UPTEC X 06 032
AUG 2006

ISSN 1401-2138

ERIK SVENSSON

Improvement of a CHO cell process by feeding peptones

Master's degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 06 032	**Date of issue 2006-08**

Author

Erik Svensson

Title (English)

Improvement of a CHO cell process by feeding peptones

Title (Swedish)

Abstract

Peptones are undefined hydrolysates of proteins. Using peptones derived from plants instead of animal derived serum to supplement mammalian cell culture media would eliminate the risk of virus, mycoplasma or prion contamination of the biopharmaceutical product. The use of plant peptones in a CHO fed-batch process was developed by studying the dose and timing of the peptone feeding using Biovitrum´s proprietary protein free medium. Different combinations of peptone cocktail and amino acids were screened in 50 ml filter tubes and spinners and the best combination was assessed in 3 L bioreactor scale. It was found that feeding the peptone cocktail significantly improved the cell growth, process longevity and antibody productivity. The beneficial effects of the peptones could not be reproduced by amino acid supplementation. Further, it was found that overfeeding the amino acids is toxic to the cells and the peptones can reduce the toxic effect of amino acid overfeeding.

Keywords

Peptones, CHO cells, fed-batch process, amino acids, mammalian cell cultivation

Supervisors

Yun Jiang, Ph.D.

Biopharmaceuticals Process Development, Biovitrum AB, Stockholm

Scientific reviewer

Prof. Lena Häggström

Dept. of Bioprocess Technology, Royal Institute of Technology, Stockholm

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **55**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala

Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Improvement of a CHO cell process by feeding peptones

Erik Svensson

Populärvetenskaplig sammanfattning

Efterfrågan på proteinläkemedel producerade av animala celler har under de senaste åren ökat markant. Detta beror till stor del på att humaniserade monoklonala antikroppar används som läkemedel i allt större utsträckning. Det finns också ett stort behov av att utveckla serum-fria medier för odling av animala celler, främst beroende på att närvaro av serum innebär en risk för kontamination av slutprodukten. Ett alternativ till serum som visat sig vara lovande är peptoner från växter. Peptoner är ett odefinierat hydrolysat av proteiner som dessutom innehåller lågmolekylära ämnen.

Den vanligaste typen av odling för produktion i den biofarmaceutiska industrin är fed-batch odling. I denna typ av odling startar man med ett basmedium och celler för att under odlingens gång tillsätta nödvändiga näringsämnen i form av ett feed medium.

I detta arbete har en Chinese hamster ovary (CHO) cell-linje använts för produktion av en monoklonal antikropp i en serumfri fed-batch process. Processen har förbättrats genom att studera inverkan av peptoner på celltillväxt, processlängd och produktivitet. Resultaten visar att tillsats av peptoner i feed mediet kan gav förbättrad celltillväxt, ökad processlängd samt högre produktivitet. Peptonerna visade sig också kunna reducera den toxiska effekt som uppkom då aminosyror tillsattes i för stor mängd.

Table of contents

Abbreviations

AA	Amino acids
AU	Arbitrary unit
BHK	Baby hamster kidney
BSA	Bovine serum albumin
BVT4	Biovitrum proprietary medium type 4
CHO	Chinese hamster ovary
D	Diameter
DO	Dissolved oxygen
DOT	Dissolved oxygen tension
G	Day of growth
Glc	Glucose
Gln	Glutamine
HRP	Horseradish peroxidase
Mvc	Million viable cells
PDT	Population doubling time
PEP	Peptone
recInsulin	Recombinant insulin
sp	Spinner
TMB	Tetramethylbenzidine
vc	Viable cells

1. Introduction

1.1 Background and aim

Over the last years there has been an increasing demand for biopharmaceuticals, especially humanized monoclonal antibodies, produced by animal cells. Chinese Hamster Ovary (CHO) cells are widely used as standard producers of complex proteins in the biopharmaceutical industry. Other cell lines like the mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human-retina-derived (PER.C6) cells are alternatives. All these cell lines have been optimized to grow in suspension cultures and are easy to scale-up using stirred tank bioreactors [1].

Mammalian cells have traditionally been grown in media supplemented with serum. Due to contamination risks, there is an increasing need to find non animal-derived substitutes for serum. Biovitrum has previously developed serum-free basal and feed media for a CHO cell fed-batch process. The process is based on Biovitrum's proprietary low protein serum-free medium and is designed with the aim of keeping the nutrients at reasonable levels and diluting the byproducts.

The aim of this project was to further improve the Biovitrum feed medium by studying the effect of plant-derived peptones as a medium component. Secondly, the effect of feeding amino acids was studied to find the optimal amino acid balance and avoid possible toxic effects of overfeeding. Parameters like cell growth, viability, culture longevity and antibody productivity were used to evaluate the different feed media formulations.

1.2 Expression systems

A number of different organisms are used to produce recombinant proteins. Bacterial systems (e.g. *Escherichia coli*, *Bacillus subtilis*) have several advantages. They are fast growing, utilize low-cost media and can relatively easily reach high expression levels. However, since proteins produced by bacteria are not post-translationally modified, these organisms can not be used to produce complex proteins (e.g. glycosylated proteins). Yeast systems (e.g. *Saccharomyces cerevisiae*, *Pichia pastoris*) can be employed to produce some proteins, but neither these organisms are equipped with a complete post-translational machinery. *S cerevisiae* has also been shown to overglycosylate proteins and *P pastoris* tends to produce proteases that may degrade the protein product [2].

Mammalian cells are equipped with a complete post-translational machinery and are thus used to produce large and complex proteins. Mammalian cells are larger and much more fragile than microbial cells, which make them sensitive to impurities and small changes in pH or temperature. They also have a more complex metabolism which means that they need more complex media containing various nutrients like vitamins, minerals, salts and amino acids in order to grow. Some cell lines can proliferate in suspension while others require a surface to grow. Traditionally anchorage dependent cells have been more successful regarding protein productivity, but lately many cell lines (e.g. NS0 and CHO) have been optimized for suspension cultures, which are easier to handle and to scale-up [3].

1.3 Batch process

A batch cultivation is initiated when adding an inoculum, a culture of cells, to the medium. No nutrients are added to the culture during the process. It is common that the cells grow slowly after inoculation due to low cell density (lag phase). After a lag phase, the cells start to grow exponentially until a maximal cell density is reached (exponential phase). When one or more nutrient components is/are depleted or the accumulation(s) of metabolic by-product(s) becomes inhibitory, the cell viability and number decline [4]. It is desirable to avoid high osmolarity in a batch culture to avoid cell disruption and termination of the culture.

1.4 Fed-batch process

A fed-batch process is often started as a batch process. Nutrients are then added over time, resulting in an increase of culture volume (Fig.1). This means that depletion of nutrients can be avoided and toxic by-products can be diluted, resulting in higher viable cell number, longer cultivation time and better productivity than the batch process. A fed-batch culture is also easy to operate due to its technical simplicity.

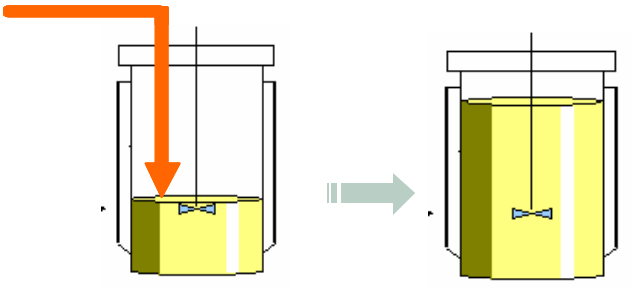

Figure 1. In a fed-batch process, nutrients are added over time, resulting in an increasing culture volume. Figure used with permission from Biovitrum AB.

1.4.1 Fed-batch process development

There are several ways to optimize a fed batch process in order to maximize culture longevity and protein production. Most essentially, a stable cell line with a high protein product secretion rate has to be developed. Further, the basal and feed media have to be optimized to find the optimal composition for the specific cell line that is to be used in the process.

Generally there are two main strategies for fed-batch process development, the “bottom-up” approach and the “top-down” approach. The bottom-up approach means that the culture is supplemented with components that are being quickly consumed. This can be done by addition of a carbon and an energy source, but most often multinutrient feeds are required in order to maximize culture longevity and productivity. “Nutrient homeostasis” is an example of a bottom-up fed-batch development strategy which aims at maintaining constant nutrient concentrations throughout the whole culture process. “Rational medium design” is another bottom-up approach where nutrient cocktails are designed based upon the metabolic requirements of the cells. Generally the bottom-up approach is very time-consuming, since a lot of analytical work has to be done in identifying important nutrients and optimizing feed cocktail composition. It is also most often cell-line specific. The top-down approach can, on the other hand, be used to quickly improve cell-line independent fed-batch processes. The principle of the top-down approach is that the culture is fed with a complete medium concentrate instead

of identifying medium components and designing cell-line specific nutrient cocktails [5]. In practical, the two approaches may be combined during the fed-batch process development.

1.4.2. Substrate component feeding

The feeding of nutrients in fed-batch cultivations is the main reason why the viable cell number and viability often are much higher than in a batch culture. To achieve maximal productivity the aim is to keep the viability as high as possible for as long time as possible. However, the accumulation of by-products like lactate and ammonia eventually cause the viable cell number and viability to decrease. Lactate accumulation can decrease the culture pH, which may lead to the addition of alkali (if a pH control is desired), hence an increase of osmolarity in the culture medium. Ammonia can permeate the cell and alter the intracellular pH. Therefore it is important to reduce the accumulation of these metabolic by-products [6].

The two major energy sources for mammalian cells are glucose and glutamine. Glucose can be utilized in two different ways depending on the concentration present in the media. At high glucose concentrations the specific glucose consumption rate is higher and most of the glucose is used as a carbon source for glycolysis and converted to lactate. At low glucose concentrations the specific glucose consumption rate is lower and more of the glucose is completely oxidized to CO_2 [6].

Glutamine can also be used as a carbon source when it is oxidized to glutamate by glutaminase. Glutamate is further transaminated to α-ketoglutarate which enters the citric acid cycle. Deamination of glutamine by glutaminase also produces the metabolic product ammonia, which can be toxic to the cells when present in high concentrations [7].

It is thus possible to minimize the production of lactate and ammonia by keeping the glucose and glutamine concentrations low. To achieve this, feeding of glucose and glutamine should ideally be done continuosly throughout the cultivation period. The sensitivities to lactate and ammonia is however cell-line specific and may vary greatly between cell-lines [8].

The essential amino acids need to be supplemented to the medium for mammalian cells to grow. It is critical to obtain a balanced supplementation of the essential and other amino acids in order to prevent possible toxic effects of overfeeding amino acids [9].

1.5 Serum-free media

Serum contains several growth-promoting compounds like growth factors, nutrients and hormones, and has been widely used as a supplement in media for mammalian cell cultivations. However, there are a number of disadvantages with the use of serum. Serum shows a variation in shelf-life and composition from batch to batch which requires extensive quality controls to be able to achieve reproducibility between batches. It also presents difficulties in the purification of the protein product and is often associated with high costs. The most important disadvantage with the use of animal-derived serum is however the risk of viral, mycoplasma or prion contamination, which may present a contagious risk to the biopharmaceutical product [7].

Because of the numerous functions serum has in culture media, substitutes for all growth-promoting components in serum has to be found. For example, the iron-carrier transferrin can be replaced by inorganic salts and chelating agents. The surfactant Pluronic F68 substitutes serum in protecting the cells against shear stress [10]. Likewise, ethanolamine and sodium selenite are considered important supplements to promote cell growth in serum-free media [11].

To successfully replace all important components in serum by chemically defined substitutes has however shown to be difficult. Growth requirements may vary widely between cell-lines and even between clones [1]. It has not been possible to design a universal serum-free medium that applies for all cell-lines. Instead serum-free media has to be designed to meet the requirements of the specific cell-line in use.

There are various ways to proceed when designing serum-free media formulations. Different combinations of serum-free basal media can be tested. Metabolic analyses may help to find important media supplementations. Microarray analysis of receptors expressed by the cells during growth can be used to identify their corresponding ligands, which can be supplemented in the media [1].

1.6 Peptones

Peptones are enzymatic or acid hydrolysates of proteins from biological material such as animal tissues, milk products, microorganisms and plants [13]. The protein hydrolysates are undefined mixtures of low-molecular weight components including amino acids, peptides, vitamins and trace elements [14].

Peptones have shown to be beneficial to cell growth and productivity in a variety of cell-lines and peptones of animal origin have been used in serum-free media since the seventies [10]. Lately much attention has been on using the animal-derived peptone Primatone RL. It has though been shown that using this peptone may decrease the sialylation at both glycosylation sites in IFN-γ in both batch and fed-batch cultures of CHO cells [14]. The primary drawback of using these peptones are their animal origin, which presents a risk of virus, mycoplasma or prion contamination.

Medium developers have recently started to focus on using plant-derived peptones as a substitute for serum. The primary reason for this is that using plant peptones instead of serum or animal-derived peptones would eliminate the risk of contamination of the biopharmaceutical product. Several reports indicate that some plant peptones have positive effect on both cell growth and productivity in both BHK and CHO cells [10,12].

2. Materials and methods

2.1 Cell line

An antibody producing Chinese Hamster Ovary (CHO) K1 cell line was used in this study.

2.2 Media

2.2.1 Peptones

The peptones used in this study were HyPep7504 and HyPep7401 (both from Kerry Biosciences, Tralee, Ireland). Additional information about the peptones can be found in Table 1.

Table 1. Information about the degree of hydrolysis and molecular weight distribution of the peptones used in this project. Information was provided by the manufacturer.

Peptone	Origin	Manufacturer	Degree of hydrolysis (%)	Molecular weight distribution			
				<1kDa	1-5kDa	5-10kDa	>10kDa
HyPep7401	Pea	Kerry	14-24	90.2	9.1	0.6	0
HyPep7504	Cotton seed	Kerry	18-28	84.7	11.9	2.3	1.1

2.2.2 Basal media

The cells were originally frozen in Ex-Cell™ 302 medium (JRH Biosciences, Kansas, US). The cells were thawed, expanded and adapted to two different serum-free media depending on the experiment.

For experiments 1-4, a proprietary serum-free basal medium developed by Biovitrum (denoted BVT4) was used. The medium was supplemented with 4 mM Glutamine (Invitrogen, Carlsbad, CA, US), 500 µg/ml Genetecin (Invitrogen), 10 mg/L recInsulin (Serologicals, Norcross, GA, US) and 2.5 g/L HyPep7504 (Kerry) and 2.5 g/L HyPep7401 (Kerry) during the adaptation of the cells. The BVT4 medium supplemented with 2 mM Glutamine (Invitrogen), 10 mg/L recInsulin (Serologicals) and 2.5 g/L HyPep7504 (Kerry) and 2.5 g/L HyPep7401 (Kerry) was used in the experiments 1-4.

For experiment 5, Dulbecco´s Modified Eagle´s Medium Nutrient mixture F12 Ham (DMEM/F12; Sigma-Aldrich, St.Louis, MO, US) basal medium was used. The medium was supplemented with 4 mM Glutamine (Invitrogen), 10 mg/L recInsulin (Serologicals), 500 µg/ml Genetecin (Invitrogen), 2.5 g/L HyPep7504 (Kerry) and 2.5 g/L HyPep7401 (Kerry) during the adaptation. The DMEM/F12 medium (Sigma-Aldrich) supplemented with 2 mM Glutamine (Invitrogen), 10 mg/L recInsulin (Serologicals), 2.5 g/L HyPep7504 (Kerry) and 2.5 g/L HyPep7401 (Kerry) was used in experiment 5.

2.2.3 Feed media

In experiments 1-4 Biovitrum proprietary serum-free feed medium was used. The feed medium was supplemented with 1 mM Glutamine (Invitrogen) and 10 mg/L recInsulin (Serologicals), except for the experiment 4, where no glutamine was added to the feed medium.

In experiment 5, a feed medium based on 3X concentrated DMEM/F12 (Sigma-Aldrich) was used. The medium was also supplemented with 0.3 g/L Serine, 0.075 g/L Methionine and 0.04 g/L Tryptophan (all from Sigma-Aldrich).

Both feed media were sterile filtered using 0.2 µm bottle filters (Nalgene, Rochester, NY, US).

2.2.4 Stock solutions

A list of stock solutions fed to the cultures can be found in Table 2. The concentrations of the stock solutions were based on the studies performed earlier at Biovitrum. Peptone stock solutions were prepared in BVT4 medium. Glucose stock solutions were prepared in destilled water. All stock solutions prepared in-house were sterile filtered using 0.2 µm bottle filters (Nalgene) before use. The RPMI1640 amino acids solution was supplemented with L-Asparagine (Sigma), L-Serine (Sigma) and L-Tryptophan (Sigma). Final concentrations of these amino acids were 2X, 2X and 1.5X the original concentrations in RPMI1640, respectively. The supplemented RPMI1640 solution is denoted RPMI1640+. The complete composition of the RPMI1640 amino acids solution can be found in Table 13, Appendix I.

Table 2. Stock solutions used for feeding and pH adjustments.

Stock solution	Concentration
Glucose	400 g/L
Glutamine	200 mM
HyPep7504	40 g/L
HyPep7401	40 g/L
Na_2CO_3	0.5 M
RPMI 1640+	50X

2.3 Cultivation setup and control

2.3.1 Small scale experiments

In all small scale experiments (experiments 1-3 and 5) cells were grown in a humidified CO_2 incubator, with standard settings: 37°C, 5% CO_2, 90% humidity. During the adaptation and expansion, the cells were grown in 75 cm^2 non-tissue culture treated T-flasks (BD Biosciences, San José, CA, US) and 125 ml spinners (Techne, Burlington, N.J, US).

In experiments 1, 3 and 5, cells were grown in 250 ml and 500 ml spinner bottles (Techne) with working volumes of 80-150 ml and 150 ml-300 ml respectively. Spinner bottles were changed from 250 ml to 500 ml size when reaching a working volume of 150 ml. Spinner tables (Belach Bioteknik, Solna, Sweden) with agitation rate 45 rpm were used in all spinner experiments.

In experiment 2, 50 ml filter tubes (Techno Plastic Products, Trasadingen, Switzerland) were used. The tubes were placed on a shaker table (Belach Bioteknik) with agitation 45 rpm.

pH was manually controlled upwards in all small scale cultivations, except for experiment 2, by the addition of 0.5 M Na_2CO_3 when needed. The pH was adjusted to 7.1.

2.3.2 Bioreactor experiment

In experiment 4 cells were grown in a 3 L glass bioreactor (Applikon, Schiedam, Netherlands). ADI 1030 Bio Controller (Applikon) was used to control the systems. Two bioreactors were run in parallel. Before sterilization of the bioreactors by autoclaving, the reactors were pressure tested. Before inoculation the DO-, pH- and temperature probes were calibrated. The starting volume for both reactors were 850 ml. The general reactor set-up with set-points are shown in Table 3 and the headplate setup is shown in Figure 2.

Table 3. General 3 L bioreactor set-up and set-points.

Item	Purpose	Set-point / Comment
DO-probe	On-line DO measurement	40%
pH-probe	On-line pH measurement	7.1
Temperature probe	On-line temperature measurement	37°C
Triple inlet	Alkali and medium feeds	As needed
Sampling pipe	Vacutainer sampling device	N/A
Dip tube	Inoculation, medium filling and emptying	N/A
Marine impeller (D=60 mm)	Agitation	100-150 rpm
Microbial sparger	Oxygen supply	Pulsing as needed
Gas inlet to headspace	Headspace gassing	Air 200 ml/min; N_2 as needed
Air out	Air out	Through a wet exhaust bottle

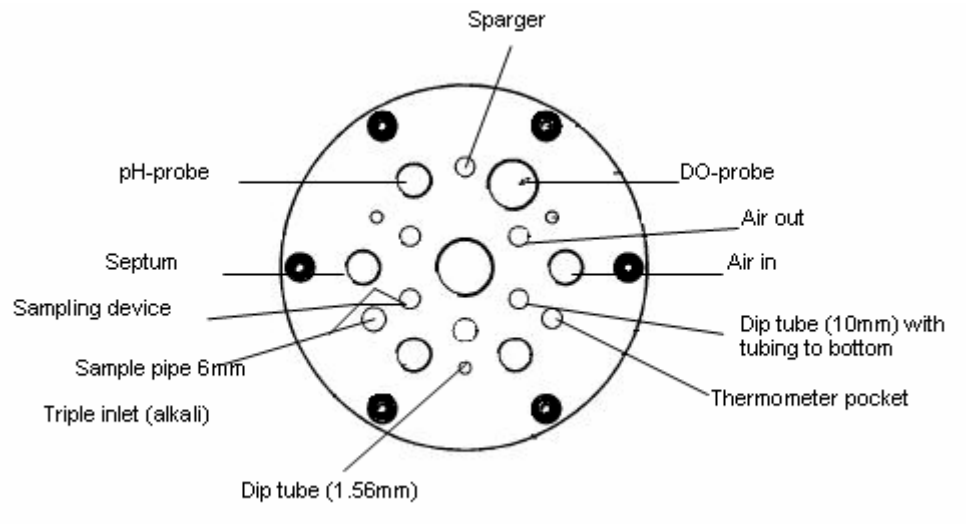

Figure 2. Headplate setup for 3 L glass Applikon bioreactor. Figure adapted from *Applikon User Manual Autoclavable Bioreactor 2-7 L.*

Air continuosly flew into headspace at a rate of 200 ml/min to maintain a slight overpressure in the bioreactor. At low cell density, air was mixed with nitrogen in order to achieve a DOT of

40% as soon as possible. DOT was further automatically controlled upwards by pulsed addition of oxygen through the sparger. The starting agitation rate was 100 rpm. Later it was increased to 120 rpm and 150 rpm to match the current working volumes. pH was automatically controlled upwards by pumping 0.5 M Na_2CO_3 into the bioreactor when needed and downwards by pulsed addition of CO_2 to headspace. All automatic regulation was monitored using a computer equipped with BioXpert® software.

2.4 Analytical methods

2.4.1 In-process analyses

Cells were counted using a Cedex™ Cell counter (Innovatis, Bielefeld, Germany), which gives viable and total cell density, viability, average cell diameter and aggregation rate.

Glucose/Lactate and Glutamine/Glutamate concentrations were measured with YSI 2700 Select™ Biochemistry Analyzer using supernatant samples. pH was measured off-line using a gas blood analyzer, ABL (Radiometer, Copenhagen, Denmark). Osmolarity was measured off-line using supernatant samples with a Roebling Automatic osmometer (type 12/12DR). Ammonia was measured using supernatant samples with a Bioprofile (Nordic Biolabs, Täby, Sweden).

2.4.2 ELISA

Antibody productivity was analyzed using a Human-IgG ELISA assay developed by Biovitrum. A 96-well Nunc™ microtiter plate (Fisher Scientific, Hampton, N.H, US) was coated with goat anti-human IgG (Fab specific). After washing using a washer (Tecan, San José, CA, US) the plate was blocked with blocking buffer containing 3% BSA and incubated for one hour at 37°C. After washing, controls and supernatant samples were diluted and added to the plate. After incubation for 2 hours at 37°C, the plate was washed before adding the F(ab`)2 goat anti-human IgGγ (heavy chain) conjugated with HRP. The plate was then incubated with the detection antibody for 2 hours. After washing, the plate was incubated with the TMB substrate for 20 min at room temperature with agitation at 300 rpm. The reaction was stopped with 1 M H_2SO_4 and the colour read photometrically at 450 nm using a microplate reader (THERMOmax, Molecular Devices, Union City, CA, US). A computer program (ACE version 4.1 for PC) was used for the calculation of the sample concentrations.

2.4.3 Productivity calculations

The following equations were used to calculate different productivity parameters. The titer, Cp, was derived from ELISA analyses.

Daily production:

$$C_d = \frac{C_{p2} \cdot V_2 - C_{p1} \cdot V_1}{\Delta t} \qquad \text{(Eq.1)}$$

Cell specific productivity:

$$q_p = \frac{\left(C_{p2} \cdot V_2 - C_{p1} \cdot V_1\right) \cdot 2}{\left(C_{v1} \cdot V_1 + C_{v2} \cdot V_2\right) \cdot \Delta t} \qquad \text{(Eq.2)}$$

Volumetric productivity:

$$Q_p = \frac{C_{p2} \cdot V_2 - C_{p1} \cdot V_1}{V_2 \cdot \Delta t} \quad \text{(Eq.3)}$$

where C_{p1} and C_{p2} are the titers at time-points 1 and 2; V_1 and V_2 are the culture volumes at time-points 1 and 2, Δt is the time difference between time-points 1 and 2, C_{v1} and C_{v2} are the viable cell number at time-points 1 and 2.

2.5 General cultivation procedures

2.5.1 Cell thaw and expansion

The ampoule was swirled gently in a 37°C water bath until the suspension had just thawed completely. The cell suspension was transferred to a 50 ml centrifuge tube (BD Biosciences) and the first 2 ml of non-selective Ex-Cell™ 302 medium (JRH Biosciences) supplemented with 4 mM glutamine (Invitrogen) was added dropwise over a 2 min interval. Totally, 10 ml medium was added. The cells were centrifuged (900 rpm, 5 min, room temperature) and the supernatant discarded. The first 2 ml of totally 10 ml fresh non-selective medium was added dropwise to the cells before adding the remaining 8 ml. The cell suspension was mixed and 300 µl was sampled for cell count using Cedex™ (Innovatis). The cell suspension was transferred to a non-tissue culture treated T75 cm^2 flask (BD Biosciences) and diluted to a cell density of 0.5 Mvc/ml in non-selective Ex-Cell™ 302 medium (JRH Biosciences) supplemented with 4 mM glutamine (Invitrogen). The flask was placed in a CO_2 incubator at 37°C, 5% CO_2 overnight.

On the first day after thaw, the cells were counted using Cedex™ (Innovatis), centrifuged (900 rpm, 5 min, room temperature) and re-suspended in pre-warmed selective Ex-Cell™ 302 medium (JRH Biosciences) containing 500 µg/ml Genetecin (Invitrogen). The culture was then transferred to a 125 ml spinner bottle (Techne) and placed on a spinner table in a CO_2 incubator. The cells were thereafter sub-cultured in a 250 ml spinner bottle (Techne) with complete medium change every second or third day with an inoculation density of 0.3 Mvc/ml.

2.5.2 Cell adaptation

In the beginning of this study, the cells were adapted to the BVT4 medium by stepwise exchanging one part of Ex-Cell™ 302 (JRH Biosciences) for BVT4 medium. It was found later that the Ex-Cell™ 302 (JRH Biosciences) could be completely replaced by BVT4 medium without affecting viability and cell growth.

The adaptation to DMEM/F12 medium (Sigma-Aldrich) showed to be more difficult. It took about 2 weeks for the cells to adapt to the new medium by gradually exchanging Ex-Cell™ 302 (JRH Biosciences) for DMEM/F12 (Sigma-Aldrich) (Table 4).

Table 4. Pattern of adaptation to DMEM/F12 medium.

Ex-Cell™ 302	DMEM/F12	Passages
100%	0%	N/A
75%	25%	1
50%	50%	2
25%	75%	2
0%	100%	N/A

2.5.3 Inoculation

A seeding cell density of 0.3 Mvc/ml was used in all the experiments. The cells that were needed to set up the experiment were pooled, centrifuged and resuspended in fresh medium.

2.5.4 Sampling

In the small-scale experiments all sampling was done in LAF-bench. A total of 1.2 ml was sampled each time. 500 µl cell-broth was taken for direct pH measurement and cell count. The remaining 700 µl was centrifuged 5 min, 900 rpm and the supernatant was taken for glucose/lactate and glutamine/glutamate measurements. 2x300 µl of supernatant was transferred to 1.5 ml plastic tubes (Eppendorf, Hamburg, Germany) and stored at -70°C for ELISA, osmometer, Bioprofile and amino acid analyses.

In the bioreactor experiment, approximately 13 ml was sampled using Vacutainer® system (BD Biosciences). The first 8 ml was discarded. 500 µl was taken for direct pH measurement and cell counting. 4 ml was centrifuged 5 min, 900 rpm and the supernatant was taken for glucose/lactate and glutamine/glutamate measurements. 3x1 ml was aliquoted in 1.5 ml plastic tubes (Eppendorf) and stored at -70°C for later ELISA, osmometer, Bioprofile and amino acid analyses.

Sampling was done every day for experiments 1 and 4 and every second day for experiments 2, 3 and 5.

2.5.5 Calculation of cell growth

Cell specific growth rate:

$$\mu = \frac{\ln \frac{N_2}{N_1}}{\Delta t} \qquad \text{(Eq.4)}$$

Population doubling time:

$$PDT = \frac{\Delta t \cdot \ln 2}{\ln \frac{N_2}{N_1}} \qquad \text{(Eq.5)}$$

where Δt is the time difference between cell counts, N_2 is the number of viable cells on the actual time of counting, N_1 is the number of viable cells on the last time of counting.

2.6 Feeding strategies

2.6.1 Feeding of glucose and glutamine

Glucose and glutamine were fed separately to maintain concentrations of 1.0 g/L and 1.0 mM, respectively. A stock solution of 400 g/L was prepared and used for the addition of D-glucose (Sigma-Aldrich) and a 200 mM stock solution of glutamine (Invitrogen) was used for the addition of glutamine.

The glucose and glutamine consumption rates, Q_{glc} and Q_{gln}, was calculated using the following mass - balance equation:

$$Q_{glc} = \frac{[glc]_0 \cdot V_0 - [glc] \cdot V + [SS]_{glc} \cdot V_{glc,lastfeed}}{\Delta t} \qquad \text{(Eq.6)}$$

where $[glc]_0$ and $[glc]$ are the measured glucose concentrations at the last time of feeding and the actual time of feeding, V_0 and V are the cultivation volumes at the last time of feeding and the actual time of feeding, $[SS]_{glc}$ is the concentration of the glucose stock solution, $V_{glc,lastfeed}$ is the volume of glucose added at the last time of feeding and Δt is the time between the last time of feeding and the actual time of feeding. Exchanging all glucose concentrations for glutamine concentrations gives Q_{gln}.

When calculating the volume of glucose (or glutamine) to feed, a compensation for cell growth until the next time, N_{est}, was included. The estimation in experiment 1 was made by linear regression based on the viable cell number for the last three time-points. In all other experiments the estimation was based on population doubling time (PDT) simply by looking at the PDT and approximating how many viable cells there would be on the next time of feeding. The calculation of the volume of glucose (or glutamine) to feed was made using:

$$V_{feed} = \frac{([glc]_{sp} - [glc]) \cdot V + \frac{N_{est}}{N} \cdot Q_{glc} \cdot \Delta t_{nextfeed}}{[SS]_{glc}} \qquad \text{(Eq.7)}$$

Where $[glc]_{sp}$ is the glucose concentration to be maintained, N is the total viable cell number and $\Delta t_{nextfeed}$ is the time until next feed. Exchanging all glucose concentrations for glutamine concentrations gives the feeding volume of glutamine.

2.6.2 Feeding of amino acids

Amino acids were fed using the RPMI1640+ solution. The amount of amino acids to feed was based on the cell specific consumption, q_{aa}, of each amino acid and the average of the current viable cell number and an estimated number of viable cells at the next time of feeding. Since amino acid consumption evaluations could not be done during the cultivation, the addition of amino acids in experiment 1 and 2 were based on earlier amino acid consumption studies

performed at Biovitrum. When cell growth reached the stationary phase, the amount of amino acids to add was reduced to 2/3 of the calculated value.

Average C_v:

$$\overline{C_v} = \frac{C_{v2} - C_{v1}}{2} \qquad \text{(Eq.8)}$$

Cell specific amino acid consumption for each amino acid:

$$q_{aa} = \frac{[aa]_2 - [aa]_1}{\overline{C_v} \cdot \Delta t_{1-2}} \qquad \text{(Eq.9)}$$

Total volume of RPMI1640+ to feed to compensate for the consumption of one specific amino acid:

$$V_{add} = \frac{q_{aa} \cdot (N_{est} + N) \cdot \Delta t}{2 \cdot [aa]_{RPMI1640+}} \qquad \text{(Eq.10)}$$

where C_{v1} and C_{v2} are the viable cell number at time-point 1 and 2, $[aa]_1$ and $[aa]_2$ are the amino acid concentrations at time-point 1 and 2, $\Delta t_{1\text{-}2}$ is the time between time-point 1 and 2, Δt is the time between feedings, $[aa]_{RPMI1640+}$ is the concentration of the specific amino acid in the RPMI1640+ solution, N_{est} is the estimated viable cell number at the next time of feeding and N is the viable cell number at the time of feeding.

2.6.3 Feeding of peptones

The feeding of peptones HyPep7504 (Kerry) and HyPep7401 (Kerry) was always done using a fixed feeding volume. The initial fixed volume was decreased by a factor 2/3 or 1/2 (depending on the experiment) when the viable cell number and viability started to decline. Earlier studies at Biovitrum had recommended a dose of 0.2 g/L – 2.0 g/L of the cultivation starting volume. Complete feeding scheme for each experiment can be found in chapter 3.

2.6.4 Feeding of feed medium

In experiments 1-3 feed medium was fed according to Table 5. In experiment 4, the bioreactors were fed continuously according to Table 16, Appendix V. In experiment 5 the cells were fed according to Table 12.

Table 5. Basic feeding strategy for a spinner with 100 ml starting volume. The same feeding strategy was also applied for 50 ml filter tubes.

Day	Volume in spinner (ml)	Added volume (ml)
0	100	0
3*	172	72
6	244	72
9	250	36**

*Change spinner size from 250 ml to 500 ml
**Removal of 30 ml cell broth resulting in a final culture volume of 250 ml

3. Results

3.1 Experiment 1 - Peptone feeding

The main purpose of this experiment was to investigate if feeding peptones is beneficial to cell growth and productivity. The experiment included 5 spinners. Glucose, glutamine and amino acids were fed every day while peptones were fed every other day. The feed medium was added every third day according to the scheme in Table 5. pH was adjusted every day to 7.1. A batch spinner and a semi-batch spinner, where only glucose and glutamine were fed, were included as controls. The seeding cell density was 0.3 Mvc/ml in all spinners. Spinner size was changed from 250 ml to 500 ml on G3 to obtain sufficient aeration. The experimental setup is shown in Table 6.

Table 6. Experimental setup for experiment 1.

Spinner	Process	Peptones in basal medium	Glucose	Glutamine	Amino acids	Feed medium	Peptone feed
1	Batch	pea+ cot 5 g/L	no	no	no	no	no
2	Semi-batch	pea+cot 5 g/L	yes	yes	no	no	no
3	Fed-batch	pea+cot 5 g/L	yes	yes	yes	yes	no
4	Fed-batch	pea+cot 5 g/L	yes	yes	yes	yes	Pea + cot 0.4 g/L G2,G4 0.2 g/L G6,G8,G10
5	Fed-batch	pea+cot 5 g/L	yes	yes	no	yes	Pea + cot 0.8 g/L G2,G4 0.4 g/L G6,G8,G10

3.1.1 Cell growth and viability

Not surprisingly, spinner 1 (batch) showed the lowest maximal number of viable cells as well as the shortest cultivation time (Fig.3). Spinner 2 (semi-batch) reached a higher maximum number of viable cells than the batch culture, but crashed abruptly on G6. Glucose was almost depleted in spinner 2 on G4. This however could not explain the drop of the viable cell number and viability, since glucose was depleted also in all other spinners on G4 or G5, but the cells continued to grow despite this (Fig.6). More likely, the reason was depletion of other nutrients than glucose and glutamine and accumulation of by-products. Spinner 2 had the highest accumulation of ammonia and the highest osmolarity (Fig.31, 32, Appendix II), both of which might contributed to the cell death. Spinner 1 was terminated on G9 and spinner 2 on G7.

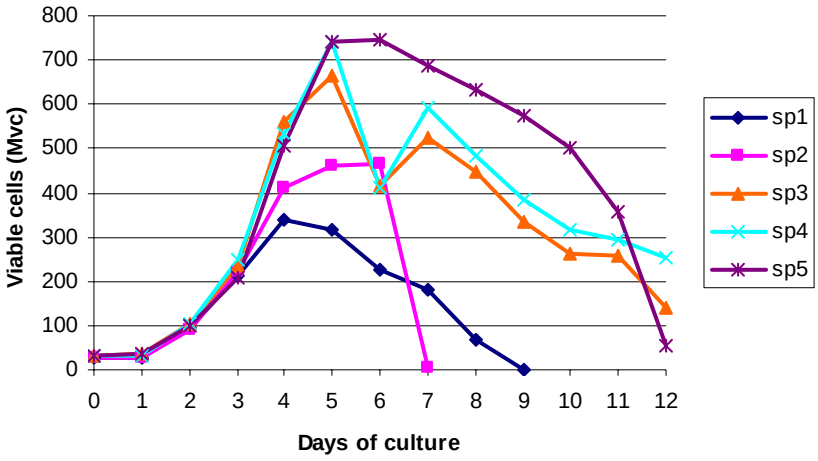

Figure 3. Number of viable cells (Mvc) in experiment 1.

Spinners 3, 4 and 5 showed higher number of viable cells, better viability and longer culture longevity compared to the batch and semi-batch spinners. This indicated that the fed-batch process was superior to the batch process. Spinner 5 had the highest maximal number of viable cells, the longest cultivation process and the best viability. Spinners 3 and 4 were very similar, although spinner 4 was slightly better. The results suggested that feeding peptones had a positive effect on all the cell growth parameters. The dose of peptone feeding in spinner 5 was doubled as compared to that in spinner 4, and the cell growth was improved. This suggested that higher dose of peptone feeding was beneficial.

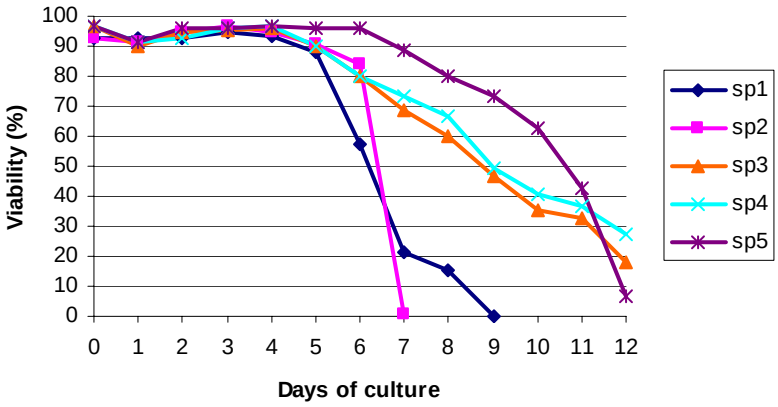

Figure 4. Viability in experiment 1.

3.1.2 Productivity

The same pattern as for growth was found regarding the productivity (Fig.5). Spinner 5 had clearly the highest accumulated productivity, followed by spinners 3 and 4. The batch and semi-batch spinners had much lower productivity than the fed-batch cultures. The reason why spinner 5 had the best total productivity can be correlated to the culture longevity and the maximal number of viable cells. The cells in spinner 5 still had a viability of 73% on G9 compared to 49% and 46% in spinners 3 and 4, respectively. The cell specific productivity was very similar in all spinners (Table16, Appendix II).

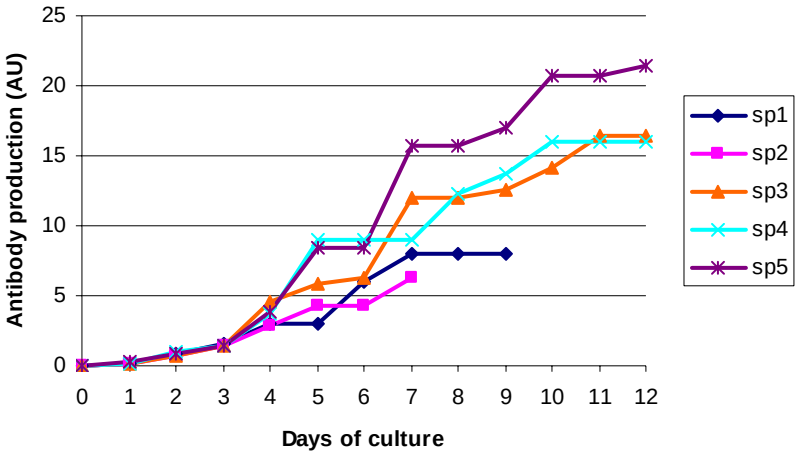

Figure 5. Accumulated antibody production in experiment 1.

3.1.3 Feeding, metabolite and osmolarity analyses

In this experiment, a linear regression model based on the last three time-points was used to estimate the number of viable cells the day after. This number was used to calculate the amount of glucose, glutamine and amino acids to feed. It showed that this linear regression model did not work satisfying, since glucose was depleted in all spinners on G4, G5 or G6 (Fig.6). The target concentration of glucose was 1.0 g/L.

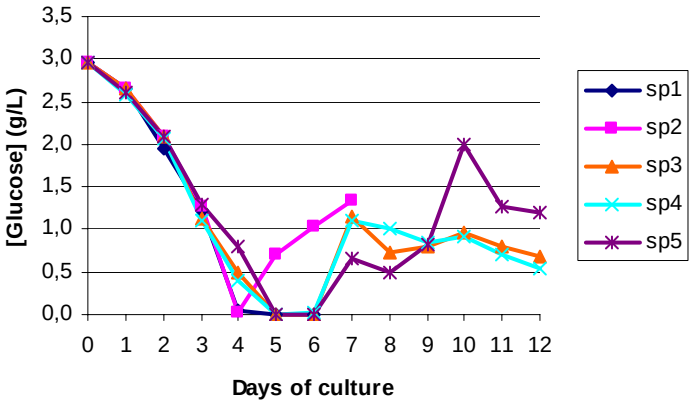

Figure 6. Glucose concentrations. Note that glucose was depleted in all spinners on G4, G5 or G6.

Table 7 shows that the model gave an underestimation of the viable cell number during the first three days of feeding, and this was the reason why too little glucose was added to the spinners. The reason why the model underestimated the number of viable cells was probably due to that the cells had a relatively long lag phase after inoculation. We conclude that the glucose regulation had to be modified for the next experiment.

Table 7. Estimation of viable cell number using the linear regression model. The model underestimated the viable cell number on G2, G3 and G4, which resulted in glucose depletion in all spinners.

G	Estimated viable cell number (Mvc)	Real viable cell number (Mvc)	Estimated/real ratio
0	N/A	30.0	N/A
1	N/A	30.9	N/A
2	32.1	104.4	0.31
3	137.7	248.5	0.55
4	339.1	534.3	0.63
5	744.8	741.6	1.00
6	890.0	411.2	2.16
7	411.2	594.0	0.69
8	712.8	485.0	1.47
9	485.0	385.4	1.26
10	308.3	246.2	1.25
11	221.6	227.6	0.97
12	204.8	195.7	1.05

As mentioned earlier, spinner 2 had the highest accumulation of ammonia (9.3 mM) and lactate (6.77 g/L), as well as the highest osmolarity (460 mOsm/L) (Fig. 31, 33, 32, Appendix II). This was probably one of the reasons why it crashed so suddenly. The concentration at which ammonia is toxic is cell-line specific. Earlier studies at Biovitrum on this cell-line had shown that the cell growth was affected negatively when the ammonia concentration was around 7-8 mM. The other spinners had comparable accumulations of lactate (~5 g/L) and ammonia (~6 mM). Spinner 5 had fairly high osmolarity at the end of cultivation (416 mOsm/L) as compared to spinners 3 and 4, which seemed not to affect cell growth, viability and productivity. The higher osmolarity in spinner 5 was the result of the addition of more alkali to this spinner to adjust the pH.

Amino acids were fed based on the average volumes for each individual amino acid calculated using Eq.10. A fixed value of 11 µl/Mvc was applied in accordance with the earlier studies at Biovitrum. As described in section 2.2.4, a number of amino acids were added to the RPMI1640 (Sigma-Aldrich) to optimize the cocktail so that enough amino acids were fed to the cells without overfeeding other amino acids. It was found in later experiments that feeding according to this scheme was not optimal for cell growth.

3.1.4 Amino acid analysis

Amino acid analysis of supernatant samples from G3, G6 and G9 was performed at Uppsala University. For spinner 3 (only amino acid cocktail feed) the analysis showed that the concentrations of all amino acids, except for asparagine, increased from G3 to G9. This suggested that the dose of 11 µl/Mvc was too high. The cell specific consumption of each amino acid was calculated using Eq.9 (Fig. 34, Appendix II) and the amount of each amino acid required to compensate the consumption was calculated (Fig.7). According to the calculations, a fixed volume of about 6 µl/Mvc should be a better choice. It was not needed to add some amino acids, especially aspartic acid, glutamic acid and glycine (negative values).

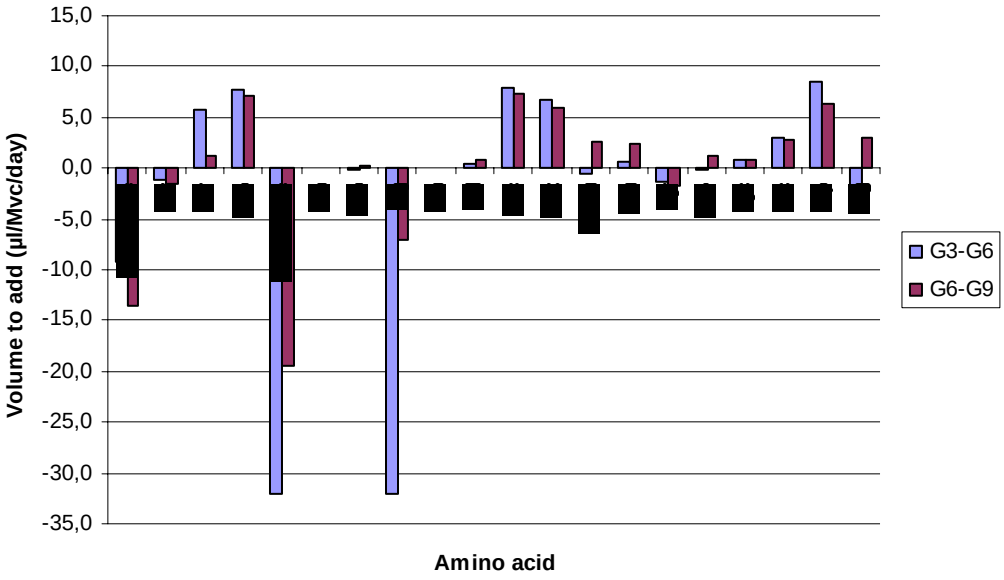

Figure 7. Calculated volume of RPMI1640+ per Mvc to add each day to cover the consumption of amino acids in spinner 3.

3.2 Experiment 2 - Peptone and amino acid dose study in 50 ml filter tubes

In this experiment we wanted to see if the positive effects from peptone feeding in experiment 1 could be repeated and if the effects could be reproduced by amino acid supplementation. Higher dosages of peptones and three different dosages of amino acid cocktail were tested. The cultivations were performed in 50 ml filter tubes (Techno Plastic Products) as described in section 2.3.1.

To simplify calculations, amino acid cocktail was fed at a fixed volume (initially 0.4 ml, 0.8 ml and 1.6 ml) every second day. The 0.8 ml feeding volume roughly corresponded to the amount of amino acids added in experiment 1. Higher and lower doses of amino acid feeding were tested because the amino acid analysis results for experiment 1 were not available when setting up the experiment. Feed medium was fed at a fixed volume (2.88 ml) every third day (the same dose per culture volume as in experiment 1). Starting volumes were 4 ml in all tubes except for 7 ml in the batch controls and 6 ml in the semi-batch controls. This was because no feed medium would be added in the batch and the semi-batch tubes, thus the volume would decrease after each sampling. All 13 conditions were run in duplicates (Table 8). Tubes 14-26 were duplicates of tubes 1-13. The duplicates were very comparable, and the average values of the duplicates are presented. The values are normalized as if the starting culture volume was 4 ml in all the tubes, so that the data may be compared. pH was not adjusted due to the small cultivation volume. The tubes in all groups, except for groups 6, 7, 11 and 12, were terminated on G8 due to low number of viable cells and low viability.

Table 8. Experimental setup for experiment 2.

Group	Tubes	Process	PEP in basal medium	Glc	Gln	AA	Feed medium	Peptone feed
1	1 and 14	Batch	pea + cot 5 g/L	no	no	no	no	no
2	2 and 15	Semi batch	pea + cot 5 g/L	yes	yes	no	no	no
3	3 and 16	Fed-batch	pea + cot 5 g/L	yes	yes	0.4 ml G2, G4, G6. 0.2 ml G8, G10	2.88 ml G3, G6 and G9	no
4	4 and 17	Fed-batch	pea + cot 5 g/L	yes	yes	0.8 ml G2, G4, G6.	2.88 ml G3, G6 and G9	no
5	5 and 18	Fed-batch	pea + cot 5 g/L	yes	yes	1.6 ml G2, G4, G6. 0.8 ml G8, G10	2.88 ml G3, G6 and G9	no
6	6 and 19	Fed-batch	pea + cot 5 g/L	yes	yes	no	2.88 ml G3, G6 and G9	Pea + cot (0.8 g/L G2, G4; 0.4 g/L G6, G8)
7	7 and 20	Fed-batch	pea + cot 5 g/L	yes	yes	no	2.88 ml G3, G6 and G9	Pea + cot (1.6 g/L G2, G4; 0.8 g/L G6, G8)
8	8 and 21	Fed-batch	pea + cot 5 g/L	yes	yes	0.4 ml G2, G4, G6. 0.2 ml G8, G10	2.88 ml G3, G6 and G9	Pea + cot (0.8 g/L G2, G4; 0.4 g/L G6, G8)
9	9 and 22	Fed-batch	pea + cot 5 g/L	yes	yes	0.8 ml G2, G4, G6. 0.4 ml G8, G10	2.88 ml G3, G6 and G9	Pea + cot (0.8 g/L G2, G4; 0.4 g/L G6, G8)
10	10 and 23	Fed-batch	pea + cot 5 g/L	yes	yes	1.6 ml G2, G4, G6. 0.8 ml G8, G10	2.88 ml G3, G6 and G9	Pea + cot (0.8 g/L G2, G4; 0.4 g/L G6, G8)
11	11 and 24	Fed-batch	pea + cot 5 g/L	yes	yes	0.4 ml G2, G4, G6. 0.2 ml G8, G10	2.88 ml G3, G6 and G9	Pea + cot (1.6 g/L G2, G4; 0.8 g/L G6, G8)
12	12 and 25	Fed-batch	pea + cot 5 g/L	yes	yes	0.8 ml G2, G4, G6. 0.4 ml G8, G10	2.88 ml G3, G6 and G9	Pea + cot (1.6 g/L G2, G4; 0.8 g/L G6, G8)
13	13 and 26	Fed-batch	pea + cot 5 g/L	yes	yes	1.6 ml G2, G4, G6. 0.8 ml G8, G10	2.88 ml G3, G6 and G9	Pea + cot (1.6 g/L G2, G4; 0.8 g/L G6, G8)

3.2.1 Cell growth and viability

Group 11 had the highest average maximal number of viable cells, followed by groups 12, 6 and 7 (Fig.8). The four groups, 6, 7, 11 and 12, were the best ones considering number of viable cells and culture longevity. Peptones were fed in all of these cultures. In groups 11 and 12, high dose of peptone cocktail was fed in combination with the addition of amino acid cocktail. The number of viable cells in these tubes declined rapidly after G6. No amino acid cocktail was added to groups 6 and 7. The viable cell number in groups 6 and 7 did not decline as fast as that in groups 11 and 12. The two doses of peptone feeding resulted in comparable cell growth in the cultures. Feeding peptones was found beneficial for cell growth. The beneficial effects of feeding peptones could not be reproduced by feeding amino acid cocktail.

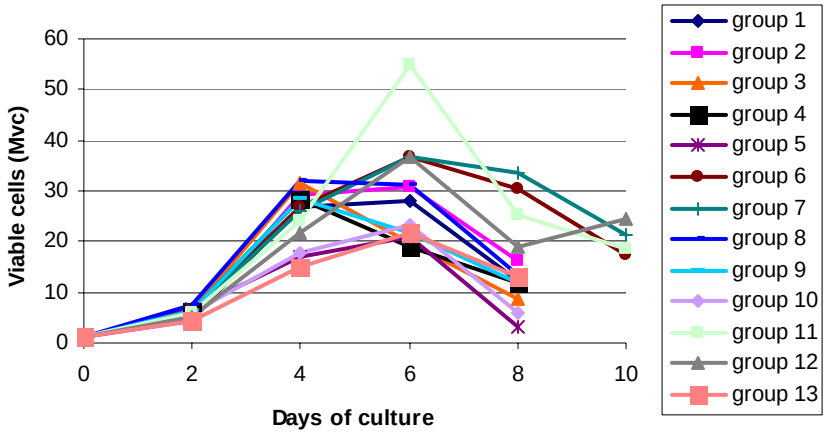

Figure 8. Average number of viable cells in experiment 2.

The viable cell number and viability in group 12 increased from G8 to G10. The cell viability in group 11 increased between G8 and G10. This could probably be explained by the reduction of the feeding dose of amino acid cocktail.

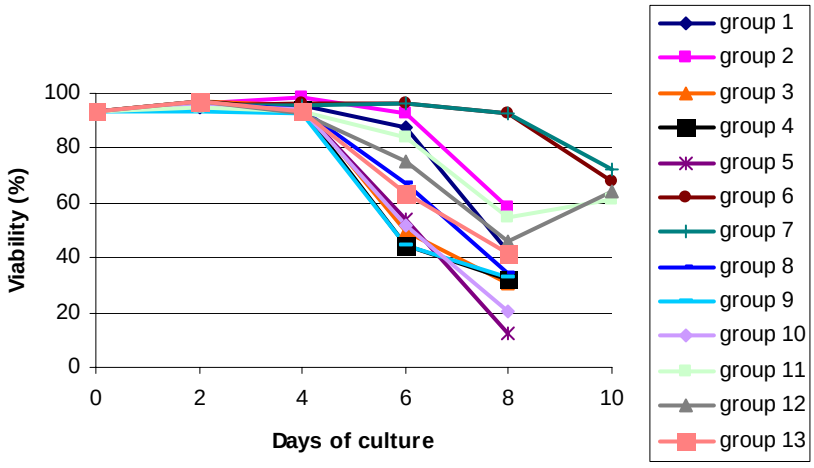

Figure 9. Average cell viability in experiment 2.

Among the groups where only amino acid cocktail was fed, the ones with the lower amino acid cocktail dosages performed better than the ones with higher amino acid dosages. This suggested that we had overfed the amino acids, which could be toxic to the cells. Groups 11 and 12 had a better cell growth than groups 3 and 4, suggesting that the toxicity of amino acid overfeeding could be partially neutralized by the peptones.

3.2.2 Productivity

Antibody productivity was measured on G4, G6, G8 and G10 (Fig.10). The four best performing groups regarding the growth also had the highest accumulated productivity. Groups 6 and 7 had the best accumulated productivity followed by groups 11 and 12. The results suggested that the addition of peptones could reduce the toxic effect from amino acid overfeeding. Higher dose of peptone feeding in groups 11 and 12 neutralized the toxic effect to a great extend, resulting in comparable viable cell number and antibody production as in groups 6 and 7.

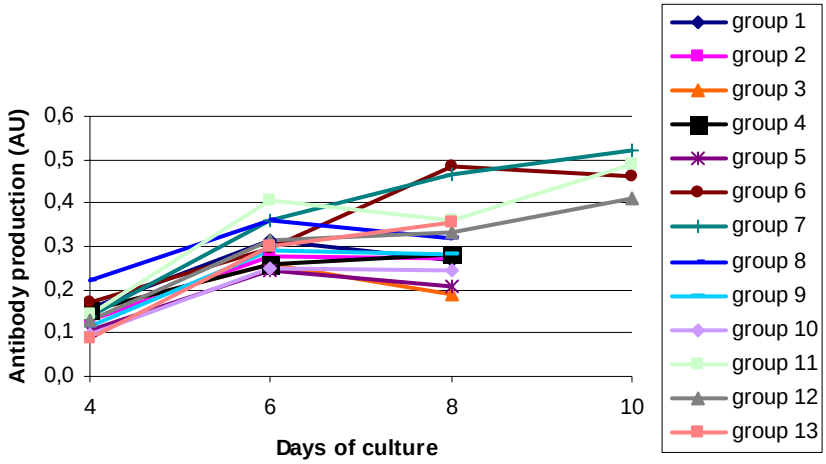

Figure 10. Average accumulated antibody productivity in experiment 2.

3.2.3 Feeding, metabolite and osmolarity analyses

Since the glucose feeding not was satisfactory in experiment 1, the estimation of the number of viable cells at the next time of feeding was based on PDT in this experiment as described in section 2.6.1. It was desirable to maintain a low glucose concentration throughout the cultivation to avoid overproduction of lactate [6]. However, it was also important to prevent a depletion of glucose. The target concentration of glucose was 1.0 g/L. In this experiment, the estimation of viable cell number was more reliable and the glucose feeding was improved (Fig.11).

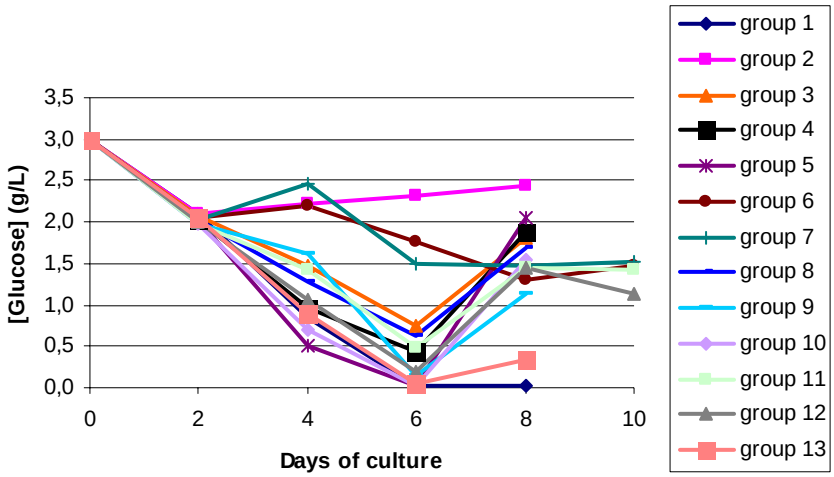

Figure 11. Average glucose concentrations. Some cultures had a depletion of glucose on G6. The glucose concentration target was 1.0 g/L.

The glutamine feeding was satisfying. Groups 6 and 7 had the lowest lactate concentrations and the concentrations were stable from G4 to G10 (Fig.12).

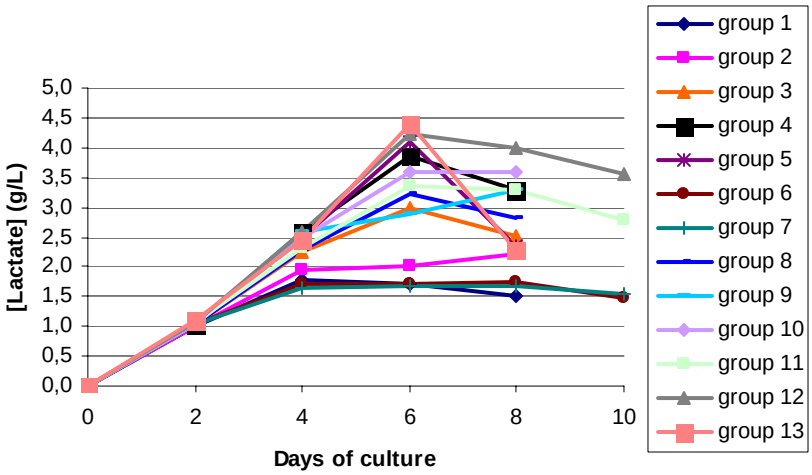

Figure 12. Average lactate concentrations in experiment 2.

Ammonia concentrations were measured on G4 and G8. The accumulation of ammonia was very similar in all tubes except for group 1 (batch) and group 2 (semi-batch) (Fig.35, Appendix III). The higher ammonia concentrations on G8 in these tubes could be explained by the small cultivation volumes. The cultures in other tubes were diluted by feeding, and had comparable ammonia concentrations.

Osmolarity was measured on G2, G6 and G10 (Fig.13). Groups 6 and 7 had the lowest osmolarities on G10. The osmolarities in groups 11 and 12 were higher. No alkali was added to any of the tubes. This suggested that the addition of amino acid cocktail somehow resulted in a higher osmolarity in the culture.

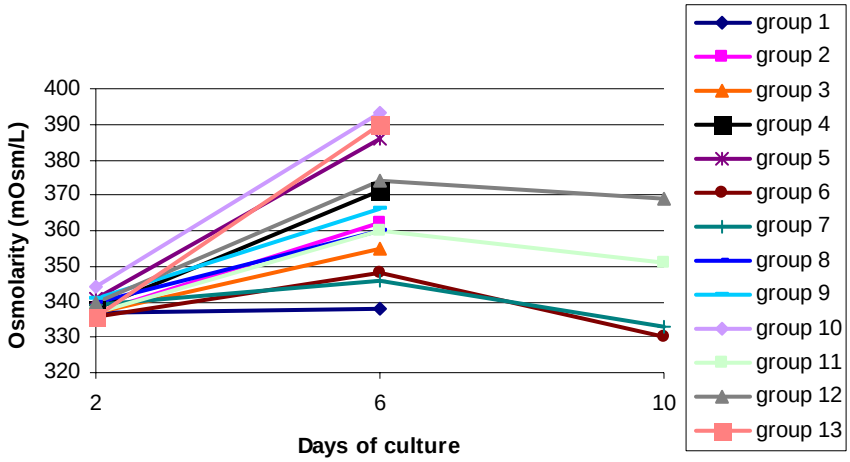

Figure 13. Average osmolarity. All groups, except for groups 6, 7, 11 and 12, were terminated on G8.

3.3 Experiment 3 - Peptone and amino acid dosage in spinners

The purpose of this experiment was to confirm the results obtained in experiment 2. Earlier studies at Biovitrum showed that the addition of peptones at the late stage of culture improved cell growth and viability. This was also tested in this experiment. The experimental setup is shown in Table 9. The amino acid feeding was modified in accordance with the results obtained from the amino acid analysis in experiment 1 (section 3.1.4). A spinner fed with feed medium only was included to better elucidate the possible positive effects of feeding peptone cocktail (spinner 3). In spinner 4 peptone cocktail was fed in late phase of cultivation. Peptone cocktail was fed in spinners 5 and 6 during the whole cultivation. Spinner 7 was fed only with amino acid cocktail. Spinners 8 and 9 were fed with combinations of peptone cocktail and amino acid cocktail. In spinner 10, the amino acid cocktail feeding was supposed to be replaced by peptone cocktail feeding from G8. Unfortunately, this spinner had to be terminated on G8 due to low viable cell number and viability. A batch spinner (spinner 1) and a semi-batch spinner (spinner 2) were also included.

Table 9. Experimental setup for experiment 3.

Spinner	Process	PEP in basal medium	Glc	Gln	AA	Feed medium	PEP feed	Comment
1	Batch	pea + cot 5 g/L	no	no	no	no	no	Batch
2	Semi batch	pea + cot 5 g/L	yes	yes	no	no	no	Semi batch
3	Fed-batch	pea + cot 5 g/L	yes	yes	no	65 ml G3, G6 and 28.4 ml G9	no	Feed only
4	Fed-batch	pea + cot 5 g/L	yes	yes	no	65 ml G3, G6 and 28.4 ml G9	1.2 g/L G8, G10	Pep feed late stage
5	Fed-batch	pea + cot 5 g/L	yes	yes	no	65 ml G3, G6 and 28.4 ml G9	1.2 g/L G2, G4, G6, 0.8 g/L G8, G10	Pep high, no aa
6	Fed-batch	pea + cot 5 g/L	yes	yes	no	65 ml G3, G6 and 28.4 ml G9	1.2 g/L G2, G4, G6, G8, G10	Pep high throughout, no aa
7	Fed-batch	pea + cot 5 g/L	yes	yes	6 µl/Mvc/day G2,G4,G6 & 4 µl/Mvc/day G8, G10	65 ml G3, G6 and 43 ml G9	no	Only aa
8	Fed-batch	pea + cot 5 g/L	yes	yes	6 µl/Mvc/day G2,G4,G6	65 ml G3, G6 and 43 ml G9	1.2 g/L G2, G4, G6 0.8 g/L G8, G10	Pep high, aa
9	Fed-batch	pea + cot 5 g/L	yes	yes	3 µl/Mvc/day G2,G4,G6	65 ml G3, G6 and 43 ml G9	1.2 g/L G2, G4, G6, G8, G10	Pep high, aa low
10	Fed-batch	pea + cot 5 g/L	yes	yes	6 µl/Mvc/day G2,G4,G6	65 ml G3, G6 and 43 ml G9	1.2 g/L G8, G10	Aa feeding followed by pep feeding
11	Fed-batch	pea + cot 5 g/L	yes	yes	1.5 µl/Mvc/day G6,G8,G10	65 ml G3, G6 and 28.4 ml G9	no	Low aa late phase
12	Fed-batch	pea + cot 5 g/L	yes	yes	1 µl/Mvc/day G6,G8,G10	65 ml G3, G6 and 28.4 ml G9	1.2 g/L G2, G4, G6, G8,G10	Pep+low aa late phase

In the middle of the experiment, it was found that feeding amino acid cocktail at a dose of 3-6 µl/Mvc/day was toxic to the cells. Therefore we expanded the experiment by adding two more spinners, 11 and 12. In spinners 11 and 12, small volumes of amino acid cocktail were fed during late phase of cultivation.

The starting volumes of the cultures in all spinners were 90 ml. Originally, the starting volumes were 100ml. Spinner 8 was terminated on G2 due to malfunction of the spinner stirrer. Therefore 10 ml from each of the remaining spinners was pooled together to form a replacement spinner. On G6, 76 ml from spinner 3 and 77 ml from spinner 4 were transferred to a new spinner 11. Likewise, 80 ml from spinner 5 and 80 ml from spinner 6 were transferred to another new spinner 12. The cultures in spinners 1, 2, 7 and 10 were terminated on G8. The rest of the cultures were studied for 10 days. The data presented below are normalized as if all the spinner cultures were started with the same volume, so that the data could be compared among the groups.

3.3.1 Cell growth and viability

Spinner 6 had the highest maximal number of viable cells and the best cell viability, followed by spinner 5 (Fig.14 and 15). This suggested that feeding peptones were beneficial to cell growth. Spinners 3 and 4 were very comparable, suggesting that feeding peptone cocktail in late phase of cultivation had no significant positive effect on cell growth. Among the spinners that were fed with amino acid cocktail, spinners 8 and 9, which were also fed with peptone cocktail, had better growth. The spinners fed with only amino acid cocktail grew significantly worse. These results indicated that the dose of amino acid feeding was toxic to the cells and the addition of peptones partially neutralized the toxic effect of overfeeding of the amino acids. The viable cell number and viability obtained in spinners 11 and 12 were not higher than those in spinners 3, 4, 5 and 6, indicating that the low dose of amino acid cocktail feeding was not beneficial. The low dose of amino acid cocktail feeding might still be toxic as indicated by the lower viable cell number and viability on G10. Spinner 12 had higher viable cell number and better viability than spinner 11, which again confirmed that the peptone feeding was beneficial to the viability (Fig.15).

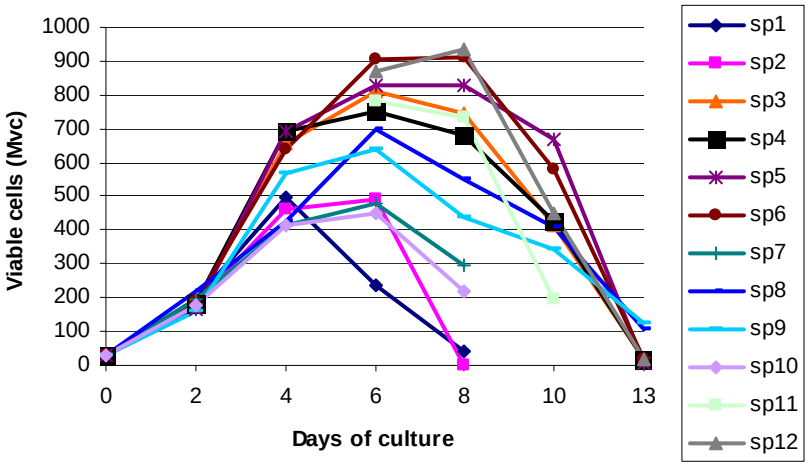

Figure 14. Number of viable cells in experiment 3.

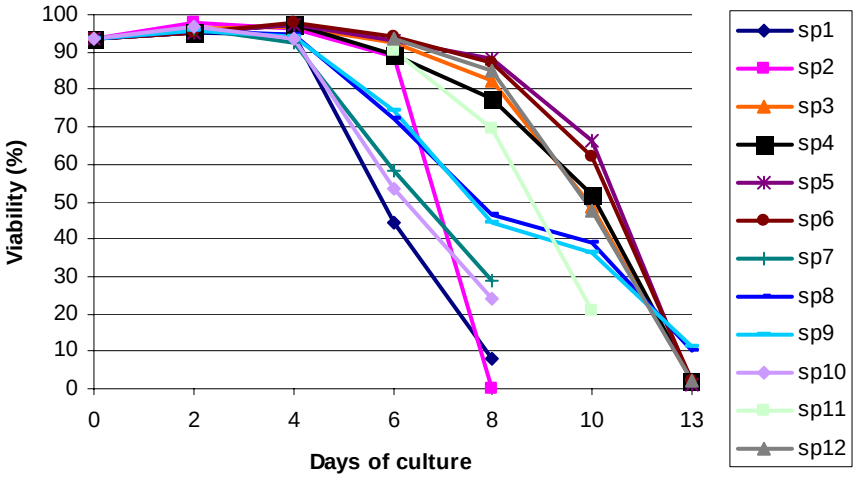

Figure 15. Viability in experiment 3.

We concluded that feeding peptone cocktail had positive effects on cell growth and viability. Higher number of viable cells and better viability was achieved in the cultures with the peptone cocktail feed than in the culture fed with the feed medium only. Lowering the dose of peptone feeding from G8 did not significantly affect the performance of the culture. We could also conclude that feeding amino acid cocktail was detrimental to the cell growth and that the addition of peptones could reduce the toxic effect of amino acid overfeeding.

3.3.2 Productivity

Antibody productivity was measured on samples from the 10 spinners originally included in the experiment. The antibody production in spinners 4, 5 and 6 was comparable and higher than in other spinners during the cultivation (Fig.16). The antibody production in spinner 3 was lower than that in the spinners 4, 5 and 6. The spinners fed with amino acid cocktail had significantly lower productivity. The addition of peptones could improve the antibody production in the cultures fed with amino acid cocktail. The cell specific productivity was comparable in both the peptone-fed spinners and the spinner fed with only feed medium.

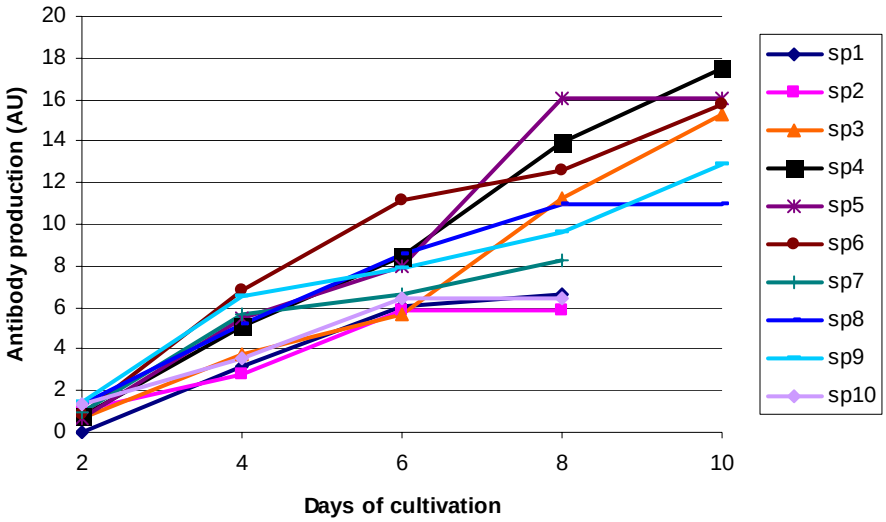

Figure 16. Accumulated antibody production in experiment 3.

3.3.3 Feeding, metabolite and osmolarity analyses

The glucose regulation was based on PDT and worked well without any depletions (Fig.36, Appendix IV). The lactate accumulation was higher in spinners 7-10, where amino acid cocktail had been fed (Fig.17).

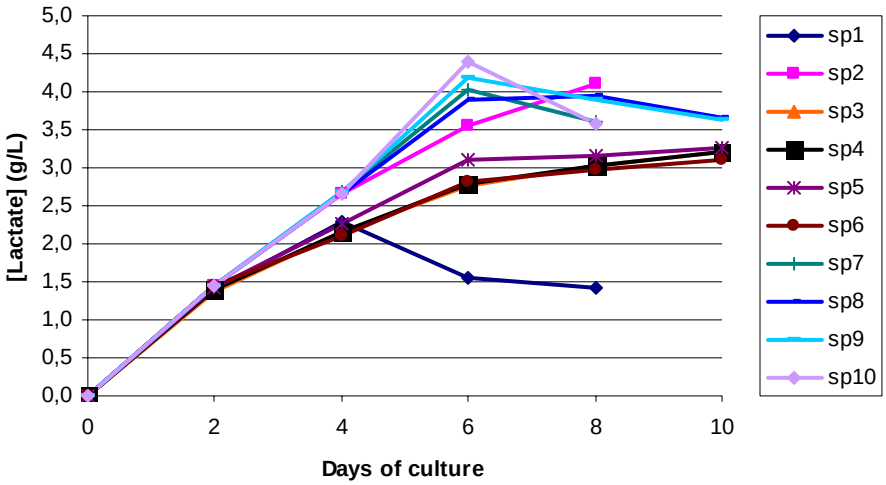

Figure 17. Lactate concentrations in the first 10 spinners in experiment 3.

Ammonia concentrations were measured on G4 and G8. The ammonia concentrations were comparable in all spinners, except for the semi-batch spinner, which had higher accumulation of ammonia (Fig.37, Appendix IV).

Osmolarity in spinners 3, 4, 5 and 6 was measured on G2, G6 and G10 (Fig.38, Appendix IV). The osmolarities were similar in all spinners. Similar amount of alkali was added to each of the spinners for pH adjustment.

3.3.4 Amino acid analysis

Supernatant samples from G2, G6 and G10 were sent to Uppsala University for amino acid analysis. The results for spinner 3 (feed medium only) indicated that only cysteine, methionine, histidine and tryptophan were depleted or nearly depleted (Fig.18). Of these amino acids, methionine, histidine and tryptophan are essential. All other amino acids had a concentration of 0.27 mM or higher on G10 (Fig.39, Appendix IV). This suggested that the amino acid content in the feed medium was sufficient to cover the cell consumption of all amino acids, except for the four mentioned above. The amino acid concentration profile of spinner 5 (peptone cocktail + feed medium) was very similar to that of spinner 3. Feeding the RPMI1640+ to the culture resulted in a toxic effect and significantly higher accumulation of amino acids (Fig.40, Appendix IV).

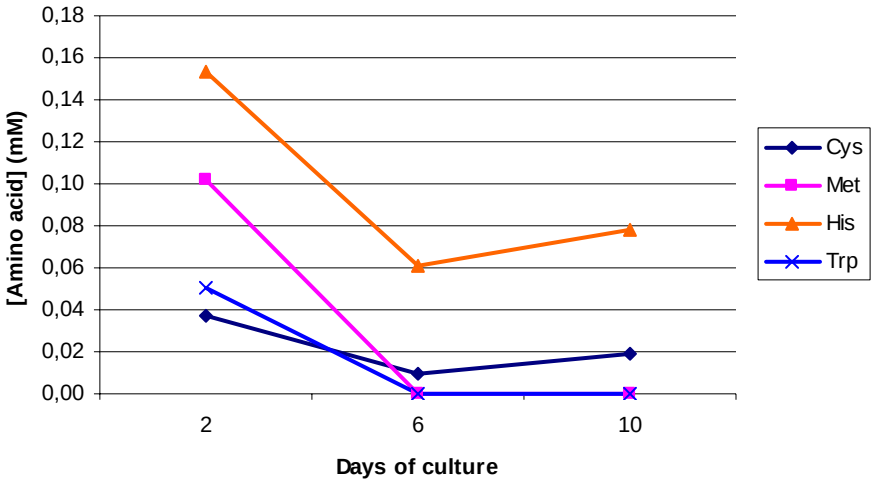

Figure 18. Depleted (or nearly depleted) amino acids in spinner 3.

3.4 Experiment 4 - Effects of peptones and amino acids in 3 L bioreactor

The aim of this experiment was to test if feeding peptone cocktail could improve the fed-batch process at bioreactor scale. The experimental setup is shown in Table 10. Two 3 L glass bioreactors (Applikon Biotechnology, Schiedam, Netherlands) were inoculated with the CHO cells in a comparable way and the process was controlled according to the parameters shown in Table 3. The target cell density for inoculation was 0.3 Mvc/ml and the culture volume after inoculation was 850 ml in both reactors. The agitation rate was set to 100 rpm at inoculation. It was then increased to 120 rpm and further to 150 rpm later during the process to obtain sufficient aeration for the cells. Both reactors were fed with glucose and glutamine as described in section 2.6.1 and with feed medium according to Table 17, Appendix V. System 1 was fed with glucose, glutamine, feed medium and the peptone cocktail (0.6 g/L/day) as well as a low dose of amino acid cocktail (0.5 µl/Mvc/day). System 2 was fed with glucose, glutamine, feed medium and a low dose of amino acid cocktail (0.5 µl/Mvc/day). No peptone cocktail was added to system 2.

Table 10. Experimental setup for experiment 4.

Bioreactor	Process	Peptones in base medium	Glc	Gln	AA	Feed medium	PEP feed
System 1	Fed-batch	pea + cot 5 g/L	yes	yes	0.5 µl/Mvc/day	yes	0.6 g/L/day
System 2	Fed-batch	pea +cot 5 g/L	yes	yes	0.5 µl/Mvc/day	yes	no

3.4.1 Cell growth and viability

The maximal number of viable cells was comparable in both reactors (Fig.19). System 1 reached the maximal number of viable cells on G6, while system 2 had the maximal number of viable cells on G5. In system 2 the viability and number of viable cells declined very rapidly after G5. In system 1 the maximal number of viable cells remained more or less constant from G6 to G8. On G8 the viability was still 91% in system 1. Both systems were terminated when the viabilities had declined below 80%. System 2 was terminated on G6, while system 1 was

terminated on G9. These results indicated that feeding peptone cocktail significantly improved the cell viability and hence the longevity of the process.

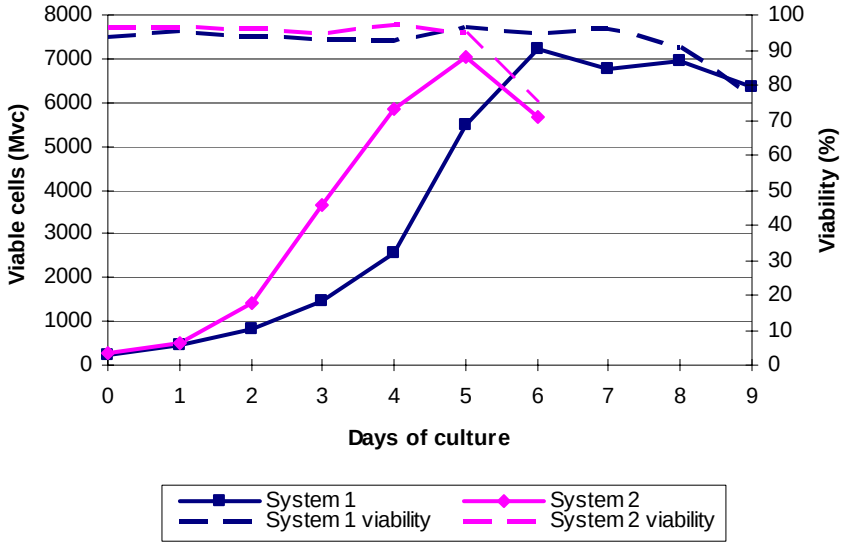

Figure 19. Number of viable cells and viability in experiment 4.

3.4.2 Productivity

The samples from the reactor cultures were analyzed in two separate ELISA analyses and the average values of the accumulated antibody titers are presented in Fig.20. It was found that 11% more antibody was produced in system 1, indicating that the peptones are beneficial for productivity. The cell specific productivity was very similar in both systems (Fig.44, Appendix V).

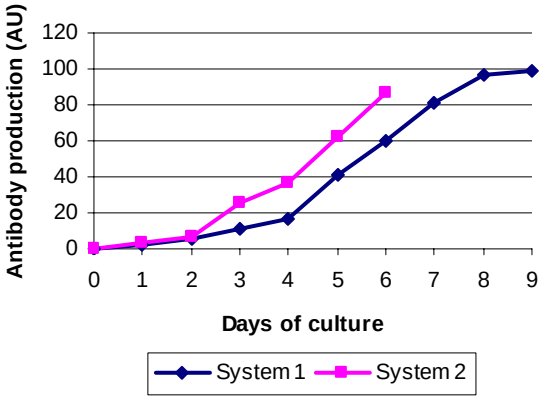

Figure 20. Accumulated antibody production in experiment 4.

3.4.3 Feeding, metabolite and osmolarity analyses

Feed medium was added according to a fixed feeding scheme (Table 17, Appendix V). The amounts of glucose, glutamine and amino acid cocktail to feed each day were calculated using

Eq.7 and Eq.10 and pooled together with the feed medium and peptone cocktail in a LAF-bench. The feed bottles were then welded onto tubings, connected to the reactors and the feed solution was pumped into the reactors continuously overnight. Twice the volume of peptone cocktail was accidently added to system 1 on G2. To compensate for this, no peptone cocktail was added on G3. Accumulated additions of nutrients can be found in Table 11. The culture volumes in both reactors were comparable throughout the cultivation (Fig.45, Appendix V).

Table 11. Accumulated additions of nutrients (ml).

Reactor	Feed cocktail	Glucose (400 g/L)	Glutamine (200 mM)	Peptones (40 g/L)	Na_2CO_3 (0.5 M)	RPMI 1640+
System 1	1171	47.2	63.9	102.4	120	20.0
System 2	710	48.3	55.9	0	100	15.1

The glucose concentration was low in system 2 on G3 and G6 (Fig.21). This could however not explain the rapid decline in viable cell number and viability in this system from G5, since the glucose concentration was not low on G4. System 2 had a higher accumulation of lactate than system 1 (Fig.21). The lactate concentration was 3.7 g/L on G6 in system 1, while system 2 on G6 had a lactate concentration of 6.3 g/L. Similarly, low accumulation of lactate was earlier observed for spinners fed with peptone cocktail in experiments 2 and 3 (Fig.12 and Fig.17).

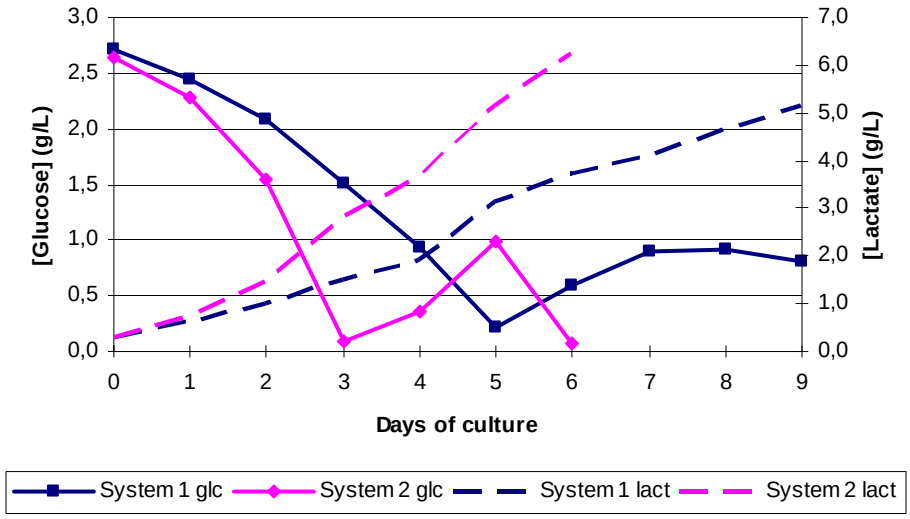

Figure 21. Glucose and lactate concentrations in both systems.

Regarding the accumulation of ammonia, system 1 had an ammonia concentration of 3.5mM on G6. System 2 had an ammonia concentration of 4.4 mM on G6 (Fig.22).

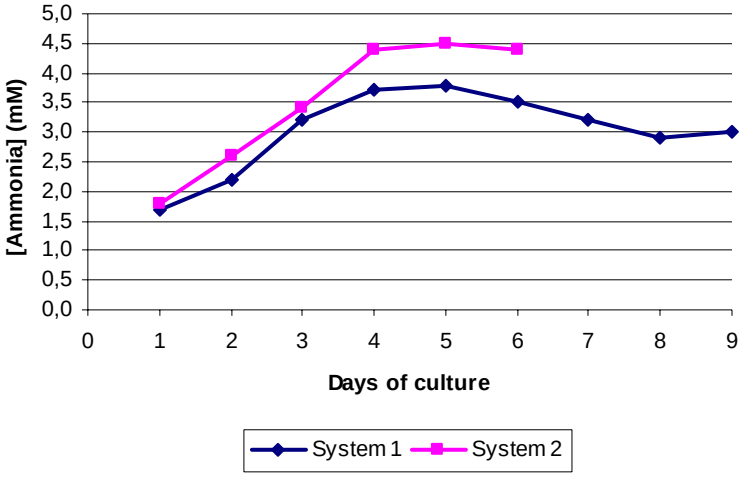

Figure 22. Ammonia concentrations in both systems.

The culture in system 2 had a higher osmolarity than that in system 1 (Fig.23). The higher osmolarity in system 2 could be explained by the higher lactate concentrations in this system. The higher lactate production resulted in more alkali addition, hence a higher osmolarity.

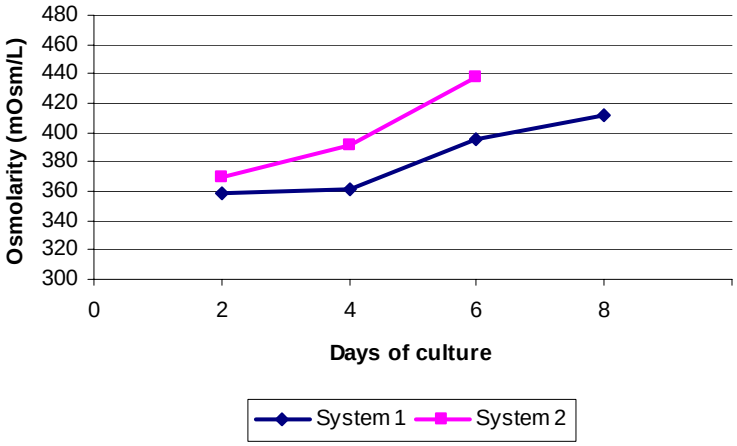

Figure 23. Osmolarity in both systems.

3.4.4 System regulation

The regulation of pH, DOT and temperature worked well in both systems (Fig.24 and Fig.25). pH was automatically regulated upwards by addition of 0.5 M Na_2CO_3 when needed using a peristaltic pump and downwards by pulsed addition of CO_2 to the headspace. Temperature was automatically controlled using a heat blanket wrapped around the reactor. DOT was automatically controlled upwards by pulsed addition of O_2 through a sparger.

Both reactors were inoculated 4 hours after the pH and temperature were stabilized at 7.1 and 37°C, respectively. It was aimed to maintain the DOT at the set point of 40%. At inoculation, the DOT was as high as 80%. It was decreased to 40% by adding N_2 to the headspace, since the consumption of oxygen by the cells was very low at a low cell density. DOT fluctuated between 35-50% at the early stage of culture. With the increase of cell density in the culture, the DOT regulation became stable.

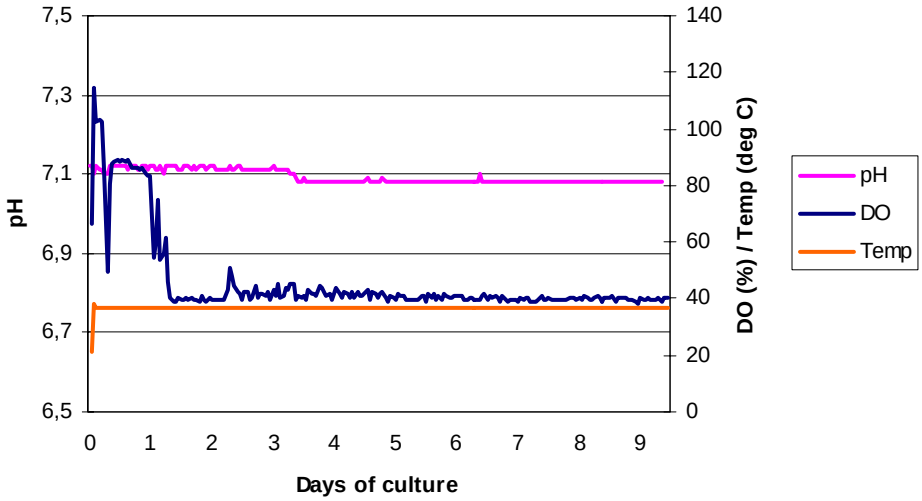

Figure 24. On-line values from BioXpert® log file for system 1.

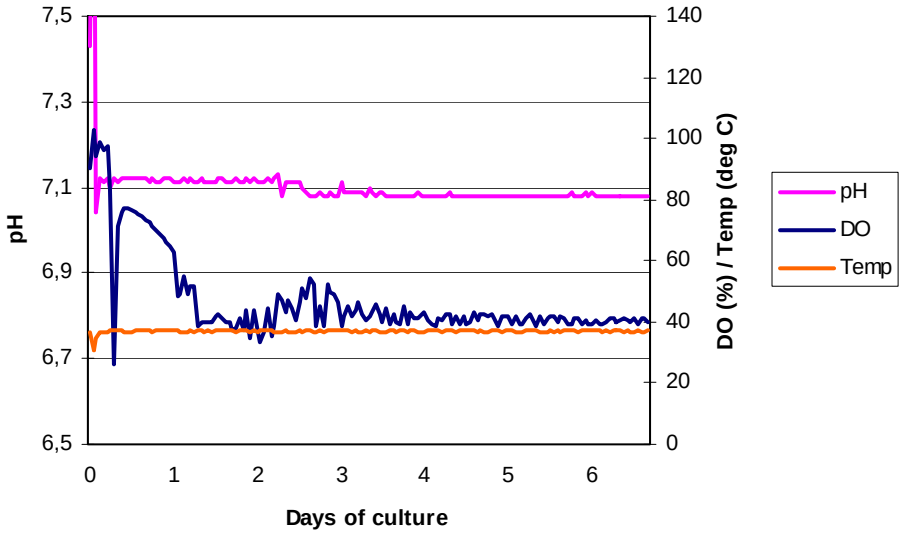

Figure 25. On-line values from BioXpert® log file for system 2.

3.5 Experiment 5 - Peptone addition in disclosed serum free DMEM/F12

The purpose of this experiment was to verify the beneficial effect of peptone feeding in a process using a disclosed medium. Serum free Dulbecco´s Modified Eagle´s Medium Nutrient mixture F-12 HAM (DMEM/F12; Sigma-Aldrich) enriched with vitamins, metals, biosynthesis precursors and pyruvate was used in this experiment. A medium based on 3X concentrated DMEM/F12 (Sigma-Aldrich) was used as the feed medium (section 2.2.2). Earlier studies at Biovitrum showed an increase in viable cell number and viability when peptones were added at a later phase of culture in the BVT4 medium. In this experiment, it was also aimed to investigate if feeding the peptones at a later phase of culture, when the cell viability had started to decline, could improve the cell viability and hence the longevity of the process.

The experiment included 7 spinners with starting volumes of 80 ml and seeding cell densities of 0.3 Mvc/ml. Spinner 1 was a batch control. The feed medium was added to the fed-batch spinners 2-7. Spinner 2 and 3 were duplicates in which no peptone cocktail was fed. Spinner 4

and 5 (duplicate) were fed with peptone cocktail during stationary phase. Spinner 6 and 7 (duplicate) were fed with peptone cocktail throughout the cultivation. All spinners except for spinner 1 were run for 16 days. The experimental setup is shown in Table 12.

Table 12. Experimental setup for experiment 5.

Spinner	Process	PEP in basal medium	Glc	Gln	AA	Feed medium	PEP feed
1	Batch	pea + cot 5 g/L	no	no	no	no	no
2	Fed-batch	pea + cot 5 g/L	yes	yes	no	15 ml G2; 40 ml G4,G6,G8; 15 ml G10,G12,G14	no
3	Fed-batch	pea + cot 5 g/L	yes	yes	no	15 ml G2; 40 ml G4,G6,G8; 15 ml G10,G12,G14	no
4	Fed-batch	pea + cot 5 g/L	yes	yes	no	15 ml G2; 40 ml G4,G6,G8; 15 ml G10,G12,G14	pea + cot (1.2 g/L G12,G14)
5	Fed-batch	pea + cot 5 g/L	yes	yes	no	15 ml G2; 40 ml G4,G6,G8; 15 ml G10,G12,G14	pea + cot (1.2 g/L G12,G14)
6	Fed-batch	Pea + cot 5 g/L	yes	yes	no	15 ml G2; 40 ml G4,G6,G8; 15 ml G10,G12,G14	pea + cot (1.2 g/L G2, G4, G6,G8,G10,G12,G14)
7	Fed-batch	pea + cot 5 g/L	yes	yes	no	15 ml G2; 40 ml G4,G6,G8; 15 ml G10,G12,G14	pea + cot (1.2 g/L G2,G4,G6,G8,G10,G12,G14)

3.5.1 Cell growth and viability

The cell growth was very similar in all the fed-batch spinners. Spinners 6 and 7 had the highest maximal number of viable cells on G12 (Fig.26). The maximal viable cell number achieved in the BVT4 medium was 30% higher than in the DMEM/F12 (Sigma-Aldrich) medium. In the BVT4 medium the maximal number of viable cells was usually reached on the 6^{th} or 8^{th} day of growth. Since the cells grew slower in the DMEM/F12 (Sigma-Aldrich) medium, the maximal number of viable cells was not reached until G12. The PDT was approximately 30 hours on G2 in DMEM/F12 (Sigma-Aldrich) medium, while it was approximately 20 hours in the BVT4 medium.

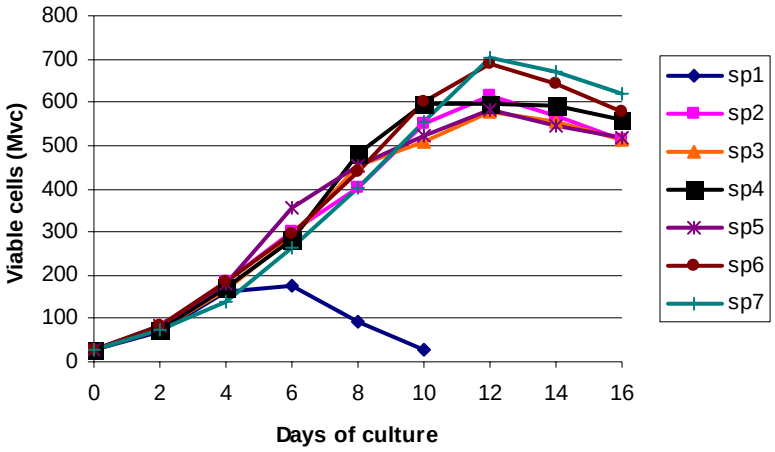

Figure 26. Number of viable cells in experiment 5.

The intention was to start feeding peptone cocktail in spinners 4 and 5 when the viable cell number and viability started to decline. Since the cells grew very slowly and due to limited time, we had to start peptone feeding on G12, when the viability was still around 80% (Fig.27). Spinners 4 and 5 were very similar to spinners 2 and 3, in which only feed medium was fed. Viability and viable cell number did not increase after feeding the peptone cocktail at the later phase of culture.

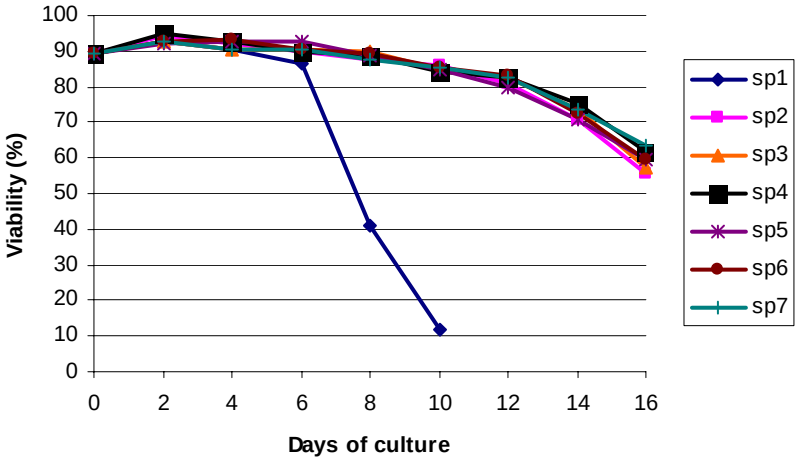

Figure 27. Cell viability in all the cultures in experiment 5.

3.5.2 Productivity

The best antibody accumulations were achieved in spinners 6 and 7. This was in accordance with the high accumulation of antibody observed in spinners fed with peptone cocktail throughout the cultivation period in earlier experiments in this study. Feeding the peptone cocktail at a later stage of the culture did not improve the antibody production (spinners 4 and 5 vs. spinners 2 and 3).

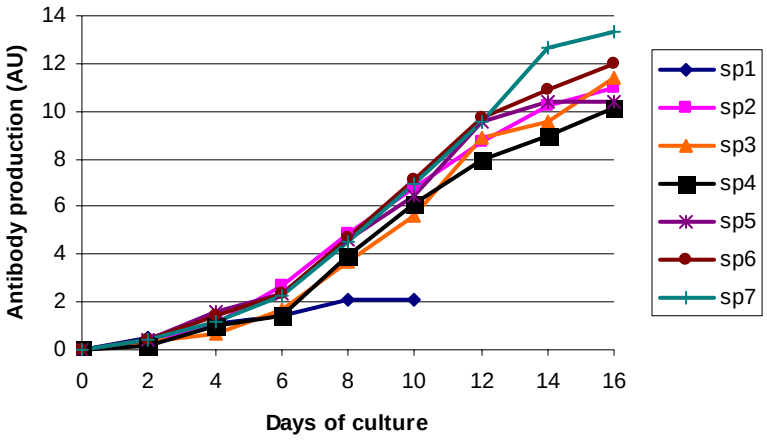

Figure 28. Accumulated antibody production in all spinners in experiment 5.

3.5.3 Feeding, metabolite and osmolarity analyses

Feed medium was fed every second day according to the feeding scheme in Table 12. Glucose was fed only on G2, since it was later noticed that the feed medium contained glucose and no additional glucose feed was needed. The glucose concentration was approximately 4.0 g/L in all the fed-batch spinners (Fig.48, Appendix VI). Glutamine was well maintained at the target concentration. The accumulated concentrations of ammonia and lactate were very similar in the fed-batch spinners. The fact of low accumulation of lactate observed in spinners fed with peptone cocktail in BVT4 medium was not observed in this experiment with the serum free DMEM/F12 (Sigma-Aldrich) medium.

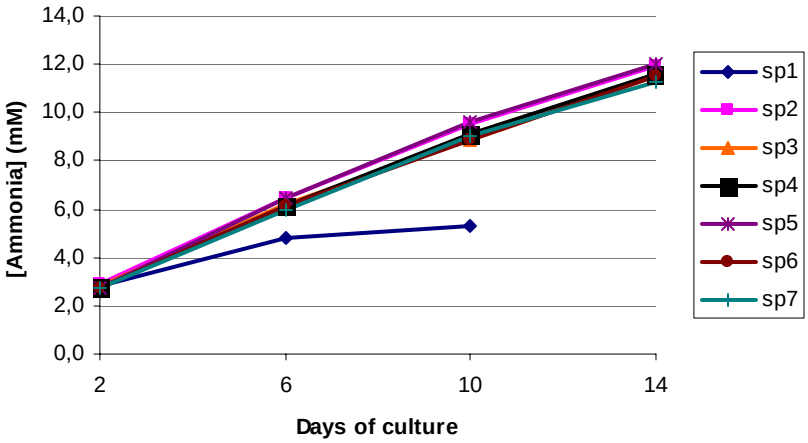

Figure 29. Ammonia concentrations in experiment 5.

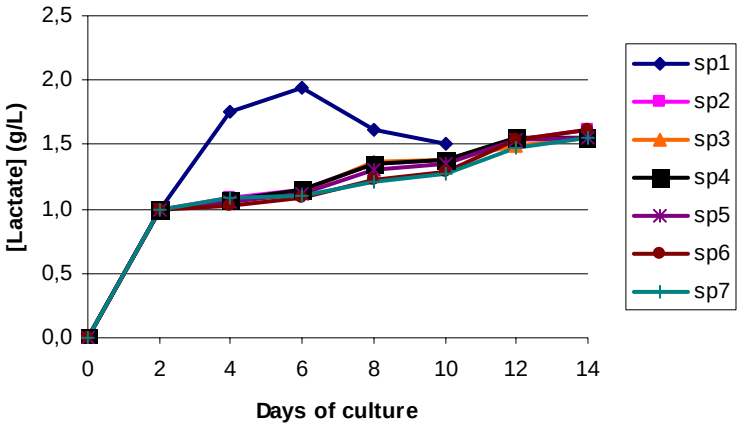

Figure 30. Lactate concentrations in experiment 5.

4. Discussion and conclusions

In the small-scale experiments, a wide range of combinations of peptone cocktail and amino acid cocktail feeding were tested. The best cell growths and productivities were achieved in cultures fed with peptones but no amino acid cocktail, suggesting that the beneficial effects from feeding peptones could not be reproduced by feeding amino acids. Feeding the RPMI1640+ amino acid cocktail at the dose of 6 µl/Mvc/day or higher was detrimental to the process. It was found that many amino acids were overfed, which could be toxic to the cells. It has been reported earlier that overfeeding of amino acids had a toxic effect on hybridoma cells [17].

The cultures that were fed with combinations of peptone cocktail and amino acid cocktail always performed better than the cultures that were fed with amino acid cocktail only. This suggested that the peptone feeding could reduce the toxic effect of the amino acid overfeeding.

The effect of peptone feeding was further investigated in fed-batch cultures in 3 L bioreactors. It was confirmed that the peptone feeding improved the cell viability and hence the longevity of the process. 11% more antibody was produced in the peptone-fed culture and the good viability in this culture might also result in production of antibody in good quality. The beneficial effect of peptone feeding was also reinforced in a process based on disclosed serum free DMEM/F12 medium (Sigma-Aldrich).

Only 4 amino acids (cysteine, methionine, valine and tryptophan) were depleted, or almost depleted, in the culture fed with feed medium only but no amino acid cocktail or peptone cocktail. Feeding the peptones did not change the amino acid concentration profile. This indicated that the amino acid content of the feed medium is enough to cover the cell consumption of all amino acids except for the four mentioned above. Therefore instead of feeding the RPMI1640+ cocktail (Sigma-Aldrich), the feed medium could be enriched with appropriate amounts of cysteine, methionine, valine and tryptophan to achieve a better balanced amino acid composition that meets the amino acid need of the cells.

Feeding peptone cocktail at an initial dosage of around 1 g/L was beneficial for cell growth, viability, and cultivation longevity. Since feeding the peptones did not change the amino acid concentration profile, the positive effect was probably not due to providing amino acids to the cells. Specialized transporters, which differ from amino acid transporters, are responsible for the uptake of peptones in mammalian cells. Inside the cells, the peptones are digested by proteases resulting in free amino acids that can be used as nutrients in a variety of ways. Bonarius *et al.* believe that the uptake of peptones in hybridoma cells is more energy efficient than amino acid uptake by the cells [15]. Burteau *et al.* suggest that some large bioactive oligopeptides supplied by the peptones may mimick growth factors or act anti-apoptotically and thus boost the cell growth and viability [10]. The peptones also contain vitamins and trace elements, which may further contribute to an enhancement of cell growth [14].

Regarding the product quality in cultures fed with peptones, Gu *et al.* observed poor glycosylation in interferon-γ produced by CHO cells in medium supplemented with peptones [14]. On the other hand, Heidemann *et al.* showed that the glycosylated protein product was intact and active when using serum-free medium containing plant peptones [12]. Unfortunately, the glycosylation profile of the antibody in this study could not be investigated due to the constraints of resource and time.

Low accumulation of by-products is considered to be of great benefit for both viability and productivity [6]. A low level of accumulated lactate is positive, since lactate buildup indirectly

causes the medium osmolarity to increase [18]. High lactate accumulation was observed in cultures fed with amino acid cocktail. The overfeeding of amino acids may stress the cells and alter the cell metabolism. The lactate accumulation was lower and comparable in the culture fed with only feed medium and in the cultures fed with feed medium and peptone cocktail.

In summation, the results obtained in this study suggest that feeding peptone cocktail is beneficial to cell growth, viability, culture longevity and productivity. These beneficial effects could not be reproduced by feeding amino acid cocktail. It was found that overfeeding the amino acids is toxic to the cells. The peptones can partially neutralize the toxic effect of amino acid overfeeding. To further improve this fed-batch process, we suggest to supplement the feed medium with cysteine, methionine, valine and tryptophan instead of feeding the RPMI1640+ amino acid cocktail (Sigma-Aldrich).

5. Acknowledgements

I would like to thank Catherine Ljung for giving me the opportunity to do my examwork at Biovitrum. A special thank to my supervisor Yun Jiang for always being very helpful and patient in answering all my questions and for interesting scientific discussions. Thank you, Véronique Chotteau and Caroline Wåhlgren, for helping me during the beginning of this project. Thanks to Nathalie Chatzissavidou for sharing your profound knowledge in mammalian cell cultivation. I am very grateful to Helena Pettersson and Jeanette Westin for all your help and guidance in the lab. Many thanks to prof. Lena Häggström, Royal Institute of Technology for scientific reviewing of this thesis. I also thank Mårten Hellberg and Björn Garpefjord for critical reading of the report. Finally, I would like to thank all the people at Biovitrum Cultivation – Expression for the friendly atmosphere and always making me feel very welcome.

We thank the company Symphogen, Denmark, for providing the antibody producing CHO cell line. A special thank to Dr. Anne Bondgaard Tolstrup and Dr. Soren Bregenholt for their kind support to get this permission and helping me in the publication release of this work. We also thank the company Kerry for providing the Kerry/Quest peptones.

6. References

1. Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl. Microbiol. Biotechnol. 68: 283-291.

2. Scott C. 2004. Microbial fermentation. BioProcess International. 2: 8-20.

3. Scott C. 2004. Animal cell culture. BioProcess International. 2: 22-32.

4. Enfors S-O, Häggström L. 1998. Bioprocess Technology Fundamentals and Applications. Royal Institute of Technology, Stockholm.

5. Bibila T.A, Ranucci C.S, Glazomitsky K, Buckland B.C, Aunins J.G. 1994. Monoclonal Antibody Process Development Using Medium Concentrates. Biotechnol. Prog. 10: 87-96.

6. Gambhir A, Europa A.F, Hu W-S. 1999. Alteration of Cellular Metabolism by Consecutive Fed-Batch Cultures of Mammalian Cells. Journal of Bioscience and Bioengineering 87(6): 805-810.

7. Freshney I.R. 2000. Culture of Animal cells - A manual of basic technique. 4th ed. Wiley-Liss, Hoboken, NJ.

8. Lao M-S, Toth D. 1997. Effects of Ammonium and Lactate on Growth and Metabolism of a Recombinant Chinese Hamster Ovary Cell Culture. Biotechnol. Prog. 13: 688-691.

9. Ducommun P, Ruffieux P-A, von Stockar U, Marison I. 2001. The role of vitamins and amino acids on hybridoma growth and monoclonal antibody production. Cytotechnology 37: 65-73.

10. Burteau C.C, Verhoeye R, Mols J.F, Ballez J-S, Agathos S.N, Schneider Y-J. 2003. Fortification of a protein-free cell culture medium with plant peptones improves cultivation and productivity of an interferon-γ-producing CHO cell line. In Vitro Cell. Dev. Biol. – Animal. 39: 291-296.

11. Hewlett G. 1991. Strategies for optimising serum-free media. Cytotechnology. 5: 3-14.

12. Heidemann R, Zhang C, Qi H, Rule J.L, Rozales C, Park S, Chuppa S, Ray M, Michaels J, Konstantinov K, Naveh D. 2000. The use of peptones as medium additives for the production of a recombinant therapeutic protein in high density perfusion cultures of mammalian cells. Cytotechnology 32: 157-167.

13. Sung Y.H, Lim S.W, Chung J.Y, Lee G.M. 2004. Yeast hydrolysate as a low-cost additive to serum-free medium for the production of human thrombopoietin in suspension cultures of Chinese hamster ovary cells. Appl. Microbiol. Biotechnol. 63: 527-536.

14. Gu X, Xie L, Harmon B.J, Wang D.I.C. 1997. Influence of Primatone RL Supplementation on Sialylation of Recombinant Human Interferon-γ Produced by Chinese Hamster Ovary Cell Culture Using Serum-Free Media. Biotechnol. Bioeng. 56(4): 353-360.

15. Bonarius H, Hatzimanikatis V, Meesters K.D, de Gooijer K, Schmid G, Tramper J. 1996. Metabolix flux analysis of hybridoma cells in different culture media using mass balances. Biotechnol. Bioeng. 50: 299-318.

16. Reddy S, Bauer K.D, Miller W.M. 1992. Determination of antibody content in live versus dead hybridoma cells: Analysis of antibody production in osmotically-stressed cultures. Biotechnol. Bioeng. 40: 947-964.

17. Stoll T.S, Mühlenthaler K, von Stockar U, Marison I.W. 1996. Systematic improvement of a chemically-defined protein-free medium for hybridoma growth and monoclonal antibody production. Journal of Biotechnology. 45: 111-123.

18. Bibila T.A, Robinson D.K. 1995. In pursuit of the Optimal Fed-Batch Process for Monoclonal Antibody Production. Biotechnol. Prog. 11: 1-13.

7. Appendices

Appendix I - Materials and methods

Table 13. Composition of RPMI1640 amino acids solution.

Amino acid	RPMI1640 (mg/L)	RPMI1640 (µM)	MW (g/mole)
Arginine-HCl	12000	56953	210.7
Asparagine-H_2O	2840	18921	150.1
Aspartic Acid	1000	7513	133.1
Cystine.2HCl	2500	7982	313.21
Glutamic Acid	1000	6798	147.1
Glycine	500	6658	75.1
Histidine-HCl-H_2O	1000	4771	209.6
Isoleucine	2500	19055	131.2
Leucine	2500	19055	131.2
Lysine-HCl	2000	10947	182.7
Methionine	750	5027	149.2
Phenylalanine	750	4540	165.2
Proline	1000	8688	115.1
Serine	1500	14272	105.1
Threonine	1000	8396	119.1
Tryptophan	250	1225	204
Tyrosine	1000	5519	181.2
Valine	1000	8540	117.1

Table 14. Additives of DMEM/F12 base medium.

Component	Concentration
DMEM/F12 (Sigma, D0547)	1X
$NaHCO_3$	1.2 g/L
Na Selenite	0.025 mg/L
β-Cyclodextrin	1 g/L
Pluronic F68	0.5 g/L
Asparagine	0.33 g/L
Ferric Citrate	0.14 g/L
Glutamic acid	0.588g/L
BME Vitamins (100X stock)	1X
recInsulin	10 mg/L

Table 15. Additives of DMEM/F12 feed medium.

Component	Concentration/volume
DMEM/F12 w/o NaCl	3X
dH_2O	200 ml
DMEM/F12 powder for 1L	1X
$NaHCO_3$	1.2 g/L
$Na2SeO_3$	0.025 mg/L
β-Cyclodextrin	2.0 g/L
Pluronic F68	0.5 g/L
Asparagine	0.8 g/L
Ferric Citrate	0.28 g/L
Glutamic acid	0.588 g/L
BME Vitamines (100X stock)	2X
Serine	0.3 g/L
Methionine	0.06 g/L
Tryptophan	0.04 g/L
recInsulin	10 mg/L

Appendix II – Experiment 1

Table 16. Average cell specific productivity from G2 to G8 for all the spinners in experiment 1.

Spinner	Average q_p (AU)
1	5.9
2	4.9
3	4.9
4	4.8
5	5.2

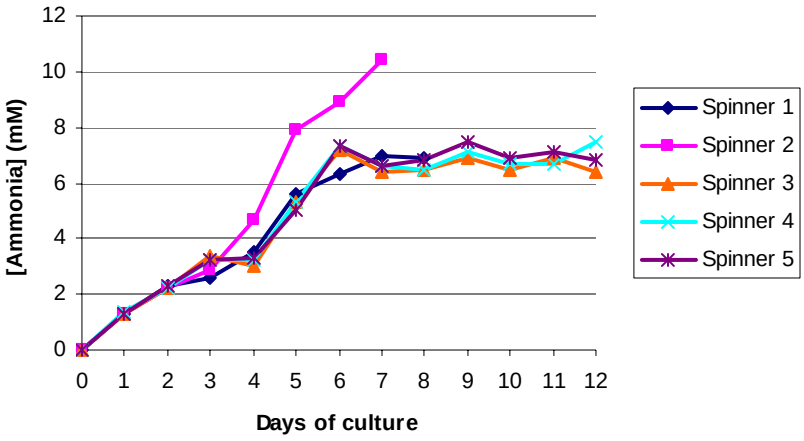

Figure 31. Ammonia concentrations in all spinners in experiment 1.

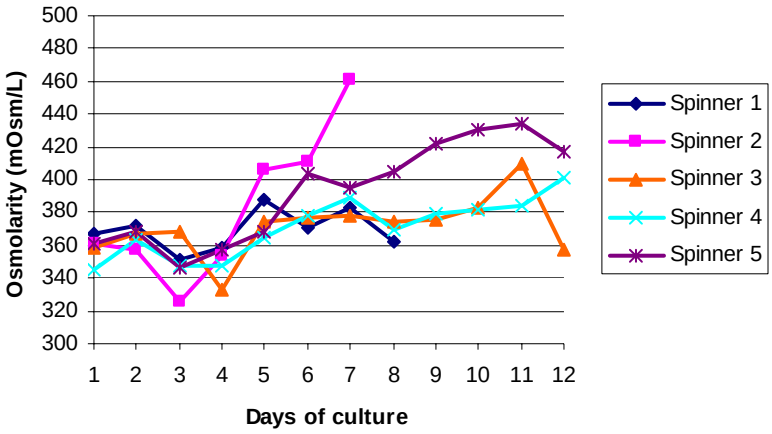

Figure 32. Osmolarity in all spinners in experiment 1.

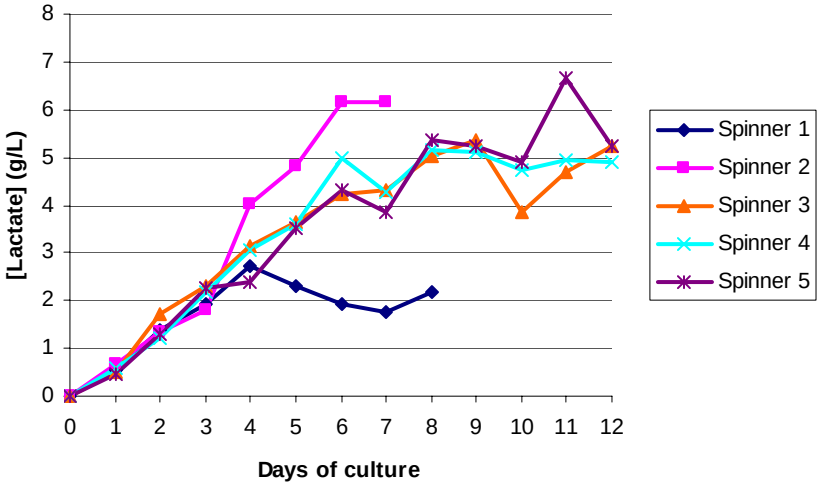

Figure 33. Lactate concentrations in experiment 1.

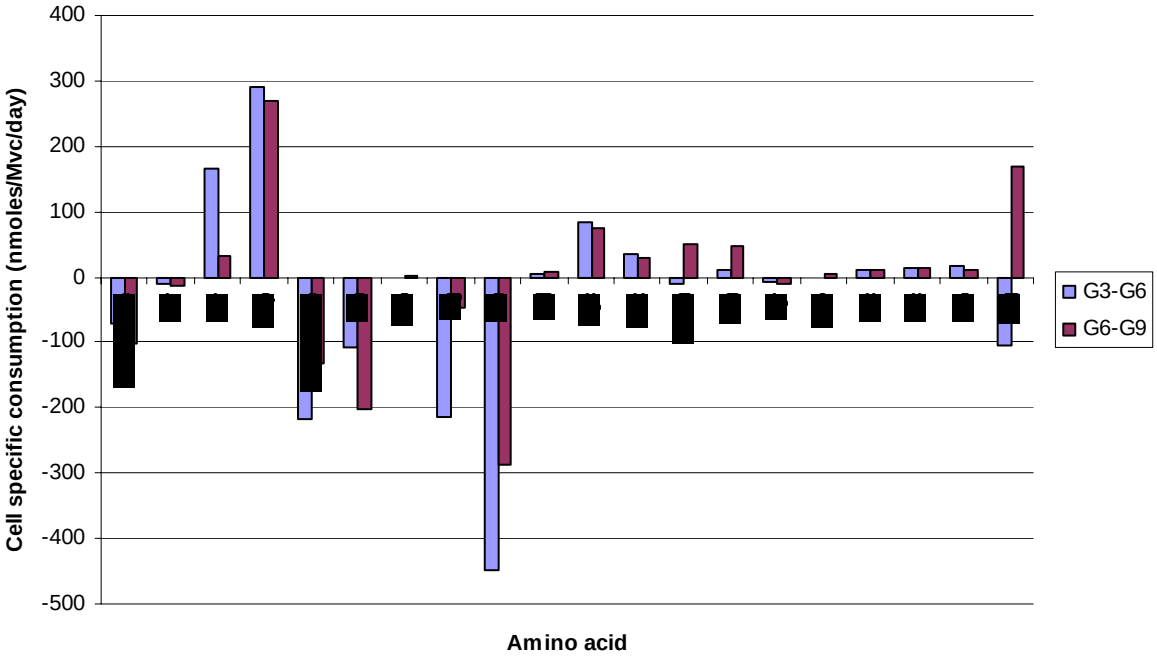

Figure 34. The cell specific consumption of each amino acid in spinner 3 in experiment 1.

Appendix III – Experiment 2

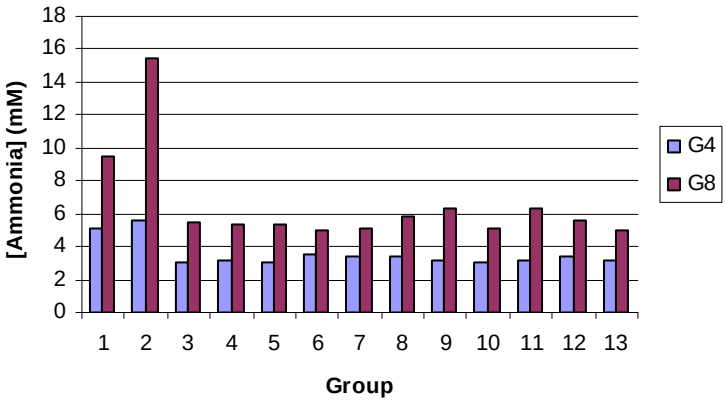

Figure 35. Ammonia concentrations in all groups in experiment 2.

Appendix IV – Experiment 3

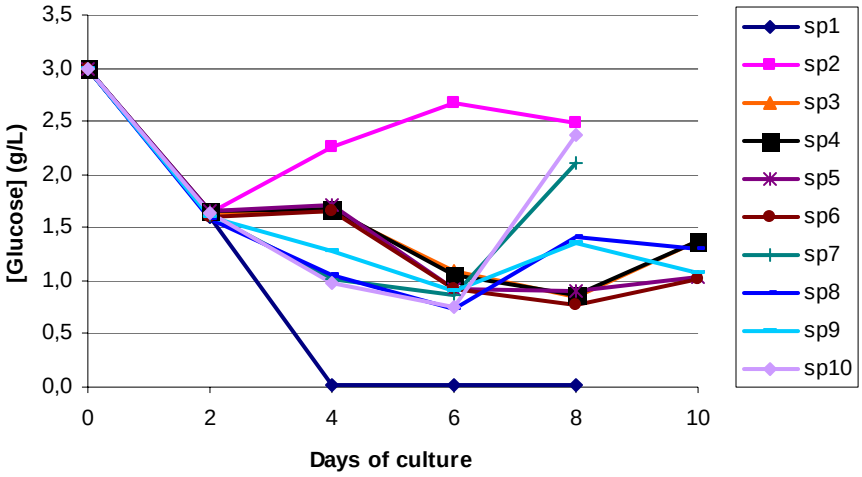

Figure 36. Glucose concentrations in experiment 3.

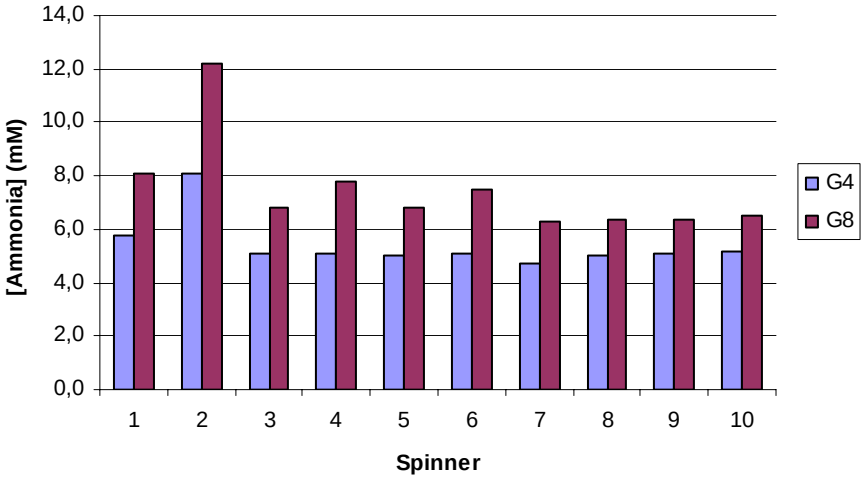

Figure 37. Ammonia concentrations on G4 and G8 in experiment 3.

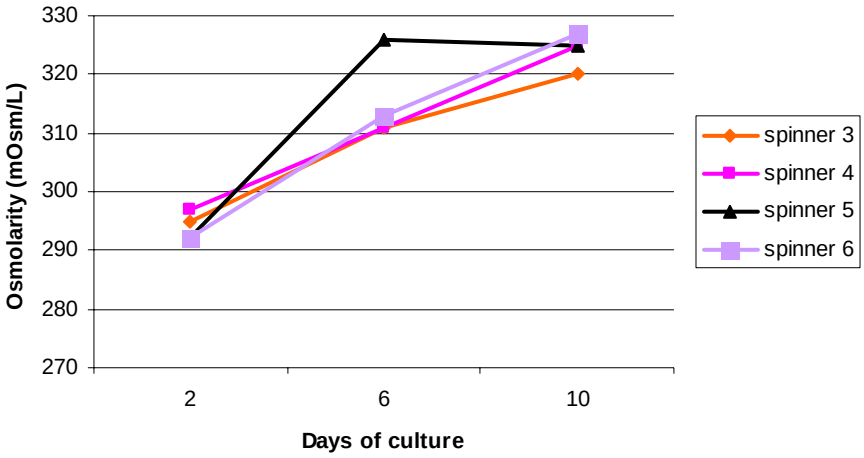

Figure 38. Osmolarity in spinners 3, 4, 5 and 6 in experiment 3.

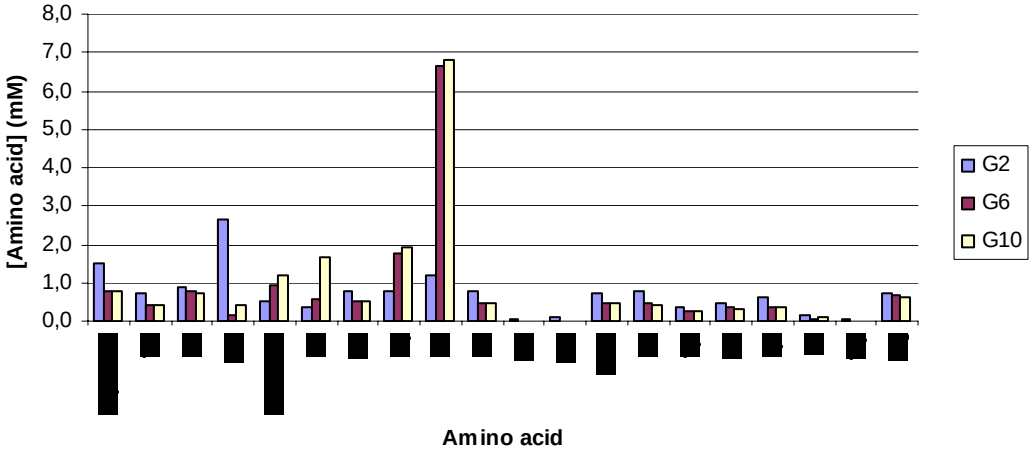

Figure 39. Amino acid concentrations in spinner 3 (only feed cocktail feeding) on G2, G6 and G10. Note that only Cys, Met, His and Trp are depleted.

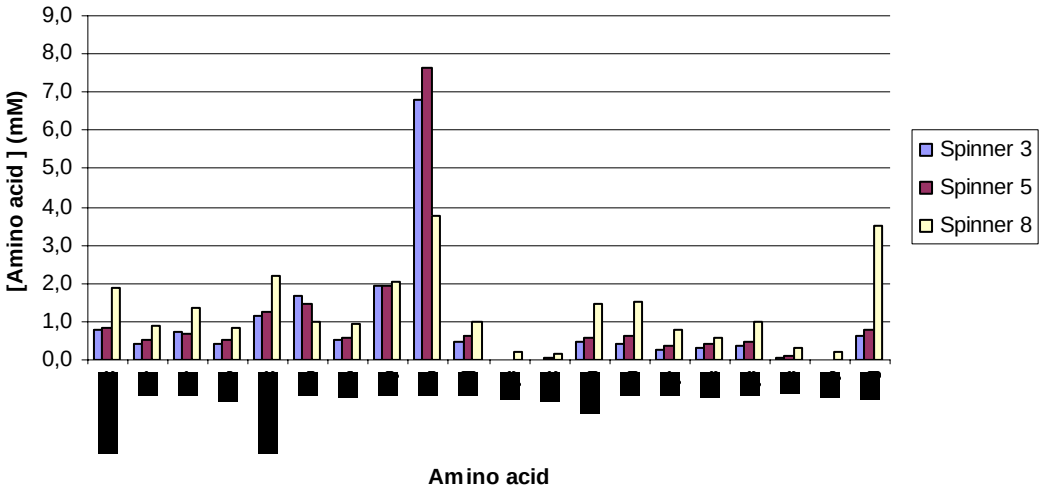

Figure 40. Amino acid concentrations in spinner 3 (only feed medium feeding), spinner 5 (feed medium + peptone cocktail feeding) and spinner 8 (feed medium, peptone cocktail and amino acid cocktail feeding) on G10. Feeding the amino acid cocktail resulted in a significantly higher accumulation of amino acids.

Appendix V – Experiment 4

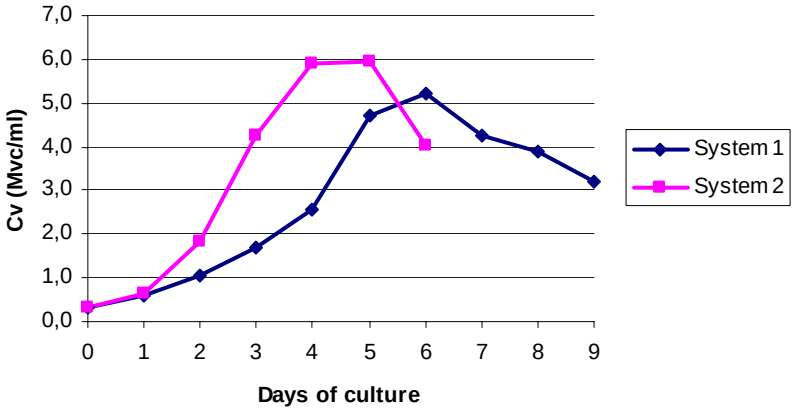

Figure 41. Viable cell densities in the systems in experiment 4.

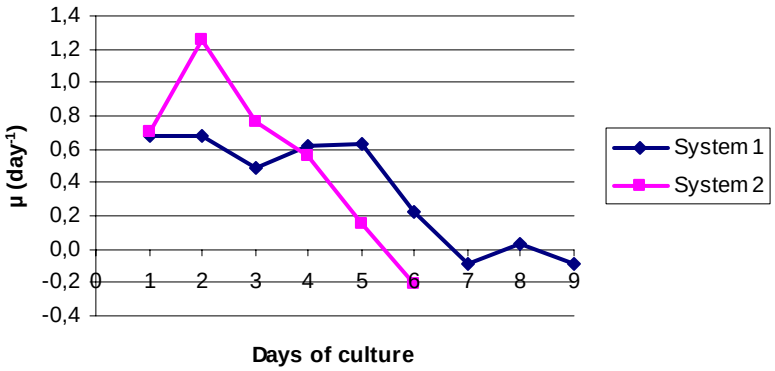

Figure 42. The cell specific growth rate, μ, in the systems in experiment 4.

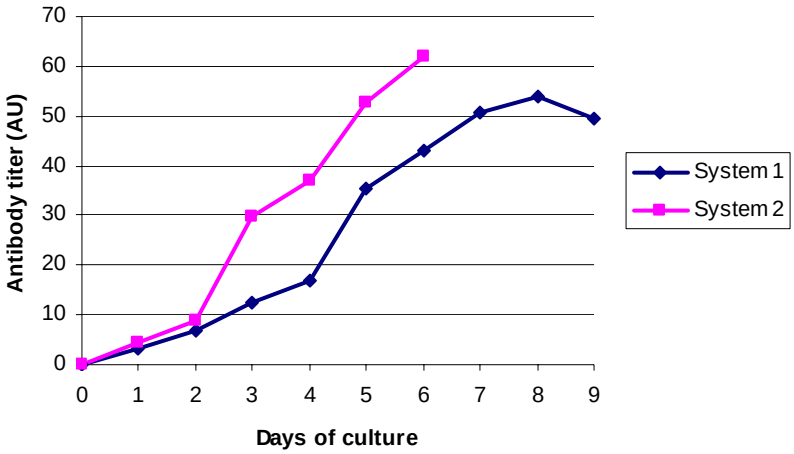

Figure 43. Antibody titers in the systems in experiment 4.

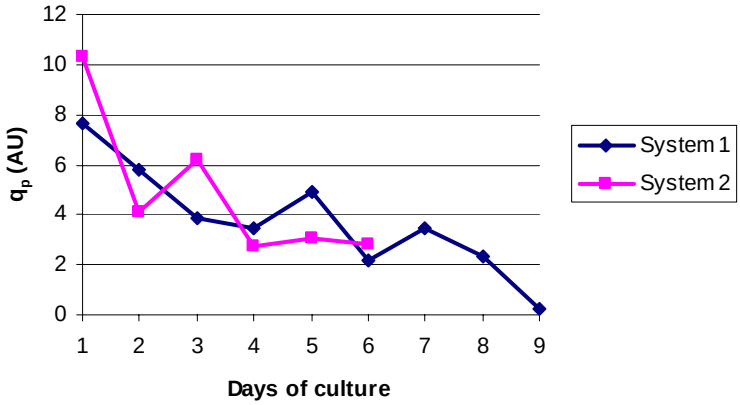

Figure 44. Cell specific productivity, q_p, in experiment 4.

Table 17. Feed medium feeding scheme for the systems in experiment 4 (ml).

G	System 1	System 2
0	0	0
1	0	0
2	100	100
3	120	120
4	144	144
5	173	173
6	173	173
7	173	N/A
8	173	N/A
9	115	N/A
Total	**1171**	**710**

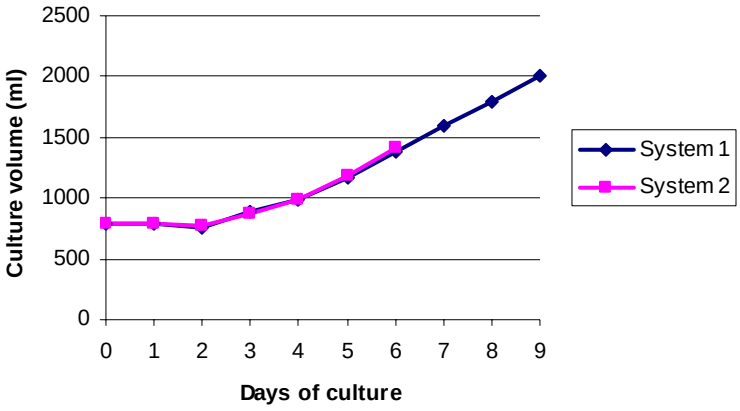

Figure 45. Culture volumes in both systems in experiment 4.

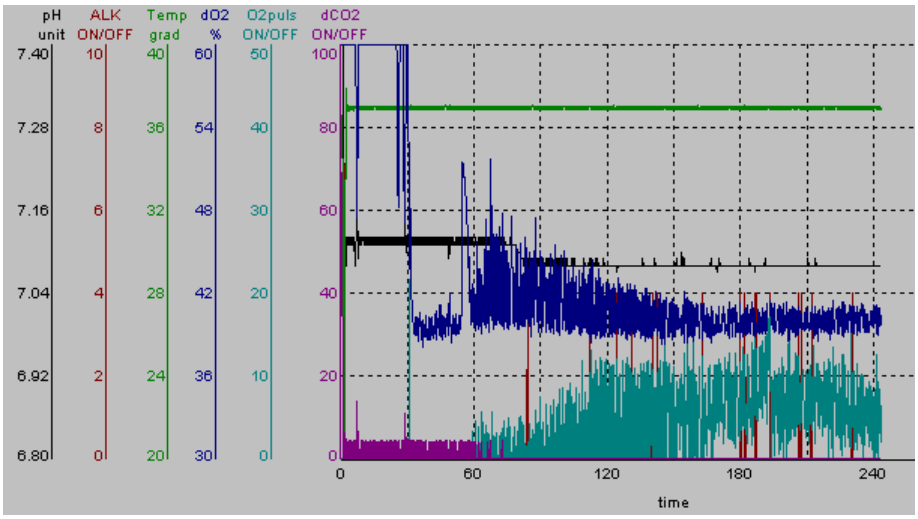

Figure 46. Data from the BioXpert log file for system 1 showing pH, alkali pump activity, temperature, DOT, O_2 pulsing and CO_2 pulsing.

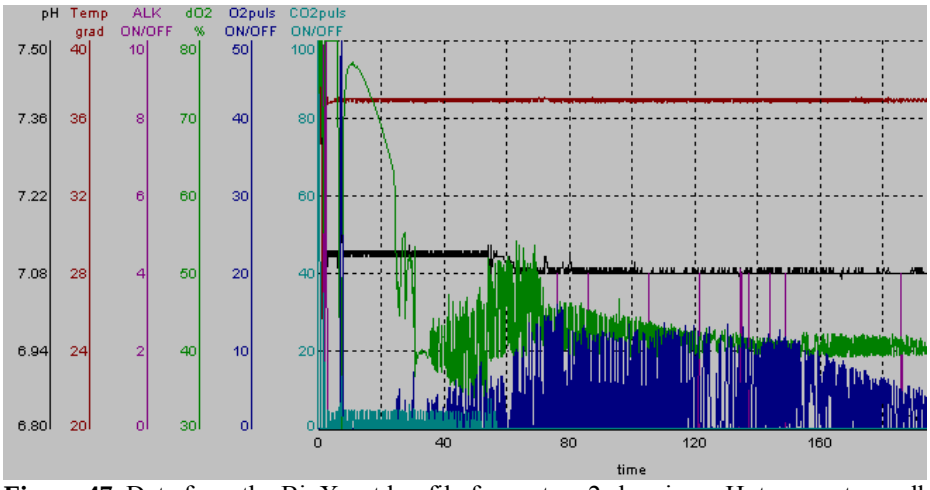

Figure 47. Data from the BioXpert log file for system 2 showing pH, temperature, alkali pump activity, DOT, O_2 pulsing and CO_2 pulsing.

Appendix VI – Experiment 5

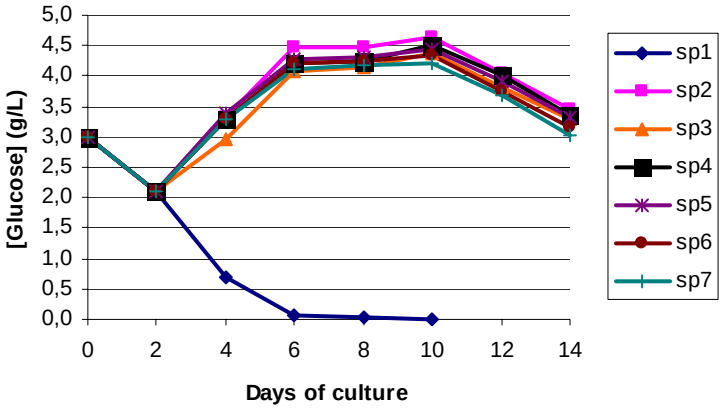

Figure 48. Glucose concentrations in all the spinners in experiment 5.