.1	111	0.73	
	1	102	0.73	58
	5	98	1.7	
	10	109	3.6	54
	15	91	7.5	
	20	86	13	59
220	0.1	102	0.76	
	1	101	1.1	60
	5	108	3.8	
	10	91	12	61
	15	62	28	
	20	39	63	67
500	0.1	105	0.42	
	1	119	1.1	56
	5	99	4.4	
	10	85	14	59
	15	62	34	
	20	30	74	68
2000	0.1	118	0.77	
	1	99	1.3	66
	5	103	7.7	
	10	67	28	64
	15	27	75	
	20	17	185	69

added concentrations of antifoam were small enough (<0.05%) to be able to neglect the increasing effect of silicone antifoam emulsion on the death rate of animal cells in sparged cultures (20).

In control experiments the effect of adding dextran to PFU-83 cultures in T-flasks was tested to exclude the possibility that adding dextran causes changes in cell concentration or viability in the absence of sparging. The added concentrations of dextrans (5-20%) had no effect on the cell numbers or viability of PFU-83 cells growing in static culture, during the maximum time (3 h) of a sparging experiment (data not shown).

Viscosity

For the combinations of concentration and molecular weight of dextrans tested in the sparging shear-stress experiments, the viscosity was determined in the DMEM/Ham's F12 culture medium. The results of the dynamic viscosity measurements with the Ubbelohde are shown in Table 1. Dextran 40 kDa gave a maximum increase in dynamic viscosity of 13 mPa s at the highest concentration of 20%. Addition of 2000 kDa dextran caused an increase of the viscosity to 28 mPa s at 10% concentration and a maximum value for the dynamic viscosity of 185 mPa s at 20%. The values for the viscosities of dextran 222 kDa and 500 kDa lie between these two curves. As with the effect on the death rate in Figure 1, the data of dextran 222 kDa and 500 kDa are close together, indicating again that the size distribution of these dextrans is less different than expected based on the given average molecular weight. The relation between the death rate constant k_d and the viscosity is shown in figure 2. From this graph it can be concluded that an increase of the dynamic viscosity above 20 mPa s of the culture medium corresponds to a decrease of k_d greater than 20%. The measurement of the apparent viscosity of dextran 2000 kDa at 10% and 20% concentrations in a Haake rotoviscometer showed a linear increase of the apparent viscosity with an increasing shear rate (figure 3). This shows that these solutions of 2000 kDa dextran in culture medium are real Newtonian fluids, for which the dynamic viscosity is a constant depending only on pressure and temperature. Therefore

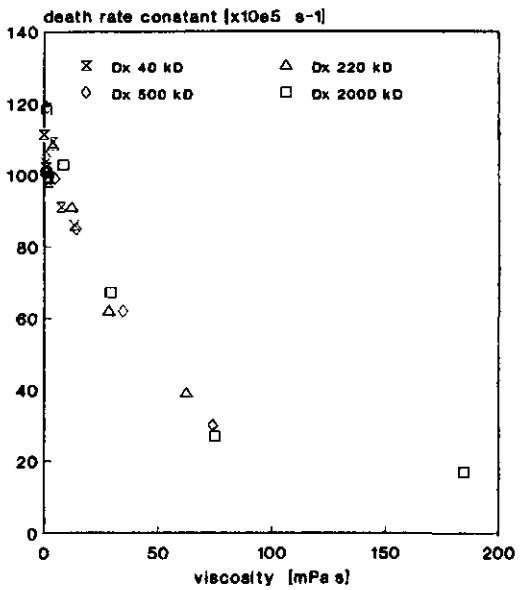

Figure 2 First-order death rate constant of hybridoma cells in a bubble column versus the viscosity of culture medium containing added dextran.

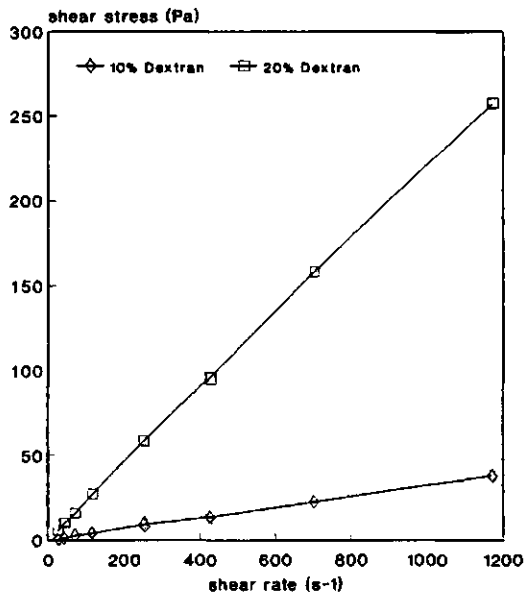

Figure 3 Shear stress and shear rate of 10% and 20% (w/v) dextran 2000 kDa solutions in culture medium, determined in a Haake rotoviscometer.

there is a linear correlation between shear stress and shear rate. In a system that creates a forced increase in shear rate an increase in viscosity will cause a higher shear stress and possibly greater damage to animal cells in the stressed suspension. However, the results of Goldblum et al. (3) show that an increase in viscosity of the culture medium from 1.4 to 8.0 $x10^{-3}$ mPa s by extra addition of dextran 476 kDa does not simply increase the rate of cell lysis in a rheogoniometer at comparable shear rates. For the bubble column the situation is even more complex. The rate of energy dissipation associated with the bursting of a gas bubble at the surface of the liquid is estimated at 9000 $m^2 s^{-3}$ (1). This corresponds to a Kolmogorov eddy length of 3.2 μm, assuming a turbulent energy dissipation. This eddy length is small enough to cause a detrimental effect on the cell viability. In this case an increase in viscosity could increase the size of the smallest eddies and reduce the detrimental effects caused by bubble bursting. If the energy dissipation is laminar, the corresponding shear stress is estimated at 95 N m^{-2} (1), which is also great enough to cause cell death. In this case an increase in viscosity could provide protection if it greatly reduces the shear rate associated with the bursting of the bubble. The results of this study show that if the viscosity of the culture medium is increased above 20 mPa s by addition of dextran the damaging fluid dynamic events associated with bursting of bubbles are damped to such an extent that a substantial (20 - 80%) damping of the lethal effects occurs.

Surface tension

The surface tension was determined as a control parameter because the protective effect of many polymers is associated with its surface active properties (15). For PEG polymers the values of the surface tension indicated that the reduction in surface tension itself can not explain the protective effect but that the presence of PEG (with large molecular weight) in the gas/liquid interface is important (21). In Table 1 the values are shown for the four different molecular weight dextrans at three different concentrations (1%, 10%, and 20%). The addition of dextran polymers to culture medium caused a reduction of the surface tension for low molecular weight dextrans or low concentrations of 222 kDa and 500 kDa dextran. The high concentrations of higher molecular weight dextrans that provided protection against shear damage have a surface tension equal to or greater than the value of culture medium.

The trend between the death rate constant k_d and the surface tension is shown in figure 4. Since the protective dextrans cause hardly a decrease in surface tension, the conclusion is that the presence of dextran in the gas/liquid interface is no requirement for its protective effect against sparging. This finding is also illustrated by the addition of antifoam and cell culture supernatant. Silicone antifoam emulsion causes a strong reduction of the surface tension (6). The addition of cell culture supernatant containing 3.3 x 10^5 mL PFU-83 cells, caused a reduction of the surface tension

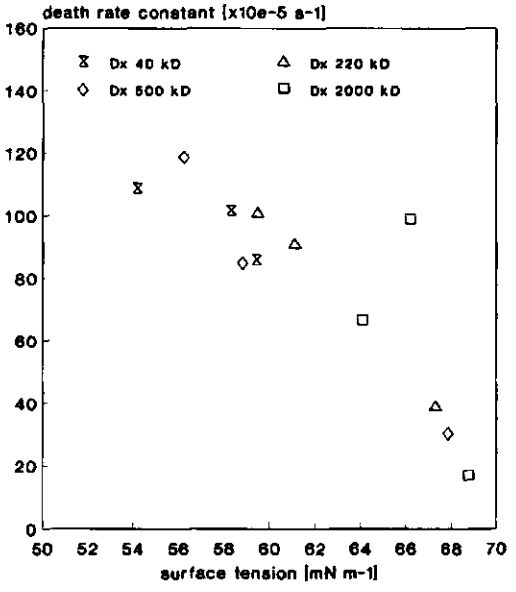

Figure 4 First-order death rate constant of hybridoma cells in a bubble column versus the surface tension of culture medium containing added dextran.

from 63.7 to 47 mN m^{-1}. Both silicone antifoam and cell culture supernatant have no protective effect against sparging.
The results with respect to the relation between the death rate in the bubble column and the surface tension obtained with dextran are the opposite of the findings obtained with PEG (21). While PEG seems to be present in the gas/liquid interface to exert its protective effect against sparging the protective dextran polymers show no affinity for this interface. The concentrations needed for a substantial protective effect with dextran are 100 fold higher for dextran than for PEG.

Oxygen transfer

The overall oxygen transfer coefficient $k_{O,l}A$ of culture medium containing dextran of different molecular weights and concentrations was determined as a second gas/liquid interface related parameter. For PEG (2%) it has been shown that its possible presence in the gas/liquid interface does not reduce the oxygen transfer (21). The addition of dextran in the concentration range that provides protection against sparging shear damage (10-20%), resulted in a drastic decrease in oxygen transfer (figure 5). Even for the medium containing 20% 2000 kDa dextran with the highest measured viscosity the response time of the oxygen electrode was such that it may

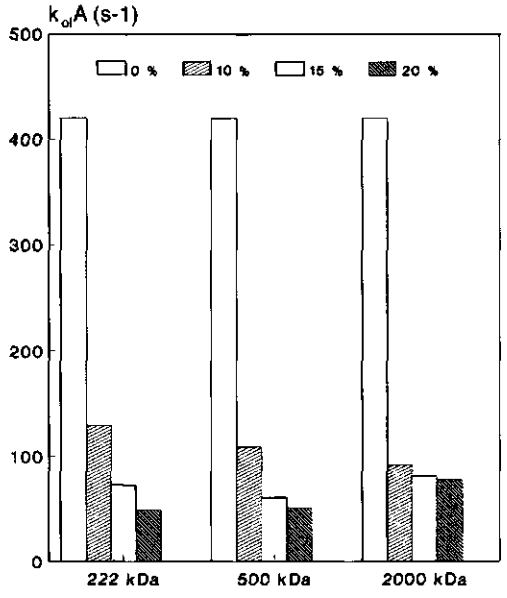

Figure 5 Overall oxygen transfer coeficient of 1 dm^3 DMEM/Ham's F12 culture medium in a 2 dm^3 stirred and sparged tank as a function of molecular weight and concentration of added dextran.

have caused a maximum deviation of 3%. The generation of micro-bubbles, however, in culture media containing dextran with a viscosity of > 15 mPa s which remain in the fluid reduced the accuracy of the dynamic measurement of oxygen transfer. The absolute values may therefore not be fully accurate but the effect of the addition of high concentrations of dextran is evident. To use high concentrations of high molecular weight dextran in serum free media to protect suspended animal cells against sparging in large scale stirred tank reactors does not seem to be feasible because of the reduction in oxygen transfer. This completely nullifies the beneficial effect of reduced cell death caused by sparging.

Conclusions

Dextran provides protection for hybridoma suspension cells in serum-free medium against the damaging events of sparging in a bubble column. For this protective effect high concentrations (10-20%) of a high molecular weight (>40 kDa) dextran have to be added to the cell suspension. The protective effect corresponds well with the increase in bulk viscosity of the medium. Under the standardized sparging shear-stress conditions in the bubble column an addition of dextran to culture medium causing a viscosity above 20 mPa s resulted in a considerable reduction (20%-80%) of the cell death rate constant k_d.
The protective effect of dextran is not correlated to the surface tension of the culture medium, indicating that its possible pressence in the gas/liquid interface is not essential for this effect. Since the addition of high concentrations of high molecular weight dextran causes a substantial reduction of the oxygen transfer dextran seems not suitable as shear protective agent in sparged animal cell cultures.

Acknowledgements

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Nomenclature

BSA	bovine serum albumin	
CSTR	continuous stirred tank reactor	
D	dilution rate	(s^{-1})
FCS	fetal calf serum	
HSA	human serum albumin	

IgG	immuno globulin type G	
k_d	death-rate constant	(s^{-1})
k_La	oxygen transfer coefficient	
MAb	monoclonal antibody	
PEG	polyethylene glycol	
vvm	volume per volume per minute	

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7. Shear sensitivity of animal cells from a culture-medium perspective

Summary

Recently several groups have published data on the shear sensitivity of suspended animal cells and the protective effect of certain polymers. These findings did not, at the time, seem to have great practical application, because shear sensitivity did not cause great problems for large-scale applications in sparged and stirred-tank reactors using current standard culture media and fermentation procedures. However, two recent developments might require renewed attention in sparged animal-cell cultures - protein-free media and new fermentation techniques.

Introduction

For the production of biopharmaceuticals, the culture of animal cells has become increasingly popular, mainly because they can produce a correctly processed protein that can be secreted into the culture medium, which greatly facilitates the purification process. The traditional disadvantages of animal-cell culture include slow and variable growth, low and unstable productivity, and shear sensitivity. However, most of these disadvantages were overcome during the 1980s, resulting in the use of animal cells for large-scale protein production.

The preferred technique for the large-scale production of biopharmaceuticals from animal cells is as homogeneous cultures in stirred-tank reactors. The reasons for this are the superior process control that is possible with homogeneous systems and the relative ease of scale-up from 1 to 1000 litre reactors. An additional advantage is that most pharmaceutical companies are already familiar with this fermentation system at different scales. Despite the fact that animal cells are relatively large in comparison to bacteria and yeast and also lack a cell wall, the shear sensitivity of these cells has not proved to be a serious concern when scaled up to an efficient commercial production process, using culture media that contains both proteins and protective components. Standard animal-cell bioreactor runs are executed with total cell densities in the range 1 - 5 $x10^6$ mL^{-1} for systems without cell-retention devices and 5 - 20 $x10^6$ mL^{-1} for reactors with a cell-retention device. To maintain a homogeneous suspension and provide a sufficient supply of oxygen, the overall power input for these stirred cultures is considerably less than for aerobic fermentations with bacteria or yeast, because animal cells grow more slowly and thus consume considerably less oxygen.

Design criteria for stirred-tank reactors for animal cells include the use of round-

bottomed fermenters, mild type impellers and the prevention of regions with close clearance. Mild type impellers, such as marine or pitched blade, generate a mild, low energy containing flow pattern. A region of close clearance, as can exist between an impeller with a large diameter and a relatively narrow vessel, can cause detrimental flow patterns for animal cells. Additional requirements are the use of culture media containing protective components against sparging damage, such as Pluronic F68, and to minimize sparging gas flow in reactors with a low aspect ratio (height:diameter). These features allow the culture of animal-cells in stirred-tank reactors greater than 1000L working volume, provoking some to question whether the fragile animal cell is just a myth[1] .

The conditions under which animal-cell culture reached a status of robust maturity is challenged by the introduction of protein-free media that contain less protective components. To successfully apply optimal production conditions for animal cells, that is at high cell density and low growth rate, a robust culture medium is required to ensure a minimal cell death rate.

Protein-free media

When components of human or animal origin are added to the culture medium there is the potential risk of the transfer of viruses and/or prions. If these components are to be used in production media, the source has to be identified and the material extensively tested and any viruses inactivated. Despite the use of serum from disease-free countries, the reliability of testing and inactivation procedures has caused regulatory authorities such as the US Food and Drug Administration (FDA) first to advise the use of serum-free media[2] and, recently, even to ban certain albumin-containing fractions. Several recalls of transferrin batches have occurred in recent years, primarily related to the prion diseases Bovine Spongiform Encephalopathy (BSE) and Creutzfeldt Jacob Disease (CJD). Manufacturers have been urged to investigate alternative sources of insulin, albumin and transferrin or to use completely protein-free media. For the use of bovine components it is possible to select material from BSE free countries like Australia and New Zealand. Recombinant transferrin or albumin seem to be not available at the right quality or expense, causing a significant increase in the unit cost of production media.

At present, many large-scale cultures use media containing both albumin and Pluronic F68 to protect animal cells against shear forces. Albumin will have to be omitted, and it is not known whether Pluronic F68 can act as a universal protective component for the large-scale production of proteins from animal cells in protein-free media. Moreover, there is concern in the literature about the interaction of Pluronic F68 with both the cells and product [3, 4], as high concentrations of F68 (> 0.2%) can be toxic.

Reduced growth conditions

An important feature of animal cell lines grown in suspension is the relative independence of the specific productivity from the growth rate (figure 1)[5]. For the few cell lines showing a strict correlation between growth rate and specific productivity, the opportunity is reduced to optimize media and reactor efficiency, resulting in higher costs per gram of product. The relative independence of the specific production rate and growth rate might therefore be included as a selection criterion for cell lines, in addition to a stable specific productivity.

For the majority of cell lines the most efficient production conditions are at low rates of cell growth, when most of the nutrients are used for product synthesis and not for actual cell growth. The use of animal cells at low growth rates pushed the development of traditional simple batch cultures to extended fed-batch strategies [6]. For continuous-perfusion bioreactor runs, it stimulated the reduction of the perfusion-medium flow rate. Therefore, the traditional 'batch-versus-continuous' discussion is becoming less relevant as both fed-batch as continuous perfusion try to mimic more or less identical conditions, that is, maintaining a high density of viable cells at a low growth rate and relatively high productivity.

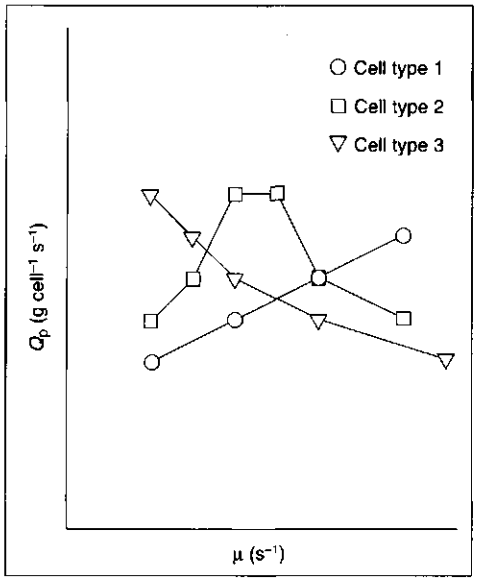

Figure 1 A schematic graph of the correlation between the specific protein productivity (Q_p) and the growth rate μ of animal cells. In general there is a relative higher Q_p at lower m for different cell types (figure adapted from reference 6).

The fed-batch strategy, originating from the simple batch culture, has developed into the extended fed-batch system, during which a high cell density is maintained at reduced growth conditions by optimising the feed stream with respect to essential nutrients, while preventing the accumulation of toxic waste products. The continuous-perfusion stirred-tank bioreactors with cell retention systems such as spinfilters also help in maintaining a high cell density and inhibitory products can be removed with the perfusion flow. The highest medium efficiency is reached with optimized fed-batch strategies, which can produce product concentrations in the final batch of 1 g L^{-1} [7], whereas the highest reactor efficiency is accomplished in a continuous-perfusion bioreactor with a cell-retention system, reaching product concentrations of 300-400 mg L^{-1} for weeks. An additional reason for the success of fed-batch and continuous-perfusion cultures is that they benefit from the 'autocrine' effect of having a high concentration of viable cells at low dilution rates. Animal cells seem to have less requirements for growth on environmental factors if they are seeded at higher cell densities[7]. Both systems have the ability to maintain a constant environment, thus assuring a constant product quality, which is important in the production of biopharmaceuticals.

Cell death

A critical requirement for the successful application of animal-cell culture at high cell density and low growth rate is that the death rate has to be considerably lower than this low growth rate. In addition to this, it can be noted that at reduced growth rate, animal cells are more sensitive to shear forces[8, 9].In animal cells there are two main pathways for cell death - apoptosis and necrosis. Considerable attention is being directed towards apoptosis and how to control it in order to prevent loss of viable cells[10]. For the successful application of protein-free media at a large scale, it will be essential that these media are able to provide a buffering capacity to neutralize toxic components such as ammonia and oxygen radicals. In this article, we will describe the impact of the use of protein-free media on the death of animal cells at high density and low growth rate caused by shear stress in both stirred and sparged bioreactors.

Production media

The culture medium should support both cell growth and product synthesis, and should provide protection against the different causes of cell death. Media for the culture of animal cells are complex when compared with most chemically defined prokaryotic media - even a minimal complete formulation contains more than 40

different components. One of the major objectives of animal cell culture has been to develop chemically defined, serum-free media, but the majority of the media used for both small- and large-scale cultures still contain undefined mixtures.
Cells alter the environment of the culture medium by removing nutrients and adding products and byproducts. These byproducts can be waste products from metabolism such as lactate, CO_2 or ammonia, but can also include excreted proteins or virus. As the viability of animal-cell cultures is rarely complete, cell death will obviously generate host-cell material from dead cells. These various 'products' will have little effect on the bulk viscosity of the culture liquid but will affect the gas-liquid interfacial properties.
The standard production medium has a kinematic viscosity (0.8×10^{-6} m^2 s^{-1}) and density (10^3 kg m^{-3}) similar to that of water. For Newtonian fluids, the shear stress (N m^{-2}) is the product of the dynamic viscosity (N s m^{-2}) and the shear rate (s^{-1}). Animal-cell suspensions are typical Newtonian fluids, because the cell density is relatively low and the culture medium contains low concentrations of solutes. Viscosity is the resistance offered by a Newtonian fluid to deformations, induced by shear stress.
The surface tension of popular basic media formulations for animal cells, like RPMI, DMEM and Ham's F12 are close to the value of water (0.070 N m^{-1}), while the surface tension of most complete media, whether serum-containing or serum-free, is lower (appoximately 0.060 N m^{-1}). A lower surface tension indicates that the gas-liquid interface is covered with surface-active components, which alters the way gas bubbles interact with other bubbles and cells.
The protective effect of certain polymers has been correlated with surface tension [11-14] and viscosity [15,16, 17]. Proteins tend to form rigid interfaces [18] by forming multiple loops attached to the interface, generating a stable foam of an hexagonal structure. A surface-active polymer such as Pluronic F68 is able to restore partial mobility of the interface in protein-containing media [12]. In such media, Pluronic F68 allows the development of a multilayer foam of 'rounded bubbles' but also prevents excessive foaming. The implementation of protein-free media requires the evaluation of the interfacial properties, because protection of animal cells against shear stress is related to the quality of the interface..

Animal cells and shear

The culture of animal cells in a homogeneous system requires mixing in order to maintain a homogeneous cell suspension and to provide effective transfer of nutrients to the cells. Mixing in animal-cell culture reactors is generated by rotating impellers (stirred-tank reactors) or by sparging (bubble-column and air-lift loop reactors). Mixing can generate shear forces, resulting from spatial differences in the pattern of liquid flow [19]. For suspended animal cells the important differences in

liquid flow (pressure, momentum) are those that occur on the scale of cells (5-20 mm). For cells adhering to microcarriers, it is the diameter of the microcarrier that is of major importance (75-200 mm).
The death of cells caused by shear forces is also related to the method of oxygen supply. The scale-up to volumes greater than 10L usually requires increased agitation combined with a low aspect ratio in order to supply sufficient oxygen to the cells. The alternative for reactors with a high aspect ratio is sparging with oxygen. This cannot be done without considering the effects of shear damage.
Laminar and turbulent flow conditions have been used to study the shear sensitivity of animal cells in different small-scale systems, such as viscometers, flow devices, bubble columns, spinner flasks and stirred and/or sparged bioreactors. From these studies, it has been concluded that the shear-induced death of animal cells is related to a range of parameters, including shear stress [20-23], shear time [8, 20], power dissipation [22, 23] and the growth phase of the cells [8, 9]. In general, sublethal cell damage or cell death were found to start in the range of shear stresses from 0.5 to 200 N m^{-2}. This was dependent on the shear time, but also on differences in cell line, culture media and shear conditions.
Other parameters that correlated with the death of animal cells caused by shear include : viscosity [15, 16, 17], surface tension [11-14], smallest turbulent eddy-length [15,16], regions of close clearance [19], regions of converging flow [24], and the generation of a deep vortex associated with bubble entrainment [25]. Turbulent eddies, smaller or of the same size as the cell can be damaging; the size of these smallest eddies can be estimated by the Kolmogoroff eddy length scale [19]. The parameters, taken from different shear systems, are listed together because shear from agitation or from sparging seem to share the same mechanisms. However, it is not known whether the different small-scale devices used for shear research represent the correct down-scaling of the large-scale fermentation process.

Stirring

Two basic mechanisms for shear-induced cell death in stirred-tank reactors are: (1) interface related [24, 26]; (2) liquid flow without interface [25, 27]. For a cell suspension stirred by an impeller, four types of flow pattern can be distinguished: (1) the generation of liquid flow by the impeller; (2) the dissipation of turbulent flow in the bulk; (3) flow in regions of close clearance; and (4) flow associated with the suspension surface.
The use of mild-type impellers combined with the relatively low power input needed for animal-cell cultures means that the liquid flow generated by the impeller and the dissipation of the turbulent flow in the bulk suspension volume are not the damaging effects of stirring. This is best illustrated by the successful use of conventional stirred tanks containing hundreds of litres of adherent cells on microcarriers.

The data that support the smallest turbulent eddy length as a critical parameter come from small-scale studies, in which an increase in viscosity results in a lower cell death [15, 16]. However, it is difficult to discriminate the effects of bulk flow and the effects of flow associated with the surface and in regions of close clearance in these small working volumes [24]. For large scale culture it was expected that the increased power input for mixing could generate small turbulent eddies if this energy would dissipate in a relative small volume around the impeller. Since no detrimental effect has been

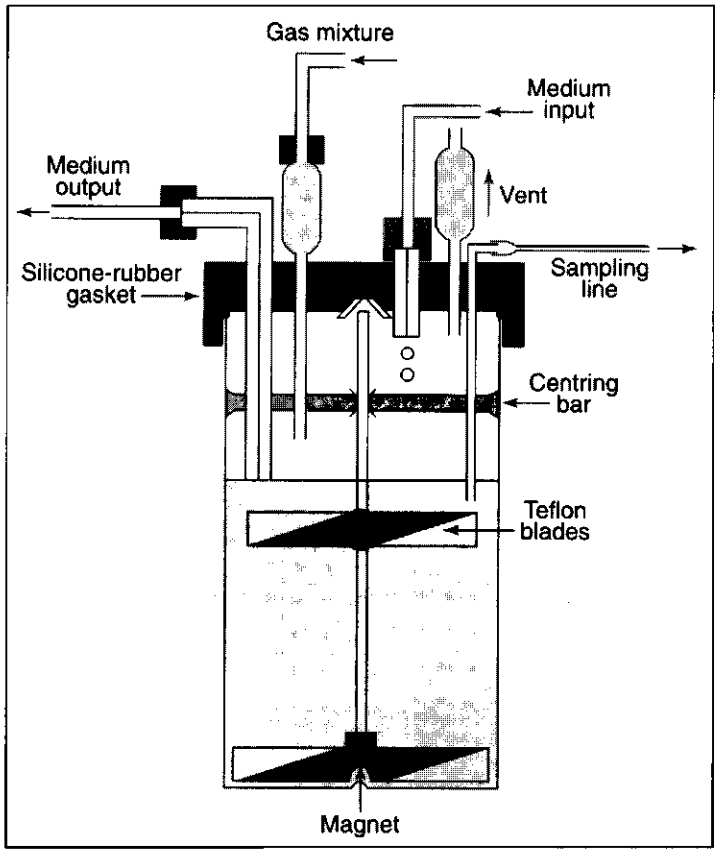

Figure 2 A design for a 1 litre chemostat reactor reported to cause cell death by shear stress at lower stirrer rotation speeds (original in reference 33). The close clearance between the lower impeller and the vessel wall and the position of the impeller just beneath the suspension surface could both create detrimental flow conditions even at moderate stirrer speeds. This is promoted by the type of blades of the impeller and the large diameter of the impeller.

reported it can be concluded that the volume in which the energy is dissipated, is sufficiently large to prevent the generation of smaller damaging eddies.
Examples of regions of close clearance include : (1) the distance from the impeller to other fixed tubing in the reactor; or (2) the distance between some types of baffle and the vessel wall. Liquid that is forced through the small openings can create converging or diverging laminar flow or turbulent flow regimes, similar to the flow devices used for shear research [23]. The effect of close clearance can be found in the integrated shear factor (ISF) model, that contains the distance between the impeller and the vessel wall [28, 29]. Usually, the design of a reactor can be adjusted to remove regions of close clearance while maintaining an effective mixing.
The last aspect of flow that can cause damage to animal cells is the liquid flow associated with the suspension surface. Using bench-scale reactors it has been concluded that cell damage caused by intensive stirring is the result of trapping and bursting of air bubbles [26]. Chinese hamster ovary (CHO) cells in medium containing 1 g L^{-1} albumin can be agitated at rates of up to 600 rpm without being damaged in the presence of bubbles if the bursting of bubbles at the suspension surface is prevented [27]. Therefore, the damaging effect of excessive agitation is primarily related to 'border' effects (wall shear, interface effects) rather than to bulk liquid flow. These effects tend to manifest themselves in small-scale systems. This conclusion is well illustrated by the 1L chemostat reactor (figure 2) for which a maximum stirrer speed of 60 to 100 rpm was reported for the culture of hybridoma cells in medium containing 10% serum [30]. The design shows small clearance between the impeller and the wall and the bottom of the vessel while the top stirrer is generating fast flow patterns near the surface.

Sparging

As for agitation shear, the shear damage caused by sparging can be divided into several types : (1) the generation of bubbles at the sparger nozzles; (2) the rising zone through the bulk liquid; and (3) the surface of the suspension. The suspension surface can be either covered with a foam layer or be free of foam. The standard condition during sparged fermentations is that a moderate foam layer is present. The damaging events of sparging are most likely to be associated with two detrimental mechanisms : (1) contact between the cell and an uncovered gas-liquid interface [31]; and (2) differences in the liquid flow pattern associated with a bubble burst at the suspension surface [19, 26].
The generation of bubbles and their rise to the surface has proved to have little effect on the death rate of cells in standard production media that contain sufficient surfactants to cover the gas-liquid interface rapidly. The differences in liquid flow associated with these events do not reach the intensity of the liquid flow that occurs following bubble burst at the surface of the suspension. What happens while the

bubbles rise is that the cells may attach themselves to the bubbles and are transported to the surface [31, 32], but this is dependent on the nature of the surfactants.
The fatal hydrodynamic effects that occur at the surface of a sparged suspension is associated with the bubble bursting [31, 33]. In the condition where no foam is present, the burst of a single bubble results in upward and downward jet streams [34]. The closer the cell is to the interface, the greater the chance is for it to get caught in the high-energy liquid flow [35]. It has been calculated that the release and dissipation of energy associated with bubble burst is several orders of magnitude greater than what has previously been described as damaging to animal cells [35, 36].
It is not known whether cells die as a result of the critical differences in liquid flow or the fast flow along the bubble interface that might also create uncovered interface areas. Because effective protection is provided by the moderately-surface-active polymer polyethylene-glycol (PEG) [14] with a molecular weight greater than 1000, the direct contact between cells and the uncovered bubble interface resulting in cell lysis, appears unlikely to be a prime mechanism of cell death. The protective effect of high concentrations of dextran that increases the viscosity seems to underline this [17].
Foam can be considered to be an indicator of the health of a sparged suspension of animal cells. A clean foam with homogeneous bubbles is a sign of suspension culture stability, whilst an inhomogeneous foam containing cell debris is associated with a dying culture. A clean homogeneous foam contains little pressure differences which is contributing to the great stability . The overall pattern of the foam is hardly disturbed by bubbles reaching the bottom layer of the foam or by the bursting of bubbles at the top layer. A controlled drainage process seems to maintain a 'foam steady-state'. A stable foam layer is associated with a low death rate [14, 33, 37]. The protective effect of a stable foam layer might be linked to reduced drainage liquid flow caused by surface-tension gradients, which create a force opposing the gravity acting on the draining fluid. The surface-tension gradient has the same units as shear stress (N m^{-2}), indicating that this gradient is able to compensate for the shear stress exerted by draining liquid in foam [18].
In a 'dirty' irregular foam, the pattern of bubbles is constantly changing because of bubble bursts throughout the foam, promoting more variation in bubble size. Fast drainage is expected in this type of foam and is correlated with a high cell-death rate [33, 37]. In bioreactor runs lasting for weeks at high cell densities, the increase in host-cell-derived molecules reduces foam stability [12], and not even Pluronic F68 seems to be able to counteract the negative effect of host cell material on the quality of the interface in longer fermentation runs.

Protection

The presence of protective polymers in the culture medium is essential for the protection of animal cells against shear. Polymers known to provide effective protection include Pluronic F68 and PEG [11, 13], and to a lesser extent, albumin and even dextran, although at higher concentrations [13, 17]. Except for dextran, all the polymers are surface active to various degrees and the only universal property seems to be in the high molecular weight.

Pluronic F68 is the most widely used and the most investigated protective polymer, and a number of protective mechanisms have been suggested : (1) the prevention of cell-bubble interaction [12, 31, 35], possibly by generating 'slippery' bubbles with a highly mobile, boundary layer [12, 33], which hampers the transport of cells to the

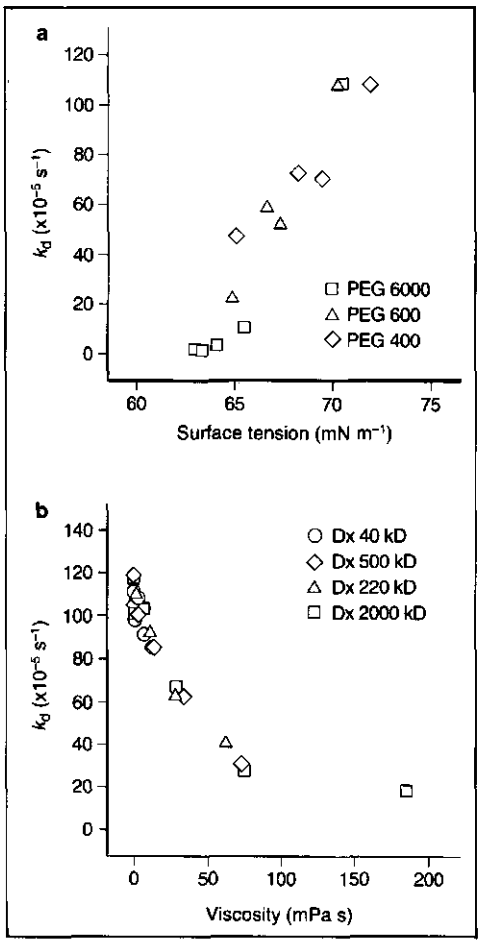

Figure 3a
First-order death rate constant (k_d) of hybridoma cells in a bubble column as a function from the surface tension of the culture medium. Differences in surface tension were generated by the addition of various concentrations of PEG with different molecular weight (figure adapted from reference 15).

Figure 3b
First-order death rate constant (k_d) of hybridoma cells in a bubble column as a function from the viscosity of the culture medium. Differences in viscosity were generated by the addition of various concentrations of different molecular weight dextran (original in reference 18).

surface and prevents them becoming caught in the jet streams following bubble burst [35]; (2) the generation of a stable foam layer with controlled slow drainage [33]; and (3) interacting with the cells [38, 39], producing more-shear-resistant cells by creating an artificial cell wall or increasing the elastic properties or mobility of the cell to resist deformation.

These protective effects seem to be related to the behaviour of Pluronic F68 at the interface, that is covering the interface but increasing the mobility of the boundary layer [12]. Pluronic F68 reduces the dynamic surface tension and appears to have no effect on the bulk viscosity.

PEG makes up the hydrophilic portion of the Pluronic F68 polymer, but the protective effect of PEG is correlated with the surface tension, indicating that its presence in the interface is required to provide protection (figure 3a). An alternative mechanism for some polymers such as albumin might be to increase the surface viscosity of the boundary layer of bubbles, comparable to the effect high concentrations of dextran can create by increasing the bulk viscosity [12, 13, 27] (figure 3b).

Conclusions

Culture media containing agents such as albumin and Pluronic F68 have proved to support large-scale cultures of animal cells at high cell densities over periods of several weeks. For the successful development of high-density, animal-cell cultures in protein-free media more information is required on the factors that initiate cell death during the production process. Improvements in protein-free media are also needed in order to increase their capacity to neutralize different toxic components. To correlate cell death caused by shear stress, it has to be clear what type of shear stress is predominantly present in a particular reactor.

However in general, cell death is proportional to the energy input and inversely proportional to the working volume of the suspension [36]. In general, the following considerations apply to scaled-up bioreactor runs : (1) minimize the energy input per unit working volume; (2) use a culture medium that is capable of maintaining interface conditions that prevent cell damage.

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Summary

Biopharmaceuticals are increasingly produced by modern biotechnological techniques. The in-vitro culture of animal cells in stirred tanks is one of the feasible systems, especially for proteins that require specific post-translational modifications to evoke a desired respons in patients. Animal cells are usually capable to accomplish these modifications in contrast to bacteria and yeast. Another advantage of animal cells is that they generally secrete their product into the culture medium, which is greatly facilitating the purification of the product.

As a disadvantage of animal cells is mentioned their sensitivity for shear stress. The high energy containing regions of liquid flow that can develop in a stirred tank can damage the cells because of their relative large size compared to yeast cells and bacteria and their lack of a protective cell wall. As a result of the scale-up of stirred tank cultures with animal cells the first detrimental shear phenomenon to become manifest is the shear stress associated with the sparging of the cell suspension. Sparging is a simple and efficient method to provide cells in large-scale stirred tanks with sufficient oxygen. The cell killing event of sparging is especially the bursting of bubbles at the surface of the suspension.

In chapter 1 an overview is given of the status of the research on the shear sensitivity of animal cells until about 1990, the starting point of the work on this thesis. The major conclusion of this overview is that because of a lack of a generic approach many fragmented data were gathered that allowed few generalized conclusions. The culture media used for shear testing was highly variable and often considered as of moderate importance. Later the central item of this thesis, the composition and features of the culture medium, emerged as the key factor to control shear sensitivity of animal cells.

Chapters 2 and 3 describe the effect of a reduced serum content of the culture medium on the shear sensitivity of animal cells for sparging. The use of culture media without serum has become a requirement for the production of biopharmaceuticals. The protective effect of serum was quantified in a bubble column under standardized conditions and the protection by serum was characterized as an immediate non-metabolic effect. In addition, in these chapters, the "killing volume" hypothesis as developed for insect cells, is validated for hybridoma cells.

In chapter 4 the effects of silicone antifoam are described. Just like serum, antifoam is preferably omitted during animal-cell fermentations. However, for some sparging experiments in the bubble column the addition of antifoam was necessary and therefore its effects needed to be quantified.

The protective effect of the polymer polyethylene-glycol (PEG) against cell death caused by sparging is described in chapter 5. The protection by PEG is correlated with the surface tension of the culture medium. It seems that the presence of PEG in the boundary layer of bubbles is essential for its protective effect.

The subject of chapter 6 is the protective effect of dextran. This polymer provides protection for animal cells against the bursting of bubbles by increasing the viscosity of the liquid. To establish the protective effect, high concentrations of high molecular weight dextran are required.
In chapter 7 an overview is given of the status of the research on the shear sensitivity of animal cells. Just like in this thesis, the focus in this trend article is determined by the composition and features of the culture medium. Trends in the production of biopharmaceuticals with animal cells, like the use of culture media without components from human or animal source, are discussed in this last chapter.

Samenvatting

Steeds meer geneesmiddelen worden geproduceerd met behulp van moderne biotechnologische technieken. Een geschikt systeem hiervoor is het kweken van dierlijke cellen die het geneesmiddel (eiwitproduct) maken terwijl ze groeien in een voedingsvloeistof (medium) in geroerde vaten. Een voordeel van dierlijke cellen is dat ze goed zijn in het maken van eiwitproducten. Aan deze eiwitproducten worden hoge eisen gesteld voor wat betreft vormgeving om bij een patient een juist effect te verkrijgen. Een ander voordeel van dierlijke cellen is dat ze normaliter het product, nadat het in de cel is gemaakt, naar buiten transporteren. Dit maakt de zuivering van het product eenvoudiger.
Een nadeel van dierlijke cellen vormt hun gevoeligheid voor mechanische krachten (afschuifkrachten). De vloeistofstromen die in een geroerd vat ontstaan, kunnen de cellen beschadigen omdat dierlijke cellen vrij groot zijn en niet worden beschermd door een celwand. Het doorblazen van luchtbellen is een simpele en efficiente manier om de cellen in grote reactoren van zuurstof te voorzien. Het zijn echter met name de afschuifkrachten die gepaard gaan met het knappen van de luchtbellen aan het oppervlak van de geroerde suspensie die de meeste celsterfte veroorzaken.
In hoofdstuk 1 wordt een overzicht gegeven van de status van het onderzoek naar de gevoeligheid van dierlijke cellen voor afschuifkrachten tot 1990, het beginpunt van dit promotie-onderzoek. Belangrijkste conclusie is dat er veel fragmentarisch onderzoek was gedaan onder verschillende omstandigheden die het moeilijk maakten tot algemene conclusies te komen. Het kweekmedium voor de cellen was de voornaamste variabele van het onderzoek en vormt de rode draad in dit proefschrift.
In de hoofdstukken 2 en 3 wordt de invloed van een dalende concentratie serumtoevoeging op de gevoeligheid van dierlijke cellen voor het doorblazen van luchtbellen behandeld. Het verwijderen van serum als toevoeging aan het medium is vereiste geworden voor de productie van geneesmiddelen. Het beschermende effect van serum wordt gekwantificeerd onder standaardomstandigheden in een bellenkolom en dit beschermend effect kan worden gekenschetst als een snel niet-metabool effect.
Ook wordt in deze twee hoofdstukken de geldigheid van het 'killing volume' model, zoals dat voor insectencellen was opgesteld, voor hybridomacellen bewezen.
In hoofdstuk 4 worden de effecten van antifoam beschreven. Net als serum is antifoam een mediumtoevoeging die men over het algemeen liever kwijt dan rijk is. Voor dit onderzoek was antifoam een onmisbare factor voor de praktische uitvoering en diende derhalve kwantitatief gekarakteriseerd te worden.
Het beschermende effect van de polymeer polyethyleenglycol tegen schade aan cellen veroorzaakt door het doorblazen van luchtbellen wordt beschreven in hoofdstuk 5. Het beschermende effect blijkt verband te hebben met de oppervlaktespanning van het kweekmedium. Kennelijk is de aanwezigheid van polyethyleenglycol in de grens-

laag van de luchtbellen nodig om een beschermende werking voor de cellen te kunnen bieden.
In hoofdstuk 6 wordt het beschermende effect van de polymeer dextraan behandeld. Dit polymeer blijkt dierlijke cellen tegen het knappen van luchtbellen te beschermen door de viscositeit van de vloeistof te verhogen. Om dit effect te bewerkstelligen zijn hoge concentraties van dextraan met een hoog molecuulgewicht nodig.
Hoofdstuk 7 biedt een overzicht van de stand van zaken voor het onderzoek naar de gevoeligheid van dierlijke cellen voor afschuifkrachten. De samenstelling en eigenschappen van het kweekmedium vormen - net als voor dit proefschrift - de bindende factor. Ontwikkelingen voor de productie van eiwitten met behulp van dierlijke cellen voor de farmaceutische markt, zoals het gebruik van kweekmedia zonder componenten van dierlijke of menselijke oorsprong, komen hier aan de orde.

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Curriculum Vitae

Leo van der Pol werd op 19 juni 1958 geboren te Raalte. In 1976 behaalde hij het Atheneum B diploma aan de Rijksscholengemeenschap 'Magister Alvinus' te Sneek. De studie Biologie aan de Rijksuniversiteit Groningen werd afgerond in 1984. Hoofdvak was moleculaire microbiologie met als bijvakken gedragsfysiologie en vakdidactiek. Na een oriëntatiejaar met diverse activiteiten werkte Leo in 1986 als onderzoeker bij de vakgroep Plantenfysiologie van de RUG. Vanaf 1987 is hij actief als microbioloog bij Bio-Intermediair in Groningen en van 1994 tot 1998 als manager van de afdeling PreProductie van Gist-brocades/Bio-Intermediair. PreProductie had als hoofdtaken ontwikkeling en overdracht van processen voor grootschalige productie volgens farmaceutische (cGMP) richtlijnen. Vanaf najaar 1998 werkt Leo als senior scientist bij DSM-Biologics, de nieuwe naam voor Gist-brocades/Bio-Intermediair en is belast met de verantwoordelijkheid voor de biotechnologische kant van de projecten met dierlijke cellen.
In 1988 werd de samenwerking gestart met de vakgroep Proceskunde van de Landbouwuniversiteit Wageningen, wat uiteindelijk heeft geleid tot dit proefschrift.