Animal-cell culture in aqueous two-phase systems

Gerben Meile Zijlstra

Promotor:	dr. ir. J. Tramper Hoogleraar in de bioprocestechnologie
Co-promotoren:	dr. ir. C.D. de Gooijer Universitair hoofddocent bij de sectie proceskunde dr. L.A. van der Pol Senior scientist DSM Biologics, Groningen

Gerben Meile Zijlstra

Animal-cell culture in aqueous two-phase systems

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 4 december 1998
des namiddags te vier uur in de Aula

ISBN 90-5485-937-7

Coverdesign: Thalen, Groningen
Lay-out: COMPUTEKST grafische tekstverwerking, Groningen
Lithography: PEACH belichtingsstudio bv, Groningen
Printed by Dijkhuizen Van Zanten bv, Groningen

Stellingen

behorende bij het proefschrift
'Animal-cell culture in aqueous two-phase systems'
van Gerben Meile Zijlstra.

1. Whereas statisticians generally overestimate the practical applicability of statistical experimental designs, experimenters underestimate its usefullness.

2. Integration of unit operations, for instance fermentation and primary recovery, not only combines the 'best of both worlds', but also the worst.

3. Visionaire wetenschappers laten hun visie prevaleren boven de bekende feiten. De weinige 'grote wetenschappers' zijn visionairen die achteraf gelijk hadden.

4. Degene die zich te druk maakt om de details komt nooit aan de grote lijn toe.

5. Zoals overal heb je ook binnen het bedrijfsleven de zogenaamde 'builders' en 'achievers'. 'Builders' zijn altijd druk met het bouwen aan een efficiente machine. 'Achievers' daarentegen houden rustig in de gaten wanneer de machine klaar is, stoppen er olie in en gaan ermee vandoor.

6. Promoveren binnen een bedrijf is een langdurige kwestie.

7. Ook in het lab geldt: beter een goede stoel dan een stel krukken.

8. Dat de mythe 'Amerika, het land van gouden bergen' na aankomst snel werd doorgeprikt door de nieuwe immigranten, blijkt duidelijk uit het volgende citaat:
   'When I went to America people told me that the streets were paved with gold. When I came here, however, I found out three things. First, that the streets were not paved with gold. Second, that they were not paved at all. And third, that I was the one who was going to pave them!'

9. De op handen zijn de geboorte van een kind stelt de aanstaande vader in staat tot ongekende prestaties.

10. De recente (bijna bijbelse) watervloeden, bosbranden, de wereldwijde ontregeling van het klimaat, te wijten aan 'El Niño' (het kerstkindje), de dreigende Millenium-problematiek en de dalende beurskoersen, zullen de speculaties over een naderende Apocalyps weer danig doen toenemen.

11. De afkeer van veel Groningers voor Friezen is voor een groot gedeelte te verklaren vanuit hun jaloezie op het feit dat Friezen beter in staat zijn geweest hun eigen identiteit te bewaren.

12. De wrâld is rûch, mar dy 't him net rêde kin is slûch!

Wageningen, 4 december 1998

Foar Aukje en lytse Johan

Voorwoord

Aan het begin van dit proefschift past een woord van dank aan iedereen die direct of indirect bij de totstandkoming betrokken is geweest.
Met name wil ik mijn begeleiders, Hans Tramper en Kees de Gooijer, bedanken voor de ruimte die ze mij hebben gegeven om te zwemmen en uiteindelijk met het hoofd boven water te komen. Als één van de eerste externe aio's en dan ook nog gedetacheerd bij een klein Biotech-bedrijfje, was het zoeken naar de juiste balans. Hun nooit aflatende steun en immer opbouwende kritiek is zeer belangrijk geweest voor de afronding van dit boekje.
Bij het bedrijf Bio-Intermediair, destijds nog gevestigd in de binnenstad, heb ik een geweldige en zeer speciale tijd beleefd. Ik ben hier getuige geweest van de geboorte en groei van een Biotech-bedrijf vanuit een 'garage', een zeldzaamheid in Europa. Ik wil met name de oude garde noemen, waarvan ik Leo van der Pol en Jos van Weperen speciaal dankbaar ben voor hun medewerking aan dit proefschrift, maar ook andere Turftoren-'giganten' als Koos Koops, Rolf Hendriks, Kees van der Graaf, Peter van Hoorn, Aidrid St. Jago, Annelies Leeuwe, Jappie Zwaagstra, Henny Schuring en Marco Lip ben ik dankbaar.
Mijn collega's in Wageningen en omstreken zijn altijd uiterst plezierig in de omgang geweest en hebben een inspirerende invloed op mij uitgeoefend, met name tijdens de excursieweken naar Zwitserland en Engeland. Frank van Lier, Fred van den End, Wilfried Bakker, Arie van Oorschot, Dirk Martens, Richard Dorrestein, Hedy Wessels, Imke Leenen, Leonie Linders, Taco Wijtzes, Joyce Krauss en alle anderen: bedankt!
Zonder studenten is het lastig om een boekje te vullen. Zonder de anderen te kort te willen doen, wil ik met name Marco Michielsen bedanken voor de fenomenale hoeveelheid data die hij heeft verzameld.
Verder bedank ik hierbij de Wageningers Sabine Cuypers, Mike Litjens, William Arts en Roeland Costenoble, en de Leeuwarders Sharon Bulstra, Vincent Munster en Elske van der Kooij.
Professor J.J. Beintema van de RUG wil ik bedanken voor zijn bijdragen aan het SGPZ-project, in het kader waarvan ik mijn promotie-onderzoek heb uitgevoerd.
Tenslotte bedankt ik DSM Biologics (voorheen Bio-Intermediair) voor het mogelijk maken van dit proefschrift.

Contents

Chapter 1

Introduction

Industrial animal-cell culture

In current industrial biotechnology, animal-cell culture is an important source of therapeutic biological products. Animal-cell-culture products range from recombinant proteins to viral vaccines, or even whole cells. Despite their slow growth rates and low productivity as compared to bacterial cells or yeast, the capacity of an animal cell to fully 'process' products (including folding, glycosilation, subunit assembly etc.), is unmatched and often decisive when choosing an appropriate expression system (Arathoon, 1988; Rhodes, 1988; Bibila, 1995; Jenkins, 1996; Hu, 1997). This introduction will focus on the production of therapeutic proteins with animal-cell culture. Typical expression systems used are hybridoma cell lines, recombinant cell lines, such as recombinant Chinese Hamster Ovary (CHO) or myeloma cell lines, or more recently the insect cell/baculovirus system. Examples of products already on the market are, erythropoietin (EPO), tissue plasminogen activator (tPA), interferons and several monoclonal antibodies. Hybridoma cell lines are established by fusion of antibody-producing beta-lymphocytes (having a limited regeneration capacity) with immortalized myeloma cell lines, and subsequent selection of high producing clones (Köhler, 1975). Recombinant cell lines are established by using expression vectors to insert one or more genes, coding for the desired product, into the genome of immortalized cell lines from primary tissues, such as CHO, Baby Hamster Kidney (BHK) or myeloma cell lines. Again, stable and high producing clones are then selected. Ingenious techniques to improve and speed up this cell-line development are continually evolving (Bibila, 1995; Trill, 1995). The insect cell/baculovirus expression system results in a transient expression. Here cells are allowed to grow to certain cell densities, after which recombinant baculovirus is added. The baculovirus takes over the insect-cell protein-production machinery to produce the recombinant protein in large amounts. The advantage of this expression system compared to the constitutively producing ones is that the time necessary to select suitable production clones can be greatly reduced (Murhammer, 1991; Van Lier, 1991; Davies, 1994).

The main driving forces for developments in current industrial animal-cell processing, beside the emergence of new technologies, are on one the hand economic considerations, such as 'the time to market' for a product or the process economy,

when the production scale is large, and on the other hand the regulatory requirements imposed on products for human-health care.

Economic considerations

The primary economic consideration in the manufacturing of biologics is the return of investment on patents, product and process development, preclinical and clinical studies and on the establishment and maintenance of production facilities. The 'time to market' is a crucial factor in this respect, because delay in market entry may compromise selling prizes, allow competitors to gain market share, and will reduce the selling period from market entry to patent expiry. Process economy is another major economic consideration (Van't Riet, 1991; Datar, 1993). However, because of the relatively small annual demands of many biologics and the subsequent small production scales involved, it's importance is often less pronounced than for other pharmaceuticals.

To reduce the time required for process development, scale-up and process validation for each individual animal-cell product, industry is working towards standardization of production processes for the major expression-system and product categories. These, so called generic processes exploit the common characteristics of cell lines and their products and provide a blueprint that can be used for similar cell lines or products, with only minor adjustments.

To optimize the economy of a production process (as a whole), the individual steps, such as fermentation, clarification, concentration and column chromatography, have to be optimized with respect to the output (yield and quality) of the process as a whole: an integral approach to process optimization is required.

As a rule of thumb process economy can be improved by increasing the volumetric productivity and/or the product concentration (compared to contaminant concentrations) of the fermentation (Van 't Riet, 1991). To achieve these goals in the first place much attention is focussed on cell-line improvement. Through genetic- and metabolic engineering improvement is pursued, not only of the cell-specific productivity, but also of other important cell characteristics like longevity and robustness (Cameron, 1997; Al-Rubeai, 1998). Secondly, much attention is paid to optimization of the cell environment, through media optimization in combination with high-yielding fermentation strategies, such as fed-batch cultivation and continuous perfusion (Bibila, 1995; Hu, 1997).

Opportunities for improvement in downstream-processing economics, through improvement of each separate unit operation, lie mainly in the emergence of new technologies like for example perfusion chromatography (Afeyan, 1990).

One step further in the integral approach to process optimization is to 'physically' integrate process steps into new unit operations (Thömmes, 1995; Born, 1996) . The rational for this approach is to reduce the number of process steps and thus to

circumvent the subsequent yield losses, and to reduce the operational and investment costs (Daugulis, 1988, 1991, 1997; Freeman, 1993).

Regulatory aspects

To obtain a marketing license for therapeutic biological products, manufacturers have to apply to the relevant regulatory authorities (such as the Center for Biologics Evaluation and Research (CBER) of the FDA) and to demonstrate, using data from extensive non-clinical and clinical studies, that their product meets the standards on safety, purity and potency, and is stable prior to its expiry date. Moreover, they have to demonstrate that the manufacturing process meets the current Good-Manufacturing-Practice (cGMP) standards to ensure a consistent product quality (21CFR 601.2).

Key issue in cGMP is to minimize the potential risks for the product quality, introduced in the manufacturing process by the people involved (human error), the facility and its equipment, or the raw materials. This comes into effect through proper design, control and validation of the manufacturing process.

Once a product is licensed and the manufacturing process is fully validated, modification of the process would not only require revalidation of the process, but also (partial) repetition of the non-clinical and the clinical studies. Generally, this is not economically feasible and manufacturing processes are more or less fixed once they are licensed. Even prior to licensing, changing (optimizing) the manufacturing process may have regulatory repercussions and is generally avoided. Therefore, if process economy is an important issue, it has to be addressed in the early stages of product and process development.

An example of regulation-driven developments in animal-cell processing is the use of serum- and 'animal'-free culture media for animal cell lines, to avoid contamination of the product with adventitious agents, such as viruses or prions from sources of animal or human origin.

Other examples are the focus on viral clearance during the process, to remove potential adventitious viruses derived from the cell lines used, as well as the focus on product quality as a process optimization parameter and the related continuing developments in analytical techniques (Andersen, 1996).

Current production processes

Current large-scale animal-cell-culture processes, where process economy is important, typically use suspended cells with constitutive protein expression and excretion of the products into the supernatant (Hu, 1997). Typically, these processes consist of high-yielding fermentations followed by a primary recovery stage, a pur-

ification stage and a polishing stage (Österlund, 1986; Scott, 1987). Since the products are excreted, a typical primary recovery would include a cell and debris removal step (clarification), by means of centrifugation, dead-end- or tangential-flow filtration, followed by a concentration- and/or buffer exchange step, for instance by tangential flow ultrafiltration, in which water and small molecules are removed. In the purification stage the objective is to remove macromolecular contaminants like lipids, DNA and contaminating proteins. In general a number of column-chromatography steps, such as affinity, ion-exchange or hydrophobic-interaction chromatography, are sufficient. If necessary these operations may be preceded by buffer-exchange steps, using for instance diafiltration. In the polishing stage, multimers, aggregates or improperly processed product are removed, for instance by gel-filtration. During the production process sufficient clearance of viruses is required. To this end, specific virus inactivation steps, such as solvent/detergent treatments, or virus removal steps, such as virus filtration, are integrated into the process.

Integration of fermentation and down-stream processing in animal-cell culture

As discussed previously, physical integration of unit operations provides an opportunity to improve the economy of existing processes. In particular *in situ* recovery, where the product is extracted from the bioreactor during fermentation, thereby combining fermentation, primary recovery and a purification step, has gained much attention as a means for such an integration (Daugulis, 1988, 1991, 1997; Andersson, 1990; Kaul, 1991; Freeman, 1993; Zijlstra, 1998).
The research described in this thesis focuses on exploring the opportunities for *in situ* recovery in animal-cell culture by means of extractive fermentations.
The concept of extractive fermentations is to use two immiscible liquid phases, one phase containing the cells and the other phase extracting the product. By recycling the cell-containing phase into the bioreactor, a continuous perfusion process is possible and if a good partition coefficient of the product into the extracting phase can be obtained, a cell-free, concentrated and partially purified harvest can be obtained straight from the bioreactor. The concept of extractive fermentation is especially useful when instable or easily degradable products are produced, because the in-line product extraction considerably reduces the residence time of the product in the fermentation broth (Daugulis, 1988, 1991, 1997; Andersson, 1990; Kaul, 1991; Freeman, 1993; Zijlstra, 1998).
For extractive fermentations with protein-excreting animal cells, polymer-polymer Aqueous Two-Phase Systems (ATPSs) seem to be the most appropriate two-liquid-phase systems (Albertsson, 1986; Andersson, 1990; Kaul, 1991; Zijlstra, 1998). These ATPSs can be made by mixing two polymers, for instance PEG and dextran, in an aqueous solution. Above certain concentrations two liquid phases will form,

the upper phase mainly consisting of water and PEG and the lower phase mainly consisting of water and dextran. Polymer-polymer ATPSs form a relatively mild, non-toxic environment to the cells in contrast with water/organic-solvent two-phase systems (Albertsson, 1986; Andersson, 1990; Kaul, 1991; Zijlstra, 1998). In addition they are suitable for protein extractions, because of their relative hydrophilicity (Kroner, 1987; Kula, 1982).
For effective extractive fermentations with animal-cell cultures, the ATPSs used, must at least be able to support animal-cell growth. Furthermore they must separate the cells from the product by selective partitioning of the cells into one phase and the product into the other phase.

Contents of this thesis

In chapter 2 of this thesis, 'Design of aqueous two-phase systems supporting animal cell growth: a first step towards extractive bioconversions', the influence of the ATPS-forming polymers PEG and dextran on hybridoma BIF6A7 cell growth is characterized and ATPSs are selected in which the cells partition to the lower phase, and can be cultured over prolonged periods of time.
In chapter 3, 'Hybridoma- and CHO-cell partitioning in aqueous two-phase systems', the partitioning of mouse/mouse hybridoma cell line BIF6A7, is investigated systematically using a statistical experimental design, to identify the key factors governing cell partitioning and to select ATPSs with suitable cell partitioning for extractive bioconversions. Furthermore, both the partitioning and cell growth of other cell lines (a mouse/rat hybridoma and a CHO cell line) in ATPSs are studied, to assess the feasibility of extractive fermentations for other animal-cell lines.
In chapter 4, 'Separation of hybridoma cells from their IgG product using aqueous two-phase systems', the partitioning of product (IgG) in ATPSs is studied systematically, to identify the key variables governing IgG partitioning, and improve the separation of hybridoma cells and their IgG product. This is done by comparing the IgG partition data with the hybridoma-cell partition data obtained in chapter 3. Because separation of cells and product is troublesome without additional means, a number of dye-resins is screened for their ability to bind the BIF6A7 IgG antibody. One particular dye-ligand coupled to PEG is tested for its ability to manipulate the IgG partitioning in an ATPS.
In chapter 5, 'IgG- and hybridoma partitioning in aqueous two-phase systems containing a dye-ligand', the effect of the previously selected PEG-dye-ligand on both IgG and hybridoma partitioning in ATPSs is characterized more extensively and selection criteria are established for effective ligands for extractive fermentations with animal cells in ATPSs.
Finally, in chapter 6, 'Extractive bioconversions in aqueous two-phase systems', the

recent developments in the field of extractive bioconversions in aqueous two-phase systems (ATPSs) are reviewed.

References

Afeyan NB, Fulton SP, Gordon NF, Maszaroff I, Varady L & Regnier F (1990) Perfusion chromatography. BIO/TECHNOLOGY 8, 203-206.

Albertsson PA (1986) In: Partitioning of cell particles and macromolecules, edn. 3. Wiley & Sons, New York.

Al-Rubeai M & Singh RP (1998) Apoptosis in cell culture. Curr. Opin. Biotechnol. 9: 152-156.

Andersen JS, Svensson B & Roepstorff P (1996) Electrospray ionization and matrix assisted laser desorption/ionization mass spectrometry: Powerfull analytical tools in recombinant protein chemistry. Nature Biotechnol. 14, 449-457.

Andersson E & Hahn-Hägerdal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb. Technol. 12: 242-253.

Arathoon WR & Birch JR (1986) Large scale cell culture in biotechnology. Science 232: 1390-1395.

Bibila TA & Robinson DK (1995) In pursuit of the optimal fed-batch process for monoclonal antibody production. Biotechnol. Prog. 11: 1-13.

Born C, Thömmes J, Biselli, Wandrey C & Kula MR (1996) An approach to integrated antibody production: Coupling of fluidized bed cultivation and fluidized bed adsorbtion. Bioproc. Bioengin. 15: 21-29.

Cameron DC & Chaplen FWR (1997) Developments in metabolic engineering. Curr. Opin. Biotechnol. 8: 175-180.

Datar RV, Cartwright T & Rosen CG (1993) Process economics of animal cell and bacterial fermentations: A case study analysis of tissue plasminogen activator. BIO/TECHNOLOGY 11: 349-357.

Daugulis AJ (1988) Integrated reaction and product recovery in bioreactor systems. Biotechnol. Prog. 4: 113-122.

Daugulis AJ (1991) Integrated fermentation and recovery processes. Curr. Opin. Biotechnol. 5: 192-195.

Daugulis AJ (1997) Partitioning bioreactors. Curr. Opin. Biotechnol. 8: 169-174.

Davies AH (1994) Current methods for manipulating baculoviruses. BIO/TECHNOLOGY 12: 47-50.

Freeman A, Woodley JM & Lilly MD (1993): *In situ* product removal as a tool for bioprocessing. BIO/TECHNOLOGY. 11: 1007-1012

Hu WS & Aunins JG (1997) Large-scale mammalian cell culture. Curr. Opin. Biotechnol. 8: 148-153.

Jenkins N, Parekh RB & James DC (1996) Getting the glycosilation right:: Implications for the biotechnology industry. Nature Biotechnol. 14: 975-981.

Kaul R & Mattiasson B (1991) Extractive bioconversions in aqueous two-phase systems. In: Extractive bioconversions. Mattiasson B & Holst O (eds.) Bioprocess Technology. 11: 173-188.

Köhler G & Milstein C (1975) Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256: 495-497.

Kroner K & Kula MR (1978) Extraction of enzymes in aqueous two-phase systems. Proc. Biochem. 13: 7-10.

Kula MR, Kroner K & Hustedt H (1982) Purification of enzymes by liquid-liquid extraction. Adv. Biotechnol. Eng. 24: 73-118.
Murhammer DW (1991) The use of insect cell cultures for recombinant protein synthesis: Engineering aspects. Appl. Biochem. Biotechnol. 31: 283-310.
Österlund C (1986) Large-scale purification of monoclonal antibodies. TIBTECH (Nov): 288-293.
Rhodes M & Birch J (1988) Large-scale production of proteins from mammalian cells. BIO/TECHNOLOGY 6: 518-523.
Scott RW, Duffy SA, Moellering BJ & Prior C (1987) Purification of monoclonal antibodies from large-scale mammalian cell culture perfusion systems. Biotechnol. Prog. 3: 49-56.
Thömmes J & Kula MR (1995) Membrane chromatography: An integrative concept in the downstream processing of proteins. Biotechnol. Prog. 11: 357-367.
Trill JJ, Shatzman A, Ganguly S (1995) Production of monoclonal antibodies in COS and CHO cells. Curr. Opin. Biotechnol. 6: 553-560.
Van Lier FLJ, Vlak JM & Tramper J (1991) Production of baculovirus-expressed proteins from suspension cultures of insect cells. In: Spier RE & Griffiths JB (eds.) Animal cell biotechnology. Vol V (pp 169-188) Academic Press, Londen
Van 't Riet K & Tramper J (1991) In: Basic bioreactor design. Marcel Dekker Inc, New York.
Zijlstra GM, De Gooijer CD & Tramper J (1998) Extractive bioconversions in aqueous two-phase systems. Curr. Opin. Biotechnol. 9: 171-176.

Chapter 2

Design of aqueous two-phase systems supporting animal cell growth: a first step towards extractive bioconversions

Summary

The design of Aqueous Two-Phase Systems (ATPSs) which support the long-term growth of animal cells is described in this paper. It was found that the increase in osmolality caused by the ATPS-forming polymers could be compensated by reducing the Na-chloride concentration of the culture medium. Cell growth was possible in culture media containing up to 0.025 g/g of PEG or up to 0.15 g/g of dextran. In ATPSs of PEG 35.000, dextran 40.000 and culture medium, the hybridoma cells partitioned to the PEG-lean phase. In two of these ATPSs hybridoma cells were successfully cultured over a period of more than two months. The MAb product, however, partitioned along with the cells in the lower phase, but preliminary experiments using PEG ligands showed improved MAb partitioning.

Introduction

A trend in the production of biopharmaceuticals is a further integration of the upstream (cell culturing) and the downstream (purification) part of the production process (Daugulis, 1988; Freeman, 1993). This development means that improvements in one part of the process that create problems in other parts of the process are avoided. In addition, the integration reduces the number of process steps, which simplifies the registration of the production process. Furthermore the increased use of serum-free and protein free media often urges a fast processing of intermediate crude product because of the greater risk of product degradation (Mohan, 1993; Kratje, 1994).
Extractive bioconversions using Aqueous Two-Phase Systems (ATPSs) seem espe-

---

This chapter has been published in: Enzyme and Microbial Technology, 1996, **19**:2-8 by the authors G.M. Zijlstra, C.D. de Gooijer, L.A. van der Pol and J. Tramper

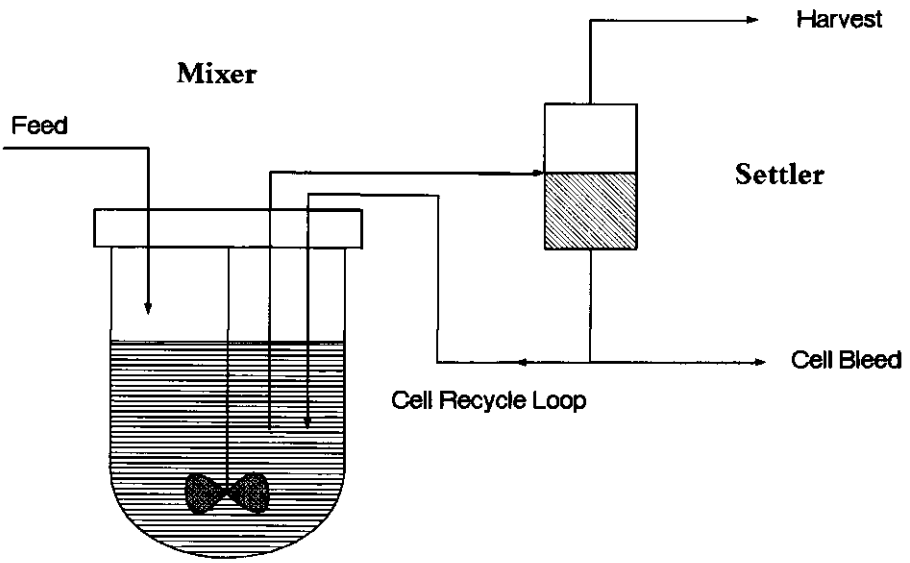

Figure 1 Schematic drawing of an extractive bioconversion with animal cells in a mixer-settler type reactor configuration.

cially suitable for integration of fermentation and downstream processing in animal-cell culture, where extracellular proteins are produced. Extractive bioconversions offer the advantages of cell retention within the bioreactor, of rapid product removal from the cell containing phase, and of a cell-free, concentrated, and partially purified effluent (figure 1). Extractive bioconversions can also be advantageous when product inhibition occurs. Aqueous Two-Phase Systems form a relatively mild environment for cells as compared to water/organic solvent two-phase systems, and they are furthermore suitable for protein extractions (Albertsson, 1986). ATPSs can be made by dissolving two incompatible polymers or a polymer and salt in water. Above certain concentrations described by a phase diagram, two immiscible phases will form (Albertsson, 1986). Using ATPSs in which the animal cells grow in one phase while the protein product concentrates in the other phase, an extractive bioconversion can be designed (Mattiasson, 1988).
Extractive bioconversions with bacteria (Andersson, 1990), some excreting a protein product, yeasts (Andersson, 1990) and even plant cells (Buitelaar, 1992) have already been reported. But, although the partitioning of several types of animal cells in ATPSs has been studied (Walter, 1982; Albertsson, 1986), no extractive bioconversions with animal cells have been reported yet.

The aim of the current research is to design aqueous two-phase systems which support the long-term growth of animal cells, as a first step towards an extractive

bioconversion with animal cells in ATPSs. To establish an aqueous two-phase system suitable for animal-cell growth, the effects of the individual ATPS-forming polymers PEG and dextran on relevant physical properties of the culture medium as well as on hybridoma cell growth have been determined. Subsequently, ATPSs in which the cells partition to a phase with tolerable polymer concentrations have been selected and these ATPSs have been tested for their ability to support long-term hybridoma cell growth. Since the current paper is only the first to describe growth of animal cells in ATPSs, and further improvements in the working values of this process are to be expected, no comparison between extractive bioconversions and conventional production processes were made at this stage.

Materials and methods

Cell line and culture media

The cell line used, BIF6A7, is a mouse/mouse hybridoma, originating from an SP2/0 myeloma. BIF6A7 produces a monoclonal antibody (MAb) binding the Factor VIII/Von Willebrand-Factor complex (Koops, 1990) which plays a role in the human blood-coagulation mechanism. BIF6A7 was supplied by DSM Biologics (Groningen, The Netherlands). A 3:1 mixture of Dulbecco's modified Eagle's and Ham's F12 (DMEM/F12) medium (Van der Pol, 1992) (both Gibco, Breda, The Netherlands) was used in the continuously stirred tank reactor (CSTR) culture from which cells were taken to inoculate the batch-growth experiments. The DMEM/F12 medium was supplemented with glutamine (0.6 g/L), Na-bicarbonate (3.5 g/L), 2-mercaptoethanole (3.9 mg/L), transferrin (Sigma 5 mg/L), selenite (2.5 μg/L), ethanolamine (0.7 mg/L), ascorbic acid (5 mg/L) and glutathione (reduced, 1 mg/L). All supplements were obtained from Merck (Darmstadt, Germany), unless stated otherwise. The osmotic pressure of this medium was about 320 mOs/kg. Polymer-containing media were made from polymer stock solutions and 4 times concentrated DMEM/F12 3:1 medium with the previously mentioned supplements, but without Na-chloride. For each polymer molecular weight and concentration, a specific amount of Na-chloride from a 1 mol/kg stock solution was added to give an osmotic pressure of about 300 mOs/kg. DMEM/F12 3:1 without Na-chloride was obtained as powder medium from Gibco (Breda, The Netherlands).

Polymer solutions

Poly(ethylene glycol) (PEG) 6000, 20.000 and 35.000 were obtained from Merck (Darmstadt, Germany). Dextran T40 and T500 were obtained from Pharmacia (Uppsala, Sweden) and Dextran 220.000 from Fluka (Buchs, Switzerland). All polymers were without further purification dissolved in distilled water to about 0.30 g/g and then sterilized by autoclaving for 20 minutes at 121°C. Dry-weight

concentrations of the polymer stocks were determined by refractometry after appropriate dilution.

Batch-growth experiments

The experiments were carried out in T-flasks in duplicate. Controls (containing media without polymers) were included. The cultures were inoculated with cells taken from a 1L CSTR culture of BIF6A7 (dilution rate 0.028 h^{-1}). Cell density, viability and specific production remained at a constant level during the CSTR culture. The cells were centrifuged (3000 rpm in a table centrifuge (MSE)) and resuspended in the polymer containing media to initial cell densities between $1.5x10^5$ and $2.0x10^5$ cells per mL. The culture flasks were incubated at 37 °C under air containing 5 % CO_2. For five days samples were taken daily for cell counts and stored at -20 °C until analysis. The cell morphology was studied directly in the culture flasks using a light microscope at 200 x magnification. At day eight of the batch culture samples were taken for final product yield determination, osmotic pressure, pH and density measurements.

Phase diagram

The phase diagram was determined by making five sterile 100 g phase systems from sterile stocks of PEG 35.000 and dextran 40.000, and sterile distilled water. The overall compositions were selected at different distances from the critical point (figure 5). They gave approximately equal top to bottom volume ratios. The systems were warmed to 37 °C and thoroughly shaken. They were left to settle for 72 h at 37 °C. From each top and bottom phase two samples of about 10 mL were carefully taken and centrifuged for 30 minutes at 4000 rpm and at 37 °C in a thermostated SIGMA 3K1 centrifuge. From the clear samples, sometimes having a small top or bottom-phase residue, quantities of about 9 mL were carefully pipetted, weighed and diluted to 25.00 mL.

Dextran concentrations (g/L) in the diluted samples were determined by optical rotation measurements. PEG concentrations (g/L) in the diluted samples were calculated using refraction measurements. First the refractions were estimated using a Zeiss hand refractometer, and after appropriate further dilution the refractions were determined accurately with an ERMA ERC-7510 refractometer. PEG concentrations (g/L) in the diluted sample were calculated from the difference between the total polymer refraction and the dextran refraction according (Forciniti, 1990):

$$[PEG](g/L) = \frac{RII(RIU) - SRII_{D_x}(RIU \cdot L/g_{D_x}) \cdot [D_x](g/L)}{SRII_{PEG}(RIU \cdot L/g_{PEG})} \quad (1)$$

Where RII is the Refractive Index Increment of the sample and RIU stands for Refractive Index Unit. $SRII_{PEG}$ and $SRII_{Dx}$ are the specific refractions for PEG and dextran. Finally from the PEG and dextran concentrations (g/L) in the diluted samples the PEG and Dextran concentrations (g/g) in the phase systems were calculated.

Long-term growth experiments

The long-term growth experiments in ATPS media were carried out in T-flasks in a repeated-batch mode. The culture volume was 40 mL. The cultures were inoculated at an initial cell density of about $3x10^5$ cells/mL with cells taken from a 1L CSTR culture of BIF6A7 as described above. Routinely the cells were diluted into fresh ATPS-culture medium every two or three days. The initial viable cell density was about $1.5x10^5$ cells/mL when the cells were intended to grow for three days and about $2.0x10^5$ cells/mL when they were intended to grow for two days. Two ATPS-culture media were tested, ATPS-1 containing 0.015 g/g PEG 35.000 and 0.105 g/g Dextran T40, and ATPS-2 containing 0.01 g/g PEG 35.000 and 0.135 g/g Dextran T40. In both ATPS media DMEM/F12 3:1 based medium without Na-chloride was used and a suitable amount of Na-chloride was added to obtain an osmotic pressure of about 300 mOs/kg. Both media were initially supplemented with 1 % v/v foetal calf serum (FCS) (Cansera, Toronto, Canada). Samples were taken daily for cell count and -20 °C storage until analysis. The doubling time was calculated according to:

$$T_D = \frac{1n2}{(1nC_0 - 1nC_t)/\Delta t} \qquad (2)$$

Where T_D is the doubling time, C_t the concentration of viable cells at time t, C_0 the concentration of viable cells at time 0 and Δt the culture time.

Analytical methods

Osmotic pressure. Osmotic pressures were measured with an Gonotec Osmomat 030 cryoscopic osmometer.

Density. Liquid densities were measured using an Anton Paar DMA-40 oscillation density meter.

Refraction. The refraction was measured against distilled water in an ERMA ERC-7510 refractometer with digital reading. The dry-weight contents were calculated using the specific refraction of 0.000139 Refractive Index Units (RIU) for a 1 g/L PEG solution and 0.000153 RIU for a 1 g/L Dextran solution (Mw > 10.000 D) (Sharp, 1985; Forciniti, 1990).

Optical rotation. Optical rotations were measured at 589 nm (Na-lamp) and 25.0 °C with a Perkin-Elmer 241 MC polarimeter. The tube length was 1.000 dm.

The specific rotation for dextran is $[\alpha]_D^{25}$= +199°(mL/g.dm) (Albertsson, 1986).
Cell count. Viable and non-viable cell densities were determined by 1:1 v/v dilution of the samples with a trypan-blue solution and subsequent counting using a Fuchs-Rosenthal haemocytometer and a light microscope.
Cell partitioning. Cell partitioning in ATPSs was determined using 10 mL quantities of a well mixed cell suspension in ATPS-culture medium. First the overall cell densities were determined by taking a 200 µL sample of the cell suspension. After diluting this 1:1 v/v with trypan-blue solution a clear one-phase solution was obtained in which the cells were counted. Then the remainder of the cell suspension in the ATPS-culture medium was left to settle for about 30 minutes at 37°C until the phases had separated. The volumes of the upper and the lower phase were determined and samples were taken from both phases. The samples were diluted 1:1 v/v with trypan-blue solution after which cell densities were measured. The number of cells adsorbed to the interphase was determined by subtracting the number of cells in both phases from the number of cells in the overall mixture.
MAb concentration. The monoclonal antibody concentration was determined by a quantitative sandwich-ELISA assay. Polyclonal rabbit anti-mouse antibodies (RAM) coated were used to bind MAb from the samples, whilst polyclonal rabbit anti-mouse antibodies coupled to horse radish peroxidase (RAM-HRP) were used to establish the 1:1 link of MAb with enzyme. Both RAM and RAM-HRP were obtained from Dakopatts (Glostrup, Denmark). The presence of polymers did not influence the results of the assay (data not shown).
MAb affinity. The affinity of the antibody was determined with the same quantitative sandwich ELISA, except that human Von Willebrand Factor (vWf) was used instead of RAM to bind MAb from the sample.

Results and discussion

Influence of ATPS-forming polymers on physical properties of the culture medium

Before studying the influence of the ATPS-forming polymers on cell growth their effect on some physical properties of the culture medium was assessed. Relevant physical parameters with respect to animal-cell culture are the temperature, pH, dissolved oxygen concentration and the medium osmolality.
It was found that the polymers did not significantly alter the equilibrium pH of the Na-bicarbonate/CO_2 buffer used in the culture medium. The polymers do however change the oxygen mass transfer rate (OTR) to the culture medium (Andersson, 1990). A four-fold reduction of the OTR compared to normal culture media was found for media containing 0.10 g/g dextran 220.000 and 0.10 g/g dextran 500.000 (Van der Pol, 1995). The reduction was correlated with the increase in viscosity caused by the dextrans. The effect of the ATPS-forming polymers therefore

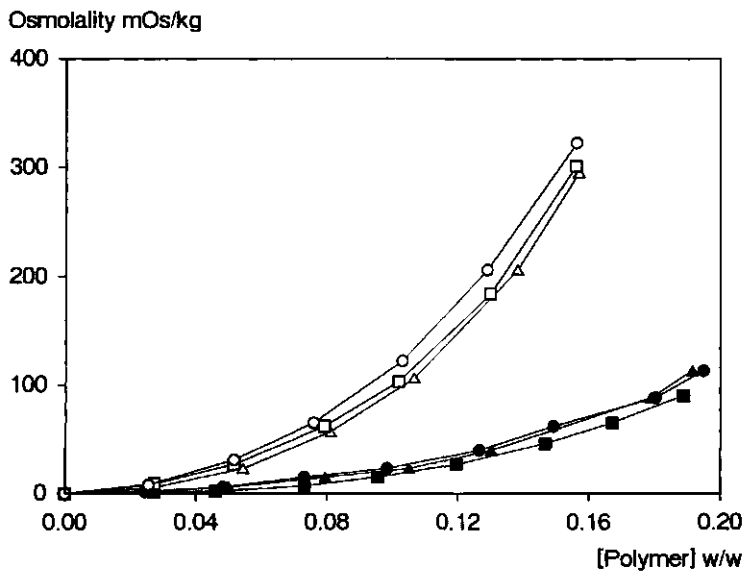

Figure 2 Osmotic pressure as a function of polymer weight fraction in distilled water. PEG 6000 (○), PEG 20.000 (□), PEG 35.000 (Δ), Dx 40.000 (●), Dx 220.000 (■), Dx 500.000 (▲)

has to be taken onto account in the design of these culture media, in particular when high cell density cultures at a larger scale are aimed for.

Dextran and especially PEG were also found to have a strong concentration dependent effect on medium osmotic pressure. The polymer molecular weight does not have a large influence on medium osmotic pressure (figure 2). Addition of PEG or dextran to culture media gave osmotic pressures of over 800 mOs/kg at 0.15 g/g PEG 20.000 and over 450 mOs/kg at 0.20 g/g dextran 220.000 (figure 3). The total osmotic pressure was more than the sum of the osmotic pressures of culture medium and polymers. Osmolality is an important process variable in mammalian cell culture. High osmolalities decrease cell growth and increase the cell death rate. Although growth up to 450 mOs/kg has been reported, most cell culture media have been designed to have an osmotic pressure of 260 to 320 mOs/kg (Marshall, 1991, Ozturk, 1991).

It was found that the osmolalities of the polymer containing media could be kept constant at a value of about 300 mOs/kg by reducing the Na-chloride content in the culture medium. Na-chloride is responsible for two-third of the culture medium osmotic pressure.

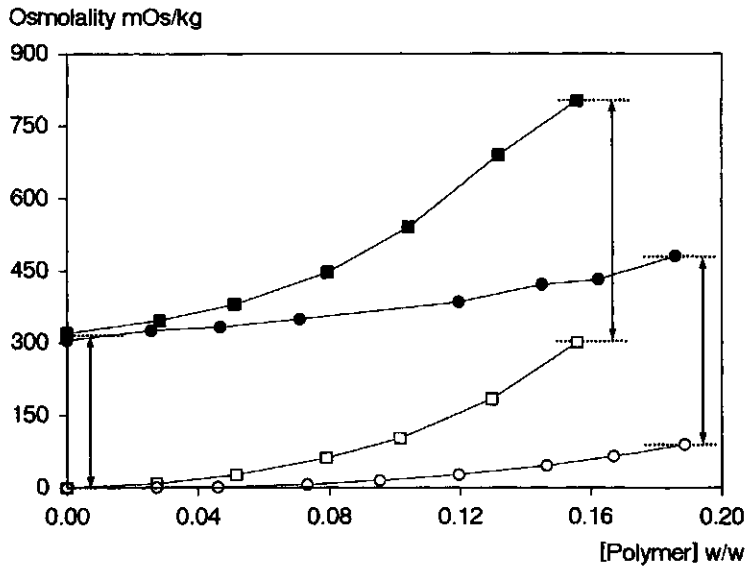

Figure 3 Osmotic pressure as a function of polymer weight fraction: solutions in distilled water compared to solutions in culture medium. Dissolving DMEM/F12 culture medium without polymers gives an osmolarity increase of about 300 mOs/kg. Dissolving DMEM/F12 in the pressence of 0.15 g/g PEG or 0.20 g/g Dx gives osmolarity increases of respectively 495 mOs/kg and 390 mOs/kg. Dx 220.000 in distilled water (○), Dx 220.000 in DMEM/F12 culture medium (●), PEG 20.000 in distilled water (□), PEG 20.000 in DMEM/F12 culture medium (■).

Influence of the individual ATPS-forming polymers on cell growth and production

To study the effect of the individual ATPS-forming polymers on cell growth, BIF6A7 hybridoma cells were cultured in media containing different molecular weights and different concentrations of PEG and dextran. PEG nominal molecular weights of 6000, 20.000 and 35.000 D were used at concentrations ranging from 0 to 0.10 g/g. Dextran nominal molecular weights of 40.000, 220.000 and 500.000 D were used at concentrations ranging from 0 to 0.20 g/g.

PEG had a strong concentration dependent effect on hybridoma product yield (figure 4a) and also on hybridoma growth. In the batch growth experiments a good correlation was found between the hybridoma product yield and the integral of the viable cells (cell growth). At concentrations of 0.025 g/g cell growth and product yield were comparable to the controls. At 0.05 g/g growth and product yield decreased. In some cases only one of the duplicates would grow exponentially. At 0.075 g/g in all cultures the cells died after a few days. At 0.10 g/g in all cultures the cells died rapidly.

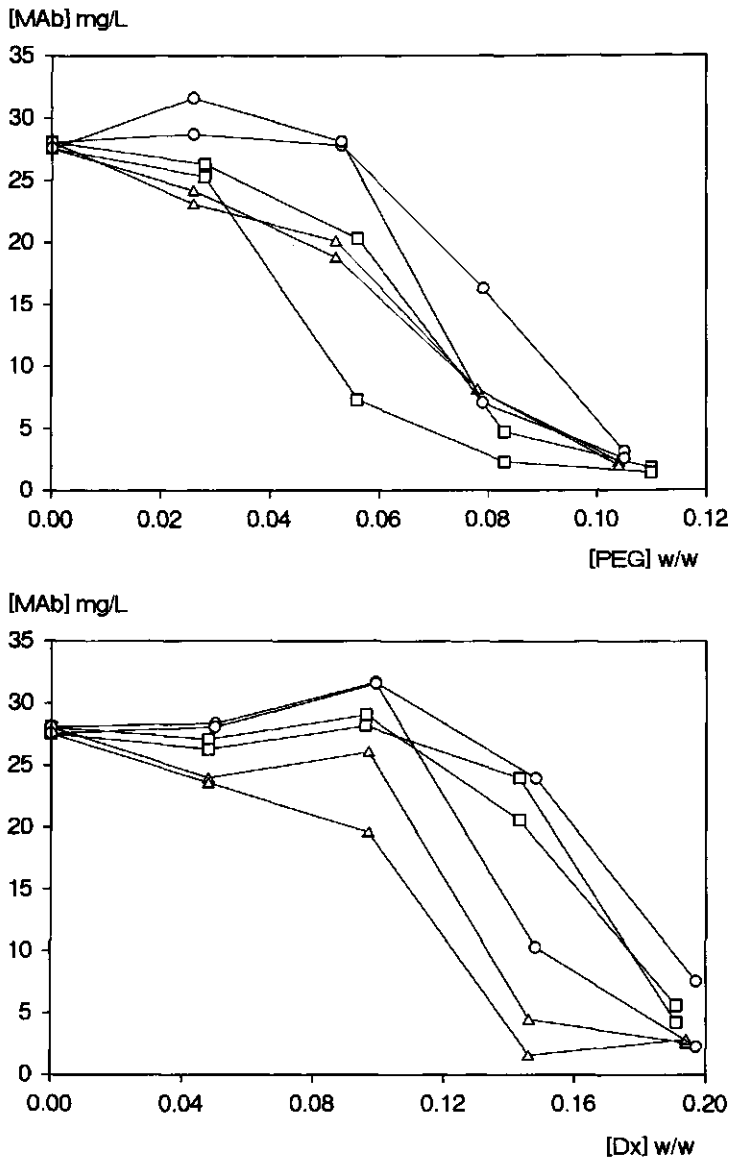

Figure 4 (a) Batch product yield as a function of PEG weight fraction. PEG 6000 (○), PEG 20.000 (□), PEG 35.000 (Δ). The points at weight fraction = 0 respresent the average of four experiments. All other points represent one experiment.
(b) Batch product yield as a function of dextran weight fraction. Dx 40.000 (○), Dx 220.000 (□), Dx 500.000 (Δ). The points at weight fraction = 0 respresent the average of four experiments. All other points represent one experiment.

No clear molecular weight dependent effect on the MAb yield was observed (figure 4a). However there was a pronounced effect of molecular weight on the cell morphology. In the cultures with PEG 6000 the viable cells were round and similar to the controls. The cells were grouped in clusters that broke up during the cell count procedure. No attachment of the cells to the culture flask bottom was observed. In the cultures with PEG 20.000 and 35.000 the cell shape was more irregular and some very large cells were present. The cells were grouped in tightly packed aggregates, in which sometimes no clear division between the individual cells could be seen. In concentrations of 0.05 g/g and higher the aggregates were attached to the culture-flask bottom.
The PEG concentrations used are in between the concentrations reported to promote cell growth (0 - 0.01 g/g PEG) (Mizrahi, 1984) and the concentrations used for cell fusion (0.40 - 0.50 g/g PEG) (Blow, 1978; Aldwinckle, 1982). The effect of high concentrations of PEG (above 0.025 g/g) on cell growth may very well be caused by changes in physical properties of the culture medium other than the osmotic pressure, or the reduced Na-chloride concentrations at elevated PEG levels. Also direct interaction of the polymers with the cell membrane or its protein constituents may have affected cell growth (Andersson, 1990). Toxic effects due to metabolization of PEG seem unlikely (Mizrahi, 1984).
Dextran did not influence the cell growth and product yield up to a concentration of 0.10 g/g (figure 4b). The viable cells looked quite similar to the ones in the controls except that they were more clustered. In the cultures containing 0.15 g/g of dextran 40.000 and 220.000 most cultures started to grow exponentially after a two days lag phase. No exponential cell growth occurred in media with 0.15 g/g dextran 500.000 and in media with 0.20 g/g dextran. In the cultures containing 0.15 and 0.20 g/g dextran (except for the cultures with 0.15 g/g dextran 220.000) the viable cells floated on top of the media after 'settling'. In the cultures containing 0.15 g/g dextran 220.000 the viable cells were suspended throughout the medium after settling, indicating that the density of the viable cells was about the same as the density of the medium (1056 kg/m^3 at 37°C). The medium densities increased with the dextran concentration from 1020 kg/m^3 at 0.05 g/g dextran to 1080 kg/m^3 at 0.20 g/g dextran. The molecular weight did not influence the medium density significantly.
The cause of the poor cell growth at high dextran concentrations (above 0.15 g/g) is unclear. It may be caused by the change in physical properties of the culture medium other than the osmotic pressure, for instance the reduced oxygen transfer rate, with the reduced Na-chloride levels, with the floating of cells, or with a combination of these effects.

Design of Aqueous Two-Phase Systems for hybridoma cell growth.
According to previous experiments with media containing PEG and dextran alone, the cells can be cultured in media containing maximally 0.025 g/g PEG or

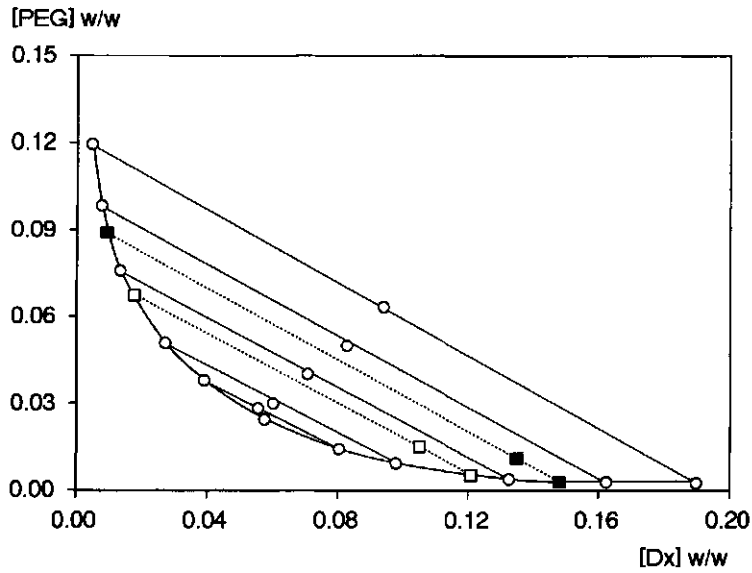

Figure 5 Phase diagram of PEG 35.000 and dextran 40.000 at 37°C in distilled water. The curved line is the binodal, the straight lines are the tielines. The markers in the middle of the tielines are the 'overall' polymer concentrations of the ATPSs that were used to determine the phase diagram (○) and the 'overall' concentrations of ATPS-1 (□) and ATPS-2 (■). The markers at the end of each tieline represent the compositions of the upper and lower phase after settling. The single marker on the binodal (○) represents the critical point.

0.15 g/g dextran. In ATPSs of PEG and dextran the coexisting phases consist of mixtures of PEG and dextran. Although it is unlikely that the effects of PEG and dextran alone can simply be added up or extrapolated to these mixtures, the concentrations mentioned do seem to present a reasonable concentration limit within which to operate.

Since the PEG concentration in the upper PEG-rich phase of virtually all ATPSs will exceed 0.025 g/g, ATPSs have to be selected in which the cells partition to the lower phase. To reduce the concentration of dextran and PEG in the lower phase of ATPSs, high molecular weight polymers should be used. The high molecular weight polymers will lower the binodal curve in the phase diagram (figure 5) so the 'overall' polymer concentrations needed to form ATPSs are lower, and lower polymer concentrations in the bottom phase become possible (Albertsson, 1986). Generally, cells will partition between one of the phases and the interface (Walter, 1982; Albertsson, 1986). In ATPSs of PEG 35.000 and Dx 40.000 with a buffered Na-chloride solution the cells were found to partition almost completely to the lower phase (table 1).

Table 1 Partitioning of hybridoma BIF 6A7 in ATPSs of PEG 35.000 and Dx 40.000.

System	[PEG] g/g	[Dx] g/g	pH	Lower phase % cells	Upper phase % cells
1*	0.035	0.050	7.4	>95	0
2*	0.050	0.060	7.4	>95	0
ATPS-1	0.015	0.10	7.0-7.4	85	0
ATPS-2	0.010	0.13	7.0-7.4	80	0

* System 1 and 2 were made up from PEG and Dx stocksolutions and a 10 mM Na-phosphate/ 154 mM Na-chloride buffer pH 7.4.

Using the phase diagram of PEG 35.000 and dextran 40.000 in distilled water at 37°C (figure 5), two systems (ATPS-1 and ATPS-2) were selected in which the cells partitioned predominantly to the lower phase (table 1) and in which the lower phase PEG and dextran concentrations should not exclude cell growth. It was found that the phase diagram did not change significantly when replacing water with the culture medium.

Long-term growth in ATPSs.

Finally the long-term cell growth of hybridoma BIF6A7 in the designed ATPS culture media was studied. Preliminary studies in which several inoculation strategies were compared, revealed that no consistent growth upon inoculation could be obtained when using serum-free ATPS culture media. Apparently the cells had difficulties adapting to the presence of the polymers. Possibly the dextran concentrations in the lower phase of the ATPS culture media (respectively 0.12 g/g and 0.15 g/g) (figure 5) were the cause of this phenomenon, because the cultures containing 0.15 g/g dextran 40.000 alone, showed inconsistent growth after inoculation as well.

The inoculation studies also revealed that when using 1 % v/v foetal calf serum (FCS), consistent growth upon inoculation could be obtained. FCS is known to contain growth factors and proteins that can enhance cell growth, and apparently they suffice to overcome the adaptational phase (Van der Pol, 1992).

After the successful inoculation of the hybridoma cells into the ATPS culture medium, the cells were cultured during more than two months (figure 6a, b, c, d). In the ATPS-1 culture the cells initially showed slow growth. The average doubling time until day 15 was 42 h and the average viable cell density was 4.4 $x10^5$ cells/mL at 84 % viability. A likely cause for the slow growth seems to be the low seeding cell density used in the beginning of this culture (averaging 1.1 x 10^6 cells/mL for days 3, 6 and 10). After higher seeding cell densities were used, the average doubling time until day 36 became 34 h. The average viable cell density

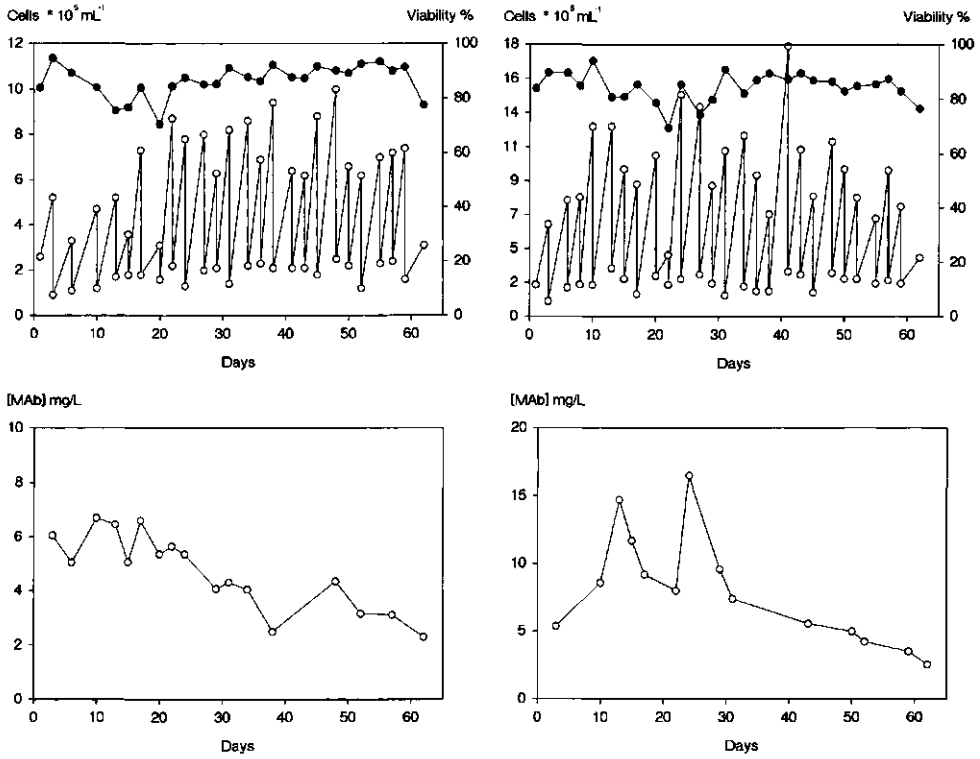

Figure 6 Repeated-batch culture of hybridoma BIF 6A7 in ATPS-1 (a) and ATPS-2 (b). Overall viable-cell concentrations (○) and viability (●). Mab concentrations during the repeated batch culture of ATPS-1 (c) and ATPS-2 (d). Overall MAb concentration (○).

was 6.7 x 10^5 cells/mL at 83 % viability. After day 36 the FCS was left out of the medium. The average doubling time until the end of the culture was 36 h, the average viable cell concentration 6.7 x 10^5 cells/mL at 87 % viability. Therefore no significant change in cell growth occurred after omission of FCS from the culture medium.

In the ATPS-2 culture the cells immediately grew well. Until day 36 they were cultured in ATPS-2 culture medium containing 1 % v/v FCS. The average viable cell density was 10.0 x 10^5 cells/mL at 84 % viability. The doubling time was 29 h. After day 36 the FCS was left out of the culture medium and the average viable cell density until the end of the culture was 9.1 x 10^5 cells/mL at 85 % viability. The doubling time became 34 h. Therefore a small reduction in growth rate occurred in serum-free culture compared to the culture with 1 % v/v FCS.

The observed growth rates did not differ significantly from the growth rates in the culture medium without polymers. Finally the cell partitioning remained the same throughout both cultures.

The initial MAb concentrations of both ATPS-1 and ATPS-2 corresponded well to MAb concentrations in control cultures with similar cell densities. However in both cultures a gradual decrease in specific production occurred. This might be caused by stresses imposed on the cells by the ATPS medium. However reinoculation of cells adapted to serum-free medium in serum containing medium may also be the cause. This has been shown to lead to loss of specific production before in CSTR cultures of BIF6A7 (Data not shown). The affinity of the antibody did not change during the culture.
Both in ATPS-1 and in ATPS-2 the MAb product partitioned mainly to the lower phase, with partition coefficients of 0.08 and 0.02 respectively. Preliminary work using a dye affinity ligand coupled to PEG 35.000 however shows promising results towards improvement of the product ATPSs coefficient. In ATPSs with PEG 35.000, Dx 40.000 and a 1 mM phosphate buffer, replacement of only 1 % of PEG 35.000 with PEG-ligand resulted in a thousandfold increase of the partitioning coefficient from 0.02 to 20 (Zijlstra, 1996). Product partitioning coefficients of this magnitude would make an efficient product extraction from the cell-containing phase possible.

Conclusions

This study for the first time reports on the growth of an animal-cell line in aqueous two-phase systems.
The influence of the individual ATPS-forming polymers PEG and dextran on physical properties of the culture medium and on hybridoma cell growth was studied. It has been shown that dextran and especially PEG increased the medium osmotic pressure. By decreasing the Na-chloride content in polymer-containing media the osmotic pressure could be kept at physiological levels. Cell growth was possible in culture media containing up to 0.025 g/g PEG or up to 0.15 g/g dextran.
For hybridoma cell growth in ATPSs, ATPSs have to be selected in which the cells partition to the lower PEG-lean phase, because the upper phase PEG concentrations do not allow cell growth. It was found that in ATPSs of PEG 35.000 and dextran 40.000 hybridoma BIF6A7 partitioned almost completely to the lower phase. In two ATPSs of PEG 35.000 and Dx 40.000 with culture medium long-term growth has been shown. The MAb product however partitioned along with the cells in the lower phase. Preliminary work using an affinity ligand coupled to PEG indicates that the partition coefficient of the MAb can be improved considerably. This study has shown that aqueous two-phase systems can support long-term animal cell growth, hereby opening the way towards continuous extractive bioconversions with animal cells.

Acknowledgements

This work was supported by DSM Biologics , Groningen, The Netherlands and by a grant under the Dutch PBTS-Biotechnology program, Ministry of Economic Affairs Nr. BIO 89041. The authors thank S. Bulstra and V. Munster for technical assistance.

References

Albertsson PA (1986) In: Partitioning of cell particles and macromolecules. Wiley & Sons, New York.

Aldwinckle TJ, Ahkong QF, Bangham AD, Fisher D & Lucy JA (1982) Effects of poly(ethylene glycol) on liposomes and erythrocytes permeability changes and membrane fusion. Biochim. Biophys. Acta. 689: 548-560

Andersson E, Hahn-Hägerdal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb. Technol. 12: 242-253.

Blow AMJ, Botham GM, Fisher D, Goodall AH, Tilcock CPS & Lucy JA (1978) Water and calcium ions in cell fusion induced by poly(etylene glycol). FEBS Letters. 94: 305-310

Buitelaar RM, Leenen EJTM & Tramper J (1992) Growth and secondary metabolite production by hairy roots of *tagetes patula* in aqueous two-phase systems. Biocatalysis 6: 73-80

Daugulis AJ (1988) Integrated reaction and product recovery in bioreactor systems. Biotechnol. Prog. 4: 113-122.

Forciniti D, Hall CK & Kula MR (1990) Interfacial tension of polyethyleneglycol-dextran-water systems: Influence of temperature and polymer molecular weight. J. Biotechnol. 16: 279-296

Freeman A, Woodley JM & Lilly MD (1993) *In situ* product removal as a tool for bioprocessing. BIO/TECHNOLOGY 11: 1007-1012

Koops K, Hoff HS, Weperen JJ, Das PC & Smit Sibinga Cth (1991) Immunoaffinity purification of factor VIII / Von Willebrand factor complex. In: Developments on hematology and immunology: Coagulation and blood transfusion. Smit Sibinga CTh, Das PC & Mannucci PM (eds.) Kluwer Academic, Dordrecht 26: 103-119

Kratje RB Lind W & Wagner R (1994) Evaluation of the proteolytic potential of in vitro-cultivated hybridoma and recombinant mammalian cells. J. Biotechnol. 32: 107-125.

Marshall CJ, Boraston R & Brown ME (1991) The effect of osmolarity on hybridoma cell growth and antibody production in serum-free media. In: Production of biologicals from animal cells in culture. Spier RE, Griffiths JB & Meignier B (eds.) Butterworth, Londen, 259-261

Matiasson B (1988) Bioconversions in aqueous two-phase systems: an alterative to conventional immobilisation. In: Methods in enzymology: Immobilised enzymes and cells. Mosbach K (ed). Academic Press Inc, San Diego, 137(D): 657-668.

Mizrahi A (1984) Oxygen in human lymphoblastiod cell line cultures and effect of polymers in agitated and aerated cultures. Develop. biol. Standard. 55: 93-102

Mohan SB, Chohan SR, Eade J & Lyddiatt A (1993) Molecular integrity of monoclonal antibodies produced by hybridoma cells in batch culture and in continuous-flow culture with integrated product recovery. Biotechnol. Bioeng. 42: 974-986.

Ozturk SS & Palsson BO (1991) Effect of medium osmolarity on hybridoma growth, metabolism, and antibody production. Biotechnol.Bioeng. 37: 989-993

Sharp KA (1985) In: Theoretical and experimental studies on erythrocyte partition in aqueous two-phase systems. Ph.D. thesis, University of British Columbia, Vancouver.

Shintani Y, Iwamoto K & Kitano K (1988) Polyethylene glycols for poromoting the growth of mammalian cells. Appl. Microbiol. Biotechnol. 27: 533-537

Van der Pol L, Bakker WAM & Tramper J (1992) Effect of low serum concentrations (0%-2.5%) on growth, production, and shear sensitivity of hybridoma cells. Biotechnol.Bioeng. 40: 179-182

Van der Pol LA, Paijens I & Tramper J (1995) Dextran as protectant against damage caused by sparging for hybridoma cells in a bubble column. J.Biotechnol. 43: 103-110.

Walter H (1982) Separation and subfractionation of blood cell populations based on their surface properties by partitioning in two-polymer aqueous phase systems. In: Cell separation: Methods and selected applications. Pretlow II TG & Pretlow II ThP (eds.) Academic Press, New York, 1: 261-299.

Zijlstra GM, Michielsen MJF, De Gooijer CD & Tramper J (1996) Separation of hybridoma cells from theie IgG product using aqueous two-phase systems. Bioseparation 6: 201-210.

Chapter 3

Hybridoma and CHO cell partitioning in aqueous two-phase system

Summary

The partitioning of mouse/mouse hybridoma cell line BIF6A7, mouse/rat hybridoma PFU-83 and CHO DUKX B11 derived cell line BIC-2 in aqueous two-phase systems (ATPSs) of PEG and Dextran was studied. The partitioning of BIF6A7 was investigated systematically using a statistical experimental design. Aim was to identify the key factors governing cell partitioning and to select ATPSs with suitable cell partitioning for extractive bioconversions with animals cells. The influence of five factors i.e. the poly(ethylene glycol) molecular weight (PEG Mw), dextran molecular weight (Dx Mw), tie-line length (TLL), pH and the ratio of K-phosphate to K-chloride, defined as the fraction (KPi/(KPi+KCl)), on BIF6A7 cell partitioning was characterized using a full factorial experimental design. The cell partitioning ranged from complete partition into the interface to an almost complete partition to the lower phase. In all cases less than one percent of the cells partitioned to the top phase. The potassium phosphate fraction had the largest effect on cell partitioning. Low potassium phosphate fractions increased the proportion of cells in the lower phase. Although to a lesser extent, the other factors also played a role in the cell partitioning. The best partitioning for the BIF6A7 cell line was obtained in ATPSs with PEG Mw = 35.000 D, Dx Mw = 40.000 D, TLL = 0.10 g/g, pH = 7.4 and KPi/(KPi+KCl) = 0.1, where 93 % of the cells were present in the lower phase. The previously reported partitioning of BIF6A7 cells in ATPS culture media, corresponded well with the current findings.
The partitioning of mouse/rat hybridoma cell line PFU-83 and CHO cell line BIC-2 was studied in an ATPS culture medium with PEG 35 kD, dextran 40 kD, TLL 0.12 g/g, and hybridoma culture medium. Both cell lines partitioned almost completely into the lower phase. Moreover the PFU-83 cell line was able to grow in the ATPS hybridoma culture medium. This ATPS hybridoma culture medium therefore seems to be suitable for extractive bioconversions with a wide range of

This chapter has been published in: Biotechnology Progress, 1996, **12**: 363-370 by the authors: G.M. Zijlstra, M.J.F. Michielsen, C.D. de Gooijer, L.A. van der Pol and J. Tramper

hybridoma cell lines, provided their product can be partitioned into the upper PEG-rich phase.

Introduction

A trend in the production of biopharmaceuticals is the integration of the upstream (cell culturing) and the downstream (purification) part of the production process (Daugulis, 1988; Freeman, 1993;Van 't Riet, 1991). Extractive bioconversions using Aqueous Two-Phase Systems (ATPSs) (Andersson, 1990; Matiasson, 1988) seem to be a very attractive method for integration of fermentation and downstream processing in an animal-cell culture, where extracellular proteins are produced. Extractive bioconversions offer the opportunity for cell retention within the bioreactor and of rapid product removal from the cell-containing phase, hereby avoiding breakdown of the product by enzymes (Mohan, 1993; Kratje, 1994). Furthermore by using a two-phase system in which the cells partition to one phase and the product concentrates in the other, a cell-free, concentrated, and partially purified effluent can be obtained (Zijlstra, 1996a).
ATPSs can be used because they form a relatively mild environment to the cells compared to water/organic solvent two-phase systems (Albertsson, 1986). In addition they are suitable for protein extractions (Kroner, 1978; Kula, 1982).
For an efficient extractive bioconversion, ATPSs yielding good cell growth and production, as well as a good separation between cells and product have to be available. Previous work with mouse/mouse hybridoma BIF6A7 has shown that by using PEG 35.000 D and Dx 40.000 D, ATPSs could be obtained in which the cells partition almost completely into the lower phase. Growth experiments in ATPSs with culture medium showed a stable cell growth over a period of two months. The growth rates, cell densities and viabilities were comparable to control cultures in media without polymers. The initial MAb production levels were also comparable to the levels in control cultures. The MAb product however partitioned along with the cells into the lower phase, so the separation between the cells and its MAb product has to be improved (Zijlstra, 1996a). In a successive study we have addressed this problem (Zijlstra, 1996b).

Aim of the work presented here was to identify the key factors governing cell partitioning, by a systematic study into partition behaviour of the BIF6A7 cell line, and to establish which range of ATPSs can provide good cell partitioning and may be used for extractive bioconversions. Furthermore we wanted to study the partitioning of other cell lines, mouse/rat hybridoma PFU-83 and a CHO cell line BIC-2 , in the ATPSs giving good partitioning for BIF6A7.

The partitioning of animal cell lines in ATPSs generally depends on the interac-

tions of the surface properties of the cells with the physical properties of the two-phase system (Albertsson, 1986; Walter, 1982, 1985a; 1985b). The cell surface properties, like surface charge, lipid composition and the presence of specific components, depend on the cell type. The physical properties of the ATPS, like the interfacial tension and the electrostatic potential difference, however, can be manipulated by altering the overall composition (Albertsson, 1986; Walter, 1982, 1985a, 1985b).

Here the PEG and dextran molecular weights (PEG Mw, Dx Mw), the tie-line length (TLL), the ratio of K-phosphate to K-chloride, defined as the fraction (KPi/(KPi+KCl), and the pH have been varied using a full-factorial experimental design. These factors are the most likely to influence the cell partitioning, when no ligands coupled to the polymers are used (Albertsson, 1986; Walter, 1982, 1985a, 1985b). Statistical experimental designs have proven to be useful in studying partitioning in ATPSs (Backman, 1984). The factorial design used here is useful in identifying the important factors and the interacting factors determining cell partitioning. Furthermore they can give an indication of the optimal factor settings and of the mechanisms involved in cell partitioning (Box, 1978, Haaland, 1989).

Materials and methods

Experimental Design

The partitioning of hybridoma cell line BIF6A7 in ATPSs of PEG and dextran was studied as a function of five factors. To quantify the partitioning, the fraction of added viable cells, present in the lower phase after phase separation, was used (Walter, 1982, 1985a, 1985b). This fraction (y), the response factor, was measured as a function of the PEG Mw (x_1), the dextran Mw (x_2), the tie-line length (x_3), the K-phosphate fraction (x_4) and the pH (x_5). For each of the five factors a high (coded value: 1) and a low (coded value: -1) set point was selected. ATPSs representing all 32 ($=2^5$) set point combinations were made, as well as an ATPS representing the center point, in which the value of all factors was in-between (coded value: 0). This resulted in an orthogonal 2^5 full-factorial experimental design with center point (Box, 1978; Haaland, 1989) (table 1). All measurements were carried out in duplicate and the center point measurement was carried out fivefold.

A model containing all main factors (x_1, x_2 etc.) and all factor interactions (x_1x_2, $x_3x_4x_5$ etc.) was fitted through the data (y), using the regression analysis module of the SAS statistical-program package. Because the coded factor values (-1, 0, 1) were used to fit the data, the magnitude of the regression parameters could be compared directly (figure 2: Pareto chart).

Table 1 Experimental design: variable settings, ATPS compositions and BIF6A7 cell partitioning

System	PEG Mw (x_1) kD	code	Dx Mw (x_2) kD	code	TLL (x_3) g/g	code	KPi(KPi+KCl) (x_4)	code	pH (x_5)	code	[PEG] g/g	[Dx] g/g	[KPi] mM/kg	[KCl] mM/kg	y ** %	
1	6	-1	40	-1	10	-1	0.1	-1	6.6	-1	0.064	0.082	6.2	62.2	93	
2	6	-1	40	-1	10	-1	0.1	-1	7.4	1	0.064	0.082	6	59.8	93	
3	6	-1	40	-1	10	-1	1.0	1	6.6	-1	0.064	0.082	56.9	0	4	
4	6	-1	40	-1	10	-1	1.0	1	7.4	1	0.064	0.082	54.6	0	1	
5	6	-1	40	-1	20	1	0.1	-1	6.6	-1	0.068	0.106	3.3	32.8	7	
6	6	-1	40	-1	20	1	0.1	-1	7.4	1	0.068	0.106	3.6	35.5	96	
7	6	-1	40	-1	20	1	1.0	1	6.6	-1	0.068	0.106	37.2	0	33	
8	6	-1	40	-1	20	1	1.0	1	7.4	1	0.068	0.106	33.7	0	10	
9	6	-1	500	1	10	-1	0.1	-1	6.6	-1	0.052	0.061	8.6	85.6	68	
10	6	-1	500	1	10	-1	0.1	-1	7.4	1	0.052	0.061	8.5	85.4	72	
11	6	-1	500	1	10	-1	1.0	1	6.6	-1	0.052	0.061	82.2	0	1	
12	6	-1	500	1	10	-1	1.0	1	7.4	1	0.052	0.061	79.2	0	1	
13	6	-1	500	1	20	1	0.1	-1	6.6	-1	0.063	0.092	5.3	52.8	93	*
14	6	-1	500	1	20	1	0.1	-1	7.4	1	0.063	0.092	5.4	54.3	76	*
15	6	-1	500	1	20	1	1.0	1	6.6	-1	0.063	0.092	56.5	0	47	
16	6	-1	500	1	20	1	1.0	1	7.4	1	0.063	0.092	47.6	0	28	
17	35	1	40	-1	10	-1	0.1	-1	6.6	-1	0.037	0.068	11	110.5	85	
18	35	1	40	-1	10	-1	0.1	-1	7.4	1	0.037	0.068	9.9	98.7	89	
19	35	1	40	-1	10	-1	1.0	1	6.6	-1	0.037	0.068	105.2	0	28	
20	35	1	40	-1	10	-1	1.0	1	7.4	1	0.037	0.068	96.5	0	5	
21	35	1	40	-1	20	1	0.1	-1	6.6	-1	0.057	0.093	8.1	80.8	44	
22	35	1	40	-1	20	1	0.1	-1	7.4	1	0.057	0.093	7.5	74.9	61	
23	35	1	40	-1	20	1	1.0	1	6.6	-1	0.057	0.093	78.6	0	3	
24	35	1	40	-1	20	1	1.0	1	7.4	1	0.057	0.093	71	0	1	
25	35	1	500	1	10	-1	0.1	-1	6.6	-1	0.028	0.050	12.4	124.1	60	
26	35	1	500	1	10	-1	0.1	-1	7.4	1	0.028	0.050	12.4	124	69	
27	35	1	500	1	10	-1	1.0	1	6.6	-1	0.028	0.050	132.5	0	7	
28	35	1	500	1	10	-1	1.0	1	7.4	1	0.028	0.050	119.7	0	0	
29	35	1	500	1	20	1	0.1	-1	6.6	-1	0.054	0.088	7.4	73.9	30	*
30	35	1	500	1	20	1	0.1	-1	7.4	1	0.054	0.088	7.2	72.4	81	*
31	35	1	500	1	20	1	1.0	1	6.6	-1	0.054	0.088	74.9	0	22	*
32	35	1	500	1	20	1	1.0	1	7.4	1	0.054	0.088	69.3	0	45	*
33	20	0	220	0	15	0	0.55	0	7.0	0	0.042	0.070	55.3	43.9	41	

* Low viabilities
** The average y values are given

Cell lines

BIF6A7 is a mouse/mouse hybridoma, originating from an SP2/0 myeloma. BIF6A7 produces a monoclonal IgG (MAb) binding the Factor VIII/Von Willebrand-Factor complex (Koops, 1990), which plays a role in the human blood-coagulation mechanism. PFU-83 is a mouse/rat hybridoma, originating from a P3 myeloma. PFU-83 produces a monoclonal IgG antibody binding rat corticotropin releasing factor (Van Oers, 1989). BIC-2 is a transfected CHO-DUKX B11 derived CHO cell line, producing a human IgG. All cell lines were supplied by DSM Biologics (Groningen, The Netherlands).

A serum-free 3:1 DMEM/F12 mixture (Gibco, Breda, The Netherlands) (Van der Pol, 1992; Zijlstra, 1996a) was used to culture BIF6A7 and PFU-83. To the PFU culture 1 g/L HSA (G.P.O., CLB, Amsterdam, The Netherlands) was added. BIC-2 was cultured in SFM-S-II medium (Gibco, Breda, The Netherlands).

Polymer solutions

Poly(ethylene glycol) (PEG) 6000, 20.000 and 35.000 were obtained from Merck (Darmstadt, Germany). Dextran T40 and T500 were obtained from Pharmacia (Uppsala, Sweden) and Dextran 220.000 from Fluka (Buchs, Switzerland). All polymers were without further purification dissolved in distilled water to about 0.30 g/g and then sterilized by autoclaving for 20 minutes at 121°C. Dry-weight concentrations of the polymer stocks were determined by refractometry after appropriate dilution.

Buffer solutions

From 2 mol/kg solutions of K_2HPO_4 and KH_2PO_4 K-phosphate buffer solutions were made with pH 6.60. pH 7.00 and pH 7.40 at 37.0°C. From these buffers and from a 2.5 mol/kg K-chloride solutions, five buffer solutions were made (table 2). All salts were of analytical grade and obtained from Merck (Darmstadt, Germany).

Table 2 Composition of the buffer solutions

Buffer	K-phosphate M	K-Cloride M	pH
1	0.15	1.5	6.6
2	0.15	1.5	7.4
3	0.63	0.5	7.0
4	1.0	0	6.6
5	1.0	0	7.4

Phase diagrams

The phase diagrams (figure 1a, b, c, d, e) were determined by making five different mixtures of PEG and Dextran for each of the molecular weight combinations PEG 6000/Dx 40.000, PEG 6000/Dx 500.000, PEG 20.000/Dx 220.000, PEG 35.000/Dx 40.000 and PEG 35.000/Dx 500.000. The compositions of the mixtures are represented by the markers in the centre of each tie line. The mixtures were made using polymer stock solutions and distilled water. The mixtures were warmed to 37°C and thoroughly shaken. They were left to settle for 72 h at 37°C until the phase separation was complete. From each top and bottom phase two samples of about 10 mL were taken and centrifuged for 30 minutes at 600 x g at 37°C in a thermostated SIGMA C centrifuge to remove microdroplets of the opposite phase. Weighed quantities of the clear top or bottom phase were diluted to 25.00 mL with distilled water for polymer analysis.

The Dextran concentrations were determined by optical rotation measurements. The PEG concentrations were calculated from the difference between the total polymer refraction and the calculated dextran refraction (Bamberger, 1985; Forciniti, 1990; Zijlstra, 1996a).

Experimental design aqueous two-phase systems

The aqueous two-phase systems for the partition experiments were made from concentrated stock solutions of PEG and dextran, concentrated buffer solutions having the desired KPi/(KPi+KCl) ratio and pH, and distilled water.

From the phase diagrams in distilled water (figure 1a, b, c, d, e) the overall PEG and dextran concentrations were selected giving about equal phase volumes and the desired tie-line lengths. Buffer and distilled water were added to the polymer solutions giving ATPSs with an osmotic pressure of about 300 mOs/kg. The amount of each buffer solution to be added to each selected phase system was determined experimentally. The final composition of the phase systems used for the partition experiments of the cells is given in table 1.

Experimental design cell-partition experiments

The cells used for the partition experiments were cultured in a continuously stirred tank reactor (CSTR). The CSTR was operated at a dilution rate of 0.028 h^{-1}. The culture medium used was serum-free, low-protein DMEM/F12. During the CSTR culture the cell density, viability and specific production remained at a constant level, indicating that the cell samples taken at the beginning of the culture are comparable to the cell samples taken later on. For each partition experiment a 1 mL cell sample from the CSTR culture was centrifugated and the supernatant was discarded. The cell pellets were resuspended in 2 g of a previously prepared ATPS at 37°C, giving single-cell suspensions. The samples were then mixed for 15 minutes with a rotary mixer. A 100 µL sample of the mixture was taken to determine the overall cell density, usually being 5 to $8*10^5$ cells/mL. Subsequently the sample

was left to settle for 30 minutes at 37°C in tubes with graduation-scales, yielding primary phase separation. Then the total and lower phase volumes were determined and 100 µL samples of the upper and the lower phase were taken for cell counts. The sample of the lower phase was taken through the upper phase. By applying a positive pressure on the pipette and by removing upper phase traces from the outside of the pipette tip, contamination of the lower with the upper phase was avoided. To see whether the cells present in the lower phase were attached to droplets of the upper phase, samples were screened under the microscope without dilution. Care was taken to avoid shifts in the phase equilibrium as a result temperature changes. To the upper phase droplets present in the lower phase, generally no cells were attached. This indicates that the 'true' partition coefficient was measured.
The fraction of cells in the lower phase (y) was calculated by dividing the cell number in the lower phase by the total cell number.

ATPS culture medium

The ATPS culture media were made from concentrated stock solutions of PEG, Dextran, a DMEM/F12-based culture medium without Na-chloride (Gibco, Breda, The Netherlands), Na-chloride (Merck, Darmstadt, Germany) and water (Zijlstra et al., 1996a). The Na-chloride content was less than in culture media without polymers, to compensate for the osmolarity increase caused by the polymers. The ATPS culture medium composition is given in table 3.

Table 3 Composition of ATPS culture medium *

Component	Amount per liter	Unit
DMEM/F12 without Na-chloride	5.52	g
L-Glutamine	0.58	g
Ascorbic Acid	5	mg
$NaHCO_3$	3.20	g
Glutathione	1	mg
Transferrin	5	mg
Na-Selenite	10	nmol
Ethanolamin	7	µL
Na-chloride	3.65	G
Dextran 40.000	91	g
PEG 35.000	24	g

* For growth experiments the medium was supplemented with 1 % foetal calf serum.

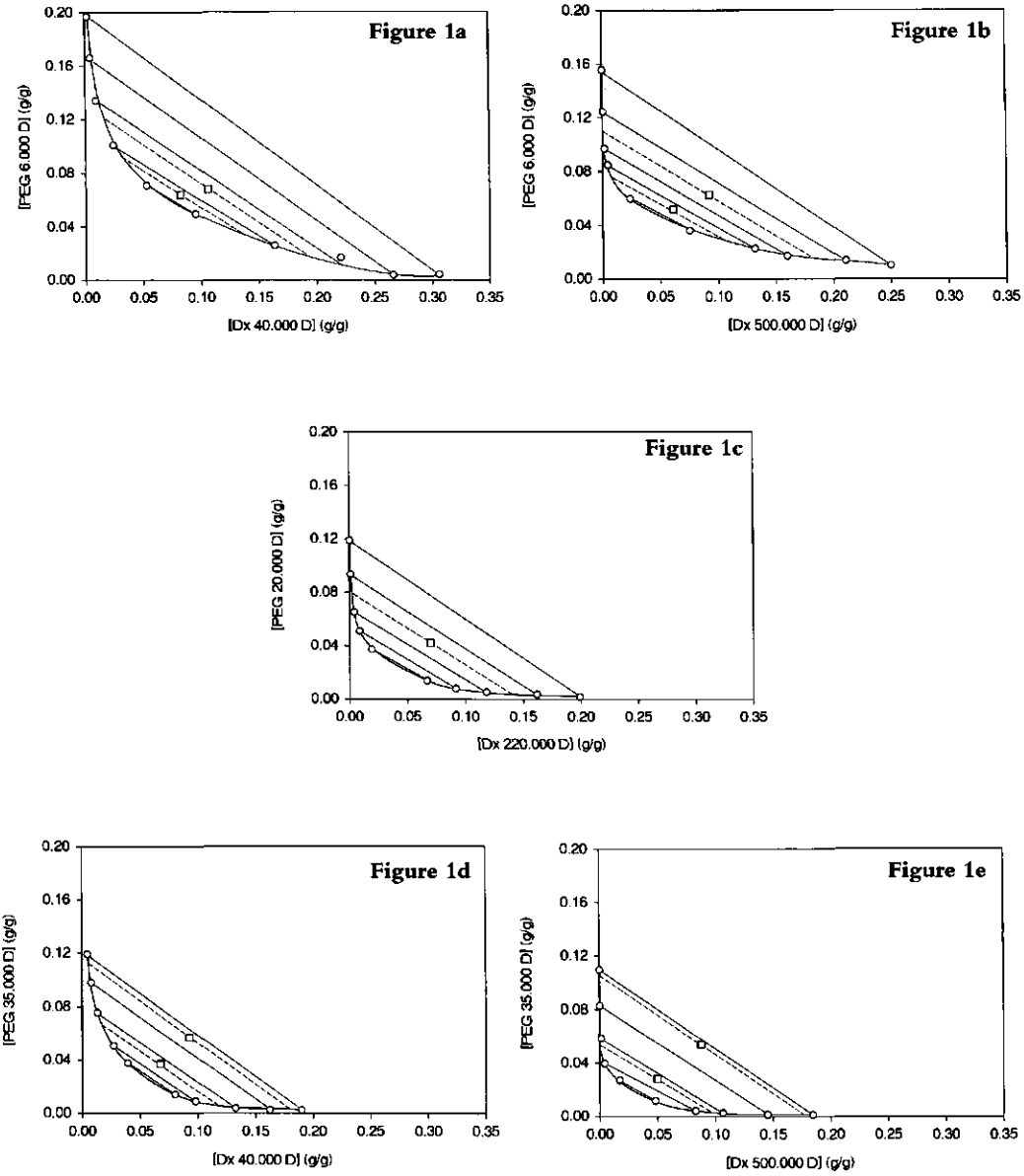

Figure 1 Phase diagrams of PEG 6000 and Dx 40.000 (a), PEG 6000 and Dx 500.000 (b), PEG 20.000 and Dx 220,000 (c), PEG 35000 and Dx 40.000 (d), and PEG 35000 and Dx 500.000 (e) in distilled water at 37°C. The squares (□) represent the selected phase system compositions giving the desired tie-line lengths (----).

Partitioning in ATPS culture media

The partition experiments were carried out in duplicate. The pH of the ATPS culture media was adjusted with H-chloride just before the partition experiment. Samples of 10 mL cell suspension were centrifuged for 3 minutes at 600 x g in a MSE minor centrifuge. The supernatant was discarded and the cells were resuspended in 10 mL ATPS culture medium, giving single-cell suspensions. The rest of the procedure was identical to the partition experiments described above.

Analytical methods

Osmotic pressure. Osmotic pressures were measured with an Gonotec Osmomat 030 cryoscopic osmometer.

Refraction. The refraction was measured against distilled water in an ERMA ERC-7510 refractometer with digital reading. The dry-weight contents were calculated using the specific refraction of 0.000139 Refractive Index Units (RIU) for a 1 g/L PEG solution and 0.000153 RIU for 1 g/L Dextran solution (Mw > 10.000 D) (Sharp, 1985; Forciniti, 1990).

Optical rotation. Optical rotations were measured at 589 nm (Na-lamp) and 25.0°C with a Perkin-Elmer 241 MC polarimeter. The tube length was 1.000 dm. The specific rotation for dextran is $[\alpha]_D^{25} = +199°$ mL/(g.dm) (Albertsson, 1986).

Cell count. Viable and non-viable cell densities were determined by 1:1 v/v dilution of the samples with a trypan-blue solution and subsequent counting using a Fuchs-Rosenthal haemacytometer and a light microscope. A 1:1 v/v dilution of the ATPS mixtures with trypan blue gave clear, one-phase solutions. The presence of the ATPS forming polymers did not influence the staining of dead cells significantly, although in some cases the staining appeared to be relatively slow.

Results and discussion

Phase diagrams

The phase diagrams of PEG 6000 and Dx 40.000, PEG 6000 and Dx 500.000, PEG 20.000 and Dx 220.000, PEG 35.000 and Dx 40.000, and PEG 35.000 and Dx 500.000 in distilled water at 37 °C are shown in figure 1a, b, c d and e. In the phase diagrams the tie-line lengths of 0.10 g/g and 0.20 g/g have been drawn (figure 1: Dotted lines). They correspond with the high and low settings of the tie-line lengths in the experimental design. In the center point phase diagram (figure 1c) a tie-line length of 0.15 g/g was drawn. The PEG and Dextran concentrations on these tie lines giving about equal phase volumes were selected (figure 1: Square markers). ATPSs for the cell-partition experiments were made by replacing the distilled water by buffers at the selected PEG and Dx concentrations. Hereby it was assumed that replacing the water with buffer did not alter the phase diagrams significantly (Albertsson, 1986).

BIF6A7 cell partitioning

BIF6A7 cell partitioning was studied in 33 ATPSs representing all factor combinations of the experimental design. The cell partition data have been listed in table 1. In all systems the cells partitioned to the lower phase or the interface. The amount of cells in the upper phase was less than 1 % in almost all cases. In ATPSs 13, 14 and 29 to 32 the cell viability dropped below 50 %. These systems were made up with dextran 500.000 and had a tie-line length of 0.20 g/g. The cause of the low viabilities is unclear. In the ATPSs with PEG 6000 and dextran 500.000 the hybridoma cells were also found to partition between the lower and the interface. This in contrast with the partitioning of several other cell types, like erythrocytes, that partitioned between the upper phase and the interface (Albertsson, 1986; Walter, 1982, 1985a).

Regression analysis of the data in table 1 resulted in a regression model (table 4). The regression model is illustrated by a Pareto chart (figure 2), in which the magnitudes of the effects of the main factors, PEG Mw (x_1), Dx Mw (x_2), TLL (x_3), KPi/(KPi+KCl) (x_4), pH (x_5), and all of their possible interactions on the cell partitioning are shown.

The Pareto chart shows that the potassium-phosphate fraction (x_4) has by far the greatest effect on cell partitioning. Furthermore the interactions between Dx Mw and the TLL (x_2x_3), the interaction between TLL and the potassium-phosphate fraction (x_3x_4), and the interaction between pH and the potassium-phosphate fraction (x_4x_5) have an important effect on cell partitioning. Finally a large number of higher-order interactions have relatively large effects on the cell partitioning and contribute statistically significant to the regression model.

Partition mechanisms

Several physical properties of ATPSs, like the electrostatic-potential difference, the interfacial tension and the phase hydrophobicities, have been shown to play a role in cell partitioning (Albertsson, 1986; Walter, 1982; Brooks, 1985). It is difficult, however, to elucidate to what extent each of the physical properties is responsible for the actual cell partitioning, by varying the composition of the ATPS. This is because changing one parameter in the composition of the ATPS can change several of its physical properties.

The paramount effect of the phosphate fraction on cell partitioning, however, is a strong indication that the electrostatic-potential difference between the two aqueous phases plays a dominant role in the partitioning of BIF6A7 hybridoma cells, within the boundaries of the experimental design.

At high phosphate fractions the potential difference will be relatively high, at low phosphate fractions it will be almost zero. The electrostatic-potential difference is caused by the unequal distribution of buffering ions like $H_2PO_4^-$ and HPO_4^{2-}

Table 4 Regression model of the BIF6A7 cell partitioning

	Parameter estimate	Factor	Significance %
y =	41.98		99.99
	-3.11	x_1	99.99
	1.19	x_2	93.59
	0.26	x_3	31.64
	-27.31	x_4	99.99
	3.46	x_5	99.99
	-1.83	x_1x_2	99.44
	-3.31	x_1x_3	99.99
	2.22	x_1x_4	99.90
	1.49	x_1x_5	97.79
	9.25	x_2x_3	99.99
	2.88	x_2x_4	99.99
	-0.23	x_2x_5	27.96
	8.65	x_3x_4	99.99
	4.07	x_3x_5	99.99
	-6.82	x_4x_5	99.99
	0.00	$x_1x_2x_3$	0.40
	2.25	$x_1x_2x_4$	99.91
	5.73	$x_1x_2x_5$	99.99
	-1.64	$x_1x_3x_4$	98.78
	2.10	$x_1x_3x_5$	99.82
	0.73	$x_1x_4x_5$	74.94
	-1.40	$x_2x_3x_4$	96.88
	-2.48	$x_2x_3x_5$	99.97
	3.33	$x_2x_4x_5$	99.99
	-3.26	$x_3x_4x_5$	99.99
	3.23	$x_1x_2x_3x_4$	99.99
	4.23	$x_1x_2x_3x_5$	99.99
	-3.53	$x_1x_2x_4x_5$	99.99
	3.47	$x_1x_3x_4x_5$	99.99
	3.18	$x_2x_3x_4x_5$	99.99
	-3.88	$x_1x_2x_3x_4x_5$	99.99

$R^2 = 0.9879$
$R^2_{Adj} = 0.9778$

between the two phases. These ions partition more to the lower phase and cause a relatively negative potential of the lower phase compared to the upper phase. Cl^- ions, which distribute evenly, can reduce the potential difference (Albertsson, 1986; Walter, 1982).

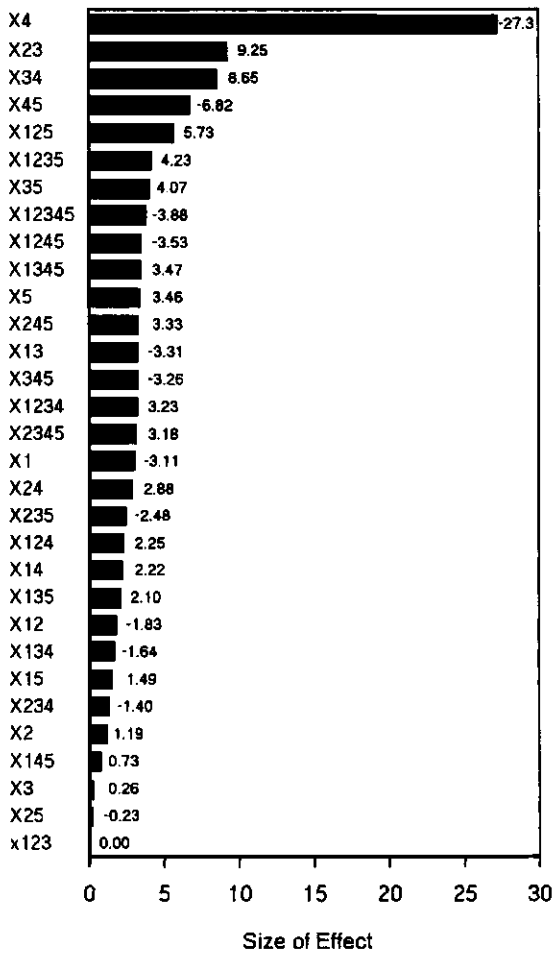

Figure 2 Pareto chart of the regression model. The size of the effects on BIF6A7 cell partitioning are given in order of magnitude.

The BIF6A7 cells are 'pushed' from the lower phase into the interface with increasing potassium-phosphate fractions (increasing relative negative potential of the lower phase). And this is exactly what was expected, because mammalian cells have a negative overall charge at a pH of around 7.0 (Albertsson, 1986; Walter, 1982, 1985a).

For thermodynamic reasons the PEG and dextran molecular weights (x_1 and x_2) are expected to have a pronounced effect on cell partitioning (Brooks, 1985). Although, within the boundaries of the experimental space, their effect is small as compared to the effect of the phosphate fraction (figure 2), the influence of Dx

Mw on cell partitioning is clear, once the phosphate fraction is set to 0.1 (figure 3a). The limited effect of PEG Mw is probably caused by the selected Mw range (6000 - 35.000 D).
In figure 3a the cell partitioning has been depicted as a function of both the dextran molecular weight and the tie-line length at a low phosphate fraction. The cell partitioning cannot be explained by simply adding the individual effects of dextran molecular weight and tie-line length. The effect of the tie-line length on cell partitioning strongly depends on the dextran molecular weight and vice versa. The factors Dx Mw and TLL interact. The positive sign of the interaction means that in the (-1,-1) (Dx Mw = -1 and TLL = -1) and the (+1,+1) situation the effect on cell partitioning will be more than the sum of the effects of the main factors. In the regression model this interaction is represented by the large parameter value (9.25) of the interaction factor (x_2x_3) (figure 2, table 4). The large percentage of cells in the lower phase at low TLL in combination with low Dx Mw is consistent with literature data (Albertsson, 1986; Walter, 1982; Brooks, 1985). The increase in cell percentage in the lower phase with high Dx Mw and high TLL was unexpected. A possible explanation might be that in the current experimental design, ATPSs with a constant osmotic pressure were used. At higher polymer concentrations (longer tie lines) therefore, less buffer was used. The reduced buffer concentrations may have decreased the electrostatic-potential difference (Albertsson, 1986).

The interaction between phosphate fraction and tie-line length (x_3x_4) is shown in figure 3b. At low phosphate fractions an increasing tie-line length will lead to more cells in the interface. This was expected because longer tie lines correspond with higher interfacial tensions as well as larger electrostatic potential differences, so the cells tend to partition more into the interface (Albertsson, 1986; Walter, 1982; Forciniti, 1990). At high phosphate fractions, larger tie lines lead to slightly more cells in the lower phase. This was not expected, because longer tie lines increase the electrostatic potential difference and therefore should 'push' the cells more into the interface (Albertsson, 1986; Brooks, 1985; Walter, 1982). Again, a possible explanation might be that in the current experimental design the buffer concentrations at the high TLL set point were lower than at the low TLL set point. The reduced buffer concentrations may have decreased the electrostatic-potential difference (Albertsson, 1986).

The interaction between phosphate fraction and pH (x_4x_5) is illustrated in figure 3c. It was not expected that the pH in the range of 6.6 to 7.4 would have a large effect on cell partitioning, because usually the major cell-surface charged components are fully ionized above pH 5.0 (Walter, 1982; Brooks, 1985; Walter, 1985a). However, the pH was set by varying the ratio of mono- to dibasic phosphates. Dibasic phosphate, relatively more abundant at pH 7.4, causes a higher electrostatic potential difference (Albertsson, 1986). This could explain why the high 'pH' and

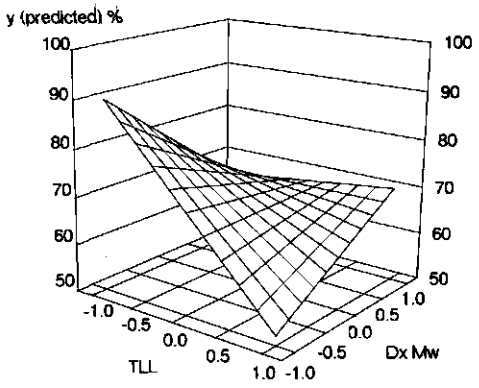

Figure 3a

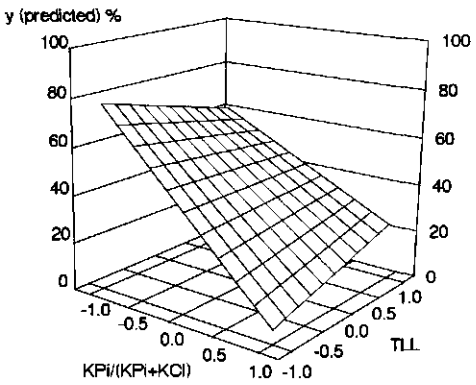

Figure 3b

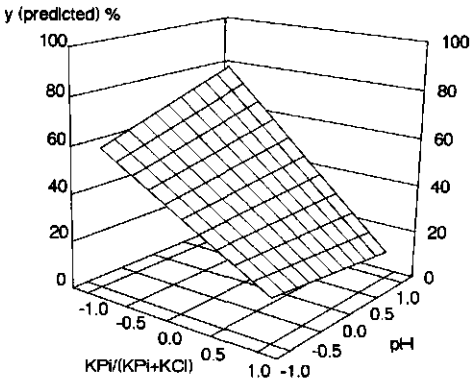

Figure 3c

Figure 3 Model predictions of BIF6A7 cell partitioning. The influence of tie-line length (TLL) and dextran molecular weight (Dx Mw) at KPi (KPi+KCl) = 0.1, PEG Mw = 20 kD and pH = 7.0 (a). The influence of KPi (KPi+KCl) and TLL at PEG Mw = 20 kD, Dx Mw = 220 kD and pH = 7.0 (b). The influence of KPi (KPi+KCl) and pH at PEG Mw = 20 kD, Dx Mw = 220 kD and TLL = 0.15 g/g (c).

high phosphate fraction augment each others effect and cause more cells to partition into the interface.

ATPSs for extractive bioconversions

For extractive bioconversions with animal cells the presence of cells in the interface is not desirable. Cells in the interface are in contact with the top phase, there-

fore cell growth could be influenced by the high PEG concentrations in the top phase (Zijlstra, 1996a). Also the phase separation could be influenced. Therefore for extractive bioconversions ATPS culture media should be designed in which the cells partition to the lower dextran-rich phase.
According to the regression model, the factor settings within the experimental design giving the highest percentage of cells in the lower phase are KPi/(KPi+KCl) = 0.1, pH = 7.4, TLL = 0.10 g/g, Mw Dx = 40 kD and Mw PEG = 35 kD, resulting in 93% of the cells in the lower phase.

BIF6A7 partitioning in ATPS culture media

In previous work it was shown that in ATPSs with PEG 35.000 and Dx 40.000 and culture medium the cells partitioned predominantly to the lower phase (table 5) (Zijlstra, 1996). The tie-line lengths used were 0.12 g/g and 0.16 g/g, the pH was 7.0 to 7.4. The buffer present in the culture medium was ± 35 mM of Na-bicarbonate buffer, the Na-chloride concentration was 60 mM, hence the Na-bicarbonate fraction was 0.37.
Despite the high buffer fraction the cells partitioned largely to the lower phase. A likely explanation is that the Na-bicarbonate buffer gives a smaller electrostatic-potential difference than a phosphate buffer (Albertsson, 1986).
The increase of cells partitioning into the interface with increasing tie-line length corresponds well with the data from the experimental design.
Based on the cell partition results from the experimental design, other suitable ATPS culture media may be designed. The PEG molecular weight for instance, being the least important main factor in BIF6A7 partitioning, may be reduced without significant consequences for the cell partitioning. Too small PEG's however will cause the need for high concentrations of PEG and dextran for phase separation (figure 1). And this may conflict with the earlier finding that to enable cell growth the PEG and dextran concentrations in the lower phase should be less than 0.025 g/g respectively 0.15 g/g (Zijlstra, 1996a).

Table 5 Partitioning in ATPS Culture Media

Cell line	[PEG 35kD] g/g	[Dx 40kD] g/g	TLL g/g	pH	y %
BIF6A7*	0.015	0.10	0.12	7.0-7.4	85
BIF6A7*	0.010	0.13	0.16	7.0-7.4	80
PFU	0.024	0.091	0.12	7.1	92
CHO	0.024	0.091	0.12	7.2	100

* (Zijlstra, 1996a)

Partitioning of other animal-cell lines in ATPS culture media

Considering the previous criteria an ATPS hybridoma culture medium was designed of which the composition is given in table 3. The top to bottom phase-volume ratio of the medium is 3:7, which should be suitable for extractive bioconversions. In table 5 partitioning of mouse/rat hybridoma cell line PFU-83 and CHO cell line BIC-2 in the designed ATPS culture medium are listed, as well as the partitioning of BIF6A7 in the similar ATPS media from previous work (Zijlstra, 1996a).

As shown in table 5, not only BIF6A7 cells partition predominantly to the lower dextran rich phase, but also the PFU-83 and the BIC-2 cells. Moreover, it has become clear from previous work, that good growth of cell line BIF6A7 in ATPS hybridoma culture medium is possible (Zijlstra, 1996a). Hybridoma PFU-83 also grew well in ATPS culture medium (data not shown). Preliminary results with the BIC-2 CHO cell line in a similar ATPS CHO culture medium have shown growth during at least one passage.

Therefore the designed ATPS hybridoma culture medium seems to be applicable to extractive bioconversions with a wide range of hybridoma cells. Furthermore, the design of ATPS CHO media may extend the use of extractive bioconversions to CHO cell lines.

Product partitioning in ATPSs

For an efficient extractive bioconversion, ATPSs are necessary that do not only support good cell growth and production, but also provide a good separation between the cells and its product. ATPSs in which mammalian cells partition almost completely to the lower phase and in which they can grow are now available. Previous work, has shown that in these ATPSs the IgG product of hybridoma BIF6A7 concentrated along with the cells into the lower phase (Zijlstra, 1996a). Preliminary work using a dye affinity ligand coupled to PEG 35.000 however shows promising results towards improvement of the product partition coefficient. In ATPSs with PEG 35.000, Dx 40.000 and a 1 mM phosphate buffer, replacement of only 1 % of PEG 35.000 with PEG-ligand resulted in a thousand-fold increase of the partition coefficient from 0.02 to 20. Product partition coefficients of this magnitude would make an efficient product extraction from the cell-containing phase possible.

Conclusions

Aim of the work described here was to identify the key factors governing cell partitioning in ATPSs of PEG and dextran by a systematic study of the partition behaviour of the BIF6A7 cell line, and to find ATPSs that are suitable for extractive bioconversions with animal cells. Furthermore we wanted to study the partitioning of

mouse/rat hybridoma PFU-83 and CHO DUKX B11 derived cell line BIC-2 in ATPSs giving good partitioning for BIF6A7, to find out whether these systems can also be used for other cell lines.

A statistical experimental design, a two-level full factorial design with center point, proved to be useful in the systematic study of the BIF6A7 cell partitioning. The potassium-phosphate fraction, which largely determines the electrostatic-potential difference, was the most influential factor affecting the cell partitioning. At high phosphate ratios the cells partitioned more into the interface, at low ratios more into the lower phase. All other factors studied, PEG Mw, Dextran Mw, TLL and pH also affected cell partitioning, although to a lesser extent. The factor settings within the experimental design giving the highest percentage of cells in the lower phase, the desired situation for extractive bioconversions, was KPi/(KPi+KCL) = 0.1, pH = 7.4, Mw PEG = 35 kD, Mw Dx = 40 kD and TLL = 0.10 g/g.

The partitioning of mouse/rat hybridoma cell line PFU-83 and CHO cell line BIC-2 was studied in an ATPS hybridoma culture medium with PEG 35 kD, dextran 40 kD, TLL 0.12 g/g, but with culture medium instead of a buffer.

Both the PFU-83 cell line and the BIC-2 cell line were found to partition almost completely into the lower phase of this ATPS hybridoma culture medium. The PFU-83 showed good growth in it. Therefore it appears that the designed ATPS hybridoma medium can be used for extractive bioconversions with a wide range of hybridoma cell lines. The design of ATPS CHO media may extend the use of extractive bioconversions to CHO cell lines. For an efficient extractive bioconversion, however, it is necessary that means are found to partition the products produced by these cell lines into the upper PEG-rich phase.

Acknowledgements

This work was supported by DSM Biologics, Groningen, The Netherlands and by a grant under the Dutch PBTS-Biotechnology program, Ministry of Economic Affairs Nr. BIO89041. The authors thank E. van der Kooy for technical assistance and G. Gort of the Mathematics Department of the Agricultural University Wageningen for the useful comments on the experimental design.

References

Albertsson PA (1986) In Partitioning of cell particles and macromolecules. 3rd edition. Wiley & Sons: New York, NY, USA.

Andersson E, Hahn-Hägerdal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb. Technol. 12: 242-253.

Backman L, Shanbhag VP (1984) Simplex optimization in biochemistry: Application of the method in two-phase partition. Anal. Biochem. 138: 372-379.

Bamberger S, Brooks DE, Sharp KA, Van Alstine JM & Webber TJ (1985) Preparation of phase systems and measurement of their physicochemical properties. In: Partitioning in aqueous two-phase systems. Walter H, Brooks DE & Fisher D (eds.) Academic Press, Orlando, 85-130.

Box PD, Hunter WG & Hunter JS (1978) In: Statistics for experimenters: An introduction to design, data analysis and model building. John Wiley & Sons: New York.

Brooks DE, Sharp KA & Fisher D (1985) Theoretical aspects of partitioning. In: Partitioning in aqueous two-phase systems. Walter H, Brooks DE & Fisher D (eds.) Academic Press, Orlando, 11-84.

Daugulis AJ (1988) Integrated reaction and product recovery in bioreactor systems. Biotechnol.Prog. 4: 113-122.

Forciniti D, Hall CK & Kula MR (1990) Interfacial tension of polyethyleneglycol-dextran-water systems: Influence of temperature and polymer molecular weight. J. Biotechnol. 16: 279-296.

Freeman A, Woodley JM & Lilly MD (1993) *In situ* product removal as a tool for bioprocessing. BIO/TECHNOLOGY. 11: 1007-1012.

Haaland PD (1989) In: Experimental design in biotechnology. Marcel Dekker Inc., New York.

Koops K, Hoff HS, Weperen JJ, Das PC & Smit Sibinga Cth (1990) Immunoaffinity purification of factor VIII / Von Willebrand factor complex. In: Developments on haematology and immunology: Coagulation and blood transfusion. Smit Sibinga CTh, Das PC & Mannucci PM (eds.) Kluwer Academic, Dordrecht, The Netherlands, 26: 103-119.

Kratje RB, Lind W & Wagner R (1994) Evaluation of the proteolytic potential of *in vitro*-cultivated hybridoma and recombinant mammalian cells. J. Biotechnol. 32: 107-125.

Kroner K & Kula MR (1978) Extraction of enzymes in aqueous two-phase systems. Proc. Biochem. 13: 7-10.

Kula MR, Kroner K & Hustedt H (1982) Purification of enzymes by liquid-liquid extraction. Adv. Biotechnol. Eng. 24: 73-118.

Matiasson B (1988) Bioconversions in aqueous two-phase systems: an alterative to conventional immobilisation. In: Methods in enzymology: Immobilised enzymes and cells. Mosbach K (ed.) Academic Press Inc., San Diego, 137(D): 657-668.

Mohan SB, Chohan SR, Eade J & Lyddiatt A (1993) Molecular integrity of monoclonal antibodies produced by hybridoma cells in batch culture and in continuous-flow culture with integrated product recovery. Biotechnol. Bioeng. 42: 974-986.

Oers JWAM van, Tilders FJH & Berkenbosch F (1989) Characterization of a rat monoclonal antibody to rat/humancorticotropin releasing factor. Endocrinology. 124: 1239-1246.

Sharp KA (1985) In: Theoretical and experimental studies on erythrocyte partition in aqueous two-phase systems. Ph.D. thesis, University of British Columbia: Vancouver.

Van der Pol L, Bakker WAM & Tramper J (1992) Effect of low serum concentrations (0%-2.5%) on growth, production, and shear sensitivity of hybridoma cells. Biotechnol.Bioeng. 40: 179-182.

Van't Riet K & Tramper J (1991) In: Basic bioreactor design. Marcel Dekker Inc.: New York.

Walter H (1982) Separation and subfractionation of blood cell populations based on their surface properties by partitioning in two-polymer aqueous phase systems. In: Cell separation: Methods and selected applications. Pretlow II TG & Pretlow II ThP (eds.) Academic Press, New York, 1: 261-299.

Walter. H (1985a) Surface properties of cells reflected by partitioning: Red blood cells as a Model. In: Partitioning in aqueous two-phase systems. Walter H, Brooks DE & Fisher D (eds.) Academic Press, Orlando, 328-376.

Walter H & Fisher D (1985) Separation and subfractionation of selected mammalian cell populations. In: Partitioning in aqueous two-phase systems. Walter H, Brooks DE & Fisher D (eds.) Academic Press, Orlando, 387-415.

Zijlstra GM, De Gooijer CD, Tramper J & Van der Pol LA (1996a) Design of aqueous two-phase systems supporting animal cell growth: A first step towards extractive bioconversions. Enz. Microb. Technol. 19: 2-8.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996b). Separation of hybridoma cells from their IgG product using aqueous two-phase systems. Bioseparation. 6: 201-210.

Chapter 4

Separation of hybridoma cells from their IgG product using aqueous two-phase systems

Summary

The partitioning of IgG in aqueous two-phase systems (ATPSs) of PEG and Dextran was studied systematically using a statistical experimental design.
Aim was to improve the separation of hybridoma cells and their IgG product, by identifying the key variables governing IgG partitioning, and by comparing the IgG partition data with the hybridoma cell partition data obtained in previous work.
The influence of five factors i.e. the poly(ethylene glycol) molecular weight (PEG Mw), dextran molecular weight (Dx Mw), tie-line length (TLL), pH and potassium phosphate fraction (KPi/(KPi+KCl)), on IgG partitioning was characterized using a full-factorial experimental design. In all of the ATPSs the IgG partitioned predominantly into the lower phase. The partition coefficient varied between 0.78 (Variable settings: PEG Mw = 6000, Dx Mw = 500.000, TLL = 0.10 g/g, KPi/(KPi+KCl) = 1.0 and pH = 7.4) and 0.0002 (Variable settings: PEG Mw = 35.000, Dx Mw = 40.000, TLL = 0.20 g/g, KPi/(KPi+KCl) = 1.0 and pH 6.6). The tie-line length, the dextran molecular weight and the PEG molecular weight had the most pronounced effect on IgG partitioning.
Matching the partition data of the IgG product with previously obtained data of the hybridoma cell partitioning, showed that within the experimental design no ATPS could be found giving a good separation of the hybridoma cells and their IgG product.
There are, however, ATPSs available in which the cells partition to, and grow in the lower dextran-rich phase. To achieve a good separation of the hybridoma cells and their IgG product in these ATPSs, the IgG product has to be specifically extracted into the PEG-rich top phase. For this purpose the use of affinity ligands coupled to PEG may offer a solution. Therefore, a number of commercially available dye-resins was screened for their ability to bind the BIF6A7 IgG antibody. The mimetic

This chapter has been published in: Bioseparation, 1996, **6**: 201-210 by the authors: G.M. Zijlstra, M.J.F. Michielsen, C.D. de Gooijer, L.A. van der Pol and J. Tramper.

green 1 A6XL dye-resin was found to bind BIF6A7 IgG. The dye-ligand coupled to PEG was used to manipulate the IgG partitioning in an ATPS. In the presence of the PEG-ligand, the IgG partitioned almost completely to the top phase. The IgG-partition coefficient increased three orders of magnitude, resulting in a 25-fold higher IgG concentration in the top phase than in the bottom phase.

Introduction

The potential of aqueous two-phase systems (ATPSs) to separate proteins and cell particles has long been recognized in biotechnology. Among the first applications were the extractions of enzymes from disrupted bacterial biomass using PEG-salt ATPSs (Kroner, 1978; Kula, 1982; Hustedt, 1988). More recently, ATPSs have been used to extract recombinant proteins from *E.coli* (Hart, 1994).
Beside the use of ATPSs in downstream-processing operations, also a number of extractive fermentations or extractive bioconversions with protein-excreting bacteria, where the extraction occurs during the fermentation, have been reported (Andersson, 1990; Park, 1991; Persson, 1992a, 1992b; Kuboi, 1995).
The use of ATPSs in animal-cell technology sofar has been rather limited. ATPSs have been used for the purification of monoclonal antibodies produced by hybridoma cells (Sulk, 1992). More recently, the feasibility of animal cell cultivation in ATPSs was demonstrated, opening the way for extractive bioconversions with animal cells in ATPSs (Zijlstra, 1996a).
In animal-cell culture, where the protein product normally is excreted by the cells and where continuous fermentations using cell-retention devices are quite common, the concept of extractive bioconversions offers a number of distinct advantages. In the first place, the concept of extractive bioconversions implies a partial integration of the fermentation and the downstream processing within the bioreactor. Provided suitable ATPSs are found, a cell free, concentrated and partially purified harvest may be obtained straight from the bioreactor. Secondly, with the cells present in one liquid phase and the product concentrated in the other liquid phase, the cells can be separated from the product by separating the liquid phases. If this separation can be performed continuously, using for instance a continuous centrifuge, a novel, scalable cell-retention mechanism is available. Finally, the yield of protein products that are instable under fermentation conditions, by nature, or because of breakdown by enzymes (Mohan, 1993; Kratje, 1994; Gramer, 1995), may be increased by rapid and selective extraction of the product.
It is clear that for an efficient extractive bioconversion, ATPSs yielding good cell growth and production, as well as a good separation of cells and their product have to be available. In previous work with ATPSs of PEG and dextran using a mouse/mouse hybridoma BIF6A7 as a model animal-cell line, it was shown that ATPSs can be designed in which the cells partition almost completely into the

lower dextran-rich phase. Furthermore, long term cell growth was demonstrated in these ATPSs. The separation of the hybridoma cells and their IgG product, however, was rather poor, because the IgG product concentrated in the lower phase, along with the cells (Zijlstra, 1996a, 1996b).
Aim of the work presented here was to improve the separation of hybridoma cells and their IgG product by doing a systematic study into the partition behaviour of the IgG, by identifying the key variables governing its partitioning, and by comparing the IgG partition data with the hybridoma cell partition data obtained in previous work.
The partitioning of proteins in ATPSs depends on the same variables as the cell partitioning, being: the type and the molecular weight of the polymers used, the polymer concentrations in each phase, the salt types, their relative concentrations, and the pH (Walter, 1982; Albertsson, 1986; Kula, 1990, Abbott, 1990). Furthermore, ligands coupled to one or both of the phase forming polymers can be used to manipulate protein partitioning (Birkenmeier, 1986, 1991).
Here the PEG and dextran molecular weights, the tie-line length (TLL), as a measure for the difference in polymer concentrations of the phases, the potassium phosphate fraction (KPi/(KPi+KCl)) and the pH have been varied using the same experimental design used earlier to characterize the cell partitioning (Zijlstra, 1996b).

Materials and methods

Experimental Design

The partitioning of IgG monoclonal antibody in ATPSs of PEG and dextran was studied using a statistical experimental design. To quantify the partitioning, the partition coefficient, defined as the IgG concentration in the top phase divided by the IgG concentration in the bottom phase, was used. This partition coefficient, being the response variable (y(IgG)), was measured as a function of the PEG Mw (x_1), the dextran Mw (x_2), the tie-line length (x_3), the KPi fraction (x_4) and the pH (x_5). For each of the five variables a high (coded value: 1) and a low (coded value: -1) set point was selected. ATPSs representing all 32 ($=2^5$) set point combinations were made, as well as an ATPS representing the center point, in which the value of all variables was in-between (coded value: 0). This resulted in an orthogonal 2^5 full-factorial experimental design with center point (Box, 1978; Haaland, 1989) (table 1). All measurements were carried out in duplicate, the center point measurement was carried out fivefold.
Regression analysis of the data using the coded values of the variables was done with the SAS statistical-program package.

Table 1 Experimental design: variable settings, ATPS compositions and IgG partitioning

System	PEG Mw (x_1) kD	code	Dx Mw (x_2) kD	code	TLL (x_3) g/g	code	KPi(KPi+KCl)(x_4)	code	pH (x_5)	code	[PEG][Dx] g/g	[KPi] g/g	[KCl] mM/kg	y(IgG)* mM/kg	y(cell)	** %
1	6	-1	40	-1	10	-1	0.1	-1	6.6	-1	0.064	0.082	6.2	62.2	0.4389	93
2	6	-1	40	-1	10	-1	0.1	-1	7.4	1	0.064	0.082	6	59.8	0.1831	93
3	6	-1	40	-1	10	-1	1.0	1	6.6	-1	0.064	0.082	56.9	0	0.1611	4
4	6	-1	40	-1	10	-1	1.0	1	7.4	1	0.064	0.082	54.6	0	0.1829	1
5	6	-1	40	-1	20	1	0.1	-1	6.6	-1	0.068	0.106	3.3	32.8	0.0395	7
6	6	-1	40	-1	20	1	0.1	-1	7.4	1	0.068	0.106	3.6	35.5	0.0306	96
7	6	-1	40	-1	20	1	1.0	1	6.6	-1	0.068	0.106	37.2	0	0.0272	33
8	6	-1	40	-1	20	1	1.0	1	7.4	1	0.068	0.106	33.7	0	0.0345	10
9	6	-1	500	1	10	-1	0.1	-1	6.6	-1	0.052	0.061	8.6	85.6	0.7603	68
10	6	-1	500	1	10	-1	0.1	-1	7.4	1	0.052	0.061	8.5	85.4	0.7244	72
11	6	-1	500	1	10	-1	1.0	1	6.6	-1	0.052	0.061	82.2	0	0.6067	1
12	6	-1	500	1	10	-1	1.0	1	7.4	1	0.052	0.061	79.2	0	0.7810	1
13	6	-1	500	1	20	1	0.1	-1	6.6	-1	0.063	0.092	5.3	52.8	0.2779	93
14	6	-1	500	1	20	1	0.1	-1	7.4	1	0.063	0.092	5.4	54.3	0.2673	76
15	6	-1	500	1	20	1	1.0	1	6.6	-1	0.063	0.092	56.5	0	0.1907	47
16	6	-1	500	1	20	1	1.0	1	7.4	1	0.063	0.092	47.6	0	0.3092	28
17	35	1	40	-1	10	-1	0.1	-1	6.6	-1	0.037	0.068	11	110.5	0.0879	85
18	35	1	40	-1	10	-1	0.1	-1	7.4	1	0.037	0.068	9.9	98.7	0.0856	89
19	35	1	40	-1	10	-1	1.0	1	6.6	-1	0.037	0.068	105.2	0	0.0148	28
20	35	1	40	-1	10	-1	1.0	1	7.4	1	0.037	0.068	96.5	0	0.0155	5
21	35	1	40	-1	20	1	0.1	-1	6.6	-1	0.057	0.093	8.1	80.8	0.0005	44
22	35	1	40	-1	20	1	0.1	-1	7.4	1	0.057	0.093	7.5	74.9	0.0016	61
23	35	1	40	-1	20	1	1.0	1	6.6	-1	0.057	0.093	78.6	0	0.0002	3
24	35	1	40	-1	20	1	1.0	1	7.4	1	0.057	0.093	71	0	0.0002	1
25	35	1	500	1	10	-1	0.1	-1	6.6	-1	0.028	0.050	12.4	124.1	0.3990	60
26	35	1	500	1	10	-1	0.1	-1	7.4	1	0.028	0.050	12.4	124	0.3811	69
27	35	1	500	1	10	-1	1.0	1	6.6	-1	0.028	0.050	132.5	0	0.2795	7
28	35	1	500	1	10	-1	1.0	1	7.4	1	0.028	0.050	119.7	0	0.3390	0
29	35	1	500	1	10	1	0.1	-1	6.6	-1	0.054	0.088	7.4	73.9	0.0157	30
30	35	1	500	1	20	1	0.1	-1	7.4	1	0.054	0.088	7.2	72.4	0.0221	81
31	35	1	500	1	20	1	1.0	1	6.6	-1	0.054	0.088	74.9	0	0.0097	22
32	35	1	500	1	20	1	1.0	1	7.4	1	0.054	0.088	69.3	0	0.0087	45
33	20	0	220	0	15	0	0.55	0	7.0	0	0.042	0.070	55.3	43.9	0.2095	41

* The IgG concentration in the top phase divided by the IgG concentration in the bottom phase: average values are given
** The percentage of cells in the lower phase: average values are given

Monoclonal Antibody (MAb)

The monoclonal antibody used is an IgG class 2a monoclonal antibody, derived from the BIF6A7 hybridoma cell line. It was obtained from DSM Biologics (Groningen, The Netherlands). The antibody was stored as a 3 mg/ml solution in a 50 mM Na-phosphate pH 7.0 buffer with 8 % maltose and 0.02 % Na-azide. The purity was larger than 95 % as estimated by SDS-gel electrophoresis.

To get an impression of the charge of the antibody as a function of pH, the iso-electric point (pI) was determined by iso-electric focusing (IEF). IEF showed 6 bands with iso-electric points ranging from pH 6.55 to pH 7.35. Each band represents a different level of glycosilation of the antibody.

Polymer solutions

Poly(ethylene glycol) (PEG) 6000, 20.000 and 35.000 were obtained from Merck (Darmstadt, Germany). Dextran T40 and T500 were obtained from Pharmacia (Uppsala, Sweden) and Dextran 220.000 from Fluka (Buchs, Switzerland). All polymers were without further purification dissolved in distilled water to about 0.30 g/g and then sterilized by autoclaving for 20 minutes at 121°C. Dry weight concentrations of the polymer stocks were determined by refractometry after appropriate dilution.

Buffer solutions

From 2 mol/kg solutions of K_2HPO_4 and KH_2PO_4, K-phosphate buffer solutions were made with pH 6.60, pH 7.00 and pH 7.40 at 37.0°C. From these buffers and from a 2.5 mol/kg K-chloride solutions, five buffer solutions were made (table 2). All salts were of analytical grade and obtained from Merck (Darmstadt, Germany).

Phase diagrams

Table 2 Composition of the buffer solutions

Buffer	K-phosphate M	K-Cloride M	pH
1	0.15	1.5	6.6
2	0.15	1.5	7.4
3	0.63	0.50	7.0
4	1.0	0	6.6
5	1.0	0	7.4

The phase diagrams of each of the polymer molecular-weight combinations (PEG 6000/Dx 40.000, PEG 6000/Dx 500.000, PEG 20.000/Dx 220.000, PEG

35.000/Dx 40.000 and PEG 35.000/Dx 500.000) in distilled water at 37°C were determined in previous work (Zijlstra, 1996b).

Experimental design aqueous two-phase systems

The aqueous two-phase systems for the partition experiments were made from concentrated stock solutions of PEG and dextran, concentrated buffer solutions having the desired KPi/(KPi+KCl) ratio and pH, and distilled water.
From the phase diagrams in distilled water the overall PEG and dextran concentrations were selected giving about equal phase volumes and the desired tie-line lengths. Buffer and distilled water were added to the polymer solutions giving ATPSs with an osmotic pressure of about 300 mOs/kg. The amount of each buffer solution to be added to each selected phase system was determined experimentally (Zijlstra, 1996b). The final composition of the phase systems used for the partition experiments of the cells is given in table 1.

IgG-partition experiments

ATPSs weighing about 2 g and containing about 50 µg/mL IgG were warmed up to 37.0°C in a waterbath and mixed by vortexing. The phases were separated by centrifugation for five minutes at 1000 g and 37.0 °C. From the clear upper and lower phase samples were taken for IgG-concentration measurements. The sample of the lower phase was taken through the upper phase. By applying positive pressure on the pipette and by removing upper-phase traces from the outside of the pipette tip, contamination of the lower with the upper phase was avoided. The IgG concentration in both phases was determined by ELISA and the IgG partition coefficient (y(IgG)) was calculated by dividing the upper-phase IgG concentration by the lower-phase IgG concentration.

Dye-ligand resins

Dye-ligand chromatography resins were obtained from ACL (The Isle of Mann, U.K.). The tested dye-ligand resins were mimetic red 2 and red 3 A6XL, mimetic orange 1, orange 2, orange 3 A6XL, mimetic yellow 1 and yellow 2 A6XL, mimetic green 1 A6XL and mimetic blue 1 and blue 2 A6XL.

Ligand screening

Samples of about 200 µL were taken from a 1:1 v/v resin/storage buffer mixture. After centrifugation the storage buffer was discarded and the resins were washed with 1.4 ml ATPS-2 culture medium (Zijlstra, 1996a). Then the resins were incubated with 1.4 ml ATPS-2 culture medium containing 0.1 mg/mL IgG and mixed with a rotary mixer during 30 minutes. Samples of the supernatant were taken for IgG measurements. Subsequently the resins were washed with 1.4 mL ATPS-2 culture medium, 1.4 mL PBS buffer and eluted with 1.4 mL 50 mM Na-phos-

phate buffer pH 7.0 and 1 M K-chloride. Again samples were taken for IgG measurements.

IgG partitioning with PEG-ligand

PEG 35.000 coupled to Mimetic Green 1 was obtained from ACL (The Isle of Mann, U.K.). In an aqueous two-phase system of PEG 35.000 and dextran 40.000 with the overall composition of 0.042 g/g PEG and 0.081 g/g dextran, resulting in an about equal phase-volume ratio and a tie-line length of 0.15 g/g, different amounts of PEG were substituted for PEG coupled to Mimetic Green. To each system approximately 50 μg/mL of IgG from the concentrated stock solution was added. The final Na-phosphate buffer concentration was 1 mM, the pH was 7.0. After warming the systems up to 37°C they were thoroughly mixed by vortexing. Then the phases were separated by centrifugation for 5 minutes at 1000 g and 37.0 °C. From the clear phases samples for IgG determination were taken as described above.

Analytical methods

Osmotic pressure. Osmotic pressures were measured with an Gonotec Osmomat 030 cryoscopic osmometer.

Refraction. The refraction was measured against distilled water in an ERMA ERC-7510 refractometer with digital reading. The dry-weight contents were calculated using the specific refraction of 0.000139 Refractive Index Units (RIU) for a 1 g/L PEG solution and 0.000153 RIU for 1 g/L Dextran solution (Mw > 10.000 D) (Sharp, 1985; Forciniti, 1990).

IgG concentration. The monoclonal-antibody concentration was determined by a quantitative sandwich-ELISA assay. Polyclonal rabbit anti-mouse antibodies (RAM) were used to bind IgG from the samples, polyclonal rabbit anti-mouse antibodies coupled to horse-radish peroxidase (RAM-HRP) were used to establish the 1:1 link of IgG with enzyme. Both RAM and RAM-HRP were obtained from Dakopatts (Glostrup, Denmark). The presence of polymers did not influence the results of the assay (data not shown).

Results and discussion

IgG partitioning

A systematic study of the IgG partition behaviour in ATPSs of PEG and dextran was done using a statistical experimental design. The IgG partitioning was studied in 33 different ATPSs, representing all variable combinations of the experimental design. The partition data have been listed in table 1. In all of the ATPSs the IgG partitioned predominantly into the lower phase ($y(IgG) < 1$). The partition coeffi-

Table 3 Regression model of the BIF6A7 IgG partitioning

	Parameter estimate	Factor	Significance %
y =	0.2087		99.99
	-0.1048	x_1	99.99
	0.1271	x_2	99.99
	-0.1314	x_3	99.99
	-0.0236	x_4	99.88
	0.0018	x_5	20.74
	-0.0490	x_1x_2	99.99
	0.0349	x_1x_3	99.99
	0.0032	x_1x_4	36.32
	0.0011	x_1x_5	13.15
	-0.0667	x_2x_3	99.99
	0.0034	x_2x_4	37.94
	0.0166	x_2x_5	98.10
	0.0189	x_3x_4	99.20
	0.0053	x_3x_5	55.99
	0.0220	x_4x_5	99.76
	-0.0046	$x_1x_2x_3$	50.35
	-0.0056	$x_1x_2x_4$	59.12
	-0.0136	$x_1x_2x_5$	94.84
	-0.0012	$x_1x_3x_4$	13.69
	-0.0074	$x_1x_3x_5$	71.75
	-0.0175	$x_1x_4x_5$	98.67
	-0.0068	$x_2x_3x_4$	67.97
	-0.0094	$x_2x_3x_5$	82.93
	0.0036	$x_2x_4x_5$	39.80
	-0.0135	$x_3x_4x_5$	94.68
	0.0068	$x_1x_2x_3x_4$	68.23
	0.0070	$x_1x_2x_3x_5$	69.35
	0.0007	$x_1x_2x_4x_5$	8.28
	0.0079	$x_1x_3x_4x_5$	75.17
	0.0031	$x_2x_3x_4x_5$	35.24
	-0.0082	$x_1x_2x_3x_4x_5$	76.57

$R^2 = 0.9705$
$R^2_{Adj} = 0.9458$

cient varied between 0.78 (Variable settings: PEG Mw = 6000, Dx Mw = 500.000, TLL = 0.10 g/g, KPi/(KPi+KCl) = 1.0 and pH = 7.4) and 0.0002 (Variable settings: PEG Mw = 35.000, Dx Mw = 40.000, TLL = 0.20 g/g, KPi/(KPi+KCl) = 1.0 and pH 6.6).

Regression analysis of the data in table 1 resulted in a regression model (table 3).

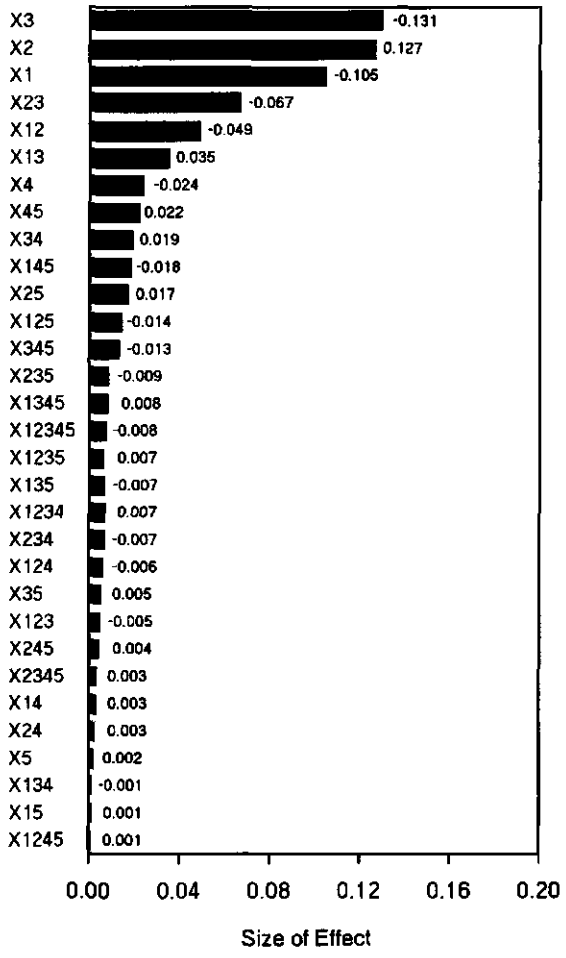

Figure 1 Pareto chart of the regression model. The effects of the main variables and their interactions on BIF6A7 IgG partitioning arranged according to their magnitude.

The outcome of the regression model is visualized in a Pareto chart (figure 1), in which the magnitudes of the effects of the main variables, PEG Mw (x_1), Dx Mw (x_2), TLL (x_3), KPi/(KPi+KCl) (x_4), pH (x_5), and all of their possible interactions on the IgG partitioning are shown.

The Pareto chart shows that the tie-line length (x_3), the dextran molecular weight (x_2) and the PEG molecular weight (x_1) have the greatest effect on IgG partitioning. When the TLL increases the IgG partitions more into the lower phase. This is consistent with partition data of other proteins. Proteins with similar sizes to IgG generally concentrate in the lower phase (Sasakawa, 1972; Johansson, 1985), and their partitioning becomes more extreme when the TLL, a measure for the differ-

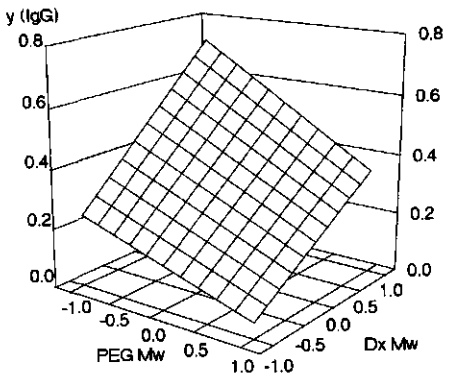

Figure 2a

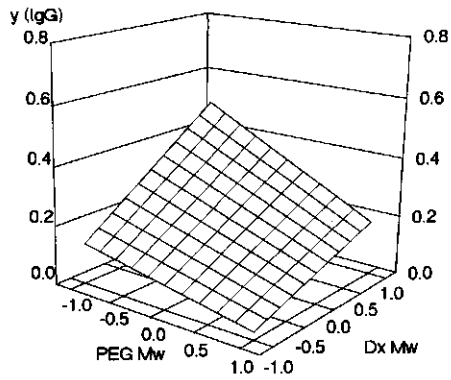

Figure 2b

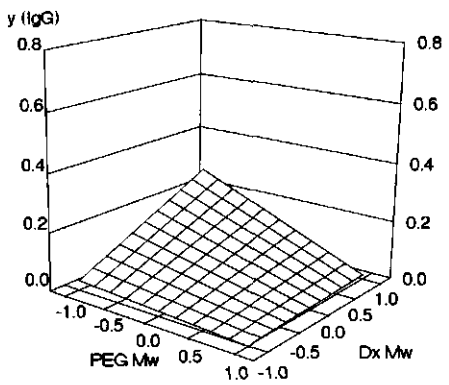

Figure 2c

Figure 2 Model predictions of BIF6A7 IgG partitioning. The influence of PEG molecular weight (PEG Mw) and dextran molecular weight (Dx Mw) on the IgG partition coefficient at different tie-line lengths (TLL) at KPi(KPi/(KPi+PCI) = 0.55 and pH = 7.0. TLL = 0.10 g/g (a), TLL = 0.15 g/g (b) and TLL = 0.26 g/g (c).

ence in polymer compositions of the two phases, increases (Johansson, 1985; Albertsson, 1986; Abbot, 1990).

When the molecular weight of PEG decreases or the molecular weight of dextran increases, the IgG partitions more into the upper phase. This is also consistent with data found for other proteins (Johansson, 1985; Albertsson, 1986; Abbott, 1990). Because an experimental design was used in the current study, it was possible to quantify the interactions between the main variables. The Pareto chart shows that the interactions x_2x_3, x_1x_2 and x_1x_3 have significant effects on the IgG partitioning.

This means that, for instance, the effect of the PEG Mw (x_1) depends on the Dx Mw (x_2). Plots of the regression model (figure 2a, b, c) show that the PEG Mw, Dx Mw and the tie-line length influence each others effect on the IgG partition coefficient, and that the highest partition coefficient can be obtained by simultaneously choosing a low TLL, a high Dx Mw and a low PEG Mw.
The Pareto chart shows that, from the phosphate fraction (x_4) downwards, the effects on the IgG partitioning gradually decrease and become statistically less significant. The pH, for instance, hardly effects IgG partitioning.
The findings that the phosphate fraction and the pH do not have a large impact on IgG partitioning indicates that within the current experimental design the charge-dependent partitioning plays a minor role. High phosphate fractions introduce an electrostatic potential difference between the two aqueous phases, that can influence the partitioning of charged particles like proteins (Albertsson, 1986; Abbott, 1990). The probable cause for this small contribution is that the pH in the current experimental design did not differ much from the isoelectric points (pI) of the IgG, so the net charge of the IgG was small.
In general the higher-order interactions, such as $x_1x_2x_3$, have a small effect on IgG partitioning.
Previous data on IgG partitioning in ATPSs correlate well with the current data. A partition coefficient of 1 was reported for sheep IgG in an ATPS with a small PEG (8000), a large dextran (500.000) and a short tie line (4.6 % Dextran, 3.9 % PEG 8000) (Buffer: 150 mM Na-chloride, 10 mM Na-phosphate, pH 7.2) (Karr, 1986). And an IgG partition coefficient of 1.1 was found for rabbit IgG in an ATPS with a small PEG (8000), a large dextran (500.000) and a short tie line (5 % dextran, 3.4 % PEG) (Buffer: 130 mM Na-chloride, 10 mM Na-phosphate, pH 7.2) (Sharp, 1986).

Separation of IgG and hybridoma cells

For extractive bioconversions with hybridoma cells producing IgG monoclonal antibodies it is important that the cells partition into one phase, while the product concentrates in the other phase. In previous work the partitioning of hybridoma cell line BIF6A7 was studied in the same ATPSs that were used for in the current study (Zijlstra, 1996b). The hybridoma cells were found to partition between the interface and the lower phase (table 1: y(cell)). In contrast to the (BIF6A7) IgG partitioning, the potassium-phosphate fraction (x_4) was the most important variable to manipulate cell partitioning, indicating that the electrostatic potential difference between the phases and therefore charge-dependent partitioning is important (Walter, 1982; Albertsson, 1986). Almost complete partitioning of the cells into the lower phase (y(cell) > 85 %) was obtained in several ATPSs (table 1).
Although the partition mechanism of the hybridoma cells and their IgG product appear different, matching the data of cell (**y(cell)**) and IgG partitioning (**y(IgG)**) (table 1) shows that within the experimental design no ATPS can be found that

gives a good separation of the cells and the IgG product. In every ATPS where the cells partition predominantly into the lower phase (y(cell) > 85 %), the IgG also predominantly partitions to the lower phase (y(IgG) < 0.5). Furthermore comparing the regression models it is unlikely that such an ATPS can be found by varying the pertinent variables.

Affinity ligands.

To improve the separation of hybridoma cells and their IgG product in ATPSs, either the cells or their IgG products have to be specifically extracted into the PEG-rich top phase, while the other remains in the dextran-rich lower phase. Since ATPSs are readily available in which hybridoma cells partition to, and grow in the lower phase (Zijlstra 1996a), preferably the IgG product is to be extracted into the PEG-rich top phase. In order to achieve this, affinity ligands coupled to PEG may offer a solution.

Several types of affinity ligands have been used to manipulate protein or cell partitioning in ATPSs sofar. Fatty acids, binding to hydrophobic sites, have been used to influence the partitioning of plasma proteins and erythrocytes (Shanbhag, 1974; Walter, 1982; Albertsson, 1986). Dye ligands have been used extensively to manipulate protein partitioning. Their binding affinity depends on the protein type, but also on pH, salt type and ionic strength (Koppersläger, 1990). Metal-chelate ligands generally depend on histidine residues exposed on the outside of the protein to be effective. The affinity of metal-chelate ligands is not affected by the ionic strength. They seem especially appropriate to extract recombinant proteins, where histidine tailing has been applied (Koppersläger, 1990). Immunoligands, antibodies covalently coupled to PEG, finally, have been used to extract cells (Sharp, 1986; Karr, 1986). Also cells have been extracted by antibodies that were coupled to PEG through protein A (Karr, 1988). Although highly specific, high affinity ligands for IgG, like antibodies, protein A or protein G, appear to be technically feasible, from a process economic point a view they are less favourable. For industrial scale application, low-cost dye ligands are more attractive.

In the current study, a number of commercially available dye-resins was screened for their ability to bind the BIF6A7 IgG antibody in an ATPS culture medium. Unlike the other dye resins tested, the mimetic green 1 A6XL dye resin was found to bind all IgG present in the ATPS culture medium. The mimetic green 1 dye coupled to PEG 35.000 was then used to manipulate the IgG partitioning in an ATPS with PEG 35.000, dextran 40.000 and a tie-line length of 0.15 g/g. In previous work it was shown that hybridoma BIF6A7 cells partitioned completely to the lower phase in similar ATPSs with culture medium. Without PEG-ligand the IgG product concentrated along with the cells in the lower phase (Zijlstra, 1996a, 1996b).

In figure 3, the difference in the logarithm of the partition coefficient between an ATPS without PEG-ligand and ATPSs with PEG-ligand, has been plotted. When

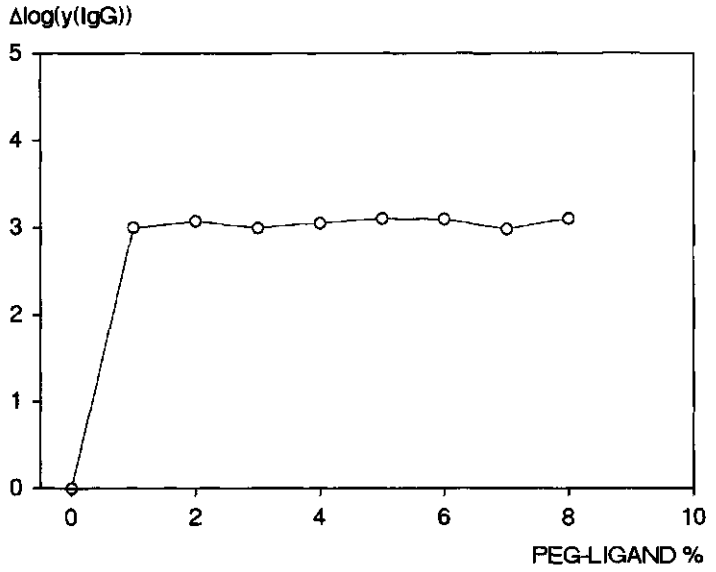

Figure 3 Effect of PEG 35.000-Mimetic Green 1 on BIF6A7 IgG partitioning. Phase system composition: PEG 35.000, Dx 40.000, TLL 0.15 g/g and 1 mM Na-phosphate buffer pH 7.0. T = 37.0°C.

no ligand was present, the partition coefficient was 0.02, and the IgG concentrated in the lower phase. The presence of PEG-ligand, even when only 1 % of the PEG was replaced by PEG-ligand, resulted in a partition coefficient of about 25, so the IgG concentrated in the top phase. Therefore, PEG-ligand caused an increase in the partition coefficient of three orders of magnitude. This partition coefficient correlates well with the partition coefficients of up to 40, that were found for rabbit IgG that was covalently coupled to different numbers of PEG 1900 molecules (Sharp, 1986).

For practical application of the PEG 35.000-mimetic green 1 in extractive bioconversions, further characterization of the affinity of the PEG-ligand is to be done, i.e. determination of the binding affinity as a function of salt type, salt concentrations, pH and host-cell protein concentrations. Furthermore, the effect of the PEG-ligand on hybridoma partitioning and cell growth should be investigated.

Conclusions

The objective of the presented study was to separate hybridoma cells and their IgG product using ATPSs. First, a systematic study into the partition behaviour of the model (BIF6A7) IgG in ATPSs was carried out, to identify the key variables gov-

erning its partitioning. Then, the IgG partition data were compared to cell partition data of model hybridoma cell line (BIF6A7), obtained in previous work.
A statistical experimental design, a two-level full-factorial design with center point, proved to be useful in the systematic study of the IgG partitioning. The tie-line length, the dextran molecular weight and the PEG molecular weight were shown to have the greatest effect on IgG partitioning. This is in contrast with the hybridoma cell partitioning where the potassium-phosphate fraction was found to be the most important variable to manipulate cell partitioning. Although the partition mechanism of the hybridoma cells and their IgG product appear different, matching the data of cell and IgG partitioning showed that within the experimental design no ATPS can be found that gives a good separation of the cells and the IgG product.
There are, however, ATPSs readily available in which the cells partition to, and grow in the lower dextran-rich phase. To achieve a good separation of the hybridoma cells and their IgG product in these ATPSs, the IgG product has to be specifically extracted into the PEG-rich top phase. For this purpose the use of affinity ligands coupled to PEG may offer a solution. In the current study, a number of commercially available dye-resins was screened for their ability to bind the BIF6A7 IgG antibody in an ATPS culture medium. One dye-resin (mimetic green 1 A6XL) was found to bind IgG in ATPS culture medium. Subsequently, the dye was chemically coupled to PEG 35.000, and the resulting PEG-ligand was used to manipulate the IgG partitioning in an ATPS with PEG 35.000, dextran 40.000 and a tie-line length of 0.15 g/g. In similar ATPSs containing culture medium, hybridoma cells partitioned to, and grew in the lower phase. The PEG-ligand caused an increase in the partition coefficient of three orders of magnitude. This resulted in a 25-fold higher IgG concentration in the top phase than in the bottom phase. Further characterization of the PEG-ligand IgG binding is to be done.

Acknowledgements

This work was supported by DSM Biologics, Groningen, The Netherlands and by a grant under the Dutch PBTS-Biotechnology program, Ministry of Economic Affairs Nr. BIO89041. The authors thank E. van der Kooy for technical assistance and G. Gort of the Mathematics Department of the Agricultural University Wageningen for the useful comments on the experimental design.

References

Abbott N, Blankschtein D & Hatton A (1990) On protein partitioning in two-phase aqueous polymer systems. Bioseparation 1:191 - 225.

Albertsson PA (1986) In: Partitioning of cell particles and macromolecules. 3rd edition. Wiley & Sons, New York

Andersson E & Hahn-Hägerdal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb. Technol. 12:242-253.

Birkenmeier G, Kopperschlaeger G & Johansson G (1986) Separation and studies of serum proteins with aid of aqueous two-phase systems containing dyes as affinity ligands. Biomed. Chromatogr. 1:64-77.

Birkenmeier G, Vijayalakshmi MA, Stigbrand T & Kopperschlaeger G (1991) Immobilized metal ion affinity partitioning, a method combining metal- protein interaction and partitioning of proteins in aqueous two-phase systems. J. Chromatogr. 539:267-77.

Box PD, Hunter WG & Hunter JS (1978) In: Statistics for experimenters: An introduction to design, data analysis and model building. John Wiley & Sons, New York.

Daugulis AJ (1988) Integrated reaction and product recovery in bioreactor systems. Biotechnol. Prog. 4:113-122.

Forciniti D, Hall CK & Kula MR (1990) Interfacial tension of polyethyleneglycol-dextran-water systems: Influence of temperature and polymer molecular weight. J. Biotechnol. 16:279-296.

Freeman A, Woodley JM & Lilly MD (1993) *In situ* product removal as a tool for bioprocessing. BIO/TECHNOLOGY. 11:1007-1012.

Gramer MJ, Goochee CF, Chock VY, Brousseau DT & Sliwkowski MB (1995) Removal of sialic acid from glycoprotein in CHO cell culture supernatant by action of an extracellular CHO cell sialidase. BIO/TECHNOLOGY 13:692-698

Haaland PD (1989) In: Experimental design in biotechnology. Marcel Dekker Inc., New York.

Hart RA, Lester PM, Reifsnyder DH, Ogez JR & Builder SE (1994) Large scale, *in situ* isolation of periplasmatic IGF-I from *E.coli*. BIO/TECHNOLOGY 12:1113-1117

Hustedt H, Kroner KH & Papamichael N (1988) Continuous cross-current aqueous two-phase extraction of enzymes from biomass: automated recovery in production scale. Process Biochem. 23:129-37.

Johansson G (1985) Partitioning of proteins. In: Walter H, Brooks DE & Fisher D (eds.) Partitioning in aqueous two-phase systems. (pp 161-226) Academic Press, Orlando.

Karr LJ, Shafer SG, Harris JM, Van Alstine JM & Snyder RS (1986) Immuno-affinity partition of cells in aqueous polymer two-phase systems J. Chromatogr., 354:269-282

Karr LJ, Van Alstine JM, Snyder RS, Shafer SG & Harris JM (1988) Cell separation by immunoaffinity partitioning with polyethylene glycol- modified protein A in aqueous polymer two-phase systems J. Chromatogr., 442:219-227

Koops K, Hoff HS, Weperen JJ, Das PC & Smit Sibinga CTh (1990) Immunoaffinity purification of factor VIII / Von Willebrand factor complex. In: Smit Sibinga CTh, Das PC & Mannucci PM (Eds.) Developments on haematology and immunology: Coagulation and blood transfusion. Vol. 26 (pp. 103-119) Kluwer Academic, Dordrecht, The Netherlands.

Koppersläger G & Birkenmeier G (1990) Affinity partitioning and extraction of proteins. Bioseparation 1:235-254.

Kratje RB, Lind W & Wagner R (1994) Evaluation of the proteolytic potential of *in vitro*-cultivated hybridoma and recombinant mammalian cells. J. Biotechnol. 32:107-125.

Kroner KH & Kula MR (1978) Extraction of enzymes in aqueous two-phase systems. Process Biochem. 13:7-10.

Kuboi R, Umakoshi H & Komasawa I (1995) Extractive cultivation of *Escherichia coli* using poly(ethylene glycol)/phosphate aqueous two-phase systems to produce intracellular ß-galactosidase. Biotechnol.Prog. 11:202-207.

Kula MR, Kroner K & Hustedt H (1982) Purification of enzymes by liquid-liquid extraction. Advances in Biotechnological Engineering 24:73-118.

Matiasson B (1988) Bioconversions in aqueous two-phase systems: an alterative to conventional immobilisation. In: Mosbach K (ed.) Methods in enzymology: Immobilised enzymes and cells.Vol 137(D) (pp. 657-668) Academic Press Inc, San Diego.

Mohan SB, Chohan SR, Eade J & Lyddiatt A (1993) Molecular integrity of monoclonal antibodies produced by hybridoma cells in batch culture and in continuous-flow culture with integrated product recovery. Biotechnol. Bioeng. 42:974-986.

Park KM & Wang NS (1991) α-Amylase fermentation with *Bacillus amyloliquefaciens* in an aqueous two-phase system. Biotechnol.Prog. 7:439-444

Persson I, Staalbrand H, Tjerneld F & Hahn-haegerdal B (1992) Semicontinuous production of celluloytic enzymes with Trichoderma reesei Rutgers C30 in an aqueous two-phase system. Appl. Biochem. Biotechnol., 27(1):27-36.

Persson I, Tjerneld F & Hahn-haegerdal B (1992) Influence of cultivation conditions on the production of cellulolytic enzymes with Trichoderma reesei Rutgers C30 in aqueous two-phase systems. Appl. Biochem. Biotechnol., 27(1):9-25.

Sasakawa S & Walter H (1974) Partition behaviour of native proteins in aqueous dextran-poly(ethylene glycol) phase systems. Biochemistry 11:2760-2765.

Shanbhag VP & Johansson (1974) Specific extraction of human serum albumin by partition in aqueous biphasic systems containing poly(ethylene glycol)-bound ligand. Biochem. Biophys.Res. Commun. 61:1141-1146.

Sharp KA (1985) In: Theoretical and experimental studies on erythrocyte partition in aqueous two-phase systems. Ph.D. thesis. University of British Columbia, Vancouver, Canada.

Sharp KA, Yalpani M, Howard SJ & Brooks DE (1986) Synthesis and application of a poly(ethylene glycol)-antibody affinity ligand for cell separations in aqueous polymer two-phase systems. Anal. Biochem., 154(1):110-117.

Sulk B, Birkenmeier G & Kopperschlaeger G (1992) Application of phase partitioning and thiophilic adsorption chromatography to the purification of monoclonal antibodies from cell culture fluid. J. Immunol. Methods, 149(2):165-171.

Van't Riet K & Tramper J (1992) In: Basic bioreactor design. Marcel Dekker Inc., New York.

Walter H (1982) Separation and subfractionation of blood cell populations based on their surface properties by partitioning in two-polymer aqueous phase systems. In: Pretlow II TG & Pretlow II ThP (eds.) Cell separation: Methods and selected applications.Vol 1 (pp. 261-299) Academic Press, New York.

Zijlstra GM, De Gooijer CD, Van der Pol LA & Tramper J (1996a) Design of aqueous two-phase systems supporting animal cell growth: A first step towards extractive bioconversions. Accepted by Enzyme and Microbial Technology.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996b) Hybridoma and CHO cell partitioning in aqueous two-phase systems. Accepted by Biotechnology Progress.

Chapter 5

IgG and hybridoma partitioning in in aqueous two-phase systems containing a dye-ligand

Summary

The effect of the important ATPS- and buffer parameters on IgG and hybridoma partitioning in ATPSs containing a PEG-dye-ligand was studied. Objective was to establish selection criteria for effective ligands for extractive fermentations with animal cells in ATPSs.
In the presence of 1% PEG-dye-ligand the binding of IgG to the PEG-ligand was affected severely by the Na-chloride concentration. The tie-line length and pH affected IgG partitioning to a lesser extent. The desired partitioning of IgG into the top phase, was only obtained when, in addition to the 10 mmol/kg K-phosphate buffer, no Na-chloride was present. In an ATPS culture medium, with ± 35 mmol/kg Na-bicarbonate and 60 mmol/kg Na-chloride, increasing the PEG-dye-ligand concentration up to 100% did increase the partition coefficient, but was not effective in concentrating the IgG in the top phase of ATPS culture medium at a pH of 7.8.
Furthermore, addition of the PEG-dye-ligand to ATPS culture medium changed the hybridoma cell partitioning from the bottom phase to the interface.

Introduction

Ongoing issues in the manufacturing of biopharmaceuticals are scale-up, rationalization and optimization of the production processes. Strategies to fulfill these objectives are on one hand the optimization of existing unit operations and on the other hand the development of alternative production methods. Our research focuses on exploring the opportunities for extractive fermentations in animal-cell culture, as an alternative for conventional processing.
In recent years extractive fermentations have gained increasing attention in prok-

---

This chapter has been published in Bioseparation 1998, **7**:117-126 by the authors: G.M. Zijlstra, M.J.F. Michielsen, C.D. de Gooijer, L.A. van der Pol and J.Tramper

aryotic cell culture with protein excretion (Mattiasson, 1988; Andersson, 1990; Park, 1991; Persson, 1992a, 1992b). The idea behind extractive fermentations is to use two immiscible liquid phases, one phase containing the cells and the other phase extracting the product. The concept of extractive fermentation is especially useful when instable or easily degradable proteins are produced, because extraction considerably reduces the residence time of the product in the fermentation broth (Andersson, 1990; Mohan, 1993; Kratje, 1994, Gramer, 1995). An additional advantage of extractive fermentations is that the cells are retained in one liquid phase. Recycling this cell-containing phase into the bioreactor therefore, would make a continuous perfusion process possible (Mattiasson, 1988; Andersson, 1990). This is a well-established culture mode in animal-cell culture (Himmelfarb, 1969; Avgerinos, 1990; Schmid, 1992). Furthermore, provided a good partition coefficient of the product into the extracting phase can be obtained, a cell-free, concentrated and partially purified harvest can be obtained straight from the bioreactor.

For extractive fermentations with protein-excreting prokaryotic cells, polymer-polymer Aqueous Two-Phase Systems (ATPSs) have been the two-liquid-phase systems of choice (Mattiasson, 1988; Park, 1991; Persson, 1992a, 1992b). These ATPSs can be made by mixing two polymers, for instance PEG and dextran, in an aqueous solution. Above certain concentrations two liquid phases will form, the lighter phase mainly consisting of water and PEG and the denser phase mainly consisting of water and dextran. Polymer-polymer ATPSs form a relatively mild environment to the cells compared to water/organic-solvent two-phase systems (Albertsson, 1986). In addition they are suitable for protein extractions (Kroner, 1978; Kula, 1982).

For effective extractive fermentations in animal-cell culture, the ATPSs used, have to support animal-cell growth and to separate the cells and their protein product. In previous work, using an IgG-producing hybridoma as model cell line, both the effect of ATPS forming polymers on cell growth, as well as the partitioning of animal cells and their product in APTSs, were studied extensively (Zijlstra, 1996a, 1996b,, 1996c). It was shown that cell growth is possible in media containing up to 0.025 g/g PEG or up to 0.15 g/g dextran (Zijlstra, 1996a). This implies that support of animal-cell growth is only to be expected in ATPSs in which the cells partition to the PEG lean bottom phase, because the PEG concentrations in the top phase usually exceed 0.025 g/g. Subsequently, it was shown that in ATPSs with PEG 35.000, dextran 40.000 and culture media, cells partitioned to the bottom phase. Moreover, they could be cultured in these ATPSs over a period of months. The product, however, partitioned predominantly along with the cells to the bottom phase (Zijlstra, 1996a). Extensive work on cell and product partitioning showed that the salt composition is the most dominant factor affecting cell partitioning, because it can cause an electrostatic potential difference between the phases (Zijlstra, 1996b). The PEG and dextran molecular weight and the tie-line length are the main factors affecting the product partitioning. Despite the differ-

ence in partition mechanisms, no ATPS could be found in which both cell growth and separation between the cells and their product occurred simultaneously (Zijlstra, 1996c).
Partitioning of the IgG product into the PEG-rich top phase could be achieved, however, by using a dye-ligand coupled to PEG (Zijlstra, 1996c). In an ATPS similar to the ones in which cell growth was shown, but with a 1 mmol/kg Na-phosphate buffer instead of culture medium, addition of the PEG-ligand raised the IgG-partition coefficient three orders of magnitude, from 0.02 to 25 (Zijlstra, 1996c). Despite their effectiveness in extracting the product into the top phase in the presence of a low salt buffer, the usefulness of these PEG-ligands under process conditions remains to be demonstrated.
Objectives of the work presented here were to establish the effect of the important ATPS- and buffer parameters on IgG and hybridoma partitioning in ATPSs containing a PEG-dye-ligand. And secondly, to establish selection criteria for effective ligands for extractive fermentations with animal cells in ATPSs.

Materials and methods

Cell line

BIF6A7 is a mouse/mouse hybridoma, originating from an SP2/0 myeloma. BIF6A7 produces a monoclonal IgG antibody binding the Factor VIII/Von Willebrand-Factor complex (Koops, 1990), which plays a role in the human-blood-coagulation mechanism. The cell line was supplied by DSM Biologics (Groningen, The Netherlands). A serum-free 3:1 DMEM/F12 mixture (Gibco, Breda, The Netherlands) (Van der Pol, 1992; Zijlstra, 1996a) supplemented with 0.5 g/L Pluronic (BASF, Arnhem, The Netherlands) and 1 g/L human serum albumin (CLB, Amsterdam, The Netherlands) was used to culture BIF6A7.

Monoclonal Antibody (MAb)

The BIF6A7 monoclonal antibody is an IgG class 2a antibody. The antibody was stored as a 3 mg/mL solution in a 50 mM Na-phosphate pH 7.0 buffer with 0.02 % Na-azide. The purity was larger than 95 % as estimated by SDS-gel electrophoresis.
To get an impression of the charge of the antibody as a function of pH, the iso-electric point (pI) was determined by iso-electric focusing (IEF). IEF showed 6 bands with iso-electric points ranging from pH 6.55 to pH 7.35. Each band represents a different level of glycosilation of the antibody.

Polymer solutions.

Poly(ethylene glycol) (PEG) 35.000 was obtained from Merck (Darmstadt, Germany). Dextran T40 was obtained from Pharmacia (Uppsala, Sweden). The poly-

mers were without further purification dissolved in distilled water to about 0.30 g/g and then sterilized by autoclaving for 20 minutes at 121°C. Dry-weight concentrations of the polymer stocks were determined by refractometry after appropriate dilution (Zijlstra, 1996a).
PEG 35.000 coupled to mimetic green 1 was obtained from ACL (The Isle of Mann, U.K.). Stock solutions of the PEG-ligand were prepared freshly.

Stock solutions of buffers, salts, sucrose and culture medium
For the partition experiments 200 mmol/kg K-phosphate-stock solutions of pH 6.60, pH 6.76, pH 7.00, pH 7.24 and pH 7.40 (at 37.0°C) were used. They were prepared from 2 mol/kg solutions of K_2HPO_4 and KH_2PO_4. Furthermore, a 1 mol/kg Na-chloride stock solution, a 2 mol/kg sucrose stock solution and a two fold concentrated culture medium stock (2 x DMEM/F12 3:1 without Na-chloride, with Na-bicarbonate, pH 7.0 (Zijlstra 1996b)). All chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany), except for the DMEM/F12 culture medium that was obtained from Life Technologies (Breda, The Netherlands).

Experimental Design
The partitioning of BIF6A7 IgG was studied systematically in ATPSs of PEG 35.000 and dextran 40.000, with 1 % of the PEG 35.000 replaced by PEG-ligand. To quantify the IgG partitioning, the partition coefficient, defined as the IgG concentration in the top phase divided by the IgG concentration in the bottom phase, was used. A statistical experimental-design approach was used, taking the IgG partition coefficient as the response factor (y(IgG)). The latter was measured as a function of three factors: the tie-line length (x_1), the pH (x_2) and the Na-chloride concentration (x_3). For each of these three factors five set points were selected using a central-composite experimental design. (Box, 1978; Haaland, 1989) (table 1). The IgG partition measurements were carried out in duplicate.
A quadratic model containing all main factors (x_1, x_2 etc.), all factor interactions (x_1x_2, $x_1x_2x_3$ etc.) and higher-order factors and interactions (x_1^2, $x_1x_2^2$) was fitted through a transformation of the data set (10log(y(IgG)+0.01)), using the regression-analysis module of the SAS statistical-program package. Transformation of the data set was necessary to fulfil the conditions of constant variance and symmetrical distribution around zero of the residuals (being the observed y(IgG) minus the predicted y(IgG)) (Haaland, 1989). The transformation used gave the best result. Because the coded factor values (-1.68, -1, 0, 1, 1.68) were used to fit the data, the magnitude of the regression parameters could be compared directly (figure 2: Pareto chart).

Experimental design aqueous two-phase systems
The aqueous two-phase systems for the partition experiments were made from

concentrated stock solutions of PEG, PEG-ligand, dextran, buffer, salt and sucrose, and distilled water.
From the phase diagram of PEG 35.000 and dextran 40.000 in distilled water the overall PEG and dextran concentrations were selected giving about equal phase volumes and the desired tie-line lengths (figure 1). Buffer, salt, sucrose and distilled water were added to the polymer solutions giving ATPSs with an osmotic pressure of about 300 mOs/kg. The amount of sucrose solution to be added to each selected phase system was determined experimentally. The final composition of the phase systems used is given in table 1.

ATPS culture media with PEG-ligand

The ATPS culture media were made from concentrated stock solutions of PEG, PEG-ligand, dextran, culture medium (including Na-bicarbonate) and Na-chloride, and distilled water.
From the phase diagram of PEG 35.000 and dextran 40.000 in distilled water, the tie line with a length of 0.12 g/g was selected, and the overall PEG and dextran concentrations on this tie line giving about equal phase volumes were determined. Five ATPSs with different percentages of PEG replaced by PEG-ligand were made. The final compositions of these ATPSs are listed in table 2.
To reduce shifts in pH, due to diffusion of CO_2 into the surrounding air and the subsequent change in CO_2/bicarbonate buffer ratio, the caps of the test tubes were kept closed as much as possible. Although the initial pH, determined by the culture medium, was 7.0, the actual pH during the experiments was 7.8 ± 0.1, as determined by measurement of the pH in the test tubes, directly after the top- and bottom phase samples were drawn.

IgG-partition measurements

ATPSs weighing about 2 g and containing about 50 µg/mL IgG were warmed up to 37.0°C in a water bath and mixed by vortexing. The phases were separated by centrifugation for five minutes at 1000 g and 37.0 °C. From the clear upper and lower phase samples were taken for IgG-concentration measurements. The sample of the lower phase was taken through the upper phase. By applying positive pressure on the pipette and by removing upper-phase traces from the outside of the pipette tip, contamination of the lower with the upper phase was avoided. The IgG concentration in both phases was determined by ELISA and the IgG partition coefficient (y(IgG)) was calculated.

Cell-partition measurements

The cells used for the partition experiments were cultured in T-flasks. A 1 mL cell sample was centrifugated and the supernatant was discarded. The cell pellets were washed in 0.5 mL PBS buffer and resuspended in 2 g of a previously prepared ATPS at 37°C, giving single-cell suspensions. The samples were then mixed for 15

Table 1 Experimental design: variable settings. ATPS compositions and IgG partitioning

System	TLL (x_1) g/g	code	pH (x_2)	code	[NaCl] (x_3) mmol/kg	code	[PEG-ligand] g/g	[PEG] g/g	[Dx] g/g	[KPi] mmol/kg	[Sucrose] mmol/kg	y(IgG)*
1	0.10	-1	6.76	-1	10	-1	0.00039	0.035	0.068	10	91	1.566
2	0.10	-1	6.76	-1	40	1	0.00039	0.035	0.068	10	62.5	0.194
3	0.10	-1	7.24	1	10	-1	0.00039	0.035	0.068	10	92.5	0.490
4	0.10	-1	7.24	1	40	1	0.00039	0.035	0.068	10	62	0.148
5	0.20	1	6.76	-1	10	-1	0.00059	0.053	0.093	10	61.5	0.250
6	0.20	1	6.76	-1	40	1	0.00059	0.053	0.093	10	34.5	0.000
7	0.20	1	7.24	1	10	-1	0.00059	0.053	0.093	10	62	0.050
8	0.20	1	7.24	1	40	1	0.00059	0.053	0.093	10	31.5	0.025
9	0.07	-1.68	7.00	0	25	0	0.00029	0.026	0.063	10	92	0.199
10	0.23	1.68	7.00	0	25	0	0.00066	0.060	0.104	10	28	0.018
11	0.15	0	6.60	-1.68	25	0	0.00045	0.041	0.077	10	70.5	0.303
12	0.15	0	7.40	1.68	25	0	0.00045	0.041	0.077	10	70	0.086
13	0.15	0	7.00	0	0	-1.68	0.00045	0.041	0.077	10	90.5	7.184
14	0.15	0	7.00	0	50	1.68	0.00045	0.041	0.077	10	50.5	0.049
15	0.15	0	7.00	0	25	0	0.00045	0.041	0.077	10	72.5	0.087
16	0.15	0	7.00	0	25	0	0.00045	0.041	0.077	10	72.5	0.091
17	0.15	0	7.00	0	25	0	0.00045	0.041	0.077	10	72.5	0.080
18	0.15	0	7.00	0	25	0	0.00045	0.041	0.077	10	72.5	0.096

* The IgG concentration in the top phase divided by the IgG concentration in the bottom phase: average values are given

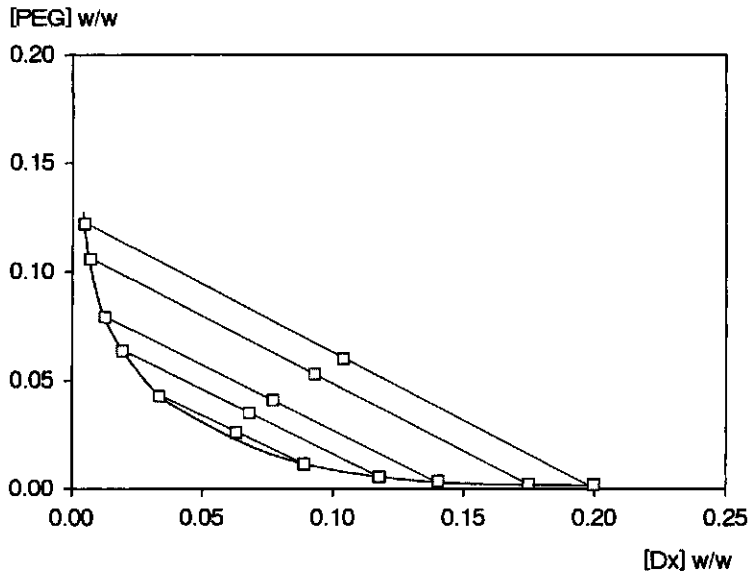

Figure 1 Phase diagram of PEG 35.000 and Dx 40.000 in distilled water at 37°C. The squares (□) represent the selected phase system compositions, with about equal phase volumes and the desired tie-line length.

minutes with a rotary mixer. A 100 µL sample of the mixture was taken to determine the overall cell density, of about $10*10^5$ cells/mL. Subsequently the sample was left to settle for 30 minutes at 37°C in tubes with graduation-scales, yielding primary phase separation. Then the total, top- and lower-phase volumes were determined and 100 µL samples of the upper and the lower phase were taken for cell counts. The sample of the lower phase was taken through the upper phase. By applying a positive pressure on the pipette and by removing upper-phase traces from the outside of the pipette tip, contamination of the lower with the upper phase (and the interface) was avoided.

The fraction of cells in the lower phase was calculated by dividing the cell number in the lower phase by the total cell number. The fraction of cells present in the interface was calculated by subtracting the fraction of cells present in both the top- and bottom phase from the total cell number.

Analytical methods

Osmotic pressure. Osmotic pressures were measured with an Gonotec Osmomat 030 cryoscopic osmometer.

Refraction. The refraction was measured against distilled water in an ERMA ERC-7510 refractometer with digital reading. The dry-weight contents were cal-

Table 2 Composition of ATPS culture media with PEG-ligand

System	[PEG] total g/g	PEG-ligand %	[Dextran] g/g	[Medium]* mg/g	Na-bicarbonate mg/g	Na-bicarbonate mmol/kg	Na-chloride mg/g	Na-chloride mmol/kg
1	0.0375	0	0.0689	6.10	3.10	37	3.51	60
2	0.0375	10	0.0689	6.10	3.10	37	3.51	60
3	0.0375	25	0.0689	6.10	3.10	37	3.51	60
4	0.0375	50	0.0689	6.10	3.10	37	3.51	60
5	0.0375	100	0.0689	6.10	3.10	37	3.51	60

* Composition given in Zijlstra 1996b

culated using the specific refraction of 0.000139 Refractive Index Units (RIU) for a 1 g/L PEG solution and 0.000153 RIU for 1 g/L Dextran solution (Mw > 10.000 D) (Sharp, 1985; Forciniti, 1990).

Cell count. Viable and non-viable cell densities were determined by 1:1 v/v dilution of the samples with a trypan-blue solution and subsequent counting using a Fuchs-Rosenthal hemacytometer and a light microscope.

IgG concentration. The monoclonal-antibody concentration was determined by a quantitative sandwich-ELISA assay. Polyclonal rabbit anti-mouse antibodies (RAM) were used to bind IgG from the samples, polyclonal rabbit anti-mouse antibodies coupled to horse-radish peroxidase (RAM-HRP) were used to establish the 1:1 link of IgG with enzyme. Both RAM and RAM-HRP were obtained from Dakopatts (Glostrup, Denmark). The presence of polymers did not influence the results of the assay (data not shown).

Results and discussion

Systematic study of IgG partitioning in ATPSs with PEG-ligand

Purpose of the systematic study was to characterize the effects of the relevant ATPS- and buffer parameters, i.e. the tie-line length, the Na-chloride concentration and the pH, on IgG partitioning in ATPSs with a PEG-dye-ligand. The partitioning was studied in 18 different ATPSs. The PEG-dye-ligand concentration was fixed at 1% of the total PEG present in each ATPS, because under good binding conditions this is sufficient to extract all IgG into the top phase (Zijlstra 1996c). The ATPS compositions used, as well as the partition data generated, have been listed in table 1. The average partition coefficient varied between 7.2 (Variable settings: TLL = 0.15 g/g, pH = 7.00 and [NaCl] = 0 mmol/kg) and 0.00 (Variable settings: TLL = 0.20 g/g, pH = 6.76 and [NaCl] = 39.9 mmol/kg).

Regression analysis of the data in table 1 resulted in the regression model listed in table 3. Before regression analysis was done, the data set was transformed

(10log(y(IgG)+0.01), to fulfil the conditions of constant variance and symmetrical distribution around zero of the residues (Observed y(IgG) - predicted y(IgG)). The outcome of the regression model is visualized in a Pareto chart (figure 2), in which the magnitudes of the effects of the main variables, TLL (x_1), pH (x_2), [NaCl] (x_3), and all of their possible interactions and quadratic interactions on the IgG partitioning are shown.

The Pareto chart shows that the salt concentration (x_3 and x_{33}) and the tie-line length (x_1) have the most prominent effect on IgG partitioning. Furthermore, the interaction between pH and [NaCl] (x_{23}) is relevant. Graphic representations of the regression model (figure 3a, b, c) give a clear picture of the IgG partition behavior. Figure 3a illustrates the effects on IgG partitioning when the Na-chloride concentration and tie-line length are varied simultaneously. By combining a low TLL with a low Na-chloride concentration, beneficial IgG partitioning (y(IgG)>1) can be obtained. By combining a high TLL and a high Na-chloride concentration, extreme partitioning of the product into the bottom phase is obtained. Figure 3b shows that by combining a low Na-chloride concentration with a low pH very beneficial partitioning of the IgG into the top phase can be obtained. Furthermore it illustrates the pH dependency of the IgG partitioning at low ionic strengths. Figure 3c shows the dependency of the IgG partitioning on the Na-chloride concentration at pH 7.0 and a tie-line length of 0.15 g/g, as predicted by the model. Under the same conditions, but in a 1 mmol/kg Na-phosphate buffer an IgG partition coefficient of 25 was reported earlier (Zijlstra 1996c). In the presence of 10 mmol/kg K-phosphate the y(IgG) already drops to 3.14. At 10 mmol/kg K-phosphate and 25 mmol/kg Na-chloride the partition coefficient is 0.09 and upon further increase of the Na-chloride concentration it levels off between 0.05 and 0.07.

Table 3 Regression model of the IgG partitioning

	Parameter	Variable	Significance (%)
10log(y(IgG)+0.01) =	-1.005		99.99
	-0.401	x_1	99.99
	-0.114	x_2	98.74
	-0.473	x_3	99.99
	-0.107	x_1x_1	96.54
	0.050	x_2x_2	72.84
	0.251	x_3x_3	99.99
	0.058	x_1x_2	69.70
	-0.038	x_1x_3	49.82
	0.196	x_2x_3	99.83

R^2 = 0.9102
R^2_{adj} = 0.8778

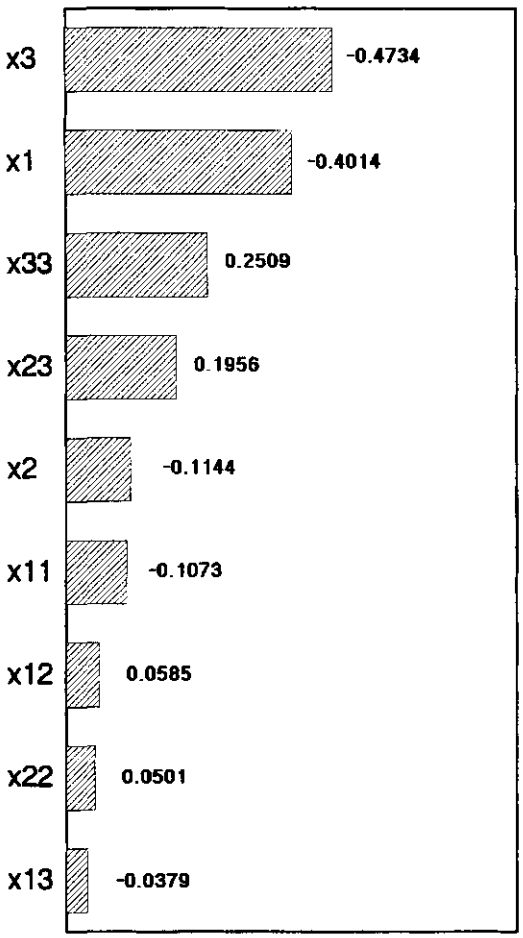

Figure 2 Pareto chart of the regression model. The size of the effects on BIF6A7 IgG partitionong are given in order of magnitude.

Partition mechanisms

The partitioning of IgG in ATPSs with a PEG-ligand in the first place will depend on whether the IgG is bound to the PEG-ligand or not. Subsequently, it will depend on the partition coefficient of the IgG-PEG-ligand complex, or the free IgG. The binding is influenced by the ionic strength, the pH and steric effects due to the size and number of PEG molecules coupled to the ligand (Birkenmeier, 1986; Karr, 1986, 1988; Koppersläger, 1990).. The partition coefficients of the free and complexed IgG, depend on the physical properties of the ATPS and, in case of the complexed IgG, on the size and number of the PEG molecules bound to the ligand (Albertsson, 1986; Koppersläger, 1990; Karr, 1986, 1988).

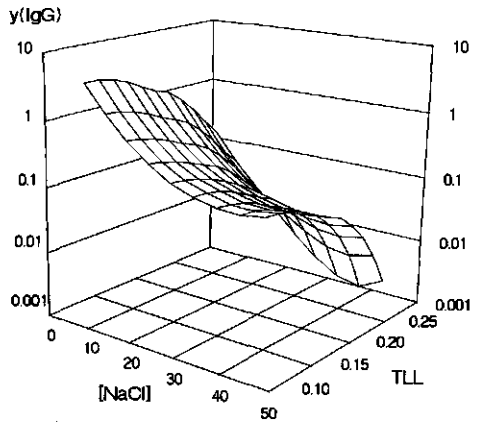

Figure 3a

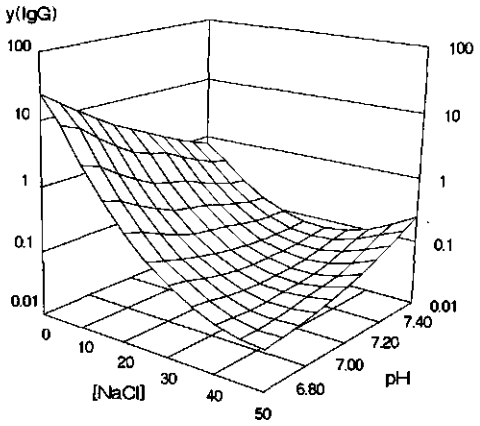

Figure 3b

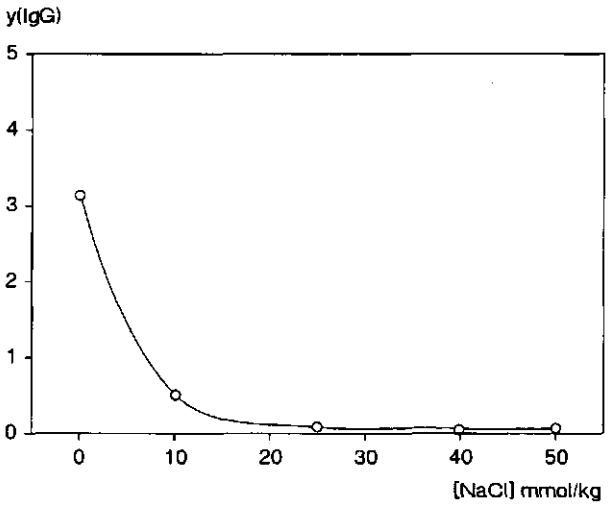

Figure 3c

Figure 3 Model predictions of BIF6A7 IgG parttioning in ATPSs with PEG 35.000, Dextran 40.000 and 1% PEG-mimetic green 1 ligand. The influence of the Na-chloride concentration ([NaCl]) and tie-line length (TLL) at pH = 7.0 (a). The influence of Na-chloride concentration and pH at TLL = 0.15 g/g (b). (Note: The Y-axes have a different scale.) The influence of [NaCl] at pH 7.0 and TLL = 0.15 g/g (c).

In a previous study using an ATPS with 1 mmol/kg Na-phosphate buffer, it was shown that the use of only 1% PEG-ligand was sufficient to increase the IgG partition coefficient from 0.02 to 25 (Zijlstra, 1996c). An increase of the PEG-dye-ligand concentration did not increase the partition coefficient any further. These data suggest that the PEG-dye-ligands affinity to the IgG is very high under the conditions used, and that full complexation occurs when using only 1 % PEG-dye-ligand. Furthermore they show that a single, large (35 kD) PEG molecule is effective in improving the IgG partition coefficient by three orders of magnitude.
In the current study, however, it was found that the IgG partition coefficient dropped one order of magnitude when going from 1 mmol/kg Na-phosphate to 10 mmol/kg K-phosphate. When the ionic strength was increased further using Na-chloride, at ± 25 mmol/kg the partition coefficient was back to the same order of magnitude as in ATPSs without PEG-dye-ligand. These data suggests that the IgG-PEG-ligand complex already dissociates at low ionic strengths.
At high ionic strengths, therefore, the IgG partition coefficient will be determined by the free IgG, which tends to concentrate in the bottom phase of PEG 35.000/dextran 40.000 ATPSs (Zijlstra, 1996a, 1996c). Oppositely, at low ionic strengths, the partition coefficient will be dominated by the IgG-PEG-ligand complex, that tends to concentrate in the top phase (Zijlstra, 1996c).
Increasing the tie-line length and thereby increasing the differences in physical properties between the phases, will normally lead to a more extreme partitioning of macro molecules (Sasakawa, 1974; Albertsson, 1986; Abbot, 1990). From figure 3a it can be seen that at high ionic strengths the (free) IgG indeed concentrates more in the bottom phase, when the TLL is increased. At low ionic strength, however, there is no increase of the IgG concentration in the top phase when the TLL is increased. On the contrary, the concentration decreases. The most likely explanation for this phenomenon is that although the buffer and salt concentrations remain the same, their activities increase at elevated polymer concentrations. This is caused by the 'excluded volume' associated with the polymers, that can 'not' be accessed by buffer salts (Albertsson, 1986). Since the ion activity is higher, a larger fraction of the IgG-PEG-ligand complex will dissociate.
Another observation done in a previous study (Zijlstra 1996c), was that the same dye-ligand coupled to sepharose beads instead of PEG 35.000 was able to bind all IgG in a 'high salt' ATPS culture medium (with 35 mmol/kg Na-bicarbonate and 60 mmol/kg Na-chloride). This can probably be explained by two phenomena. First, the close proximity of the ligands on the sepharose beads may allow multi point attachment of the IgG, which on its turn enables binding at higher ionic strengths. Secondly, the affinity of the ligand for the IgG may be greater in the absence of a large randomly coiled PEG 35.000 molecule, that shields the ligand (Karr, 1988).

IgG partitioning in ATPS culture medium with PEG-ligand

From the systematic study it has become clear that for a beneficial IgG partitioning, where the IgG concentrates in the PEG-rich top phase, a high degree of IgG-PEG-ligand complex formation is necessary. This can be achieved in three different ways:

I) By improving the affinity of the PEG-ligand for IgG by reducing the ionic strength and by reducing the pH. However, in extractive fermentations with animal cells, these parameters can only be modified to a limited extent.

ii) Another option is to improve the affinity of the PEG-ligand by reducing the size of the PEG molecule coupled to the ligand, hereby reducing the steric hindrance (Karr, 1988; Koppersläger, 1990). Reduction of the PEG molecular weight would, however, probably also decrease the partition coefficient of the complexed IgG (Karr, 1988; Koppersläger, 1990). To counteract that effect the use of multiple smaller PEG molecules might be considered. Instead of PEG, alternative 'tailing molecules' might also be considered.

iii) The most straight-forward approach to obtain a higher degree of complex formation, however, is to increase the PEG-ligand to IgG ratio by increasing the PEG-ligand concentration.

Here, the effect of increasing the PEG-ligand concentration on IgG partitioning, was studied in an ATPS culture medium in which previously cell growth was shown (Zijlstra, 1996a). The Na-chloride concentration in this ATPS was 60 mmol/kg and the Na-bicarbonate concentration was approximately 35 mmol/kg. The pH was 7.8.

In figure 4 it can be seen that, as expected, the partition coefficient increases with increasing PEG-ligand concentrations. The increase, however, was relatively small, and the final partition coefficient at 100% replacement of ordinary PEG with PEG-ligand was still less than one. Since the pH in this experiment was relatively high (7.8), the partition coefficient might be improved by lowering the pH.

Cell partitioning in ATPS culture medium with PEG-ligand

For application of the PEG-ligand in extractive fermentations with hybridoma cells, not only the effect of PEG-ligand on the IgG partitioning is important, but also the effect of the PEG-ligand on the cells. Here, using the same ATPS culture medium as in the IgG partition experiments, the effect of PEG-ligand on cell partitioning was studied.

Figure 5 shows that when no PEG-ligand is present, the cells partition predominantly to the bottom phase. When PEG-ligand was added the cell partitioning changed dramatically, and the cells partitioned almost completely into the interface.

The change in cell partitioning by the addition of PEG-ligand can either be caused by non-specific changes in the physical properties of the ATPS, or by a specific interaction of the PEG-ligand with the cells (Walter, 1982; Mattiasson, 1983;

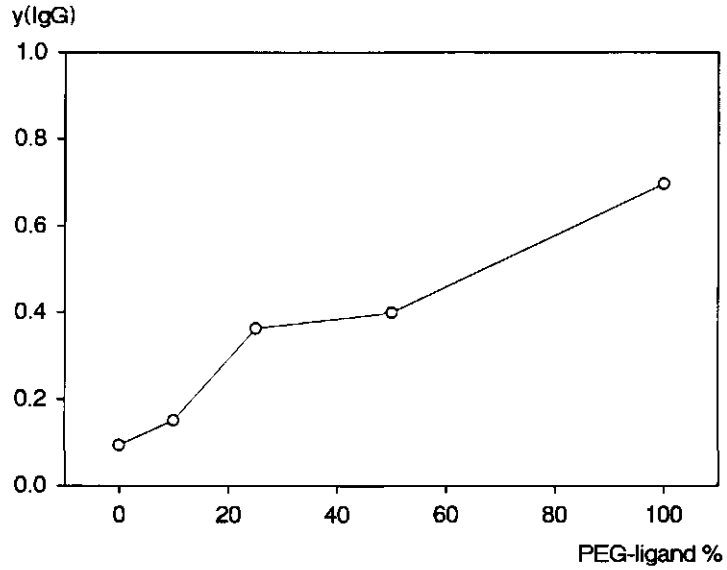

Figure 4 The effect of the PEG-ligand concentration on IgG partitioning in an ATPS with PEG 35.000, Dextran 40.000, TLL = 0.12 g/g and culture medium (containing: ± 35 mmol/kg Na-bicarbonate and 60 mmol/kg Na-chloride) at pH = 7.8.

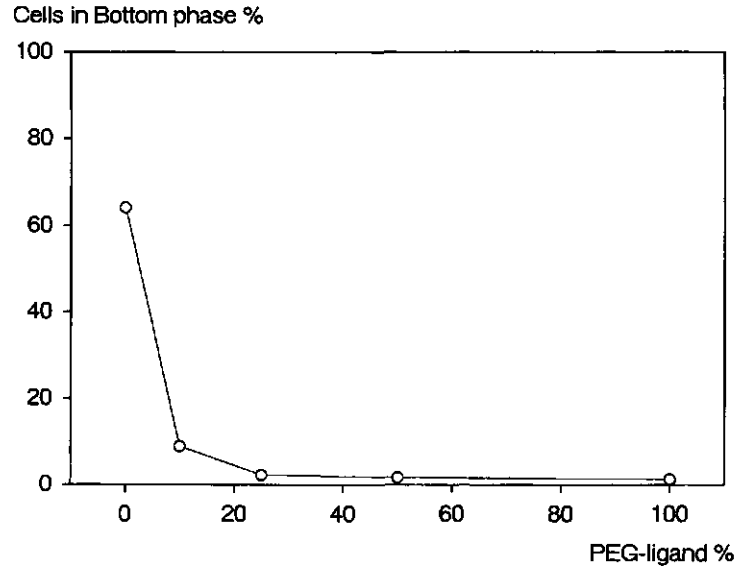

Figure 5 The effect of the PEG-ligand concentration on BIF6A7 cell partitioning in an ATPS with PEG 35.000, Dextran 40.000, TLL = 0.12 g/g, culture medium and pH = 7.8.

Albertsson, 1986; Karr, 1986; 1988). Physical properties of ATPSs that play an important role in the cell partitioning are the electrostatic-potential difference (EPD) and the interfacial tension (Walter, 1982; Albertsson, 1986) For hybridoma cells it was found that the ionic composition, causing the magnitude of the EPD, was the most important factor affecting cell partitioning (Zijlstra, 1996b). It is not unlikely that the ligand, because it is charged, and because it partitions almost exclusively to the top phase, alters the EPD.
Another matter that has to be taken into account is that hybridoma cells can display (IgG) antibodies on their cell surface (Cherlet, 1995). This could lead to a specific interaction of the PEG-ligand with the cells. Under the current circumstances, however, this seems unlikely, since the (IgG) antibodies in solution do not bind to the PEG-ligand either.
A further elucidation of the mechanisms behind this change in cell partitioning brought about by the PEG-ligand, remains necessary.

Conclusions

Objectives of the work presented here were to establish the effect of the important ATPS- and buffer parameters on IgG and hybridoma partitioning in ATPSs containing a PEG-dye-ligand. And secondly, to establish selection criteria for effective ligands for extractive fermentations with animal cells in ATPSs.
A systematic study of the IgG partitioning in the presence of 1% PEG-dye-ligand showed that the binding of IgG to the PEG-ligand was affected severely by the Na-chloride concentration. Binding of IgG to the PEG-ligand, and subsequently the desired partitioning of IgG into the top phase, was only obtained when no Na-chloride was present (additional to the 10 mmol/kg K-phosphate buffer). Possibly, the affinity of the ligand for IgG has decreased by coupling it to the large PEG 35.000 molecule.
Increasing in PEG-dye-ligand concentration up to 100% was not effective in concentrating the IgG in the top phase of ATPS culture medium (with ± 35 mmol/kg Na-bicarbonate and 60 mmol/kg Na-chloride).
Furthermore, it was demonstrated that the dye-ligand coupled to PEG affects the cell partitioning, probably by changing the electrostatic potential difference. Further research on this matter remains necessary however.
Summarizing, in order to be an effective ligand in extractive fermentations with animal-cell lines in ATPSs, ligands should be selected that bind under 'high' salt culture conditions. Furthermore, the ligands should be coupled to a 'tail' molecule that does not alter its affinity significantly, but that is able to extract the ligand-tail-protein complex into the PEG-rich top phase. To avoid changes in the cell partitioning through changes in the EPD, the net charge of the ligand-tail should be zero. Furthermore, the ligand-tail should have no specific interactions with the

cells. This might prove to be difficult if the excreted products are also displayed on the cell membrane.

Acknowledgements

This work was supported by DSM Biologics, Groningen, The Netherlands and by a grant under the Dutch PBTS-Biotechnology program, Ministry of Economic Affairs Nr. BIO89041. The authors thank E. van der Kooy and C. Schlukebir for technical assistance and G. Gort of the Mathematics Department of the Wageningen Agricultural University for the useful comments on the experimental design.

References

Abbott N, Blankschtein D & Hatton A (1990) On protein partitioning in two-phase aqueous polymer systems. Bioseparation 1:191 - 225.

Albertsson PA (1986) In: Partitioning of cell particles and macromolecules. 3rd edition. Wiley & Sons, New York

Andersson E & Hahn-Hägerdal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb. Technol. 12:242-253.

Avgerinos GC & Drapeau D (1990) Spin filter perfusion systems for high cell density culture: production of recombinant urinary type plasminogen activator in CHO cells. BIO/TECHNOLOGY. 8: 54-58

Birkenmeier G, Kopperschläger G & Johansson G (1986) Separation and studies of serum proteins with aid of aqueous two-phase systems containing dyes as affinity ligands. Biomed. Chromatogr. 1:64-77.

Box PD, Hunter WG & Hunter JS (1978) In: Statistics for experimenters: An introduction to design, data analysis and model building. John Wiley & Sons, New York.

Cherlet M, Kromenaker SJ & Srienc F (1995) Surface IgG content of murine hybridomas: Direct evidence of variation of antibody secretion rates during the cell cycle. Biotechnol. Bioeng. 47: 535-540

Forciniti D, Hall CK & Kula MR (1990) Interfacial tension of polyethylene glycol-dextran-water systems: Influence of temperature and polymer molecular weight. J. Biotechnol. 16:279-296.

Gramer MJ, Goochee CF, Chock VY, Brousseau DT & Sliwkowski MB (1995) Removal of sialic acid from glycoprotein in CHO cell culture supernatant by action of an extracellular CHO cell sialidase. BIO/TECHNOLOGY 13:692-698

Haaland PD (1989) In: Experimental design in biotechnology. Marcel Dekker Inc., New York.

Himmelfarb P & Martin HE (1969) Spin filter culture: the propagation of mammalian cells suspension. Science. 164: 555-557

Karr LJ, Shafer SG, Harris JM, Van Alstine JM & Snyder RS (1986) Immuno-affinity partition of cells in aqueous polymer two-phase systems J. Chromatogr., 354:269-282

Karr LJ, Van Alstine JM, Snyder RS, Shafer SG & Harris JM (1988) Cell separation by immunoaffinity partitioning with polyethylene glycol- modified protein A in aqueous polymer two-phase systems J. Chromatogr., 442:219-227

Koops K, Hoff HS, Weperen JJ, Das PC & Smit Sibinga CTh (1990) Immunoaffinity purification of factor VIII / Von Willebrand factor complex. In: Smit Sibinga CTh, Das PC & Mannucci PM (Eds.) Developments on hematology and immunology: Coagulation and blood transfusion. Vol. 26 (pp. 103-119) Kluwer Academic, Dordrecht, The Netherlands.

Koppersläger G & Birkenmeier G (1990) Affinity partitioning and extraction of proteins. Bioseparation 1:235-254.

Kratje RB, Lind W & Wagner R (1994) Evaluation of the proteolytic potential of *in vitro*-cultivated hybridoma and recombinant mammalian cells. J. Biotechnol. 32:107-125.

Kroner KH & Kula MR (1978) Extraction of enzymes in aqueous two-phase systems. Process Biochem. 13:7-10.

Kula MR, Kroner K & Hustedt H (1982) Purification of enzymes by liquid-liquid extraction. Advances in Biotechnological Engineering 24:73-118.

Mattiasson B (1983) Partition affinity ligand assay (PALA) for quantifying haptens, macromolecules, and whole cells. Methods in Enzymology 92: 498-522

Matiasson B (1988) Bioconversions in aqueous two-phase systems: an alterative to conventional immobilization. In: Mosbach K (ed.) Methods in enzymology: Immobilized enzymes and cells. Vol 137(D) (pp. 657-668) Academic Press Inc, San Diego.

Mohan SB, Chohan SR, Eade J & Lyddiatt A (1993) Molecular integrity of monoclonal antibodies produced by hybridoma cells in batch culture and in continuous-flow culture with integrated product recovery. Biotechnol. Bioeng. 42:974-986.

Park KM & Wang NS (1991) α-Amylase fermentation with *Bacillus amyloliquefaciens* in an aqueous two-phase system. Biotechnol. Prog. 7:439-444

Persson I, Staalbrand H, Tjerneld F & Hahn-hägerdal B (1992) Semicontinuous production of cellulolytic enzymes with *Trichoderma reesei Rutgers C30* in an aqueous two-phase system. Appl. Biochem. Biotechnol., 27(1):27-36.

Persson I, Tjerneld F & Hahn-hägerdal B (1992) Influence of cultivation conditions on the production of cellulolytic enzymes with *Trichoderma reesei Rutgers C30* in aqueous two-phase systems. Appl. Biochem. Biotechnol., 27(1):9-25.

Sasakawa S & Walter H (1974) Partition behavior of native proteins in aqueous dextran-poly(ethylene glycol) phase systems. Biochemistry 11:2760-2765.

Schmid G, Wilke CR & Blanch HW (1992) Continuous hybridoma suspension cultures with and without cell retention; kinetics of growth, metabolism and product formation. J. Biotechnol. 22: 31-40

Sharp KA (1985) In: Theoretical and experimental studies on erythrocyte partition in aqueous two-phase systems. Ph.D. thesis. University of British Columbia, Vancouver, Canada.

Van der Pol L, Bakker WAM, Tramper J (1992) Effect of low serum concentrations (0%-2.5%) on growth, production, and shear sensitivity of hybridoma cells. Biotechnol. Bioeng. 40: 179-182

Walter H (1982) Separation and subfractionation of blood cell populations based on their surface properties by partitioning in two-polymer aqueous phase systems. In: Pretlow II TG & Pretlow II ThP (eds.) Cell separation: Methods and selected applications. Vol 1 (pp. 261-299) Academic Press, New York.

Zijlstra GM, De Gooijer CD, Van der Pol LA & Tramper J (1996a) Design of aqueous two-phase systems supporting animal cell growth: A first step towards extractive bioconversions. Enz. Microb. Technol. 19: 2-8

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996b) Hybridoma and CHO cell partitioning in aqueous two-phase systems. Biotechnol. Prog. 12(3): 363-370

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996c) Separation of hybridoma cells from their IgG product using aqueous two-phase systems. Bioseparation. 6: 201-210

Chapter 6

Extractive bioconversions in aqueous two-phase systems

Summary

Although extractive bioconversions in aqueous two-phase systems (ATPSs) have been studied for over a decade, this has not resulted in widespread industrial application so far. The main reasons are the costs of the phase-forming polymers and the complexity of ATPS behaviour.
A number of recent developments may give a new impetus to this technology. First of all the use of extractive bioconversions in ATPSs has recently been extended to high value protein products, while in the meantime the development of low cost ATPSs is ongoing. Furthermore, novel analytical techniques, as well as statistical experimental designs have recently been employed for improved modelling of ATPS behaviour.

Introduction

Extractive bioconversions are one of the techniques for *in situ* product recovery (ISPR), the separation of products from their biocatalyst immediately after they are formed (Freeman, 1993). In extractive bioconversions two immiscible liquid phases are employed, the objective being to run a bioconversion in one liquid phase, while the product is extracted into the other phase. Aqueous two-phase systems are formed by mixing two aqueous polymer solutions (or a polymer and a salt solution). Above certain concentrations two immiscible aqueous phases will form, each enriched in one of the polymers (Albertsson, 1986). ATPSs are especially useful in extractive bioconversions, because they constitute a relatively mild environment for biocatalysts (Albertsson, 1986; Andersson, 1990) and they are suitable for extraction of hydrophilic products like proteins (Kroner, 1978; Kula, 1982; Hustedt, 1988).

This chapter has been published in Current Opinion in Biotechnology 1998, **9**: 171-176 by the authors: G.M. Zijlstra, C.D. de Gooijer and J. Tramper

The benefits of *in situ* product recovery techniques are to be found mainly in their potential for improving yield and productivity of existing processes. They are obligatory, however, when highly toxic or highly instable products are made (Daugulis, 1988; Daugulis, 1991; Freeman, 1993). Extractive bioconversions have the additional advantage of scaleability (Andersson, 1990; Kaul, 1991). Yield improvements may be obtained by reduction of product inhibition, through immediate product removal, or by prevention of product degradation, through minimization of the product residence time in the vicinity of the biocatalyst. ISPR techniques furthermore offer the opportunity for continuous processing with biocatalyst retention, which may greatly improve the overall volumetric productivity. Finally, through integrating part of the downstream processing with biocatalysis, the number of subsequent DSP steps may be reduced (Freeman, 1993).
The major disadvantages that have hampered widespread industrial application of extractive bioconversions with ATPSs are the costs of the polymers (Andersson, 1990; Kaul, 1991; Kuboi, 1994; Kwon, 1996; Planas, 1996), as well as the complexity of ATPS behaviour and the subsequent lack of adequate mathematical models to evaluate extractive bioconversions in ATPSs (Andersson, 1990; Freeman, 1993; Asenjo, 1994; Mistry, 1996).
Key issues in designing technically and economically feasible processes are the partitioning of biocatalysts and their products in ATPSs, the activity and stability of biocatalysts in ATPSs, the mode of operation, equipment design, process control, downstream processing and, what is most important, process economics.
In the present paper recent developments that may improve the technical and economical feasibility of extractive bioconversions with ATPSs are reviewed.

Partitioning

First objective in designing ATPSs for extractive bioconversions is to separate the product and its biocatalyst. For efficient processing the biocatalyst has to partition completely to one of the liquid phases, while the product concentrates in the other phase. In this manner i) the biocatalyst can be retained and reused by recycling the biocatalyst containing phase, ii) the product concentration in the vicinity of the biocatalyst is low, and iii) a biocatalyst-free product phase is available for further downstream processing.
Cells, like all larger particles, mainly partition between one of the liquid phases and the interface. The cell concentration in the other phase is typically several orders of magnitude less, so in essence it is free of cells (Walter, 1982; Albertsson, 1986; Andersson, 1990; Park, 1991; Chen, 1994; Kwon, 1996; Zijlstra, 1996). During continuous processing some leakage of cells into the extracting phase is less of a problem, because it is compensated for by cell growth. It may interfere, however, with the further downstream processing.

Enzymes, like all macro molecular sized particles, are distributed far more evenly between the two liquid phases than cells. During continuous processing, therefore, enzymes will be extracted rapidly with the product extracting phase and enzyme retention in the bioreactor is poor (Albertsson, 1986; Andersson, 1990). In contrast to cells, this leakage is not compensated for and therefore may be problematic. To achieve a one-sided partitioning of enzymes, whole-cell enzyme preparations (Tomaska, 1995), immobilized enzymes (Kondo, 1994) and enzymes chemically coupled to PEG (Mukataka, 1992) have been used.

Typical products of extractive bioconversions in ATPSs so far have been on one hand low molecular weight inhibitory products like lactic acid (Dissing, 1994; Kwon, 1996; Planas, 1996, 1997), sugars from particulate substrates like starch (Larsson, 1989; Tjerneld, 1991; Liakopoulou, 1996; Min, 1996), cellulose (Taguchi, 1996) or chitin (Chen, 1994, 1995), and easily degradable products like antibiotics (Chang, 1992; Paquet, 1994). On the other hand, high-molecular weight products like extracellular enzymes (Park, 1991; Persson, 1992a, 1992b) or even high-value (recombinant) proteins (Kuboi, 1995; Umakoshi, 1996; Zijlstra, 1996a, 1996c, 1998) have been produced in ATPSs. The partition coefficient of these products between the two liquid phases generally differs more from unity with increasing molecular weight (Albertsson, 1986).

Despite numerous theoretical models (Abbott, 1990), to date no practical models exist to predict the partitioning of biocatalysts and their products in a given ATPS, based on their physical and chemical properties. Moreover, the partitioning of biocatalysts, products, but also polymers may change as bioconversions progress, because biomass and metabolites can alter the composition of the aqueous phases (Park, 1991; Planas, 1997).

Two recently employed approaches may greatly assist in generating dynamic models that describe the partitioning in ATPS media for extractive bioconversions more accurately, also taking biomass and metabolite effects into account. In the first place the use of analytical tools to directly determine the composition of each phase in complex mixtures (Planas, 1997), constitutes a significant improvement compared to the traditional situation where phase diagrams in water were used to simulate those in complex mixtures. Secondly, the use of statistical experimental design (Hart, 1995, Zijlstra, 1996b, 1996c, 1998) may provide empirical partition models in which more relevant factors, like metabolite and biomass concentrations can be included, while the required number of experiments to generate data for these models is reduced to a minimum.

Because selecting ATPSs with the proper biocatalyst partitioning is the starting point in ATPS design, the opportunities for maximizing product partitioning to the opposite phase are sometimes limited. Essentially two approaches can be used for improving product partitioning in a given system. One is to add a specific extractant to the phase system, like a ligand coupled to a phase forming polymer (Koppersläger, 1990; Birkenmeier, 1991; Garg, 1996; Zijlstra, 1998). This may, how-

ever, affect the partitioning of other components, for instance cells, as well (Hamamoto, 1996; Zijlstra, 1998). Furthermore, these additives may be expensive. The other approach is to alter the product in such a way that its partitioning improves. With recombinant proteins 'tagging' is a common procedure to improve further downstream processing. This procedure has also been used in ATPSs (Köhler, 1991; Carlsson, 1996). Here the consequences for further downstream in the process have to be considered.

Activity and stability of biocatalysts

The second major objective in ATPS design is to allow biocatalysis at similar specific activities and stabilities compared to reference media.
Extractive bioconversions with enzymes have been executed so far in polymer/polymer ATPSs. The specific enzyme activities in ATPSs are generally not affected by the phase-forming polymers and are similar to those in reference media (Larsson, 1989; Andersson, 1990; Tjerneld, 1991; Tomaska, 1995, Mukataka, 1992). Reports on the effects of ATPSs on enzyme stability vary from a slight increase (Larsson, 1989; Tjerneld, 1991; Taguchi, 1996) to a slight decrease (Chen, 1995). Cell growth has been reported in ATPSs with neutral polymers, with charged polymers and even with polymers and salts. Bacterial- and fungal-cell growth appears to be hardly affected in ATPSs with moderate concentrations of neutral polymers (Andersson, 1990; Park, 1991; Persson, 1992a, 1992b; Chen, 1994; Katzbauer, 1995; Planas, 1996), unless oxygen transfer due to the increased viscosity becomes rate limiting. However, changes in morphology, like cell aggregation, but also changes in cell-surface hydrophobicity have been reported (Andersson, 1990). Charged polymers can offer the advantage of improved product partitioning as well as ease of polymer recovery. Recently the cultivation of lactic acid producing bacteria in ATPSs containing a charged polymer was described (Dissing, 1994; Kwon, 1996). The inhibitory effect of the charged polymer was largely overcome by adaptation of the cells to the ATPS culture medium, although cell aggregation was still observed. The production rates were similar to those of the control cultures.
A major breakthrough is the recently reported cultivation of several bacterial species in polymer/salt ATPSs (Kuboi, 1994, 1995). Polymer salt ATPSs can be cost effective for protein extractions (Hustedt, 1988; Kula, 1990; Kuboi, 1994). Furthermore their suitability for large scale extractions of products with a wide range of hydrophobicities has long been recognized (Hustedt, 1988; Kula, 1990). Cell growth was found to be largely unaffected by PEG when the molecular weight was 4000 kD or higher. It was inhibited exponentially, however, with increasing salt concentrations Kuboi, 1994).
Another major breakthrough is the recently reported cultivation of animal cell

lines excreting high value proteins in ATPSs (Zijlstra, 1996a). In ATPSs of PEG and dextran, hybridoma cell growth was shown over a period of months with similar growth rates to control cultures.
Finally some preliminary work on plant cell cultivation in ATPSs with neutral polymers has been reported (Buitelaar, 1992; Ilieva, 1996).
To date no models are available to describe the effect of the phase forming constituents on cell growth and production. Again, like the partition behaviour, empirical correlations need to be developed from experimental data.

Mode of operation

The mode of operation of extractive bioconversions can vary from simple batch processes, to full continuous processes with biocatalyst recycling or even polymer recycling.
Batch extractive bioconversions have been reported most frequently in recent literature (Chen, 1994, 1995; Dissing, 1994; Kondo, 1994; Kuboi, 1994; Ilieva, 1996; Kwon, 1996; Liakopoulou, 1996; Min, 1996; Zijlstra, 1996a, 1996b). Their main advantage is simplicity. Once an ATPS has been designed that provides adequate separation between the biocatalyst and its product and supports adequate activity and stability of the biocatalyst in small-scale experiments, the process can easily be scaled up in conventional stirred tanks. The product can be harvested by removing the product-containing phase by settling or centrifugation. The time required for batch process development therefore is minimal. Disadvantages are that both the product-biocatalyst interaction and the final product yield in the extracting phase depend strongly on the product partition coefficient. Furthermore, since the residence time of the product in the reaction mixture is equal to the batch cycle time, the yield of instable or easily degradable products may be compromised. Finally, especially for bioconversions with cells, the volumetric productivity of batch processes is low compared to fed-batch processes or continuous processes with cell retention.
Continuous processing with biocatalyst and polymer recycling may compensate for most of the disadvantages of batch processes. This highly integrated approach, however, inherently leads to a higher level of complexity and it poses stringent requirements on both equipment design and process control. Many reports so far therefore only describe repeated extractive batch processes, where the product extracting-phase is periodically replaced with fresh extracting phase (Tjerneld, 1991; Kuboi, 1995; Planas, 1996, 1997; Umakoshi, 1996). Also continuous processes in which the extracting phase is continuously replaced and the biocatalyst containing phase is recycled by means of an in-line settler are described (Park, 1991; Katzbauer, 1995; Tomaska, 1995; Taguchi, 1996).
In-line recycling of polymers from the product containing phase, after removal of the product, has been reported scarcely (Larsson, 1989).

Equipment design

Important issues to take into account during the equipment design for extractive bioconversions are i) the mass transfer in each phase, as well as ii) the mass transfer between the phases, iii) the phase separation, iv) the separation of the product from the extracting phase and v) the recycling of polymers.
Stirred tanks, operated at moderate stirring speeds, generally have been found to provide adequate mass transfer for extractive bioconversions in ATPSs. Because of the low interfacial tensions in ATPSs, emulsions with high interfacial areas, and thus with high mass transfer rates, will already occur at low power input (Andersson, 1990; Huddleston, 1990; Joshi, 1990; Kaul, 1991). To separate the ATPS emulsions, however, long settling times are required. In batch extractions phase separation can be obtained by settling in the stirred tank itself. For continuous extractive bioconversions with biocatalyst recycling, however, dedicated settlers are required. Several settler designs have been described in literature, however, none of them seems to be very effective at high flow rates (Park, 1991; Katzbauer, 1995; Tomaska, 1995; Taguchi, 1996). Moreover, the residence time in these settlers may cause oxygen depletion when growing cells are used (Park, 1991; Katzbauer, 1995) and when cells partition to the interface, they can accumulate in the settler and compromise the effectiveness of the phase separation (Katzbauer, 1995). Complete cell partitioning to one of the liquid phases therefore is of paramount importance.
Although for large-scale ATPSs extractions the use of continuous disk-stack centrifuges has been reported (Hustedt, 1988), and meanwhile specific continuous centrifuges for cell recycling have become available on the market, so far their use in extractive bioconversions with ATPSs has not been reported.
To avoid phase-separation difficulties and moreover to obtain multistage extraction of the product, the opportunities for counter-current extraction using spray- and other types of column extractors have been investigated (Joshi, 1990). For polymer/polymer ATPSs, however, the mass-transfer coefficients were extremely low. Furthermore, to avoid flooding, the residence time in these extractors has to be long, which practically excludes their use for extractive bioconversions with cells.

Process control

In batch processes only some basic process parameters in the stirred tank need to be controlled. Typically the temperature, pH, stirrer speed, and in case of cells, the dissolved oxygen concentration, are important.
In continuous processes, beside the process parameters of the stirred tank, also the parameters of the phase separator, as well as the parameters of incoming and outgoing fluxes need to be controlled.
For effective continuous extractive bioconversions the aim is to obtain and main-

tain a steady-state situation, under optimal process conditions, usually after an initial dynamic phase, where for instance the biomass increases. Recent studies have shown, however, that shifts in the ATPS composition can occur during extractive bioconversions, that may finally lead to a system breakdown (Katzbauer, 1995; Planas, 1997). Controlling the ATPS composition, therefore, is vital in continuous extractive bioconversions. Novel on-line analytical methods may be used for this purpose (Planas, 1997). Furthermore, process models consisting of mass balances of all phase-system constituents, models describing the partitioning, as well as models describing the bioconversion in ATPSs, seem to be necessary for process control, especially during the dynamic phase (Jarzebski, 1992; Baughman, 1994).

Downstream processing

Depending on the product type and the required purity, one or a combination of conventional DSP techniques like extraction, evaporation, filtration, ultrafiltration, chromatography etc. can be used for further purification.
The first purification step generally includes separation of the polymers from the product. Recently new polymer types have been employed that form a precipitate (Kamihara, 1992) or an immiscible liquid phase highly concentrated in the polymer (Farkas, 1996; Kwon, 1996; Lu, 1996; Planas, 1996), after a shift in pH or temperature. The polymers can be recovered or even recycled after phase separation, while the polymer-lean-product phase is available for further downstream processing.

Process economics

Obviously, for the breakthrough of extractive bioconversions with ATPSs it is essential that the costs of implementation are favourable compared to the revenues of the product, and that the extractive bioconversion is more cost effective than conventional processing.
Previous economical evaluations have shown that the polymer costs largely determine the process economics. Especially fractionated dextran is too expensive for industrial application in extractive bioconversions (Datar, 1986; Andersson, 1990; Huddleston, 1990; Kaul, 1991). To tackle this problem several approaches have been employed. In the first place low-cost alternatives to dextran are being developed, mainly based on starch derivatives (Tjerneld, 1986; Szlag, 1990; Kwon, 1996). Secondly, the possibilities for using low cost PEG/salt systems for extractive bioconversions are being explored (Kuboi, 1994, 1995) and polymer recycling schemes are being developed (Larsson, 1989; Farkas, 1996; Kwon, 1996; Lu, 1996; Planas, 1996). Finally, the opportunities for using extractive bioconversions in

high-value-product manufacturing are being explored (Kuboi, 1995; Umakoshi, 1996; Zijlstra, 1996a, 1996b, 1996c, 1998). Also, scale appears to be crucial for the economically effective implementation of extractions (Asenjo, 1994).
Once the knowledge of extractive bioconversions has increased to a level where rational process design can take place and in the meantime low-cost ATPSs have become available, extractive bioconversions with ATPSs may become an important tool for the large-scale manufacturing of biologicals.

Conclusions

Extractive bioconversions with ATPSs offer a number of unique features which make them exceptionally useful for large-scale manufacturing of biologicals. The current knowledge of this technology, however, is not sufficient for rational process design. Moreover, the costs associated with implementing extractive bioconversions are still too high.
The continuing developments in the field of aqueous two-phase systems, in combination with important recent findings, may greatly improve the practical applicability of extractive bioconversions with ATPSs in the near future.

Acknowledgements

This work was supported by DSM Biologics, Groningen, The Netherlands and by a grant under the Dutch PBTS-Biotechnology program, Ministry of Economic Affairs Nr. BIO89041.

References

Abbott N, Blankschtein D & Hatton A (1990) On protein partitioning in two-phase aqueous polymer systems. Bioseparation 1: 191-225.

Albertsson PA (1986) In: Partitioning of cell particles and macromolecules, edn.3. New York: Wiley & Sons.

Andersson E & Hahn-Hägerdal B (1990) Bioconversions in aqueous two-phase systems. Enzyme Microb. Technol. 12: 242-253.

Asenjo JA (1994) Industrial prospects of aqueous two-phase processes. J. Chem. Technol. Biotechnol.59: 109

Baughman DR & Liu YA (1994) An expert network for predictive modeling and optimal design of extractive bioseparations in aqueous two-phase systems. Ind.Eng.Chem.Res. 33(11): 2668-2687.

Birkenmeier G, Vijayalakshmi MA, Stigbrand T & Kopperschläger G (1991) Immobilized metal ion affinity partitioning, a method combining metal- protein interaction and partitioning of proteins in aqueous two-phase systems. J. Chromatogr. 539: 267-277.

Buitelaar RM, Leenen EJTM & Tramper J (1992) Growth and secondary metabolite production by hairy roots of *tagetes patula* in aqueous two-phase systems. Biocatalysis 6: 73-80.

Carlsson M, Berggren K, Linse P, Veide A & Tjerneld F (1996) Effects of fused tryptophanrich peptides to a recombinant protein A domain on the partitioning in polyethylene glycol/dextran aqueous two-phase systems. J. Chromatogr. A. 756: 107-117.

Chang HN, Lee YH & Lee CY (1992) Continuous production of 6-APA in an aqueous two-phase system. Ann. N.Y. Acad. Sci. 672: 643-648.

Chen JP & Lee MS (1994) Simultaneous production and partition of chitinase during growth of Serratia marcescens in an aqueous two-phase system. Biotechnol. Tech. 8: 783-788.

Chen JP & Lee MS (1995) Enhanced production of Serratia marcescens chitinase in PEG/dextran aqueous two-phase systems. Enz. Microb. Technol. 17: 1021-1027.

Datar R (1986) Economics of primary separation steps in relation to fermentation and genetic engineering. Proc. Biochem. Feb: 19-26.

Daugulis AJ (1988) Integrated reaction and product recovery in bioreactor systems. Biotechnol. Prog. 4: 113-122.

Daugulis AJ (1991) Integrated fermentation and recovery processes. Curr. Opin. Biotechnol. 5: 192-195.

Dissing U & Mattiasson B: (1994) Cultivation of Lactococcus lactis in a polyelectrolyte-neutral polymer aqueous two-phase system. Biotechnol. Lett. 16: 333-338

Farkas T, Stalbrand H & Tjerneld F (1996) Partitioning of beta-mannanase and alpha-galactosidase from *Aspergillus Niger* in Ucon/Reppal aqueous two-phase systems and using temperature induced phase separation. Bioseparation 6: 147-157.

Freeman A, Woodley JM & Lilly MD (1993) *In situ* product removal as a tool for bioprocessing. *BIO/TECHNOLOGY*. 11: 1007-1012

Garg N, Galaev Iyu & Mattiasson B (1996) Dye-affinity techniques for bioprocessing: recent developments. J. Mol. Recognit. 9: 259-274.

Hamamoto R, Kamihira M & Iijima S (1996) Specific separation of animal cells using aqueous two-phase systems. J. Ferm. Bioeng. 82: 73-76.

Hart RA, Ogez JR & Builder SE (1995) Use of multifactorial analysis to develop aqueous two-phase systems for isolation of non-native IgF1. Bioseparation 5: 113-121.

Huddleston JG & Lyddiatt A (1990) Aqueous two-phase systems in biochemical recovery. Appl. Biochem. Biotechnol. 26: 249-280.

Hustedt H, Kroner KH & Papamichael N (1988) Continuous cross-current aqueous two-phase extraction of enzymes from biomass: automated recovery in production scale. Proc. Biochem. 23: 129-137.

Ilieva MP, Bakalova A, Mihneva M, Pavlov A & Dolapchiev L (1996) Production of phosphomonoesterases by Nicotiana tabacum 1507 in an aqueous two-phase system. Biotechnol. Bioeng. 51: 488-493.

Jarzebski AB & Malinowski JJ (1992) Analysis of continuous fermentation processes in aqueous two-phase systems. Bioproc. Eng. 7: 315-317.

Joshi JB, Sawant SB, Rao KSMSR, Patil TA, Rostami KM & Sikdar SK (1990) Continuous counter-current two-phase aqueous extraction. Bioseparation 1: 311-324.

Kamihara M, Kaul R & Mattiasson B (1992) Purification of recombinant protein A by aqueous two-phase extraction integrated with affinity precipitation. Biotechnol. Bioeng. 40: 1381-1387.

Katzbauer B, Cesi V, Narodoslawsky M & Moser A (1995) Extractive lactic acid fermentation using aqueous two-phase systems. Chem. Biochem. Eng. 9: 79-87. Kaul R & Mattiasson B (1991) Extractive bioconversions in aqueous two-phase systems. In: Extractive bioconversions. Mattiasson B & Holst, O (eds.) Bioprocess Technology 11: 173-188.

Köhler K, Ljungquist C, Kondo A, Veide A & Nilsson B (1991) Engineering proteins to enhance their partition coefficients in aqueous two-phase systems. BIO/TECHNOLOGY. 9: 642-646.

Kondo A, Urabe T & Higashitani K (1994) Bioconversions in an aqueous two-phase system using enzymes immobilized on ultrafine silica particles. J.Ferm. Bioeng. 6: 700-703.

Koppersläger G & Birkenmeier G (1990) Affinity partitioning and extraction of proteins. Bioseparation 1: 235-254.

Kroner K & Kula MR (1978) Extraction of enzymes in aqueous two-phase systems. Proc. Biochem. 13: 7-10.

Kuboi R, Maruki T, Tanaka H & Komasawa I (1994) Fermentation of Bacillus subtilis ATCC 6633 and Production of Subtilin in Polyethylene Glycol/Phosphate Aqueous Two-Phase Systems. J. Ferm. Bioeng. 78: 431-436.

Kuboi R, Umakoshi H & Komasawa I (1995) Extractive cultivation of *Escherichia coli* using poly(ethylene glycol)/phosphate aqueous two-phase systems to produce intracellular ß-galactosidase. Biotechnol. Prog. 11: 202-207.

Kula MR, Kroner K & Hustedt H (1982) Purification of enzymes by liquid-liquid extraction. Adv. Biotechnol. Eng. 24: 73-118.

Kula MR (1990) Trends and future prospects of aqueous two-phase extraction. Bioseparation 1: 181-189.

Kwon YJ, Kaul R & Mattiasson B (1996) Extractive lactic acid fermentation in poly(ethyleneimine)-based aqueous two-phase system. Biotechnol. Bioeng. 50: 280-290.

Larsson M, Arasaratnam V & Mattiasson B (1989) Integration of bioconversion and downstream processing: starch hydrolysis in an aqueous two-phase system. Biotechnol. Bioeng. 33: 758-766.

Liakopoulou-Kyriakides M, Karakatsanis A & Stamatoudis M (1996) Enzymic hydrolysis of starch in agitated PEG-dextran aqueous two-phase system. Starch. 48: 291-294.

Lu M, Albertsson PA, Johansson G & Tjerneld F (1996) Ucon-benzoyl dextran aqueous two-phase systems: protein purification with phase component recycling. J.Chromatogr. B Biomed. Appl. 680: 65-70.

Min KH, Chang WJ & Koo YM (1996) Increased production of beta-cyclodextrin using an aqueous two-phase system. Biotechnol. Tech. 10: 395-400

Mistry SL, Kaul A, Merchuk JC & Asenjo JA (1996) Mathematical modeling and computer simulation of aqueous two-phase continuous protein extraction J.Chromatogr. 741: 151-63

Mukataka S, Haynes CA, Prausnitz JM & Blanch HW (1992) Extractive bioconversions in aqueous two-phase systems: enzymatic hydrolysis of casein proteins. Biotechnol. Bioeng. 40: 195-206.

Paquet V, Myint M, Roque C & Soucialle P(1994) Partitioning of pristinamycins in aqueous two-phase systems: a first step toward the development of antibiotic production by extractive fermentation. Biotechnol. Bioeng. 44: 445-451.

Park KM & Wang NS (1991) α-Amylase fermentation with *Bacillus amyloliquefaciens* in an aqueous two-phase system. Biotechnol. Prog. 7: 439-444.

Persson I, Staalbrand H, Tjerneld F & Hahn-hägerdal B (1992a) Semicontinuous production of cellulolytic enzymes with *Trichoderma reesei Rutgers C30* in an aqueous two-phase system. Appl. Biochem. Biotechnol. 27: 27-36.

Persson I, Tjerneld F & Hahn-haegerdal B (1992b) Influence of cultivation conditions on the production of cellulolytic enzymes with *Trichoderma reesei Rutgers C30* in aqueous two-phase systems. Appl. Biochem. Biotechnol. 27: 9-25.

Planas J, Radstrom P, Tjerneld F & Hahn-Hagerdal B (1996) Enhanced production of lactic acid through the use of a novel aqueous two-phase system as an extractive fermentation system. Appl. Microbiol. Biotechnol. 45: 737-743.

Planas J, Lefebvre D, Tjerneld F & Hahn-Hägerdahl B (1997) Analysis of phase composition in aqueous two-phase systems using a two-column chromatographic method: application to lactic acid production by extractive fermentation. Biotechnol. Bioeng. 54: 303-311.

Ramadas M, Holst O & Mattiasson B (1996) Production of amyloglucosidase by Aspergillus niger under different cultivation regimens. World J. Microbiol. Biotechnol. 12: 267-271.

Szlag DC, Guiliano KA & Snyder SM (1990) A low cost aqueous tow-phase system for affinity extraction. In: Bioseparations. Downstream processing in biotechnology. Hamel JF, Hunter JB & Sikdar SK (eds.) ACS Symposium series. 419: 71-86.

Taguchi F, Yamada K, Hasegawa K, Taki-Saito T & Hara K (1996) Continuous hydrogen production by Clostridium sp. strain no. 2 from cellulose hydrolysate in an aqueous two-phase system. J. Ferm. Bioeng. 82: 80-83.

Tjerneld F, Berner S, Cajarville A & Johansson G (1986) New aqueous two-phase systems based on hydroxypropyl starch useful in enzyme extraction. Enz. Micribiol. Technol. 8: 417-423.

Tjerneld F, Persson I & Lee JM (1991) Enzymatic cellulose hydrolysis in an attrition bioreactor combined with an aqueous two-phase system. Biotechnol. Bioeng. 37: 876-882.

Tomaska M, Stredansky M, Tomaskova A & Sturdik E (1995) Lactose hydrolysis in aqueous two-phase system by whole-cell beta-galactosidase of Kluyveromyces marxianus. Bioproc. Eng. 12: 17-20.

Umakoshi H, Yano K, Kuboi R & Komasawa I (1996) Extractive cultivation of recombinant Escherichia coli using aqueous two-phase systems for production and separation of intracellular heat shock proteins. Biotechnol. Prog. 12: 51-56

Walter H (1982) Separation and subfractionation of blood cell populations based on their surface properties by partitioning in two-polymer aqueous phase systems. In: Cell separation: Methods and selected applications. Pretlow II TG & Pretlow II ThP (eds.) Academic Press, New York, 1: 261-299.

Zijlstra GM, De Gooijer CD, Van der Pol LA & Tramper J (1996a) Design of aqueous two-phase systems supporting animal cell growth: A first step towards extractive bioconversions. Enz. Microb. Technol. 19: 2-8.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996b) Hybridoma and CHO cell partitioning in aqueous two-phase systems. Biotechnol. Prog. 12: 363-370.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996c) Separation of hybridoma cells from their IgG product using aqueous two-phase systems. Bioseparation. 6: 201-210.

Zijlstra GM, Michielsen MJF, De Gooijer CD & Tramper, J (1998) IgG and hybridoma partitioning in aqueous two-phase systems containing a dye-ligand. Bioseparation. Accepted for publication.

Summary

In current industrial biotechnology, animal-cell culture is an important source of therapeutic protein products. The conventional animal-cell production processes, however, include many unit operations as part of the fermentation and downstream processing strategy.

The research described in this thesis focuses on exploring the opportunities for physical integration of fermentation and part of the downstream processing by means of extractive fermentations in Aqueous Two-Phase Systems (ATPSs). This *in situ* recovery concept, where the product is extracted from the bioreactor during fermentation, combines fermentation, primary recovery and part of the purification in a single step. The subsequent reduction of process steps and of processing time may provide an opportunity to improve the process economy or even the product quality. ATPSs are thought to be appropriate, because they form a relatively mild environment to cells and they are suitable for protein extractions. ATPSs can be formed by dissolving two polymers, e.g. PEG and dextran in an aqueous solution. Above certain concentrations two aqueous phases will form, each enriched in one of the polymers.

In chapter 2 of this thesis, first the influence of the individual ATPS-forming polymers PEG and dextran on the relevant physical culture medium parameters and on the growth of a model (hybridoma) cell line were characterized. It was found that the polymers raised the osmotic pressure. This could be compensated for, however, by reducing the Na-chloride concentration of the culture medium. Subsequently, it was found that cell growth was possible in culture media containing up to 0.025 g/g of PEG or up to 0.15 g/g of dextran. Using these findings, ATPSs of PEG and dextran were selected. In ATPSs of PEG 35.000, dextran 40.000 and culture medium, the hybridoma cells partitioned to the dextran-rich phase and could be cultured over prolonged periods of time. The IgG product, however, partitioned along with the cells in the lower phase.

In chapter 3, first the partitioning of the model (mouse/mouse) hybridoma cell line, was investigated systematically using a statistical experimental design. It was found that the ionic composition had the largest effect on cell partitioning. Only at a low phosphate- to chloride-ion ratio cells partitioned into the dextran-rich phase, otherwise they were present in the interface. The cell partitioning could be optimized by choosing a low dextran Mw (40 kD), a high PEG Mw (35 kD) and a low tie-line length (10 g/g). In the second place, the cell partitioning and cell growth of other cell lines (a mouse/rat hybridoma and a CHO cell line) were studied in the ATPS culture media described in chapter 2. It was found that both cell lines, partitioned almost completely into the lower phase. Moreover the hybridoma cell line was able to grow well in the ATPS (hybridoma) culture medium. This medium therefore appears to be suitable for extractive bioconversions with a wide range of hybridoma cells.

In chapter 4, first the partitioning of product (IgG) was studied systematically. In this study the same variables were used as in the cell partition study. In all of the ATPSs the IgG partitioned predominantly into the lower phase. The partition coefficient varied between 0.78 and 0.0002, in none of the ATPSs IgG concentrated in the top-phase. The tie-line length, the dextran molecular weight and the PEG molecular weight had the most pronounced effect on IgG partitioning. In non of the ATPSs tested good separation between the cells and their product was achieved. In the second place, therefore, the opportunities to manipulate the product partitioning with a ligand coupled to PEG were explored. A number of dye-resins was screened for their ability to bind the IgG antibody. The mimetic green 1 A6XL dye-resin was found to bind IgG. The dye-ligand coupled to PEG improved the IgG-partition coefficient by three orders of magnitude (in the presence of a low-salt buffer and at 1% PEG-ligand), resulting in a partition coefficient of 25. In chapter 5, the effect of the PEG-dye-ligand on both IgG and hybridoma partitioning was characterized more extensively. It was found that the binding of IgG to the PEG-ligand was affected severely by the Na-chloride concentration. The tie-line length and pH affected IgG partitioning to a lesser extent. The desired partitioning of IgG into the top phase (with 1% PEG replaced by PEG-ligand), was only obtained when, in addition to the K-phosphate buffer, no Na-chloride was present. In ATPS culture medium increasing the percentage of PEG replaced by PEG-ligand up to 100%, did increase the IgG partition coefficient up to 0.7, but was not effective in concentrating the IgG in the top phase. Moreover, addition of the PEG-ligand to ATPS culture medium changed the hybridoma cell partitioning from the bottom phase to the interface.

This thesis has shown the feasibility of animal cell cultivation in ATPSs and it has shown the usefulness of statistical experimental design in characterizing partitioning in ATPSs. Furthermore it has pointed out the pitfalls and possibilities for the separation of animal cells from their protein products. Therefore, it presents an important step towards proof of principle for the technical feasibility of extractive fermentations with animal cells in ATPSs. Further research remains necessary, however, to amongst others improve the separation between hybridoma cells and their IgG product, for designing and implementing fermentation processes, for scale up and for establishing the economical feasibility.
In chapter 6, the recent developments in the field of extractive bioconversions in ATPSs in general are reviewed. A number of recent developments may give a new impetus to this technology and lead to a more widespread use in industry. First of all the use of extractive bioconversions in ATPSs for high value protein products, in combination with the ongoing development of low cost ATPSs, is promising . Furthermore, the application of novel analytical techniques, in combination with statistical experimental designs may lead to improved design of and control over extractive bioconversions in ATPSs.

Samenvatting

In de hedendaagse biotechnologie is de dierlijke celkweek een belangrijke bron van therapeutische eiwitten. De huidige, conventionele productieprocessen met dierlijke cellen omvatten echter vrij veel processtappen. Het onderzoek dat in dit proefschrift wordt beschreven richt zich op het onderzoeken van de mogelijkheden tot integratie van de fermentatie en een deel van de product opwerking in één stap door middel van extractieve fermentaties met waterige twee-fasen systemen (ATPSs). In dit concept wordt het product tijdens de fermentatie uit de bioreactor geëxtraheerd met een tweede, vloeibare fase, terwijl de cellen in de bioreactor aanwezig blijven. Op die manier worden de fermentatie, de celverwijdering en een deel van de zuivering gecombineerd. Als gevolg hiervan neemt het aantal processtappen af en ook de procestijd, en dat zou kunnen leiden tot een kostenreductie en mogelijk tot een verbeterde productkwaliteit. ATPSs worden geschikte tweefasen systemen geacht, omdat ze een milde omgeving voor de cellen vormen en bovendien geschikt zijn voor de extractie van eiwitten. ATPSs kunnen worden gemaakt door twee polymeren, bijvoorbeeld PEG en dextraan, in water op te lossen. Boven bepaalde concentraties vormen zich dan twee, niet mengbare waterige fasen, ieder verrijkt in één van de polymeren.

In hoofdstuk 2 van dit proefschrift, werd eerst de invloed van de individuele ATPS-vormende polymeren op de relevante fysische kweekmedium parameters en op de groei van een model hybridoma cellijn onderzocht. Gevonden werd dat de polymeren de osmotische druk vergroten, maar dat dit effect gecompenseerd kan worden door de zout concentratie van het kweekmedium te verlagen. Vervolgens werd gevonden dat celgroei mogelijk is in kweekmedium met tot 25 g/kg PEG of 150 g/kg dextraan. Met deze gegevens werden vervolgens ATPSs van PEG en dextraan geselecteerd. In ATPSs van PEG 35.000 en dextraan 40.000 werd gevonden dat de hybridoma cellen in de onderste fase verdelen en dat ze daarin gedurende lange tijd gekweekt kunnen worden. Het product echter bevond zich in dezelfde fase als de cellen.

In hoofdstuk 3 werd eerst de verdeling van de model (muis/muis) hybridoma cellijn systematisch onderzocht, met behulp van een statistisch ontworpen proefopzet. Gevonden werd dat de ion samenstelling het grootste effect op de celverdeling heeft. Uitsluitend in geval van een lage phosphaat / chloride ion verhouding kunnen de cellen in de onderste (dextraan-rijke) fase verdelen, anders gaan ze in het grensvlak zitten. De celverdeling kon worden geoptimaliseerd door een laag dextraan molecuulgewicht (40 kD), een hoog PEG molecuulgewicht (35 kD) en kort nodelijn (10 g/g) te kiezen. In de tweede plaats werd de verdeling en de groei van twee andere cellijnen (een muis/rat hybridoma en een CHO cellijn) onderzocht in het eerder beschreven ATPS kweekmedium. Gevonden werd dat beide cellijnen bijna volledig in de onderste fase verdelen en verder dat de hybridoma cellijn goed

in staat is te groeien in het ATPS kweekmedium. Dit medium lijkt daarom voor een grote groep hybridomas geschikt.
In hoofdstuk 4, werd eerst de verdeling van het product (IgG) systematisch onderzocht op basis van dezelfde proefopzet als voor de celverdeling. Gevonden werd dat IgG in alle onderzochte ATPSs in de onderste fase verdeelt. De verdelingscoefficient varieerde tussen 0.78 en 0.0002. De nodelijn lengte, het dextraan- en het PEG molecuulgewicht beinvloeden de IgG verdeling het meest. In geen van de geteste ATPSs werd een goede scheiding tussen de cellen en het product bereikt. Daarom werd in de tweede plaats, de mogelijkheid onderzocht om de productverdeling te manipuleren met een aan PEG gekoppeld affiniteitsligand. Een aantal kleurstofliganden gekoppeld aan kolomchromatografiematrix werd getest op binding met het IgG product. De mimetic groen 1 A6XL kleurstof vertoonde binding. Aan PEG gekoppeld, verbeterde de kleurstof ligand de IgG verdelingscoefficient met drie ordes van grootte (in aanwezigheid van een laag-zout buffer en bij 1% PEG-ligand), waardoor de uiteindelijke verdelingscoefficient uitkwam op 25.
In hoofdstuk 5, werd het effect van de PEG-ligand op zowel IgG als hybridoma verdeling nader onderzocht. Gevonden werd dat de binding van IgG aan het PEG-ligand uiterst gevoelig is voor zout. De nodelijn lengte en de pH beinvloeden de IgG verdeling in mindere mate. De gewenste verdeling van IgG in de bovenste (PEG-rijke) fase werd alleen bereikt (met 1% PEG-ligand), als naast de 10 mM K-phosphaat geen extra zout aanwezig was. In ATPS kweekmedium was door het verhogen van de PEG-ligand concentratie naar 100% de verdelingscoefficient nog wel naar 0.7 omhoog te brengen, maar er werd geen ophoping van het product in de bovenste fase bereikt. Een groter probleem was dat door toevoeging van het PEG-ligand de hybridoma celverdeling volledig van de onderste fase naar het grensvlak verschoof.

Dit proefschrift heeft de haalbaarheid van dierlijke celkweek in waterige tweefasen systemen aangetoond en heeft het nut van statistisch ontworpen proefopzetten voor het vastleggen van de verdeling in ATPSs laten zien. Verder heeft het de mogelijkheden en onmogelijkheden ten aanzien van het scheiden van dierlijke cellen van hun product met behulp van ATPSs duidelijk gemaakt. Daarom vertegenwoordigd het een belangrijke stap in het aantonen van de technische haalbaarheid van extractieve fermentaties met dierlijke cellen in ATPSs. Verder onderzoek blijft echter nodig om ondermeer de scheiding tussen cel en product te verbeteren, om fermentatie processen te ontwerpen en te implementeren, om op te schalen en tenslotte de economische haalbaarheid aan te tonen.

In hoofdstuk 6 zijn de recente ontwikkelingen op het gebied van extractieve bioconversies in ATPSs in het algemeen uiteen gezet. Een aantal van die ontwikkelingen zou voor een nieuwe impuls kunnen zorgen en de toepasbaarheid in de industrie kunnen vergroten. Ten eerste vertegenwoordigd de toepassing van extractieve

bioconversies voor producten met een hoge toegevoegde waarde, in combinatie met de voortschrijdende ontwikkelingen op het gebied van goedkopere ATPSs, een belofte voor de toekomst. Verder vormt de toepassing van nieuwe analytische technieken gekoppeld met het gebruik van statistisch ontworpen proefopzetten, een sterke combinatie om tot verbetering in het ontwerp van en de controle over extractieve bioconversies in ATPSs te komen.

Bibliography

Publications

Van der Pol LA, Zijlstra GM, Thalen M, & Tramper J (1990) Effect of serum concentration on production of monoclonal antibodies and on shear sensitivity of a hybridoma. Bioproc. Engin. 5: 241-245.

Zijlstra GM, Van der Pol LA, De Gooijer CD & Tramper J (1994) Integration of fermentation and downstream processing: Extractive bioconversions with hybridoma cells in aqueous two-phase systems. In: Proceedings of the Cell Culture Engineering IV conference, 1994, 8-9, Engineering Foundation, New York, USA.

Zijlstra GM, Van der Pol LA, De Gooijer CD & Tramper J (1994) Extractive bioconversions with hybridoma cells in aqueous two-phase systems. In: Abstract boo of the 207th ACS National Meeting, March 13-17, 1994. ACS, Washington, USA.

Zijlstra GM, De Gooijer CD, Van der Pol LA & Tramper J (1996) Design of aqueous two-phase systems supporting animal cell growth: A first step towards extractive bioconversions. Enz. Microb. Technol. 19: 2-8.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA &Tramper J (1996) Hybridoma and CHO cell partitioning in aqueous two-phase systems. Biotechnol. Prog. 12: 363-370.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper J (1996) Separation of hybridoma cells from their IgG product using aqueous two-phase systems. Bioseparation. 6: 201-210.

Zijlstra GM, Michielsen MJF, De Gooijer CD, Van der Pol LA & Tramper, J (1998) IgG and hybridoma partitioning in aqueous two-phase systems containing a dye-ligand. Bioseparation. 7:117-126.

Zijlstra GM, De Gooijer CD & Tramper J (1998) Extractive bioconversions in aqueous two-phase systems. Curr. Opin. Biotechnol. 9: 171-176.

Patent

Tramper J & Zijlstra GM (1993) Procedure for the cultivation of animal cells (Werkwijze voor het kweken van dierlijke cellen). Dutch Patent Application No. NL 93.01447.

Curriculum Vitae

Gerben Meile Zijlstra werd op 7 September 1965 geboren in Reduzum, Friesland. Na afronding van het Stedelijk Gymnasium (β-richting) te Leeuwarden begon hij in 1983 met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. Deze studie werd in 1989 afgerond, waarbij afstudeervakken gedaan werden in de Moleculaire Biologie bij de groep van dr. T. Bisseling, in de Proceskunde bij de groep van dr. C.D. de Gooijer en in de Biochemie bij de groepen van dr. R. Hilhorst en dr. I. Rietjens. Het afstudeervak Proceskunde werd uitgevoerd bij Bio-Intermediair in Groningen onder leiding van dr. L. van der Pol. Tevens werd een stage Industriele Microbiologie gedaan bij DSM Andeno in Venlo bij dr. V. Elferink..

In de periode 1990 - 1994 was Gerben werkzaam als assistent in opleiding bij de Bioprocesstechnologie groep van de sectie Proceskunde van Prof. J. Tramper. Het onderzoek werd uitgevoerd bij Bio-Intermediair in Groningen en de resultaten van deze studie staan beschreven in dit proefschrift.

Van 1994 tot heden was Gerben als Cell Culture Scientist en later Senior Scientist bij Bio-Intermediair in Groningen, betrokken bij de ontwikkeling van productie processen voor hoogwaardige eiwitten met behulp van bacteriële, insecten en dierlijke cellen.