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Diploma Thesis

Development of cost effective media formulation for rCHO cell lines by eliminating or reducing serum

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original text except as acknowledgement in the text. I herby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other institution.

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1 Abstract

Therapeutical applications of proteins are one of the fastest growing fields worldwide.
Mammalian cell lines require a range of growth factors to allow growth and production. Traditionally foetal calf serum is used to provide these factors but cost and regulatory issues necessitates their elimination from the media.
This thesis describes a considerable effort spent to reduce and eliminate foetal calf serum from the production media of two cell lines: a CHO derivative cell line, called Lec-8, producing insulin receptor (IR) fragment and a CHO cell line producing epidermal growth factor receptor (EGFR) fragment.
Despite the fact that several commercial companies invested heavily to formulate and market serum-free media, the specific nature of these recombinant cell lines mentioned above, did not allow the direct use of commercial formulations and thus an in-house development work was required.
During the experimental work a number of formulations and additives were tested to enable the elimination or reduction of the serum:

- *sequential adaptation of cells to commercially available serum-free media*
  Additives to improve media performance were tested using Cellscreen®, a novel automated non-invasive cell monitoring system. Experiments aimed to adapt cells to suspension mode were also conducted.

- *improvement on the currently used in-house media*
  A literature survey identified potential additives that enable the use of low serum levels without the loss of yield or product quality. Several new formulations were developed during these experiments and tested for efficacy in both small and large- scale cultures.

After conducting more than 100 different experiments the following conclusions were reached:

- in-house additives improved the performance of one of the commercial media so that satisfactory growth and production was achieved under low serum or serum free conditions
- a combination of novel equipment (Cellscreen®) and Plackett Burman experimental design allowed rapid testing of the new formulations and should be the first choice for similar adaptation work
- anchorage dependent Lec-8 cells can be adapted to suspension mode to facilitate scale-up under reduced serum levels

2 Introduction

Genetically engineered Chinese Hamster Ovary (CHO) Cells are one of the most important systems to produce therapeutic proteins in large scale. Most cultured cells, especially those of the fibroblast type, require the addition of relatively high amounts of serum (5-20%) for growth.

Because serum is a complex mix of albumins, growth factors, it becomes one of the most important components of cell culture medium. The most commonly used serum is foetal calf serum.

The growing demand of the pharmaceutical industry for foetal calf serum as an important medium additive and in contrast, a decreasing source for foetal calf serum due to the appearance of Bovine Spongiform Encephalopathy (BSE), leads to an increase of the price.

Apart from an increasing price for foetal calf serum, there are further reasons to consider the use of this medium additive:

- The batch to batch variation of compounds in foetal calf serum from makes a standardization of production protocols difficult. A regular supplementation with 10% foetal calf serum provides $\approx 0.5\ g$ of protein per litre in culture medium, which complicates downstream processing.

- A wide range of serum free media formulations are commercially available. Generally serum free medium formulations should be specifically formulated to meet specific nutrient requirement of the different cell line. The performance of a serum free medium is not only particular to the cell line in culture, but also the expressed product can also play an important role in the successful adaptation of the cell line to serum free medium

The ultimate aim of this project was to develop a new in-house medium formulation for the expression of two recombinant proteins using mammalian cell cultures.

The development was conducted by using the Cellscreen® system (Innovatis,Germany) and a statistical procedure, the Plackett Burman Design.

The Cellscreen® system is non invasive image recording device. The ability to screen 96 well plates with simultaneous archiving of relevant parameters such as morphology and cell growth makes this system highly beneficial for medium optimization. With the high throughput and accuracy of the Cellscreen® in combination with the Plackett Burman Design the media optimisation was possible in a cost and efficient manner.

The growth and protein production performance of two cell lines, CHO K1 line, which express the epidermal growth factor receptor (EGFR) and the CHO derivate cell line Lec8 expressing a truncated version of the insulin receptor (IR) were used to evaluate low serum formulations. Both constructs are used for structure based drug design. This was a special requirement for the new medium formulation appeared when attempting to express the truncated IR. Because insulin, an important medium additive and generally found in serum free media and is a ligand of the IR, it blocks the binding domain of the receptor, complicates purification and assay and thus requires the development of a specific media formulation.

2.1 Serum and its position in cell culture

The advantage of serum as a single medium additive is its broad spectrum of positive influences on cell proliferation and cell viability. Serum supplies growth factors, nutrients for proliferation, attachment factors as well as factors for binding to and inactivating toxic compounds such as proteases and free radicals. The high amount of proteins in serum such as albumin and feutin protect the cells from environmental alterations, such as pH (sera are a source of natural buffers), shear stress, viscosity, osmolarity and gas delivery rate (Staines and Price 2003).(Freshney 1999)

2.1.1 Serum as cost factor

The current price for 1 litre dialyzed foetal calf serum is approximately 700 - 1000 *AUS$/l*. Dialyzed foetal calf serum is routinely added to the culture medium at 10% *v/v*. 70 - 100 *AUS$* per litre culture medium for an additive is an enormous cost factor.

Serum from countries where BSE has appeared is no longer used as a source of serum. Foetal calf serum in particular must be sourced from areas that are free of BSE, foot-and-mouth disease, and other highly infectious diseases (Staines and Price 2003).

From 2001 the entire continent of Europe reported cases of BSE. Europe with its big cattle market was not longer a source for serum manufacturers. The pharmaceutical industry relies heavily on foetal calf serum for its production of therapeutic proteins. Thus, it is anticipated that the price trend for foetal calf serum will continue to increase.

2.1.2 Variability of compounds in serum

The variability of the composition of serum can result in different growth pattern and protein production efficacy of a cell line. Enormous variations of growth factors and other compounds were determined in serum from different batches. For instance, hormones are known to effect cell growth at media concentrations in the pico- and nanogram ranges. Variations of these compounds can impact radically cell growth (Staines and Price 2003).

2.1.3 Lower level of contaminants in product purification

The purification of the target protein can be affected when the product protein is biochemically, functionally and physically related to serum proteins. The common serum concentration in Dulbecco's Modified Eagle's Medium/F12 (Coon´s modified) (DMEM/F12) is 10%, ≈ 0.5 *g/l* Protein could be contributed by the serum in addition of up to 2 *mg/l* protein produced by the CHO cells. The serum protein is a large contaminant, it complicates target protein purification (Darling D.C. and Morgan S.J. 1994).

2.1.4 Harvesting of foetal calf serum

Animal welfare campaigners have pointed at the procedure of harvesting foetal calf serum. Cattle are slaughtered at their last stage of pregnancy. After stunning the cattle are slaughtered and bled to death while the calf is still alive. When cow is being dismembered the foetus is removed and the blood is harvested via cardiac puncture. Ethical aspects of harvesting foetal calf serum suggest reconsidering the use of serum as cell culture additive.

2.1.5 Disadvantages of adopting cells into low serum or serum free conditions

The alteration of culture conditions is a labour and time consuming procedure. The cultured cells have high and specific nutritional requirements. Many commercial serum free medium formulations are offered for special applications and nutrient requirements and more formulations can be obtained by literature survey. Comparison of the serum free medium formulation from literature showed that the mutant of a particular cell have different nutrient requirements to the parentals. The chance of choosing the right medium for the cell line and its expressed product from a supplier and therefore for its special nutrient requirement is low. Some cells may not successfully adapt to serum free conditions. This implies a development of a serum free medium formulation for each cell line and its expressed product. A cell line with practical culture conditions is desired and an adaptation into suspension condition is optimal. Anchorage depended cell lines like fibroblasts require many attachment factors like fibronectin and laminin, usually provided by serum. Substituting these proteins with recombinant alternatives is very costly. Regarding the rising costs due recombinant proteins it is also suggested to develop a low serum medium formulation. Depending on the serum level, the cost reduction effect will be gained. The level of variations between serums contributes to the high cost of cell culture due to the additional testing and selecting serum batches that is required before the serum can be used as an additive. The predicament of contamination by undesired serum contents could not be avoided with a low serum formulation, but it could be decreased by a multiple (Freshney 1999).

2.1.6 Requirements to serum free medium

A major consideration in developing a new medium formulation is that it should be easy prepared with well defined compounds. A defined composition is desired. Protein content of new formulation should be lowered or eliminated to simplify downstream processing. Growth in small.- and large scale cultures should be able to be maintained by the new medium formulation (Darling D.C. and Morgan S.J. 1994;Freshney 1999).

An example of specific media requirement is for the IR fragment. For rational drug design, the structure of the insulin receptor is determined by crystallisation of IR protein. Different qualities of expressed product for crystallography purposes were examined.

The product had to be considered during medium optimisation. Furthermore the medium optimization was conducted with the exclusion of insulin, an additional complication.

2.2 CHO Lec8 and the difference from the parentals cell line

The CHO Lec8 cell line is a glycosylation mutant of its parental cell line CHO K1.
Reduction of the degree of glycosylation can change the solubility of the protein and increase sensitivity to enzymatic attack and affinity to the reaction partner. Lec8´s lack the nucleotide - sugar transporter UDP-galactose. Due to defects in the UGT gene transcripts, this complicates their growth (Oelmann; Stanley, and Gerardy-Schahn 2001). The phenotypical classification of the glycosylation mutants was achieved by determining its ability to bind or be killed by several lectins of different carbohydrate binding specificities. The nomination Lec8 is derived from lectin (Stanley 1984).

2.2.1 Functional consequences of glycosylation mutations

Cells can tolerate major changes in the structure of their carbohydrates as such changes are not often lethal. A slower growth and a higher sensitivity for environmental alterations were observed for Lec8s compared to the parental cell line. Some changes in adhesive properties and morphology have been observed to accompany certain glycosylation mutations (Stanley 1984).

2.2.2 Why Lec8 Mutant used for expression of IR fragment

Heterogeneity of the product is one of the major effects for an unsuccessful crystallisation.
A lower degree of heterogeneity of the protein can be achieved by expressing the protein in a glycosylation mutant such as Lec8. A one third lower glycosylation of the ectodomain was achieved in the Lec8 mutant compared to CHO-K1 cell line. The ectodomain of IR produced in Lec8 has not been changed in biosynthesis or Insulin binding relative to unmodified ectodomain and remains soluble at high protein concentration (Hendrickson 1996).

2.3 Function of medium additives in cell metabolism

Isolated cells from any organism require specific in vitro conditions for their survival. Cell culture medium attempts to simulate the natural environment of the organism of the extracted cells. The function of cell culture medium and its additives is to maintain the cells by supporting proliferation and metabolism.

Essential substances, which can not produced by the cells, are needed. A carbohydrate source for energy production and amino acids for protein and energy production, Vitamins as cofactors for enzymes and trace elements are added in all cell culture media. The buffer capacity of blood is imitated by using a mix of two buffer systems. $NaHCO_3$ in a 5% CO_2 environment is a popular buffer system, because it is able to capture and neutralize CO_2 as metabolic product. Hepes buffer properties are close to the desired physiological pH 6.8-7.2.

Antibiotics are not essential and were not used, but they simulate the immune system. Most of the hormonal system of an organism is provided by serum, mainly insulin, transferrin and other growth factors (Lindl T. 2002)

2.3.1 Essential additive for adaptation work (SITE)

The listed compounds insulin or long R^3 IGF, transferrin, selenium and ethanolamine were used in this work to support the adaptation of the rCHO´s into low serum and serum free conditions. These supplements and their growth promoting effects are widely recognized and available as commercial additives (Bottenstein and Sato 1979;Castro; Hayter; Ison, and Bull 1992;Kim; Kim, and Lee 1998;Kim; Kim, and Lee 1999;Kuchibhatla J. 2005;Lee; Kim; Kim; Yoon; Ahn, and Song 1999) (Kovar and Franek 1986).

2.3.1.1 Selenium

Selenium is an essential trace element as it is a component of several enzymes in both prokaryotes and eukaryotes. These selenoproteins contains selencysteine residues that are thought to participate in redox reactions such as that catalysed by the mammalian selenprotein glutathione peroxidase. Selenium aids antioxidants in this enzymatic reaction. Selenium also participates in electron transfer in mitochondria (Metzler D.E 2001).

2.3.1.2 Insulin

Insulin is added to most of the serum free medium formulation due to its action in promoting glucose uptake which increases cellular growth by supporting synthesis of RNA(Baseman; Paolini, and Amos 1974), protein, lipid and glycogen .Fatty acid biosynthesis is also affected by insulin. cAMP levels are decreased by insulin, leading to the dephosorylation and thus inactivation of hormone sensitive lipase. This reduces the amount of fatty acid available for oxidation. Insulin also activates acetyl-CoA carbaxylase (biotin depended) which is the first committed step of fatty acid biosynthesis (Voet D.; Voet J.G., and Pratt C.W. 1998).
Insulin also has an impact on the key reaction between glycolyses and TCA. Pyruvate dehydrogenase complex is regulated by Insulin (Metzler D.E 2001).

2.3.1.3 Long™R^3 IGF-1 as substitute for Insulin

Insulin and Insulin-like-growth factor-1 (IGF-1) are highly homologous in amino acid sequence and tertiary structure. IGF-1 binds to the insulin receptor with low affinity. (Adams; Epa; Garrett, and Ward 2000) Many signalling pathways are shared by both IR and IGF-1. IGFs play an important role in cell proliferation and survival. The possible use of lower IGF-1 concentrations compared to Insulin is resultant from a 5-8 times higher numbers of IGF1-R compared to IR. (Yandell)
Long™R^3 IGF-1 has been specifically engineered and manufactured by Gropep limited. It has structurally two significant modifications essential for the reduction of IGF-1 binding proteins. (JHR Bioscience 2003)
Insulin and Long™ R^3 IGF-1 are ligands of the expressed truncated IR and both will bind to the truncated protein. Purification of the truncated protein is based on an affinity purification step. Utilizing Long™R^3 IGF-1 up to a 1000-fold lower concentration compared to insulin can increase recovery of the expressed protein.

2.3.1.4 Transferrin

Transferrin is an iron binding glycoprotein which plays a role in transporting iron into the cell (Voet D. and others 1998). Iron is taken into cells by receptor-mediated endocytosis of monoferric and diferric transferrin (McClelland; Kuhn, and Ruddle 1984) (Karin and Mintz 1981). Transferrin can be substituted by Tropolone, an iron chelator, in combination with Fe^{3+} in the chemical form of ferric ammonium or transferrin which can be partially replaced by an increased concentration of $FeSO_4$ in the medium (Schroder; Matischak, and Friedl 2004) (Barnes and Sato 1980).

2.3.1.5 Ethanolamine is derivate from serine

Glycerophospholipids or phosphoglycerides are the major lipid components of biological membranes. They consists of glycerol-3-phosphate whose C1 and C2 positions are esterified with fatty acids In addition, the phosphoryl group is linked to another group – Water , Ethanolamine, Choline or Serine (Voet D. and others 1998).

2.3.2 Trace elements and ions as constitutes of media salts

Many metal ions are essential in enzymatic reactions. Almost one third of enzymes are depended on metal ions to perform catalytic reactions. For this purpose, metal ions such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Mn^{2+} or Co^{2+} are strongly bound to their enzymes. However, alkali and alkaline earth ions Na^{2+}, K^{+}, Fe^{2+}, Mg^{2+} or Ca^{2+} are loosely bound to their enzymes and play a structural role. Metal ions support catalytic reactions in different ways:

1. By binding to substrate to orient them properly for reaction
2. By mediating oxidation-reduction reactions through reversible changes in the metal ion´s oxidation state
3. By electrostatically stabilizing or shielding negative charges

(Voet D. and others 1998)

Ion that are major contributors to the osmolarity of the medium are: Na^{+}, K^{+}, Mg^{2+}, Ca^{2+},Cl^{-} ,SO_4^{2-},PO_4^{3-} and HCO_3^{-}. Mg^{2+} and Ca^{2+} should be reduced from medium for suspension cultures, as they are cofactors for attachment and cell aggregation. Phosphorus is present in nucleic acids and sulphur is a content of proteins (Darling D.C. and Morgan S.J. 1994).

2.3.2.1 Iron

Iron and copper play an important role in the cytochrome oxidase complex. An Iron-Copper centre in the cytochrome oxidase catalyzes efficient O_2 reduction (Alberts; Bray; Lewis; Raff; Roberts, and Watson 1994). Iron is an essential supplement for the energy recovery of the cell at the mitochondrial electron pump. Iron also has a important role in other catalase enzymes (Voet D. and others 1998).

2.3.2.2 Zinc

Amino acid and nucleic acid metabolism involving enzymatic reactions are zinc depended. Zinc plays a big role during DNA synthesis as transcription is zinc depended (Alberts and others 1994).

2.3.3 Amino acids catabolism

Amino acids are degraded to compounds that can be metabolized to CO_2 and H_2O or used in glycogenesis. The standard amino acids are broken down in to one of the seven metabolic mediates: pyruvate, α-ketoglutarate, succinyl-CoA, fumarate, oxalacetate, actyl-CoA or acetoacetate. Consequently the amino acids can be classified into two groups regarding their catabolic pathways:

1. Glucogenic amino acids are degraded to one of the glucose precursors such as pyruvate, α-ketoglutarate, succinyl-CoA, fumarate or oxalacetate.

2. Ketogenic amino acids are degraded to actyl-CoA or acetoacetate and therefore be converted to fatty acids or ketone bodies.

A few amino acids such as isoleucine, phenylalanine, threonine, tryptophan, and tyrosine can be degraded to glucose and fatty acid precursors and are thus characterized as being glucogenic and ketogenic

2.3.3.1 Alanine, Cysteine, Glycine, Serine and Threonine are degraded to Pyruvate

Yielding pyruvate the following amino acids were degraded: alanine, cysteine, glycine, serine, and threonine. Alanine is transaminated by alanine aminotransferase to. Serine is catalyzed by serine dehydratase and it is a pyridoxal 5'-phosphate (PLP) – depended reaction. PLP is derived from vitamin B_6 pyridoxine. Glycine is converted to pyruvate by first being converted to serine, another PLP depended enzyme catalyzed this reaction. Cysteine can be transferred into pyruvate by different ways, the sulfhydryl group has to be released as H_2S, $SO32^-$ or SCN.

Threonine belongs to both amino acid families; it can be classified to gluconic as well as ketogenic. Threonine dehydrogenase is major enzyme, which converted via an intermediate metabolic product to glycine (pyruvate) or either to acetyl-CoA.

2.3.3.2 Asparagine, Aspartate are degraded to Oxalactate

Asparagine is broken down by L-asparaginase to aspartate. Ammonia is removed from aspartate which could be derived from asparagine by aminotransferase directly into oxalacetate.

2.3.3.3 Arginine, Glutamate, Glutamine, Histidine, and Proline are degraded to α-Ketoglutarate

Arginine, glutamine, hisitidine, and proline are all degraded by conversion to glutamate, which in turn is oxidized α-ketoglutarate by glutamate dehydrogenase. The conversion or glutamine to glutamate is only a hydrolysis to glutamate. Glutamate is an intermediate product and it is converted via glutamate dehydrogenase into α-ketoglutarate.

2.3.3.4 Isoleucine, Methionine, and Valine are degraded to Succinyl-CoA

Methionine is first degraded in an ATP depended reaction to s-adenosylhomocyteine (SAM). SAM is made due its sulfonium ion´s highly reactive methyl group a very important biological reagent. Isoleucine, methionine, and valine have complex degradative pathways that all yield propionyl-CoA this is converted to succinyl-CoA in three reactions requiring biotin and coenzyme B_{12}.

2.3.3.5 Leucine, Lysine and Tryptophan are degraded Acetoacetate and to Acetyl CoA

Isoleucine, leucine and valine are belong the same sub group of amino acids, the branched chain amino acids. Leucine degradation begins with same manner as isoleucine and valine degradation, but finally it ends as Acetyl-CoA and/or Acetoacetate. Tryptophan is first degraded into alanine and the benzol residue, which converts in Acetoacetate.

2.3.3.6 Phenylalanine and Tyrosine are degraded to Fumarate and Acetoacetate

A single pathway is the first reaction of phenylalanine a hydroxylation to tyrosine. Following enzymatic steps ensure the degradation to fumarate and acetoacetate. (Voet D. and others 1998)

2.3.4 Vitamins

2.3.4.1 Biotin

Biotin is an important carboxyl group carrier. Biotin is classified as an essential medium additive as it participates in a lot of catalytic reactions. Beta carboxylation reactions are biotin dependent carboxylases using HCO_3^- with the cleavage of ATP to ADP + P_i. For example acetyl.- and pyruvate-CoA are in dependent on biotin, for activity.
The Na^+ pump is also maintained by biotin dependend enzymes such as oxaloactate decarboxylase, methylmalonyl ,- and glutaconyl-CoA decarboxylase (Voet D. and others 1998).

2.3.4.2 Vitamin B6 - Pyridoxal hydrochloride and Pyridoxine hydrochloride

Pyridoxal, pyridoxamine and pyridoxine are collectively known as vitamin B6. All three compounds are efficiently converted to the biologically active form of vitamin B6, pyridoxal phosphate. This conversion is catalyzed by the ATP requiring enzyme, pyridoxal kinase.
Pyridoxal phosphate functions as a cofactor in enzymes involved in transanimation reactions required for the synthesis and catabolism of the amino acids as well as in glycogenolysis as a cofactor for glycogen phosphorylase (Metzler D.E 2001).

2.3.4.3 Vitamin B1 or Thiamine - Thiamine hydrochloride

The active form of thiamine is thiamine pyrophaspate (TPP). TPP is a cofactor for pyruvate dehydrogenase. Pyruvate is decarboxylated to avetyl-CoA, the first molecule in the citrate acid circle. Ketoglutarate is decarboxylated by a TPP-depenent enzyme succinyl-CoA. α-Ketoglutarate dehydrogenase is responsible for this reaction. Thiamine participates in at least two important reactions for energy production (Metzler D.E 2001).

2.3.4.4 Vitamin B2 or Riboflavin

Riboflavin is an important compound of the coenzymes, FAD and FMN. Flavin proteins have a high reduction potential. FAD is used as a hydrogen carrier in the citric acid circle and FMN supports the electron transport in the mitochondria. Riboflavin, as the precursor for flavin protein, is indispensable as a medium additive (Metzler D.E 2001).

2.3.4.5 Vitamin B12 – Cobalamine and Cyanocobalamine

Several types of enzymatic reactions are depended upon alkyl corrin coenzymes. The first reaction is catalyzised by cobalamin dependent ribonucleotide reductase, a process involved in intermolecular hydrogen transfer. This is an important reaction in DNA synthesis. Secondly isomerisation reactions and at least ten different V_{B12} depended reactions are known. Methyl groups are transferred in the final cobalamin dependent reactions. An example for an essential metabolic reaction is the conversion of methylmalonyl-CoA to succinlyl-CoA by methylmalonyl-CoA mutase. This is an important step in the catalyzation of proteins and fats to energy (Metzler D.E 2001).

2.3.4.6 Vitamin C or Ascorbic acid

Vitamin C or Ascorbic acid can not be synthesised by mammalian cells and therefore Ascorbic acid or Vitamin C is an essential medium supplement (Metzler D.E 2001).

2.3.4.7 Putrescine

The polyamines spermidine and spermine and their precursor putrescine are intimately involved in and required for cell growth and proliferation. Their exact role in specific events related to cell proliferation at the molecular level is still unclear. Growth regulatory consequences of polyamine depletion suggest a link between signal transduction cascades, cell cycle machinery, and apoptosis. Several investigators have provided evidence for polyaminedependent restriction points during the G0-G1 transition and G1 phase in various cell types (Ray; Zimmerman; McCormack; Patel, and Johnson 1999).

2.3.4.8 Cortisol

Synthetic cortisol known as hydrocortisone is a corticosteroid hormone. Hydrocortisone is used in serum free medium formulations as it acts as an antagonist with insulin and carbohydrates, breaking down lipids, and proteins by enzymatic reactions depended by these hormones (Eisen; Goldfine, and Glinsmann 1973).

2.3.4.9 Niacinamide or Vitamin B3

The two important hydrogen carriers NAD and NADP are derived from niacin. Therefore Vitamin B3 an essential medium supplement.

2.3.4.10 Folic acid

Folate is necessary for the production and maintenance of new cells. Folate is needed to replicate DNA and synthesize RNA. Tetrahydrofolic acid (THF) is derived from vitamin folic acid, a doubly oxidized form of THF that must be enzymatically reduced before it becomes an active coenzyme. Both reductions were done by DHFR. Mammals cannot synthesize folic acid, so it must be provided by culture medium (Voet D. and others 1998).

2.4 Design of Experiment (DoE)

2.4.1 Introduction

The aim of the experimental design was to estimate factor effects with the highest accuracy possible. Usually, an experimental design with fixed factor levels is used and the response of the experiment is used to find which factors of high influence as possible with a few experiments as possible.

"The use of experiments in fractional replication was proposed in 1945 by Finney. He outlined methods of construction for 2^n and 3^n factorials, and described a half replicate of $4 \cdot 2^4$ agriculture field experiment that had been conducted in 1942. In 1946, Plackett and Burman gave designs for the minimum possible size of experiment with p^n factorials (p=2,3,…7) and pointed out their utility in physical and industrial research. Their chief appeal is that they enable 5 or more factors to be included simultaneously in an experiment of a practicable size, so that the investigator can discover quickly which factors have an important effect on the product" (Cochran W.G. and Cox G.M. 1957).

Plackett Burman designs are two level fractional factorial designs for studying k=N-1 variables in N runs, where N is a multiple of 4. However, for N = 12,20,24,28 and 36 the Plackett Burman are frequently useful (Montgomery D.C. 1984).

For example, assuming is a screening with seven factors and two levels of each factor means $2^7 = 128$ runs are essential, if the factors are to be checked in each possible combination. The huge reduction of essential runs or experiments is provided by using the Plackett Burman plan. Seven factors N in all possible combinations could be screened in eight runs by a utilized Plackett Burman matrix.

Some assumptions for statistical design were done prior to starting with the experiments. The measurement system, in this case the Cellscreen® system (Innovatis,Germany) provided the needed accuracy for statistical designs. The process had to be stable for the entire screening; even backup cells should not change their growth patterns. This was considered because the measurements of the multi titre plates were done at room temperature and not under culture conditions at 37°C. No impact on viability and on growth of the cells due to temperature shift was assumed as previously published (Brinkmann; Lütkemeyer; Gudermann, and Lehmann 2001).

To run experimental design for medium optimisation is very labour, cost and time intensive. Laboratories all over the world spend years finding new and better medium formulations. Limited time was a very important issue for this study. A System was required for medium optimisation that was able to measure the growth of adherent cell lines, eliminate human errors by a fully automated system, and cost saving by using small volumes in a time saving manner. All these requirements were covered by the Cellscreen® System provided by Innovatis (Germany). Eukaryotic cells grown in micro plates can be analysed using the non-invasive, automated system called Cellscreen®. Images of the entire well of a plate or regions of interests can be captured by the Cellscreen®. Following the images are processed and stored in a database. Growth curves and other parameters are generated by the software. Archiving of the data allows the recall of the experiments at any time for reviewing or reanalysing. (Innovatis 2004).

2.4.2 Selection of DoE

The selection of an experimental design depend heavily on the number of factors which are to be investigated and the purpose such as accuracy, interaction of variables, main effects, time for experiments, complexity and practicability (Giesbrecht F. and Gumbertz L. 2004). For medium optimisation the number of screenable compounds is presumably quite high and experimental design is required, which allows the investigation of many factors.

In this project the number of screened factors was up to 20 factors per matrix. The most economically method is a factorial fractional design – the Plackett-Burman design.

The Plackett Burman design provides an efficient way of screening a large number of variables and identifying the most important ones. Plackett Burman design allows the estimation of main effects but not interactions. The principle of Plackett Burman is all effects are compared with one basic experiment and all variables or factors are added at the lowest level.

2.4.3 Selection of Variables for DoE

The selection of factors or process variables which should be screened is very important. Many additives are available such amino acids, salts, trace elements, vitamins and growth factors. The choice of 20 amino acids, 12 inorganic. - and 27 organic compounds was given. All these additives were added to medium formulations.

The development of a low serum formulation for CHO Lec8 was based on DMEM/F12 (Coon´s modified) supplemented with GS-supplements (see: Appendix 9.1) and sodium selenite, transferrin and ethanolamine. The medium formulation DMEM/F12 (Coon´s modified) and additional GS-supplements is by it self an enriched medium formulation with a quite high level of amino acids, therefore was the first step in the development of additives which are not components or only available in very low concentrations of the current medium. This medium was compared with serum free or low serum formulation using published data(Kim and others 1998;Kim and others 1999;Lee and others 1999) and it was found that the medium has a lack of biotin, hydrocortisone, hypoxanthine, long R^3 IGF as substitute for insulin, linoleic acid, putrescine, transferrin and trace elements. Each of these additives has an important function in medium, especially when serum as main supplier of these supplements, (chapter 2.3) was reduced. It was recommended to test independently the amino acids involved in the major metabolic pathways as the metabolism of one amino acid may be influenced by the levels of others (Kim and others 1999). The amino acid consumption investigations (Table 5.4-4) and comparison of medium formulations were taken to estimate the importance of a factor chosen for screening experiments. The branched chained amino acids (chapter 2.3.3.5) were usually screened as one variable, but it was decided after consideration of amino acid consumption (Table 5.4-4) and the recommendation of individual screening of amino acids, that leucine (>70% was consumed after four days) should be taken as a single variable. Further grouped amino acids in the literature were proline and histidine (chapter 2.3.3.3) as well as phenylalanine and tyrosine (chapter 2.3.3.6). Proline was a chosen as variable because of the auxotroph rCHO´s requirements for exogenous proline for growth due to a block in de novo synthesis of proline from glutamate. (Austin and Winkler 1988) Histidine consumption (Table 5.4-4) was to low to be adducted as variable. Phenylalanine was to be (Table 5.4-4) the preferred amino acid.

3 Materials

3.1 General lab ware

10, 100, 1000µl tips	Eppendorf AG, Germany
10, 100, 1000µl tips	Molecular Bio Products, USA
1.5 ml tubes	Eppendorf AG, Germany
PP- 15 and 50 ml Falcon tubes	Becton Dickinson, USA
1, 5, 10, 25 ml disposable pipettes	Becton Dickinson, USA
General cell culture dishes and flasks	Nalge NUNC International, USA

3.2 General lab equipments

Incubator	Forma Scientific, USA
Cabinet	Biological Safety Cabinet BH 2000 lass 2
Waterbath	Selby, Austarlia
Oven	Thermoline , Australia
Auto vortex mixer MT19	CHILTERN
OM6 Orbital Mixer	Ratek, Australia
Microscope CK2	Olympus,Germany

3.3 Cell Culture Media and Additives

3.3.1 Cell culture medium DMEM/F12 (Coon´s modified)

The standard medium was DMEM/F12 (Coon´s modified) supplemented according to the requirement of the Glutamine Synthetase Expression System. The contents of DMEM/F12 medium + Glutamine Synthetase Supplements (GSS) are listed in the Appendix 9.1.

3.3.2 Concentrated DMEM/F12 (Coon´s modified)

Concentrations of DMEM/F12 (Coon´s modified) and supplements were chosen according 9.1. The targeted osmolarity was 340 *mOsm/(kg H_2O).* Contents of the DMEM/F12 dry powder such as Hepes and inorganic compounds increase the osmotic pressure. Osmolarity was determined at almost final volume of prepared medium. After osmolarity determination Sodium Chloride was added as needed amount to achieve 340 *mOsm/(kg H_20).*Till a value of 400 *mOsm/(kg H_2O)* is a linear coherence between Sodium Chloride concentration and osmolarity exists. Millipore water was used for medium preparation. A maximal solubility of DMEM/F12 (L6077) was 35.2 *g/l* determined. The only compound which was used with a

factor 1, 1.5 or 2 was DMEM/F12 (L6077). $NaCO_3$, hepes, methionine sulphoximine (MSX), phenol red and nucleosides were used in single concentrations (see: 9.1). The glucose level was increased from 4.5 *g/l* to 6 *g/l*. A higher glucose level of 6 *g/l* is a standard concentration in serum free medium. GS-supplement accessorized amino acids such as asparagine, alanine, aspartic acid, glycine, serine and L-proline were obmitted, because an icreased level of amino acid was reached by the foctorized use of DMEM/F12 (L6077). 250 μl MSX (100 *mM*) was added per litre medium. Nunc filter (0.2μm) was used for sterile filtration of medium.

Table 3.3-1: Medium formulations of concentrated DMEM/F12 (Coon´s modified) Medium.

Compounds	Product.-nr.	1x DMEM/F12	1.5x DMEM/F12	2x DMEM/F12
DMEM/F12	L6077	16.0 *g/l*	24.0 *g/l*	32 *g/l*
$NaCO_3$	S5761	2.15 *g/l*	2.15 *g/l*	2.15 *g/l*
Hepes	H6147	3.57 *g/l*	3.57 *g/l*	3.57 *g/l*
MSX [100*mM*]	M3443	0.25 *ml*	0.25 *ml*	0.25 *ml*
Glucose	47829	6.00 *g/l*	6.00 *g/l*	6.00 *g/l*
Phenol red	P5530	8.0 *mg/l*	8.0 *mg/l*	8.0 *mg/l*
Nucleosides 100 x	-	10.0 *ml/l*	10.0 *ml/l*	10.0 *ml/l*

DMEM/F12 was stored at 4°C with out light contact. DMEM/F12 was aliqouted in 250 ml Schott bottles as required to avoid excess heat degradation due to warm-up prior to contact with cells.

3.3.3 Trace elements stock solution (100 x)

The commercial chemicals contain foreign ions which cause contamination of the medium but as concentrations were very low and it was decided to proceed with their use.

Table 3.3-2: Final concentration of trace elements and stock solution 100 fold concentrated.

Compounds	Product.-nr.	1 x [*µg/l*]	100 x [*mg/l*]
Cupric Sulfate 5·H2O	BDH 10091.4Q	1.5	0.150
Ferric Sulfate 7·H2O	BDH 10112 4V	500	50.0
Potassium Nitrate	M&B Lot 58848	90	9.0
Zink Sulfate	BDH 10302.3G	600	60.0

Cupric Sulfate 5 · H_2O was prepared in a 10^4 x stock (15 *mg/l*), from which an aliquot was added to the 100 x Trace stock prepared using Millipore water. The Trace elements were solved in Milllipore water. The solution was stored in the dark at 4°C.

3.3.4 Nucleoside solution stock solution (100 x)

Nucleosides are soluble at room temperature in water in a 100 fold concentration of the final concentration required for culture conditions (Appendix 9.1). Nucleosides stock solution was divided in aliquots and stored at -20°C.

Table 3.3-3: Concentration of nucleosides in it final concentration and stock concentration 50 and 100 fold.

Compounds	Product.-nr.	1 x [*mg/l*]	100 x [*mg/l*]
Adenosine	A4036	7.0	700
Guanosine	G6264	7.0	700
Cystidine	C4654	7.0	700
Uridine	U3003	7.0	700
Thymidine	T1895	3.6	360

3.3.5 STE stock solution (100x)

Selenium, Transferrin and Ethanolamine (STE) as the most essential supplement for adaptation work into low serum or serum free medium was prepared in a 100 fold concentration. Sodium selenite is a powder and is regarded as toxic in high concentrations. Sodium selenite was prepared in a 10^4 fold concentration in PBS and from there reduced to 100 fold final stock concentration. Transferrin was directly dissolved in PBS. Ethanolamine was added as liquid to the stock solution.

Table 3.3-4: Concentrations of additives for the most essential supplement for adaptation work "STE" in its final concentration and 100 fold concentrated stock solution.

Compounds	Product.-nr.	1 x [*mg/l*]	100 x [*mg/l*]
Sodium Selenite	S9133	$5.0 \cdot 10^{-3}$	0.5
Transferrin	T1428	55.0	550
Ethanol amine	E0135	2.04	204

3.3.6 Non water soluble additives

Some additives are not soluble in water. Solvents such as 96% ethanol and 1N sodium hydroxide are utilized as solvents for these supplements. Ethanol was avoided as a solvent when possible, because if its negative effect on cell viability. Sodium hydroxide as a solvent affected the stock solution by increasing the pH, which was neutralized with hydroxyl chloride prior to adjusting to the final volume. After pH and volume was adjusted, the stock

solution was sterile filtered with 0.2 *µm* pore size filter in the biosafety cabinet. A stepwise increase in solvent volume was chosen until the entire amount of substance was dissolved.

Table 3.3-5: Non soluble medium additives and their solvents.* L-Aspartic acid and L-Phenylalanine a small increase in pH to solubilize.

Substance	Product.-nr.	Solubilize in	Solubility [*mg/µl*]
L-Asparagine	A4159	1N NaOH or 1N HCl	0.06
L-Aspartic acid*	A4409	1N NaOH or 1N HCl	-
D-Biotin	B4639	1N NaOH	0.01
L-Glutamic acid	G5638	1N NaOH	0.09
Hydrocortisone	H0888	Ethanol	-
Hypoxanthine	H9636	1N NaOH	0.05
Linoleic acid	L1012	Ethanol	-
L-Phenylalanine*	P5030	1N NaOH	-

Following dissolving the substance in solvents and adding to the basal medium (DMEM/F12 (Coon´s modified), GS-supplements) pH was adjusted with hydroxyl chloride. The highest possible final Ethanol concentration in the medium was 0.08% *v/v*, which was assumed to have no affect on the cells. The stock solutions were stored after sterile filtration (Nunc,0.2 µm) in 15 ml falcon tubes at 4°C.

3.3.7 BD CHO Medium (Cat.220229)

BD CHO medium is an optimized medium for CHO cell lines and parentals, including CHO-K1, DG44 and DBX11. This medium is supplemented with a plant derived hydrolysate (DS 100) to eliminate the risk of contamination of by animal adenovirus agents and simplify downstream purification. 250 *µl* MSX (100 *mM*) was added per litre BD CHO Medium.

3.3.8 Peptones

Stock solutions of hydrolysates were prepared in a concentration of 100 *g/l* in PBS buffer. An aliquot mass of peptones was added under stirring at room temperature. The peptones showed varying times required to dissolved completely. The stock solution was sterile filtered utilizing a pore size of 0.2 *µm* in the biosafety cabinet and stored at 4°C.

3.3.8.1 Difco™ Springer DS 100 Soy Peptone UF (220515)

Difco™ Springer DS 100 Soy Peptone UF (ultra-filtered) is an enzymatic digest of soy protein. Only enzymes of microbial origin are used in the digestion process. Difco Springer DS100 Soy Peptone, UF is ultra-filtered to enhance solubility and reduce endotoxin content in the final product. Difco™ Springer DS 100 Soy Peptone UF is a medium tan, free-flowing, homogenous powder.

3.3.8.2 Difco Select Phytone™ Peptone UF (210193)

Phytone Peptone, UF is an ultra-filtered enzymatic digests of soybean meal. The nitrogen source in the soy peptones contains naturally occurring high concentrations of vitamins and carbohydrates of soybean. It has an endotoxin level of less than 500 EU/g, which makes it an ideal substitute or supplement for foetal bovine serum in cell culture applications. Difco Select Phytone™ Peptone UF is a light tan, free-flowing, homogeneous powder.

3.3.8.3 TC Yeastolate UF (292804)

TC Yeastolate products are animal-free and water-soluble portions of autolyzed yeast or Saccharomyces cerevisiae. TC Yeastolate is a mixture of peptides, amino acids, carbohydrates as well as vitamins. TC Yeastolate, UF has been ultrafiltered at a 10,000 MWCO (Molecular Weight Cut-Off). It has an endotoxin value of less than 500 *EU/g*.

Table 3.3-6: Information about contents in Hydrolysates.

	Endotoxin level *EU/g*	Osmolarity *µOsm*	Hypoxanthine *µg/g*	Thymine *µg/g*
Difco™ Springer DS 100 Soy Peptone UF (220515)	< 300	45	14	< 10
Difco Select Phytone™ Peptone UF (210193)	< 300	52	< 2	< 10
TC Yeastolate UF (292804)	< 500	64	32	< 10

3.3.9 Dialyzed Foetal Calf Serum (Gibco, cat.26400-044)

Sourced from the United States the serum is dialyzed by tangential flow filtration utilizing a 10,000 MW cut-off. Dialyzed serum is essential for expression utilizing the Glutamine synthetase system in CHO cells. The serum was tested by Gibco for performance, chemical and physical and adventitious agents. Growth and cloning performance was confirmed.
The following chemical tests were approved:

Table 3.3-7: Parameter dFCS cat.:26400-044.

Endotoxin level [*EU/ml*]	≤ 50
Glucose [*mg/dl*]	≤ 5.0
Hemoglobin [*mg/dl*]	≤ 25
Osmolarity [*mOsm*]	280 - 310
pH	6.9 - 78
Total Protein [*g/ml*]	3.0 - 5.0

3.4 Cell lines

3.4.1 CHO Lec 8

CHO Lec8 is referred to as a glycosylation mutant, which has not a molecular basis of mutation (Stanley 1984). The selection of the mutant CHO Lec8 cells, unable to galactosylate glycoproteins was undertaken utilizing lectin. Mutants were differentiated by their glycoproteins on the cell surface. After binding with carbohydrates on the cell surface lectin enables biochemical reactions, which is lethal for non mutated cells like CHO K1. (Stanley 1989) The cells were cultivated in DMEM/F12 medium containing 1 – 10% dialyzed foetal calf serum. The CHO Lec8 cell line was obtained from the American Type Culture Collection, USA (Cat. No. CRL-1737).

3.4.2 CHO K1

The CHO K1 cell lines which are Chinese Hamster Ovary cells were derived as a sub clone from the parental CHO cell line originally from a biopsy of an ovary of an adult Chinese hamster by T.T. Puck in 1957. These anchorage depended cells show the morphology of epithelial cells growing as monolayer. The cells were cultivated in DMEM/F12 medium containing 0.5 – 10% dialyzed foetal calf serum.The CHO K1 cell line was obtained from the American Type Culture Collection, USA (Cat. No. CCL- 61).

4 Methods

4.1 Maintenance conditions

4.1.1 Cell culture conditions

All cell culture procedures were performed in a class II ultraviolet biohazard cabinet (Biological Safety Cabinet BH 2000) for personnel, environment and product protection with strict adherence to aseptic technique. Adherent cells were routinely cultivated as monolayer in disposable T flasks (25-175 cm^2) and suspension cells were cultivated in siliconized Shott bottle agitated on an orbital mixer (Ratek,Australia) incubated at 37^oC and 5% CO_2 without humidity control in the Incubator. Daily observations of growth and morphology were performed under microscopically (Olympus,Germany). The cells were kept in the midlog phase and confluence of over 90% was avoided. To passage cells, the spent medium was removed and the cell monolayer rinsed with 1x PBS. The cells were than stripped from the flask surface by incubation with 1x trypsin/versene solution. After an incubation time of approximately 5 minutes (depending on cell line and cell density) at 37˚C in the incubator, the cells were detached. Trypsin inhibtor (Sigma) was used to neutralize trypsin in a ratio of 1:1. If cell clumps were appeared after using trypsin inhibitor (Sigma) the cell suspension was gently mixed with a pipette.

4.1.2 Maintenance Spinner Basket® with Fibra-Cel® Support

These spinners has a 0.5 l. working volume, stirred and aerated and kept in the incubator at 37°C and 5% CO_2 environment. In scale up experiments the cells were grown in DMEM/F12 in Spinner Basket® with Fibra-Cel® Support. Depending on the glucose consumption of the culture an aliquot of medium was replaced with fresh medium as required. The Lactic acid concentration was kept < 2 *g/l*. The spinner flask was aerated by stirring and was monitored using medium colour and glucose consumption. The pH was kept at 7.2.

4.1.3 Freezing cells

Cells were proliferated in T175 flasks until 80 - 90% confluence than stripped of the surface in the midlog phase. The cells were centrifuged at 1000 *rpm*, 20°*C* and 4 minutes. The cell pellet was resuspended in pre warmed freeze mix (37°C) by keeping a desired freeze cell concentration at 10^6 *cells/ml*. 1 *ml* aliquots of cell suspension were added into Nunc cryotubes (3-63401). The tubes were placed in a NALGENE Cryo freezing container warmed to room temperature. The Nalgene Cryo freezing container was kept overnight at -70°*C*. The cells were kept at this temperature for short term storage.

4.1.4 Thawing cells

The Nunc tube was carried in dry ice into biosafety cabinet and added directly from -80°*C* or nitrogen storage into 70% pre warmed ethanol. While the cells thawing and 15 *ml* Falcon was filled with 9 *ml* pre warmed culture medium. The cells were resuspended in culture medium and spun down for 2 *min* at 1000 *rpm*. Supernatant was discarded and the pellet was resuspended again in pre warmed culture medium. The cell suspension was added to a medium containing T flask.

4.1.5 Adaptation into serum free or low serum conditions

Adaptation into serum free conditions utilizing BD CHO Medium: the cells were added from 10% dFCS DMEM/F12 (Coon´s modified) directly in BD CHO Medium 5% dFCS. The cells were kept for one – two passages at each stage. The cells were weaned from serum in three steps. 5, 2, 1% dFCS and then serum free.

Weaning the cells from 10% serum in DMEM/F12 (Coon´s modified) was done in four steps for allowing the cells to re-establish at each stage. Four steps 5, 2, 1 and 0.5% dFCS were needed for achieving low serum conditions.

For both adaptation work at a serum concentration of 1% dFCS , sodium selenite, transferrin and ethanolamine were supplemented to the medium. The three supplements were added as one additive “STE”.

4.2 Set up cell culture vessels

4.2.1 Siliconizing of glassware

Suspension culture of fibroblasts requires a silconized layer of the inner reactor wall. Sigmacote® was added into the glass vessel and the surface was distributed over the entire inner surface evenly. After the heptane had evaporated (in a chemical hood) from the Sigmacote® the siliconized glassware was backed in the oven at 100°*C* for 30 minutes. Removing of the silicon layer was done by treating with 20% sodium hydroxide overnight.

4.2.2 Spinner Basket® with Fibra-Cel® Support

4.2.2.1 Set up

The entire parts of the spinner flask were rinsed with deionized water .The basket was filled with 10 *g* Fibra-Cel® disc. A Silicon tube was connected to air inlet and fixed by a cable tie. The full assembled spinner basket was put into the vessel; the magnet stirring bar was connected with the spinner basket. The silicon tube was connected to the lid. Two 0.2 *µm* filters were connected to the inlet and outlet arm and fixed with cable ties. 500 *ml* PBS was filled into the spinner and all function such as aeration and stirring bar were tested. The entire Spinner system was covered with an autoclave bag and sterilised using the fluid cycle at 121°*C* for 30 minutes in the autoclave.

4.2.2.2 Inoculation with cells

12,000 cm^2 growth area is given by using 10 *g* Fibra-Cel® disc. At least 10 x T-175 flask with 90% are needed for inoculation of one Spinner Basket. The inoculation concentration should not be less than 10^6 *cells/ml* to avoid an extend lag phase due to low cell concentrations.
The cells of ten T175 flasks were collected in one T175 flask. The cell suspension was topped up till 500 *ml* working volume of the spinner with pre warmed culture medium. The buffer was discarded through one of the arms and the spinner was refilled with the cell suspension. Analogue stirring system (Vineland,USA) was run at level 2.

4.2.3 Celligen Plus Bioreactor suspension culture

Fermentation of the suspension culture it was performed using as few probes as possible, to avoid shear forces and turbulences in the reactor. The reactor was equipped with temperature, dissolved oxygen (DO), pH probe and one pitched blade impeller was used in fermentation.
Inoculation bottle (2 litres) was connected via silicon tube to the reactor inlet. The reactor connections were covered with silicon. 1.5 l PBS buffer was used as liquid filler for the bioreactor and the entire apparatus was autoclaved for 30 min at 121°C.
The minimum inoculation volume of 1.6 l (working volume: 6.5 l; max. volume: 7.5 l) was reached by proliferation of the cell first in shaker (Schott,USA) and then in 2 x 1 *l* Spinner (Corning,USA).

4.3 Set up of Experiments

4.3.1 Medium Performance of BD CHO Medium in T25 flasks

CHO Lec8 cells were weaned stepwise to serum free conditions, and kept for two passages at each stage. The viability was 88.0% and the seeding concentration was $5 \cdot 10^4$ *cells/ml*. 50 *ng/ml* long R³ IGF and STE [1x] were added as further supplements to BD CHO Medium. The cells were cultured in 6 T25 flasks with a working volume of 10 *ml*. Each day a T flask was sacrificed to obtain the cell parameter and the experiment lasted 140 hours.
The cell counting was performed after adding 0.5 *ml* versene to the T25 flask and resuspending the cells in 9.5 ml in-house medium supplemented with 10% dFCS. The cell sample was taken from 10 *ml* working volume. Calculations are shown in Appendix 9.7.

4.3.2 Peptone screening in T25 flasks

In the early stage of this project Difco Select Phytone™ Peptone UF and Difco™ Springer DS 100 Soy Peptone UF were screened in a concentration range of 1-10 *g/l*. The cells were weaned from serum according to the described procedure and kept for two passages under the following conditions: DMEM/F12 (Coon´s modified), GS-supplements, 1% dialyzed foetal calf serum. The supplementation of SITE or STE was omitted. 2 x 175 cm^2 of confluent growth area was needed to provide the sufficient cell number for screening of 1*1* different conditions in T25 flasks. Peptones were dissolved at a concentration of 100 *g/l* in PBS buffer. The working volume for T25-flaks was 10 *ml*. At a peptone concentration of 5 *g/l* a dilution of 5% was reached. The viability was 81.8% and the inoculation concentration was $4 \cdot 10^4$ *cells/ml*. The flasks were stored in the incubator and after 20 min, when inner temperature of T25 flasks and incubator temperature was even; the lid of the T25 flaks was loosened for aeration purposes.

4.3.3 Peptone screening in 96 well plates

The working volume per chamber of a 96 well plate was 200 *µl* and the volume for each peptone concentration and basic medium volume was added according to the following table:

Table 4.3-1: Composition of volumes per chamber on a 96 well plate with a working volume of 200 *µl*.

Compounds	w/o Peptone	1 *g/l*	2 *g/l*	3 *g/l*	4 *g/l*	5*g/l*	6 *g/l*	7 *g/l*	8 *g/l*	9 *g/l*
Peptone Stock [*µl*]	0	2	4	6	8	10	12	14	16	18
Basic Medium [*µl*]	195	193	191	189	187	185	183	181	179	177
Cell Suspension [*µl*]	5	5	5	5	5	5	5	5	5	5

Prior 5 *µl* cell suspension in basal medium was added to the plates with an array pipette, medium as well as peptones stocks (100 *g/l*) were added into the chambers and the plate was kept in the incubator for 20 min. The 96 well plates were measured immediately after inoculation, when all the cells were settled. The screening lasted 100 *h*.

4.3.4 Plackett Burman preparing of experiments

Every experiment was conducted with a volume of 10 *ml* in a 15 *ml* Falcon tube and the stock solutions of the variables were 100 fold concentrated relative to the difference between high and low level (Appendix 9.5). After addition of 0.1 *ml* stock solution per variable and 5 *µl* dFCS (0.5% dFCS final concentration) to the experiments the final volume adjusted by adding 8.8 *ml* basal medium (DMEM/F12 (Coon´s modified), GS-supplements, STE) to 10 *ml*. The experiments for the Plackett Burman application with CHO Lec8 were performed according the same procedure but with 1% dFCS as final concentration.

4.3.5 Plackett Burman Application setup of 96 well Design 1-3 (Lec8)

Two 96 well plates were prepared for the Plackett Burman screening. Each experiment was performed in 5 replicates on each plate. A high viability was established when the screening was started. After addition of medium into the chambers, the plate was kept in the incubator for 20 min to warm up the medium prior to inoculation of the plates. 5 *µl* cell suspensions were added per chamber with an array pipette. The final cell concentration in the chamber was $2.5 \cdot 10^4$ *cell/ml*. The plates were cultured for 50 *h*.

4.3.6 Plackett Burman Application setup of 96 well/ CHO K1

24 experiments were distributed over three 96 well plates. The screening with 24 experiments was conducted twice and a plate was filled with eight experiments in triplicates. The experiments were prepared according to chapter 4.3.4. 190 *µl* of medium was added per well and 10 *µl* cells suspension resulted a final seeding cell density of $2 \cdot 10^4$ *cell/ml*. The cells were cultured for 69 *h*.

4.3.7 Plackett Burman Evaluation of Data

The possibility of a statistical procedure was enabled by the Cellscreen® system. 96-well plates (Nunc,Denmark) were used for the entire screening of the experiments. The Plackett Burman matrix was generated in an excel sheet. The required concentration of each factor was estimated by comparison of published concentration, prior analytical investigation of amino acid consumption. Each experiment was set in 15 *ml* Falcontube.

4.3.7.1 Development of Plackett Burman Matrix

A Plackett Burman Design only exists if the number of experiments “n” is a multiple of four and the number of variables is n-1. The factors were in the horizontal row and the experiments are shown in the column of the matrix. High levels and low levels of factors are represented by “+” and”-“. The standard in the matrix is represented by the experiment containing only low levels of the variables. The first row was always utilized as generation row. From there an orthogonal matrix was developed by shifting the next row one position to the right and placing the last character in the first position of the row. The sum of each column after the whole matrix is developed has to be zero.

4.3.7.2 Response parameter

The kinetic of cell growth was described as:

$$\mu = \frac{\ln(2)}{dt}\left[\frac{1}{d}\right]$$

Equation 4-1 specific growth rate

Where μ = specific growth rate [*1/d*] and dt = doubling time [*d*] (provided by Cellscreen).

The production of the receptor fragment expressed by Lec8 can be described as:

$$c_p = \frac{m_{product}}{l}\left[\frac{mg}{l}\right]$$

Equation 4-2 specific product yield per percentage growth area

Where $c_{product}$ = concentration of receptor [*mg/l*] was determined by ELISA (Ahuja; Ferreira, and Moreira 2004). The product yield was computed independently of the volume, because of the difference in volume per area between a 96 well plate and T Flasks.

4.3.7.3 Selection of Dummies

Another important part of the Plackett Burman screening design was the choice of dummies. The dummies are used to obtain an estimate of error. A dummy variable could be any substance known to have no effect on the response parameter (Ahuja and others 2004). In the experiment DMEM/12 medium was used as a dummy, as the experimental design is based on DMEM/F12 medium. Dummy variables should not have any effect, their values should be zero. A deviation from zero may indicate an analytical error, or deficiency in experimental precision. Interactions not detectable by the Plackett Burman Design may also cause dummy effects (Castro and others 1992). Dummy variables also should not have a significant level (Castro and others 1992). (Ahuja and others 2004;Ganne and Mignot 1991)And for statistical designs it is important that dummies are uniformly distributed over the entire matrix (Giesbrecht F. and Gumbertz L. 2004).

4.3.7.4 Data Analysis for the Plackett Burman Design

Analysis for the Plackett Burman experiment was carried out as follows. First, for all components, including dummies, their effect on the growth and production was calculated, which was the difference between the average response for variables having a higher level "+" and its response having a lower level "-". Thus, the effect of a given variable on the response parameter R could be written as:

$$Effect = \frac{[\Sigma R(H) - \Sigma R(L)]}{N}$$

Equation 4-3 single effects of each variable

Where R(H) = response parameter of a variable in the screening that contains the higher concentration and analogue R(L) = response parameter of a variable with lower quantity of the component, and N = number of experiments (Ahuja and others 2004).

The experimental error was determined as the average square of the dummy effects E_d:

$$V_E = \frac{\Sigma(E_d)^2}{n}$$

Equation 4-4 experimental error

Where V_E is the variance of the effects and n is the number of dummy variables.

The standard error of the effects is determined as the square root of the variance:

$$S.E._{eff} = \sqrt{V_E}$$

Equation 4-5 standard error

Where $S.E._{eff}$ is the standard error of the effects and V_E is variance of the effects

Finally, the significance level of each variable effect was determined using Students´s test:

$$t - value = \frac{E}{S.E._{eff}}$$

Equation 4-6 t-value for estimating the significance level

These analyses allow evaluation of the probability of finding the observed effect purely by change. Confidence level was accepted only to the 80% level. The variables with significantly effects were ranked either positively or negatively (Kim and others 1999)(Castro and others 1992;Kim and others 1998).

4.3.8 Medium Performance SF1-4 and BD CHO Medium

96 well plates contains the tested medium formulation with 0.5% dFCS were inoculated with CHO cells to a final cell density of $2 \cdot 10^4$ *cells/ml* and cultured fro 130 *h*. The cells were added to the different medium formulations from in-house medium with STE and 0.5% dFCS. STE and Nucleosides (BD CHO STE N) were chosen as supplements for BD CHO medium.

4.3.9 Production performance T75 flask with CHO K1

CHO K1 cells were cultured in T75 flask under different medium conditions as shown in table 5.4-6. SF1 and SF2 were passaged 3 times, and then from SF2 the cells were put in the new media formulations SF3 and SF4.The cells remained in this media for two passages. BD CHO Medium supplemented with STE and Nucleosides (N) were kept for 5 passages under the conditions.

Once 100% confluence was reached in every T75 flask the old medium was replaced by fresh medium. After 72 *h* in production phase with 100% confluent growth area the medium was harvested and product yield determinated by ELISA.

4.3.10 Medium Performance with Insulin as additive

The developed media SF1-4 were compared with addition of SITE instead of STE. SITE was available in a 100 fold concentration from Sigma. STE was prepared at the same concentration as SITE. 2 *µl* SITE or STE and 188 *µl* different media including 0.5% dFCS were added to the chambers. The chambers were inoculated with 10 *µl* cell suspension to a final density of $2 \cdot 10^4$ *cells/ml*.

4.3.11 Enhanced productivity using MSX

Both cell lines (K1; Lec8) were cultured for 60 *h* in a 96 well plate under different levels of MSX pressure in DMEM/F12 (Coon´s modified) contains GSS, STE and 1% dFCS. MSX concentration was increased in 12.5 *µM* increments from 25 *µM to*100 *µM*.

Table 4.3-2: Composition of volumes per chamber on a 96 well plate with a working volume of 200 *µl*.

	MSX						
Compounds	**25*µM***	**37.5*µM***	**50*µM***	**62.5*µM***	**75*µM***	**87.5*µM***	**100*µM***
Basic Medium [*µl*]	190	189	188	187	186	185	184
MSX (1.25 *mM*) [*µl*]	0	1	2	3	4	5	6
Cell Suspension [*µl*]	10	10	10	10	10	10	10

4.3.12 Medium performance with increased MSX levels

CHO K1 were added from the backup culture conditions DMEM/F12 (Coon´s modified) supplemented with GSS, STE and 1% dFCS directly in four screenings. Basic medium in the table 4.3-3 is either:

- DMEM/F12,GSS, STE
- SF1
- SF2
- BD CHO Medium,STE

The plate was inoculated with $2 \cdot 10^4$ *cells/ml* and cultured for 90 *h*. Four replicates were chosen for this screening.

Table 4.3-3: Composition of volumes per chamber on a 96 well plate with a working volume of 200 *µl*.

Compounds	**25 *µM* MSX**	**50 *µM* MSX**
Basic Medium [*µl*]	186	184
MSX (1.25*mM*) [*µl*]	0	2
dFCS [*µl*]	2	2
STE 100 x [*µl*]	2	2
Cell Suspension [*µl*]	10	10

4.3.13 Influence of ammonium on cell growth and productivity

A 400 *mM* stock solution of ammonium chloride was prepared in PBS (2.14 *g* NH_4Cl (M_{NH4Cl} = 53.46 *g/mol*) in 100 *ml* PBS). The seeding cell density was $2.25 \cdot 10^4$ *cell/ml* and the plate was screened for 99 *h*.

The culture medium consisted of in-house medium supplemented with 1% dFCS and STE. The following table shows the composition of the 96 well plate:

Table 4.3-4: Composition of volumes per chamber on a 96 well plate with a working volume of 200 *µl*.

	NH_4Cl					
Compounds	**0 *mM***	**2 *mM***	**4 *mM***	**6 *mM***	**8 *mM***	**10 *mM***
Basic Medium [*µl*]	195	194	193	192	191	190
NH_4Cl (400*mM*) [*µl*]	0	1	2	3	4	5
Cell Suspension [*µl*]	5	5	5	5	5	5

4.3.14 Influence of Sodium Butyric Acid productivity

A 400 *mM* stock solution of ammonium chloride was prepared in PBS (440.4 *mg* Sodium Butyrate ($M_{Sodium\ Butyrate}$ = 110.1 *g/mol*) in 10 *ml* PBS). The seeding cell density was $2.25 \cdot 10^4$ *cell/ml* and the plate was screened for 99 *h*.

The culture medium was in-house medium supplemented with 1% dFCS and STE. The composition of the 96 well plate is shown in the following table:

Table 4.3-5: Composition of volumes per chamber on a 96 well plate with a working volume of 200 *µl*.

	Sodium Butyrate					
Compounds	**0 *mM***	**2 *mM***	**4 *mM***	**6 *mM***	**8 *mM***	**10 *mM***
Basic Medium [*µl*]	195	194	193	192	191	190
Sodium Butyrate (400 *mM*) [*µl*]	0	1	2	3	4	5
Cell Suspension [*µl*]	5	5	5	5	5	5

4.3.15 Cell Proliferation for Purification Purposes

The product quality of truncated IR can only be detected after the ion exchange chromatography. The yield of the IR fragment obtained from three different medium formulations were purified.

4.3.15.1 DMEM/F12 supplemented with STE and 1% dFCS

Scale up was done with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE to achieve the required amount of truncated IR for the purification of the product. A spinner flask (NBS; USA) was inoculated with Lec8 to a final cell density of $1 \cdot 10^6$ *cells/ml*. Glucose and lactate concentrations and medium colour were monitored every day. When glucose level reduced to 1.5 *g/l* or lactate increased to 1.6 *g/l* then 250 -500 *ml* of medium was replaced. Medium colour indicates the acidic of media. . The harvested medium was stored at 4°C with the addition of (0.002%) azide. Finally 0.9 litre of harvested medium with 5 *mg/l* truncated IR was purified according to the described procedure (chapter 4.5) in methods. After purification on an affinity column and gel filtration, 3.9 *mg* (quantisation see: 4.5-2) of product was obtained. Finally 1mg truncated IR was injected to the ion exchange column.

4.3.15.2 BD CHO Medium (sf) supplemented with STE

Cells producing IR fragment were not grown in spinner flasks due to problems previously encountered in prior trials. T175 flasks were used to culture CHO Lec8 in BD CHO Medium supplemented with STE. By increasing of the cell number in the T flasks under this condition, the amount of floating cells in the supernatant was increased. The cell suspension harvested from the supernatant was divided in aliquots into fresh T flasks and medium added to a final volume of 50 *ml*. Cell parameter were routinely determined and the cell viability did not drop below 80%. A final volume of 1.2 *l* was harvested from 25 T175 flasks. The harvest was centrifuged for 5 *min* at 1000 *rpm* prior storing at 4°*C* supplemented with (0.002%) azide. The purification procedure is described in methods (chapter 4.5).

4.3.15.3 DMEM/F12 with 4% dFCS and 0.1% Pluronic (F68)

The CHO Lec8 cultured in suspension mode were scaled up from 50 *ml* shaker bottle to a 3 litre bioreactor (NBS,USA) in order to yield enough truncated IR for investigation of the product quality. The scale up work was performed in 250 *ml* shaker bottles (Schott,USA), 150 *ml* Spinner (Corning,USA) 1 *l* Spinner (Corning,USA) and a Bioreactor (NBS,USA) . Stirring platform (Vineland,USA) was set on level 2 during scale up work in 250 *ml* and 1 *l* spinners. Viability was constantly >90% and shearing effects from the stirring bars or baffles in the 1 *l* spinner were not observed. Growth was stopped at a filling volume over 800 *ml* in a 1 *l* Spinner. Presumably the oxygen or gas exchange could not be maintained at this volume, however the viability was not affected. The cell growth was recovered after decreasing the filling volume to 600 *ml* and was kept at this level until achieving the required cell concentration for inoculation of the reactor. The reactor was inoculated with 1.7 *l* cell suspension $0.8 \cdot 10^6$ *cell/ml* with a viability of 91%. After two days in the bioreactor the culture died. The cell death could not be explained.

The harvest obtained from Scale up in spinner flasks was used for the purification of truncated IR.

4.4 Assays

4.4.1 Glucose and Lactic acid determination

The biochemical analyser YSI 2700 Select Biochemistry Analyzer was used for Glucose and Lactic acid determination of the culture medium. The measurement principle is based on a specific enzymatic reaction. The enzymes are immobilized between two membranes. The target substances such as glucose and lactate are oxidated and the H_2O_2 is generated. This H_2O_2 is measured by a platinum electrode. The electron flow is linearly proportional to the steady state H_2O_2 concentration and, therefore, to the concentration of substrate (YSI Inc. 1995).

4.4.2 Cell count with Cedex HiRes® (Innovatis,Germany) and ViCell™ (Coulter & Beckman,USA)

The determination of the number of viable cells (*cells/ml*) and cell viability of cell suspensions is a standardized procedure based on the manual tryphan blue exclusion method.
The tryphan exclusion method is based on the principle that viable cells will not take up the dye, while dead or dying cells have a compromised membrane that allows for the uptake of the tryphan blue. Thus, dead cells will appear darkly stained when viewed with a microscope in a microscope (Innovatis 2002).
Both the Cedex HiRes® and ViCell™ were used for cell parameter determination such as viability, cell number, diameter, circularity and compactness (only Cedex HiReS®,Innovatis). Both systems follow the tryptophan exclusion principle, but the techniques differ.
Cedex HiRes® mixes cell suspension and tryphan blue in the beaker and the stained cells are pumped via capillary tubes into precision flow chamber and the images were recorded with a scanner after the cells have settled. The scanned image of the flow chamber is then divided into smaller images for evaluation.
Vicell™ system (Coulter & Beckman,USA) utilized a flow cell and recorded up to 100 images per measurement. The cell images were taken while flowing. Required sample volumes were 300 μl for Cedex HiRes® and 1ml for ViCell™ (Coulter & Beckman,USA).

4.4.2.1 Viability

Measurement of the overall health of cell cultures requires accurate measurements of both cell density and percentage of viable or live cells. This data is essential to the decision making process for basic tissue culture cell growth and maintaining optimum culture conditions (Beckmann & Coulter).

4.4.2.2 Diameter and circularity

Diameter and circularity are very important parameters for medium optimisation and adaptation work because they provide information about the morphology of the culture.

4.4.2.3 Compactness

This unique parameter measured by the Cedex HiRes® describes the ratio between circumference and cell area. A circle has the value of one. Each cell line is unique therefore has its own unique compactness value, for example for CHO K1 this compactness factor is ≈1.32. This value describes a situation when the cells are healthy. Deviation from the ideal value can be used to judge the health of the culture and thus helps to identify suboptimal culture conditions (Innovatis 2002).

4.4.3 Cellscreen® system (Innovatis,Germany)

Eukaryotic cells grown in micro plates can be analysed using the non-invasive, automated system called Cellscreen®. Images of the entire well of a plate or regions of interests can be captured by the Cellscreen®. Following the images are processed and stored in a database. Growth curves and other parameters are generated by the software. Archiving of the data allows the recall of the experiments at any time for reviewing or reanalysing.(Innovatis 2004).

4.4.3.1 Experimental setup

CS-experiment preparation module

Prior to the measurement starteding the system requires specific details about the experiment. Three different kinds of proliferation studies are provided by the system. Proliferation studies with suspension cells, adherent cells and monoclonal detection for adherent and suspension cells. The adherent module (PA) was chosen for proliferation studies with CHO K1 and its mutant Lec8.

Culture system

The following culture system was chosen: dimensions of common culture plates from known manufacturers were stored in the system.6 - 96 well-plates were accepted as screening plates. Nunc 96 well plate was the plate of choice for proliferation studies with adherent cells.

Chambers

For growth studies in 96-well plates the use of the outer wells is not recommended by Innovatis. Outer wells were filled with sterile water or PBS to humidify the plates and minimise evaporation of the medium. Chambers which were utilized for experiments has to be marked as active chambers on an interactive 96 well plate in the software.

Regions of Interests

Per well, up to 16 images can be taken by using the adherent screening modus. This number can be reduced to save time and space on the hard disc drive. If a homologues distribution of the cells in the well could be guarantied, the reduction of taken images can be dropped to one image per well. Due the low inoculation volume of 5-10 μl in the experiment a homologues distribution could not be guarantied so the entire well was recorded with 16 *images/well* or 25 *s/well* were acquired.

Definition of groups

Each condition was screened in at least triplicates and the software provides a feature which allows the declaration groups. Growth curves were displayed as average of pooled wells in a group in the result view.

Input of experiment information

Addition information can be added to each experiment. Information such as filling volume, cell density, cell line, excepted doubling time and cell diameter were added. These parameters are for user information only and not included in the calculations performed by the software. Further information concerning the experiment was entered in an additional field called "experiment comments".

4.4.3.2 Data acquisition

Images of the complete or only part of the well were recorded by starting the CS-acquisition module. All potential sources of error such as finger prints, medium or liquid drops on the inner or outer side of the plate were avoided. Refraction from these artefacts would disturb the measurement. The plate lids were secured with sticky tape to avoid accidental movement. The plates were inserted in the motor x-y-stage table as programmed. For cloning and adherent studies the objective has to be set 4 times. When CS-progressing operator signalled "IDLE", the acquisition was started. CS processing is responsible for the evaluation of recorded images.

Checking of correct positioning is the first step performed by the cell screen system. At first, the two active chambers furthest from each other in the culture-vessel were determined and the assumed centre of the chamber on the far top left of the culture vessel was positioned with the motor stage of the microscope. When the position was set, the calibration starts with the

determination of the actual centre point. The centre point was determined by computing the perimeter of a chamber. The centre point was determined with a precision of ±15 pixles, waning from a 4 x objective the error is ± 25µm. Average diameter of a CHO Lec8 is ~15 µm. Once the centre point is evaluated the measurement is started.

4.4.3.3 Result view with Cellscreen® software

A very user friendly results view is provided by the Cellscreen® software. Growth characteristics can be observed and interpreted by growth curves or images of the cells. Experiments or single wells can be selected to display doubling time, regression quality of the graph and minimum growth area. The exportation of data into Excel for further investigations or combination with external created data such as product yield obtained from ELISA is easily practicable. The legend displays beside the well or experiment name the doubling time in days.

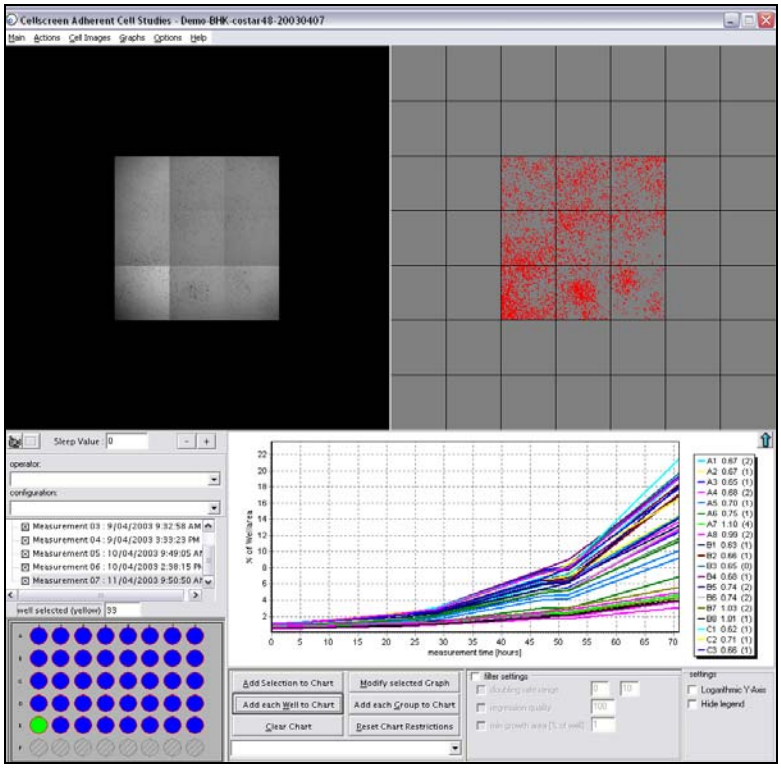

Figure 4.4-1: PA result view Cellscreen® System (Innovatis;Germany).

4.4.4 Product yield determination principle

The technology is based on the use of lanthanide chelate labels with unique fluorescence properties. Among the possible lanthanide chelate Europium (Eu^{3+}) is used. In time resolved fluorescence, the label is measured at a time when the background fluorescence has completely died away. Due longer stokes shift and decay time enabled due properties of lanthanide chelates. Eu^{3+} is connected via DTDA to a ligand or IGG protein of the target protein. Europium labelled protein is aimed at the target protein. After incubation time the Eu^{3+} was released by pH shift and counted by Wallac Victor^{2}™ 1420 (EG&G Wallac).

IR and EGF

A 96- well plate (Greiner,Germany) was coated with 100 *µl* protein G (Sigma,USA) in bicarbonate buffer overnight at 4 *ºC* and blocked with 125 *µl* of 0.5% ovalbumin in TBS for two hours at room temperature. The plates were frozen at -20°*C* and thawed required. The blocking solution was discarded and 100 *µl* diluted samples and standardised protein in culture medium was added in triplicates on the plate. After incubation for three hours at room temperature or for 4°*C* overnight liquid was removed and the wells were washed three times with 1 x TBST buffer using a well-wash 4MK2 system (Labsystems,Finnland).

100 *µl* of europium labelled protein G for detection of IR and europium labelled ligand for EGF in ligand binding buffer, pH 8.0, were added, incubated for a further three hours at room temperature or 4°*C* overnight and washed again.

100 *µl* Enhancement solution (Perkin Elmer,Finnland) was added to each well and incubated for 10 minutes at room temperature. Europium time- resolved fluorescence was measured using a Wallac Victor^{2}™ 1420 multiple counter (Perkin Elmer, Finnland). Culture media with out target was taken for background estimation.

4.4.5 Ammonium Test Kit (Art. 91315, Riedel-de Haen)

Ammonium a catabolic product can have an impact on growth, viability and productivity.
A simple Ammonium test kit was used with measuring range of 0-400 *mg/l* NH_4^+. 5 *ml* sample volume was required. The sample was centrifuged for 5 *min* at 4000 *rpm*. Following the sample was added in a beaker and mixed with three drops of NaOH. The colour of the sample was compared to a colour scale.

4.4.6 Osmolarity determination Osmometer 800 Cl (Slamed, Germany)

Measuring principle

The osmolarity of a solution is a function of the number of particles – molecules and ions of the substances, which are dissolved in 1 *kg* of the solvent. The osmotic concentration or osmolarity does not depend on the molecular weight of the solved substances. There are several ways to detect osmotic pressure, but the method of choice was the freezing point detection method. The relationship between freezing point and osmolarity in diluted solutions is a linear function.

Measurement

The one point calibration requires distilled water for the evaluation of the point of origin.
100 *µl* sample was filled into a 1.5 *ml* tube and then attached to the measuring head. The measuring head with attached test tube was inserted in to the cooling chamber. After the measuring cycle was finished the results were displayed in *mOsm/1kg* H_2O. (Measuring range: 0-2000 *mOsm/1kg* H_2O)

4.4.7 Amino acid determination HPLC

The system is a Waters Alliance HPLC, controlled via Waters proprietary Millenium software.
A Waters Cation exchange column (Wat080002) was employed with two eluting buffers

- Buffer A pH 2.96 [0.2M] Na+
- Buffer B pH 6.5 [1.2M] Na+

With a elution temperature of 65°*C* .

Ninhydrin post column reaction with reaction temperature of 125°*C* was used to develop a chromophore detected by a Waters 2487 UV/Vis detector. Primary amino acids were detected at 570 *nm* and secondary amino acids at 440 *nm*. Millenium software was used for data collection and calculation.

Samples have to be prepared for amino acid determination with Waters Alliance HPLC according to the following procedure. The samples were collected in 1.5 *ml* tubes and treated with trichloric acid at a concentration of 5 *Vol%*. The well agitated samples were stored in the fridge for 30 minutes at 4°*C*. After coagulation of the proteins the sample was centrifuged for 5 minutes at 10000 *rpm*. 10 *µl* of the prepared samples were injected.

4.5 Purification of IR

4.5.1 Affinity chromatography

Soluble truncated IR protein was recovered from harvested fermentation medium by affinity chromatography columns prepared from BOC Insulin covalently connected to divinyl sulfone-activated-agarose beads (Mini Leak; Kem En Tec, Denmark).

A pre column was filled one third with S300 sephracryl-packing material. The packing material was washed with three column volumes of TBSA-buffer. The pre column and the main column were connected by tubes, the entire system was hermetically sealed. The maximum throughput of the affinity column is 250 *ml/h*. The flow rate was calculated in harvest volume in *ml* divided by time in hours for the single purification run. The entire affinity purification step was performed at 4°*C* environment.

After the harvest has passed through the pre column and the main BOC-Insulin-Mini Leak affinity column, the content of the pre column is not supposed to reuse. The S300 Sephracryl-packing material was discarded and the column rinsed with deionised water.

To wash out unspecific proteins the BOC Insulin Minileak (BIML) column is washed with three column volumes of TBSA pH 8 at room temperature. The elution of the truncated IR protein was done at pH 5 with Elution buffer 1 (0.4 *M* NaCL, 0.2 *M* TriNACiT) , one column volume (200-300 *ml*) of Elution buffer 1 was required for the elution of the product.

The BIML column is reusable and reconditioned to remove any unspecifically bound material with two to three column volumes of 0.5 *M* Citrate with (0.005%) azide. The column is stored at pH8. The pH is confirmed by colour of the column changing to pink and by checking the eluate with pH strips (Merk,Germany). Once pH 8 was reached the column was stored at 4°C.

The eluted receptor fragment from the affinity chromatography step was concentrated in a "stirred cell" concentrator (Vol. 150 *ml*, Cat.nr.0C050C60) (Pall,USA) with a cut off of 50 *kDa*. The concentrator was kept in a bowl of crushed ice, to keep the product cold. N_2 gas was used to press the liquid through the membrane. The volume was reduced to ~10 *ml*, which is the maximum injection volume for the next gel filtration step.

The concentrated eluate was aliquotted in 1.5 *ml* tubes. Those tubes were spun for 5 *min* at 14000 *rpm* (Sigma,Germany).

The supernatant was separated very gently from the unspecific protein pellet with a 1 ml disposable transfer pipette (Bio Rad Style L; Cat: 223-9562) and the clean supernatant containing the receptor fragment was collected is a 15 *ml* Falcon tube.

4.5.2 Gelfiltration

Size exclusion chromatography is the principle of gel filtration and this step provide further reduction of aggregated proteins residues.

A Bio Rad working station with S200 26/60 Superdex™ (Amersham Pharmacia Biotech,Sweden) as packing material were used for the gel filtration step. The injection volume of the injection valve AV7-3 (Bio Rad,USA) was 10 *ml*. Purified receptor fragment was detected by UV/Vis detector (BioRad,USA) at λ=280 *nm* and collected with a fraction collector (Bio Rad Module 2128).

The capacity of the ion exchange column is limited. The measurement of optical density was taken by a photometer (Varian 50-BOO UV Visible). The measured absorbance minus the background of TBSA was divided by a specific factor (0.129) to give the concentration of receptor fragment. The concentration multiplied by the fraction volume gives the total amount of truncated receptor in the fraction.

4.5.3 Ion exchange chromatography

For crystallography proposes the protein purity in regards to charges, glycosylation pattern and structure has be guaranteed. The purity of a protein is proportional to the expected yield of protein crystals. The separation of receptor fragment with different gycolysation patterns and balancing of unspecific charges was performed by ion exchange chromatography.
Following a further concentration step, 1 *mg* of receptor fragment was diluted in 10 *ml* TBSA and injected on an Anion column (UnoQ2 , Volume 2 *ml*). Buffer A (40 *mM* Tris pH8) was used to run the column. Elution was with Buffer B (40 *mM* Tris pH8, KCl). Truncated receptor fragment was detected at $\lambda = 280$ *nm* and collected in fractions.

5 Results

5.1 BD CHO Medium in T25 flasks (Lec8)

After testing several media formulations BD CHO Medium was founded with the best performance for the used cell lines. The growth and production performance was tested in T25 flasks. The adaptation of CHO Lec8 cells in serum free condition utilizing BD CHO Medium succeeded very well under supplementation with STE and long R³ IGF compared to medium alternatives available from Sigma. The performance of BD CHO Medium as shown in Table 5.1-1 is remarkable. While the doubling time of these cells in serum free conditions was increased from approximately 1.25 days to 2.03 days compared to the in-house medium with 10% dFCS, high productivity was achieved utilizing BD CHO Medium. Based on this experience with this medium, BD CHO Medium was tested further.

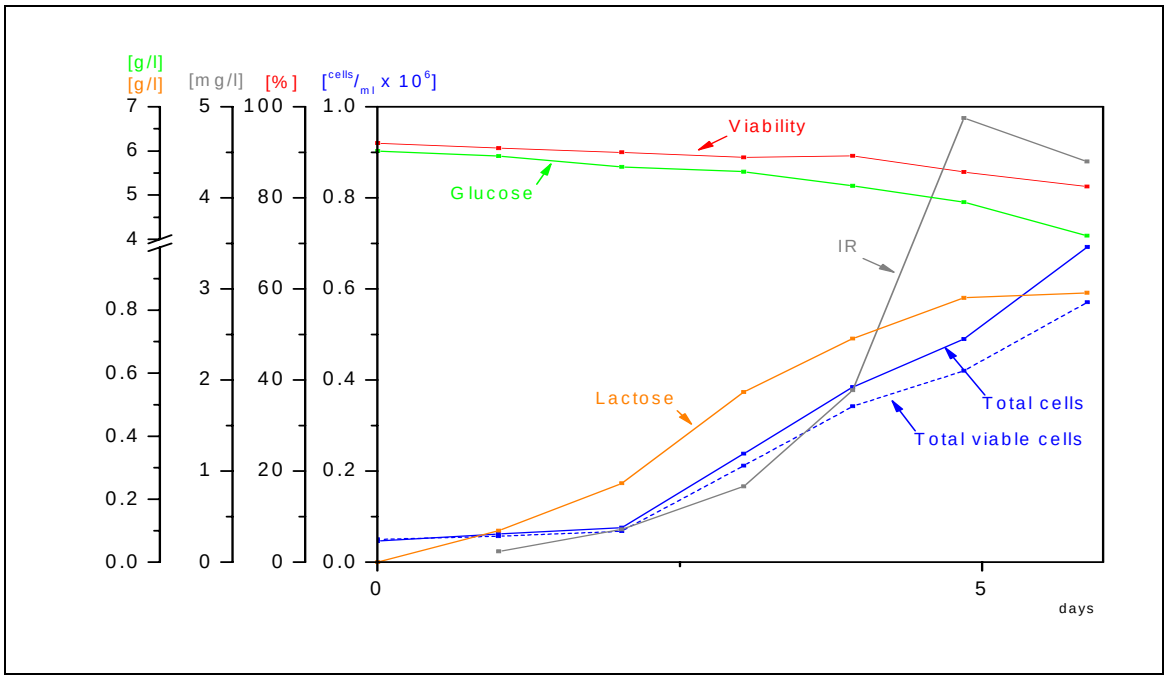

Figure 5.1-1: Shows the growth curve of CHO Lec8 in BD CHO Medium in serum free conditions.

Table 5.1-1: Performance of serum free culture in BD CHO Medium supplemented with STE and 50 *ng/ml* long R³ IGF. Calculations were based on viable cell number.

	µmax [*1/d*]	doubling time [*d*]	$q_{p/x}$ [*µg/(·10⁶ cells · d*]
CHO Lec8	0.341	2.03	2.28

5.2 Peptone screenings

Protein hydrolysates (peptones) as substitutes for serum are used in many in commercially available media formulations, particularly in low protein or protein free formulations.
The addition of peptones might even make the process more robust and decrease product degradation rates. The use of protein hydrolysates (peptones) as a substitute for serum is widely recognized and being a low cost medium supplement it is very important for cell culture medium optimisation (Heidemann R. and Zhang C. 2000).

5.2.1 Peptone screening in T25 flasks (Lec8)

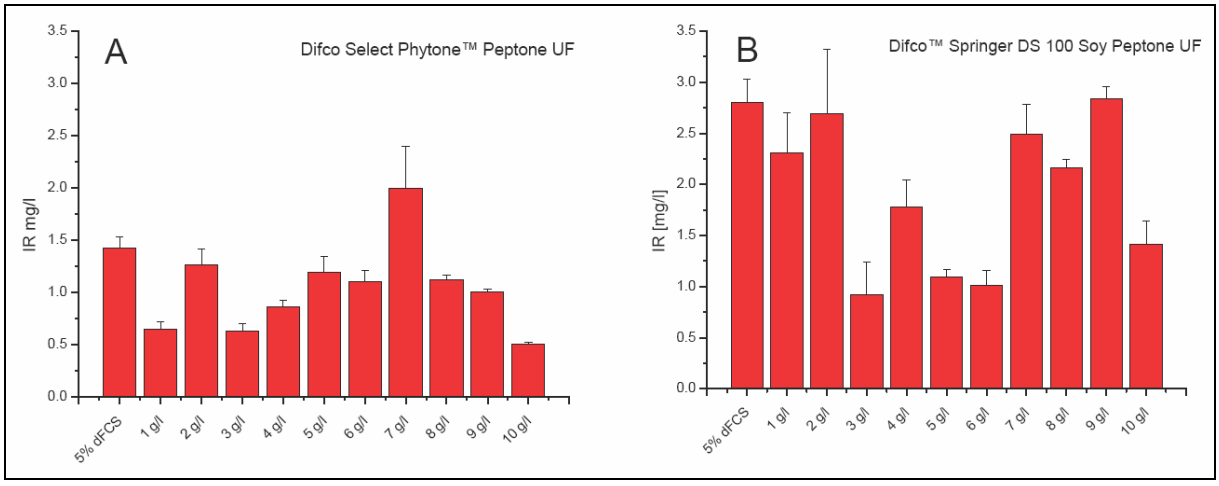

Figure 5.2-1: Peptone screening conducted in T25 flasks with Lec8 expressing truncated IR. DMEM/F12 (Coon´s modified) plus GS-supplements and 1% dFCS was the basal medium.

After monitoring the cell growth for 4 days, it was discovered that the growth was reduced by increased Peptone concentration, although cell morphology was not altered at these high concentrations. As shown Figure 5.2-1 Peptones could be use successfully as a serum substitute and its ability to increase yield was confirmed by this screening. Difco Select Phytone™ Peptone UF compared to 5% serum as standard formulation, has a peak at 7 *g/l*. This observation was confirmed by a second screening under the same conditions. The screening with Difco™ Springer DS 100 Soy Peptone UF was done with a different batch of cells. Cell viability was 94.8% and an inoculation cell density was of $4 \cdot 10^4$ *cells/ml*, which was higher compared to Difco Select Phytone™ Peptone tests. The higher viability of initial seeded culture may explain the higher product yield. DS100 (Figure 5.2-1 chart B) shows better product yields in lower concentrations while Phytone (Figure 5.2-1 chart A) should be

used at a concentration of about 7 *g/l*. All cells were grown for at least two passages after weaning from serum and then added directly added into the T flasks as shown in the histograms (Figure 5.2-1). In both screenings 100% confluence was reached at first with 5% dFCS.

5.2.2 Peptone screening 96 well plates (Lec8)

The first peptone screenings (chapter 5.2.1) Difco Select Phytone™ Peptone UF and Difco™ Springer DS 100 Soy Peptone UF were tested in a concentration range of 1-10 *g/l*. This range was chosen according the recommendation from BD Medium. The productivity enhancing properties were confirmed by using DMEM/F12 (Coon´s modified) supplemented with 1% dFCS without supplementing with STE. A subsequent peptone screening was done by utilizing the Cellscreen® System (Innovatis,Germany), which enabled the screening of 96-well-plates. DMEM/F12 media supplemented with 1% dFCS, STE and 25 *ng/ml* Long R^3 IGF was used during the experiment. Besides the screening of Difco Select Phytone™ Peptone UF and Difco™ Springer DS 100 Soy Peptone UF and TC Yeastolate UF, blends of the three peptones were prepared in the following ratios:

Table 5.2-1: The fraction of Peptones containing in the Blend 1-4.

Blend	DS 100	Phytone	TC Yeast
1	⅓	⅓	⅓
2	-	½	½
3	½	-	½
4	½	½	-

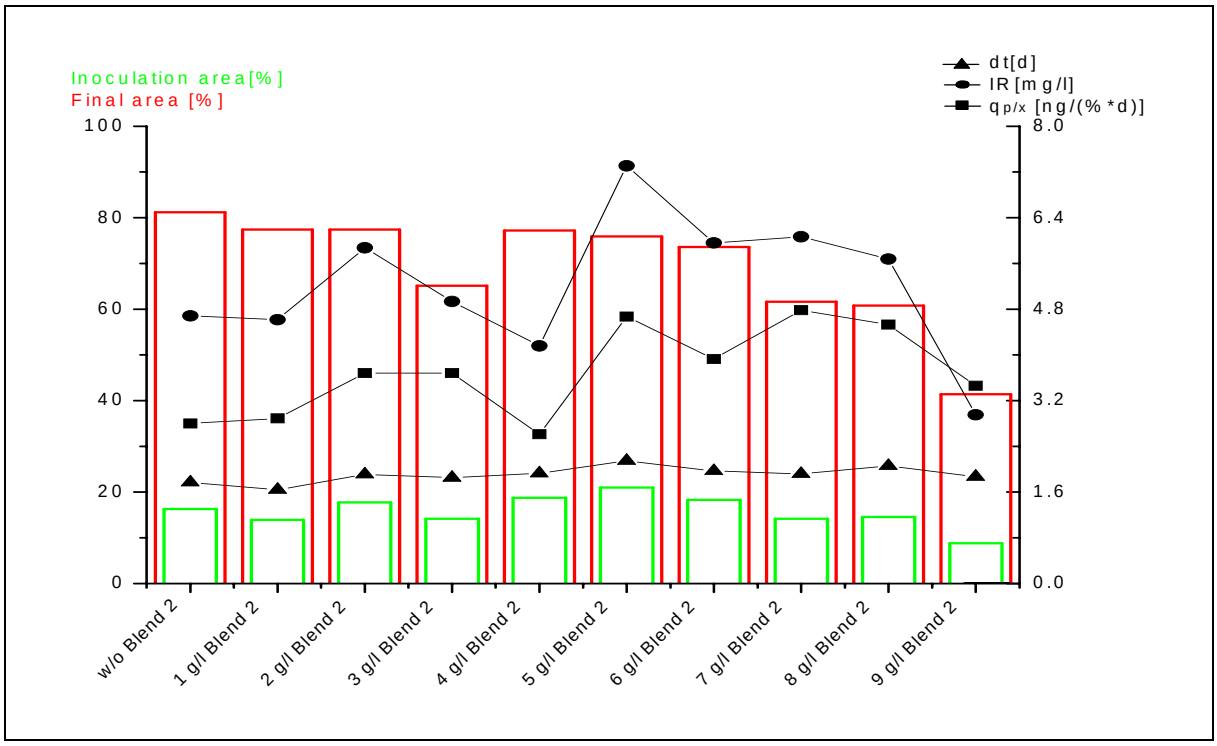

Figure 5.2-2:Blend2 (1:1 of Select,TCY) screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE.

The Figure 5.2-2 represents a chart for a peptone screening. Further charts are located in the Appendix 9.3. No obvious correlation between growth and productivity was observed in these screenings. An interesting observation was the same peaks of the specific production rate at 2 and 5 *g/l* with all three Peptones. DS100 was lowering the productivity and TCY dropped the growth significantly at higher concentrations. Select Phytone had only a slightly lower production performance compared to the standard. This peptone also produced steady productivity and growth over the complete concentration range. Blends of peptones were prepared according Table 5.2-1 and their screenings have shown that a single Peptone can be dominant in the blend (see Appendix: Blend2 (Select is dominant) Figure 9.3-5; Blend3 (TCY is dominant) Figure 9.3-6). Comparing the histograms of a blend and its peptone screened without mixing has confirmed this observation. Interesting is a plateau of productivity in higher concentration (> 5-6 *g/l*) at Blend2, 3 and 4. This fact is important and suggest regular the use of peptone. This is useful information because it seems this blends of peptones may perform production in a wide range of concentration (between 5-8 *g/l*). Thus we do not have to be concerned to pick the exact concentration.

5.3 Adaptation trials into suspension and serum free conditions with Lec8

During the adaptation trials of Lec8 cells into serum free culture conditions (BD CHO Medium) viable suspended cells were observed while most of the cells were still adherent. The ratio of floating and attached cells in the T flask shifted towards floating cells at higher cell densities. Instead of a semi adherent culture, a serum free suspension culture was desired. There were two different approaches to achieve the suspension culture:

1) First adapt cells into serum free condition and from there into a suspension mode.

2) First adapt the anchorage depended cells into suspension culture using 5% serum supplemented media.

All further trials to achieve serum free conditions were started from the newly created suspension culture with serum supplementation.

5.3.1 Adaptation trial with BD CHO Medium serum free

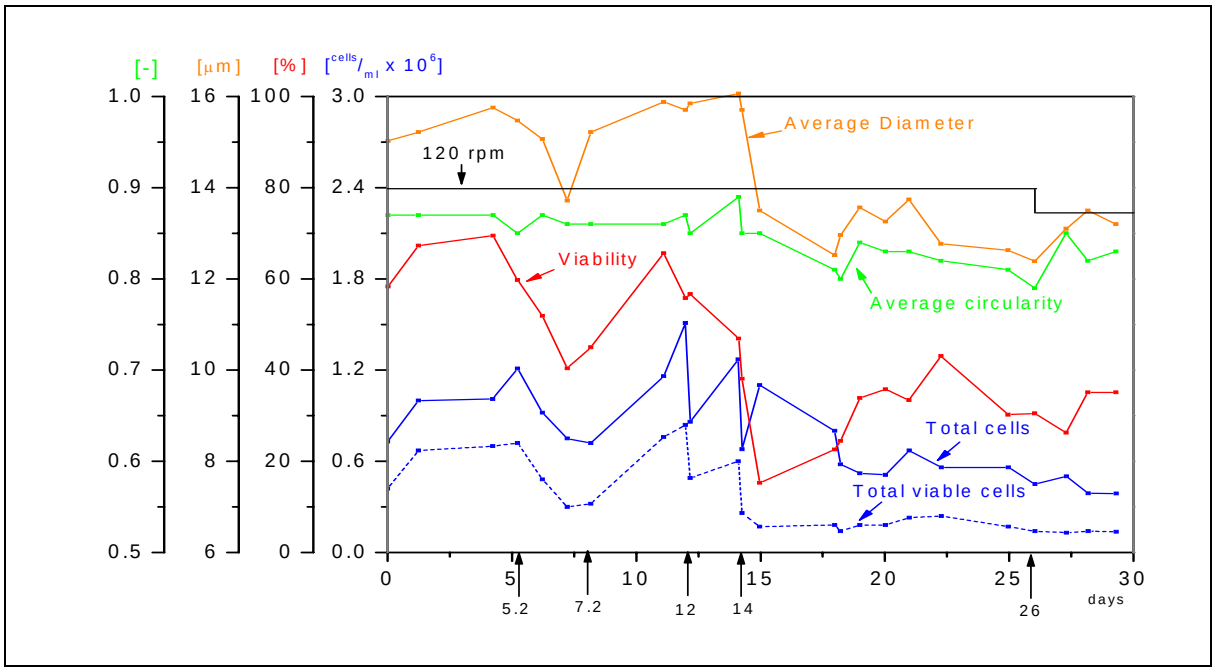

Figure 5.3-1: Adaptation trial with Lec8 in BD CHO Medium (sf) into a suspension culture.

Carried out in 50 *ml* shaker flasks, the shaker speed was kept normally at 120 *rpm*. Results from other adaptation experiments suggested that centrifugation of cells when changing media had a negative effect on cell viability. According to this media change by using a centrifuge was omitted. Medium replacement was conducted after reaching a high cell density in the shaker bottle. Cell suspension was diluted by using fresh medium to maintain a residual cell concentration of greater than $4 \cdot 10^5$ *cells/ml*. BD CHO medium was supplemented with STE, 20 *ng/ml* long R^3 IGF and 0.1% Pluronic (F68). Cell viability at the beginning of the experiment was 80% and dropped within a 24 hours till 60%.The cell growth up to medium change 1 on day 5.2 (Figure 5.3-1) was satisfied, then cell viability dropped. In addition to a medium change on day 7.2 (Figure 5.3-1) new cells were added to the culture. The addition of new cells had not been improved the adaptation. Until 14th day (Figure 5.3-1) the culture looked very promising, but afterwards the viability dropped below 10%. Shaker speed was dropped to 100 rpm on day 26 (Figure 5.3-1). During the last four days of this adaptation trial no improvement of cell viability was found.

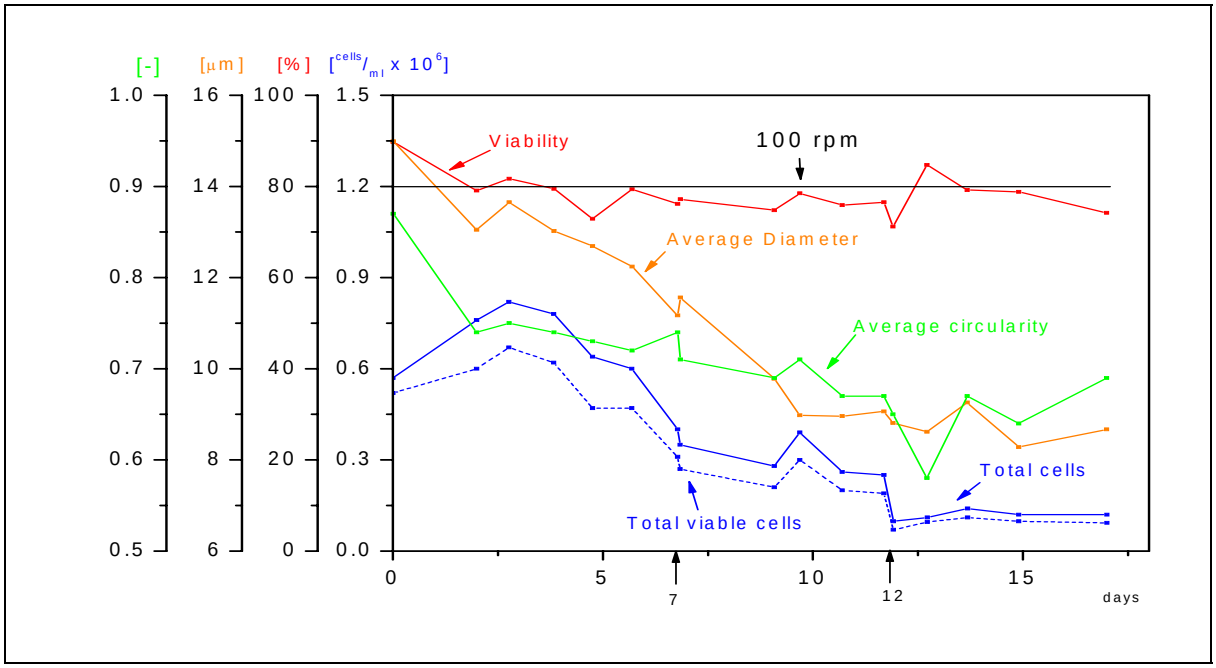

Figure 5.3-2: Adaptation trial with Lec8 in BD CHO Medium (sf) into a suspension culture at 100 *rpm*.

A second adaptation trial was conducted using the same media. Inoculums viability was 90%, shaker speed was 100 *rpm*. During the experiment the viability was constantly high. The comparison of the average diameter of Figure 5.3-1 and 5.3-2 is interesting. In Figure 5.3-1 the diameter was dropped only from 15.5 *µm* to 12.5 *µm*, while the average diameter in the

Figure 5.3-2 was dropped from 15 μm till 8 μm. Also the circularity in the previous trial (Figure 5.3-1) was at least 95% of the original starting circularity whereas in the present trial (Figure 5.3-2) it was fallen to 66% of the commencing level. Generally decreasing level of average diameter and circularity were always accompanied by dropping level of viability, however this phenomenon was not observed in this trial. This difference of the average diameter and circularity could be either a result of the decreased shaker speed or may be changes in the osmotic pressure (not measured during the experiment). Due low consumption of Glucose and low production of Lactic acid demanded only two media replacements. The metabolism was during the entire experiment on a low level.

5.3.2 Successful adaptation trial into suspension mode

Cells were cultivated in a T175 flask using 2% dFCS for this trial. Inoculation medium condition 4% dFCS, 0.1% Pluronic (F68) in CSIRO medium was chosen. Inoculation density was 1.06 $\cdot 10^6$ *cells/ml* while viability was 92.0%. Literature recommends a minimum of 5 $\cdot 10^5$ *cells/ml* for adaptations into a suspension culture. During the adaptation work the working volume varied between 30 – 50 *ml* depending on the cell concentration.

The first three days the shaker bottle was not siliconized and the dead cells stuck inside of the bottle. The viability dropped down till 20 – 30%. At the fourth day the siliconized bottles were used and 30% medium was replaced by fresh medium with 4% dFCS.

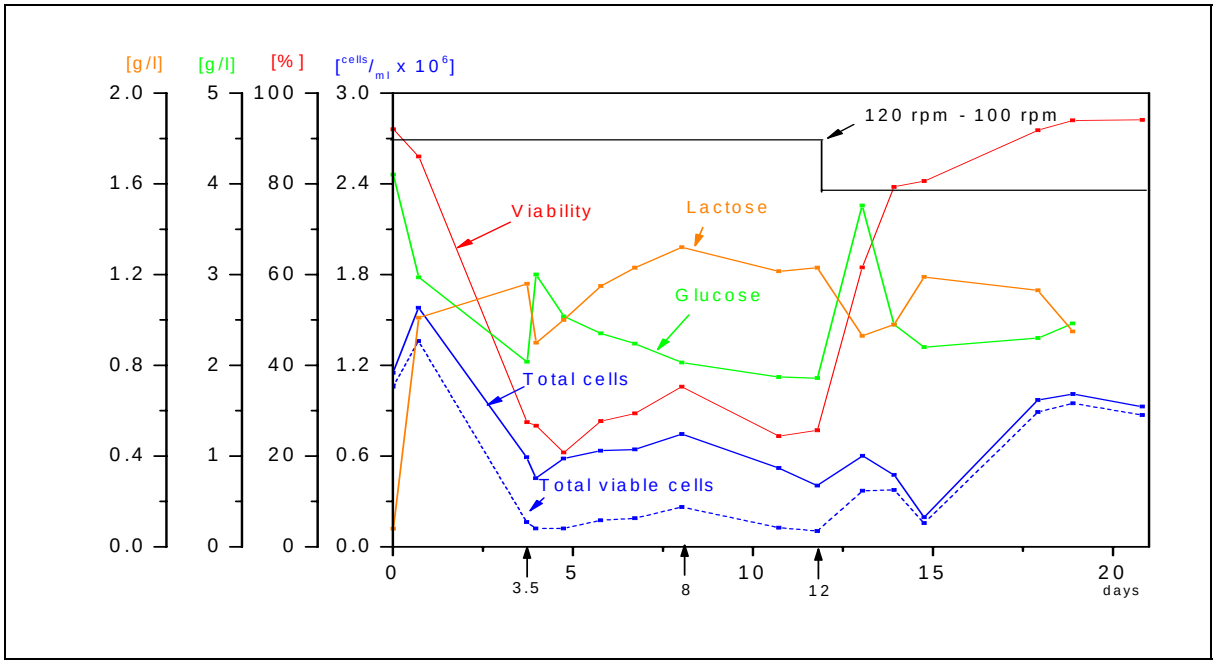

Figure 5.3-3: Successful adaptation from an anchorage dependent cell line into a suspension culture in DMEM/F12 supplemented with 4 % dFCS and 0.1 % Pluronic (F68).

After the medium change on day 3.5 (Figure 5.3-3), the viability was improved until day 8 (Figure 5.3-3).Increasing of Lactic acid production and glucose consumption was observed. The cell viability was rather constant since media change on day 3.5. A steady state of the metabolism was reached on day eight. No significant changes in culture conditions were observed after day 8 until the intervention on day 12. The shaker speed was decreased from 120 *rpm* to 100 *rpm* and a second medium change (Figure 5.3-3) was performed. After the 12th day (Figure 5.3-3) the viability rapidly increases till over 90%.This is most likely due to lowering stirrer speed, however this phenomena should be confirmed in future experiments. Adaptation into suspension condition with 4% dFCS and 0.1% Pluronic (F68) was successful.

5.3.3 Concentrated use of DMEM/F12 as enriched medium formulation

After successful adaptation into suspensions conditions a serum free culture was desired. Apart from using BD CHO Medium, a cost and time saving solution was searched for an enriched medium formulation based on DMEM/F12 medium. Literature suggests higher level of the medium components such as amino acids, vitamins and trace elements for serum free medium formulations compared to the standard formulation. DMEM/F12 (Coon´s modied) in 1.5 and 2 fold concentrations compared to standard formulation was prepared.

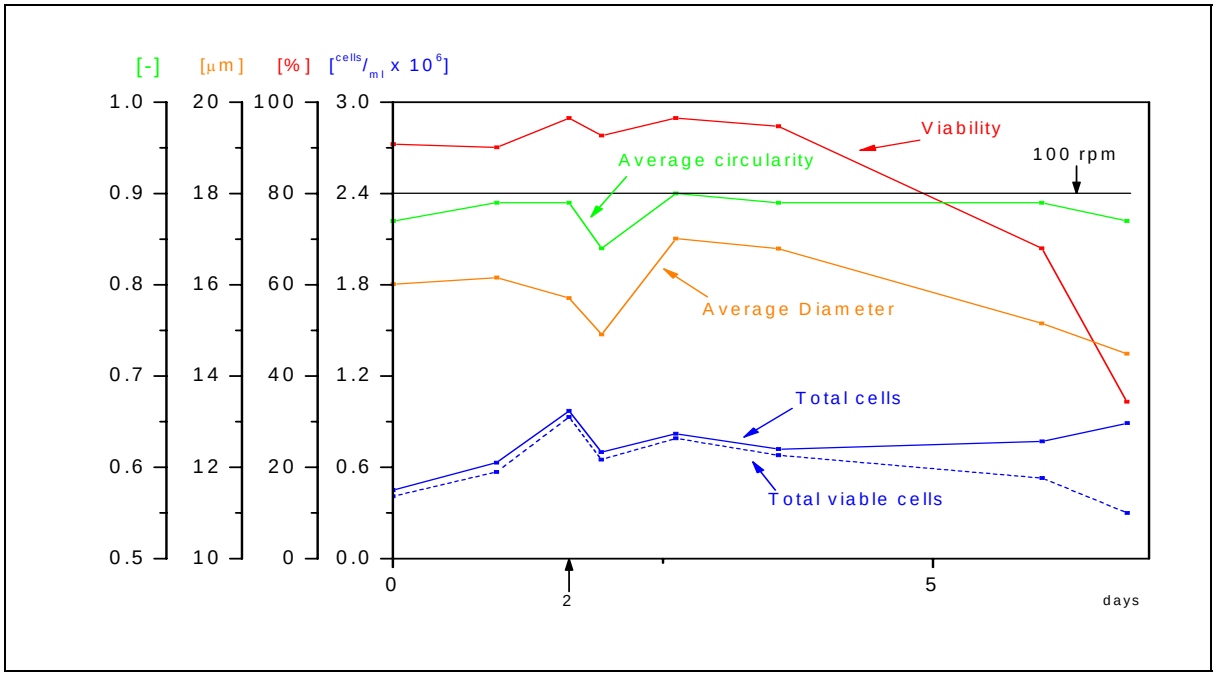

Figure 5.3-4: Adaptation of a Lec8 suspension culure from 4% FCS trial into serum free utilizing 1.5 and 2 fold concentrated DMEM/F12 (Coon´s modiefied).

Cells adapted for suspension mode (see: chapter 5.3.5) kept as Backup for experiments always at a viability >90%. Both medium formulations were tested under the same conditions. 1.5 and 2 fold DMEM/F12 (Coon´s modified) was tested in shaker supplemented with 4% dFCS and 0.1% Pluronic (F68). The experiment started with 91% cell viability. After two days 50% of the cell suspension was replaced with fresh medium. After this growth rate decreased and was stayed into a kind of a stationary phase. Lactic acid concentration was rather low during the experiment and a cell density of $0.7 \cdot 10^6$ *cells/ml* was determined, so no explanation of

growth arrest was available. The similar growth pattern was determined by using 1.5 and 2 fold concentrated in-house medium. The utilisation of concentrated DMEM/F12 (Coon´s modified) was unsuccessful.

5.3.4 Adaptation trial from suspension mode into BD CHO (sf)

A different strategy was applied to this trial. As weaning the suspension culture from 4% dFCS level to serum free conditions repeatedly failed when DMEM/F12 medium was used. However when cells were adapted to serum free conditions using BD CHO Medium in T flasks adaptation was successful. According to this observation, the serum concentration was decreased from 4% to 2% dFCS using BD CHO Medium supplemented with STE and 0.1% Pluronic (F68). After each medium replacement the viability and growth rate were decreased stepwise.

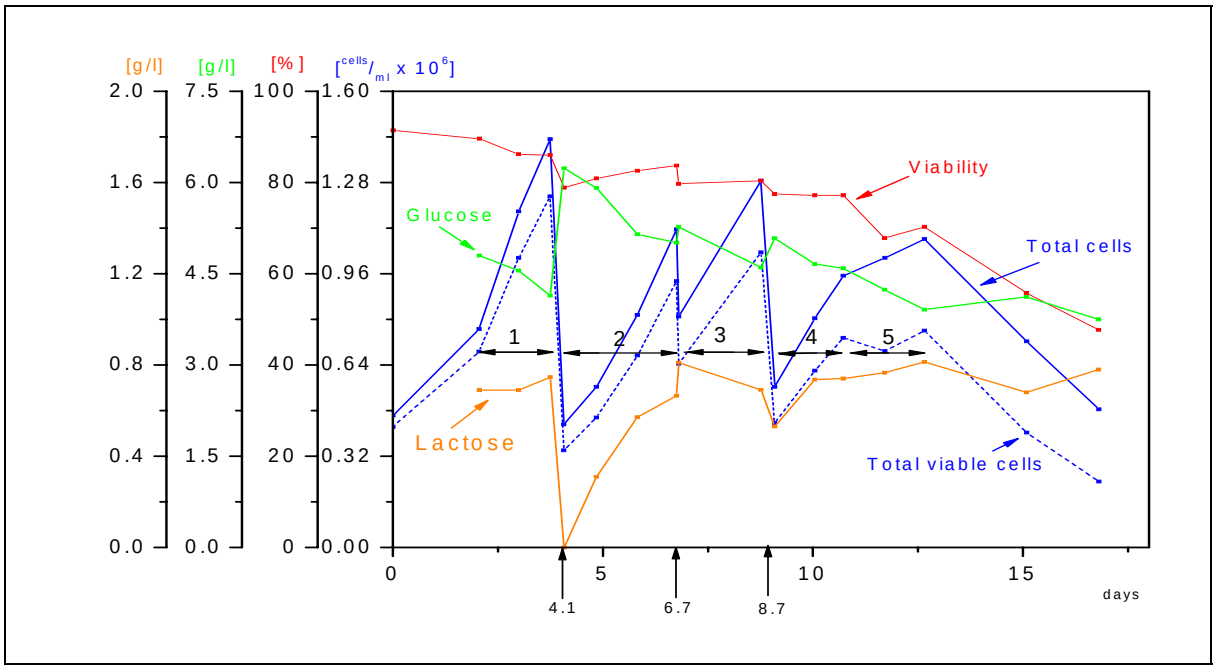

Figure 5.3-5: Adaptation trial from 4%dFCS into serum free conditions utilizing BD CHO Medium.

For the sections 1-5 (highlighted by arrows in Figure 5.3-5) the doubling time and specific glucose consumption rate was calculated based on viable cell concentration:

Table 5.3-1: Shows the evaluated parameter divided in Sections 1 - 5 from Figure 5.3-5. The sections are representing the time range for the conducted calculation of the parameter shown in this table.

Section	μmax [*1/d*]	doubling time [*d*]	$q_{s/x}$ [$g/(10^6\ cells \cdot d)$] (total cells / viable cells)
1	0.351	1.98	0.57 / 0.72
2	0.378	1.84	0.67 / 0.77
3	0.365	1.90	0.72 / 0.88
4	0.321	2.16	1.77 / 1.01
5	0.014	50.4	2.27 / 100

The doubling time was not altered significantly till reaching section four (Table 5.3-1; Figure 5.3-5). From there doubling time (dt) was increased dramatically. Until section 4 (Table 5.3-1), the specific glucose uptake was increased slightly. While normally lactic acid production was increased after each media change, after the second media change (day 8.7) lactic acid production slowed down. However glucose uptake rate was not affected much (Table 5.3-1). Normally cell viability in suspension was increasing after each medium replacement (Figure 5.3-5) this was observed during several experiments. However in this trial the culture was never recovered after a medium replacement and gradually decreased after 11 days.

5.3.5 Performance of suspension culture with basal medium

After a lot of unsuccessful trials with CHO Lec8 into suspension and serum free condition it was decided to not to pursue this goal. It was determined that a suspension culture using DMEM/F12 supplemented with 4% dFCS and 0.1% Pluronic (F68) can be maintained. Serum level cannot be further reduced under this condition. The following parameter for this medium formulation was found:

Table 5.3-2: Performance of created suspension culture in DMEM/F12 (Coon´s modified) supplemented with GSS, 4% dFCS and 0.1%Pluronic (F68). Calculations were based on viable cell number. Estimated error is 15%.

	µmax [*1/d*]	doubling time [*d*]	$q_{p/x}$ [$\mu g/(\cdot 10^6$ *cells* · *d*]
CHO Lec8	0.603	1.149	1.635

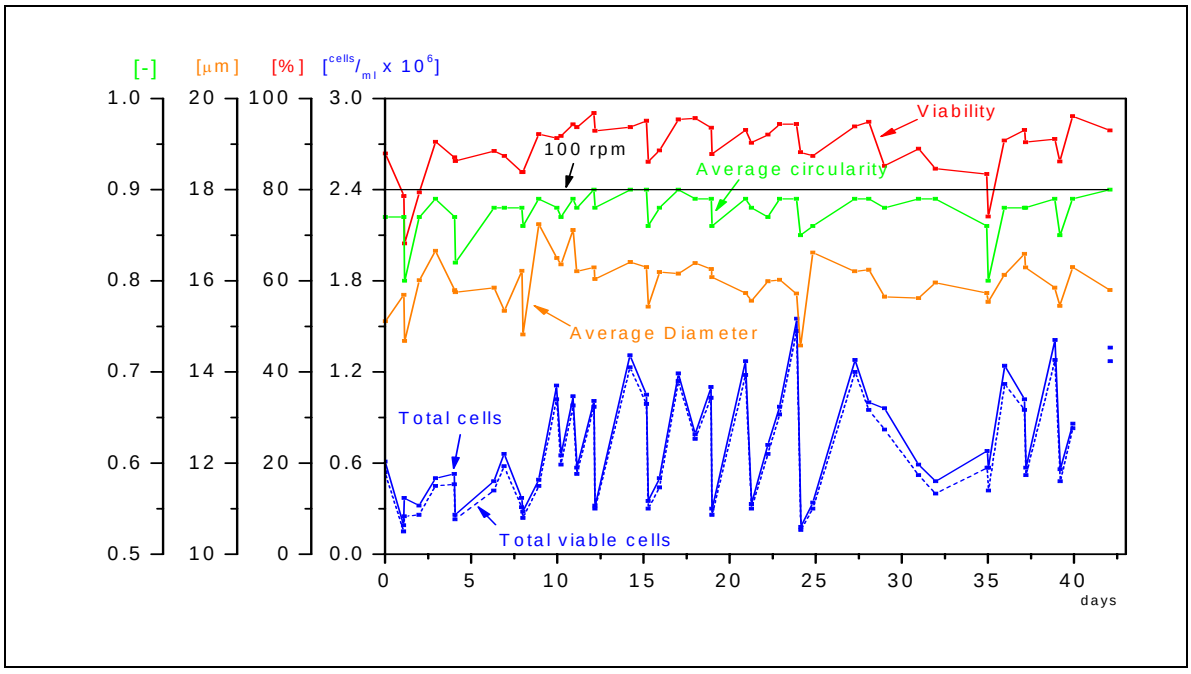

Figure 5.3-6: Show the backup cells (Lec8) in suspension supplemented with 4% dFCS and 0.1% Pluronic (F68). CHO Lec8 in suspension shown during the entire culture a high viability, average diameter and average circularity.

5.4 Results of experiments using Plackett Burman Design

5.4.1 Creating a low serum formulation by using statistical design for CHO Lec8

The concentration of certain media components in CSIRO media is relatively low compared to published medium formulations. These experiments were designed to determine the effects of these additives. The Plackett Burman Designs were conducted in three experiments and the results are shown in Table 5.4-1 and Table 5.4-2.

Table 5.4-1: Results of Plackett Burman Application (Appendix, Table 9.4-1). Effects of variables on productivity and cell growth were determined.

PB-Design 1	Effect on Growth			Effects on Productivity		
	Effect	t-value	Significance[%]	Effect	t-value	Significance[%]
Biotin	-0.045	-3.86		-0.093	-1.88	
Hydrocortisone	0.0254	2.18	84.0	0.2594	5.22	91.8
Hypoxanthine	0.0203	1.74	82.0	-0.111	-2.24	
Dummy	-0.012	-1.00		0.0497	1	77.0
Linoleic acid	0.0128	1.09	77.8	-0.01	-0.20	
Putrescine	-0.043	-3.72		0.0698	1.40	80.1
Transferrin	0.0186	1.59	81.2	0.1305	2.63	85.7
V_E	$1 \cdot 10^{-4}$			$2.47 \cdot 10^{-3}$		
$S.E._{eff}$	$1.17 \cdot 10^{-2}$			$4.97 \cdot 10^{-2}$		

The Table 5.4-1 presents results of a Plackett Burman screening done in eight experiments. The used orthogonal matrix (Appendix, Table 9.4-1) provide a screening capacity of seven variables including one dummy. Based on the dummy the experimental error and the standard error of the effects were computed.

Table 5.4-2: Results of Plackett Burman Application utilizing Matrix (Appendix, Table 9.4-2). Only μ_{max} was taken as response parameter.

	PB-Design 2			PB-Desgin 3		
Variable	Effect	t-value	Significance[%]	Effect	t-value	Significance[%]
Biotin	0.00717	0.24	64.3	-0.04051	-1.08	
Hydrocortisone	-0.02268	-0.77		-0.04242	-1.14	
Hypoxanthine	0.07563	2.57	85.5	0.03431	0.92	76.3
Long R^3 IGF	0.06381	2.17	84.0	0.02446	0.66	73.2
Linoleic acid	0.09439	3.21	87.5	0.05280	1.41	80.1
Putrescine	0.02090	0.71	73.9	0.01108	0.30	66.1
Transferrin	0.07697	2.61	85.6	0.03055	0.82	75.2
TRACE	-0.01829	-0.62		-0.02212	-0.59	
Dummy1	0.02727	0.93	76.3	0.01959	0.52	71.2
Dummy2	-0.02465	-0.83		-0.05069	-1.36	
Dummy3	-0.05363	-1.82		-0.03357	-0.90	
V_E	$8.67 \cdot 10^{-4}$			$1.39 \cdot 10^{-3}$		
$S.E._{eff}$	$2.94 \cdot 10^{-2}$			$3.73 \cdot 10^{-2}$		

The results of the Plackett Burman design utilizing matrix (Appendix,Table 9.4-2) for the study of 8 variables are shown in Table 5.4-2. Three Dummies were used in this matrix and their effects were used to estimate the experimental error. The placement of Dummies was not done according the recommendations of the literature. (Giesbrecht F. and Gumbertz L. 2004)
Two different matrixes (Appendix, Table 9.4-1 and 9.4-2) confirmed the positive effects for of the following compounds: Hypoxanthine, long R^3 IGF, linoleic acid and transferrin.
Hydrocortisone was confirmed in the screening 12 experiments matrix (Appendix, Table 9.4-2) with negative influence on cell growth. But shown in Table 5.3-1, Hydrocortisone was found with growth and productivity promoting properties.
Trace elements were determined with negatives effects on cell growth. Controversial variable were biotin with its slightly positive effect in PB-Design 2, but with a low significance level and putrescine in accompany of growth promoting effects in both results, but with also a low significance level. A repetition of the Plackett Burman Desgin with CHO Lec8 using Matix (Appendix, Table 9.4-1) could be conducted, because the used culture died.
Two low serum medium formulations (SF1 and SF2, Table 5.4-6) were prepared based on the results Plackett Burman application (Table 5.4-1 and 5.4-2) utilizing two different matrixes with a screening capacity of 11 variables (Appendix, Table 9.4-2) and 7 variables (Appendix, Table 9.4-1) .DMEM/F12 (Coon´s modified) including GS-supplements and STE were taken as basal medium. Hypoxanthine, long R^3 IGF and linoleic acid were taken for the medium formulation SF1. In addition hydrocortisone was used to formulate SF2. This decision was based on the results of PB-Design 1 (Table 5.4-1). The final concentrations of the chosen medium supplements in SF1 and SF2 are shown in table 9.5-1 (Appendix).

5.4.2 Error estimation for experiment using 96 well plates

Inoculate densities might be varied from well to well in a 96 well plate experiment. This might cause up to 20 % of error. Therefore experimental results have to be carefully examined to draw a conclusion confidently. The Plackett Burman Designs (Table 5.4-3) were conducted in 12 experiments and each experiment in 5 replicates.

Table 5.4-3: The doubling time and the Standard Deviation computed by 5 replicates of each experiment.

	PB-Design 2		**PB-Design 3**	
Experiment	dt [*d*]	STDEV [%]	dt [*d*]	STDEV [%]
1	0.939	4.6	0.885	6.8
2	0.789	8.9	0.848	6.0
3	0.783	3.7	0.907	1.2
4	0.778	6.8	0.801	3.5
5	0.702	8.1	0.801	3.5
6	0.754	1.1	0.824	4.9
7	0.723	9.2	0.771	8.4
8	0.732	5.6	0.818	4.1
9	0.768	2.7	0.821	4.9
10	0.826	4.4	0.823	3.2
11	0.770	9.0	0.872	4.2
12	1.007	1.5	1.005	2.5

To calculate the error caused by different seeding cell densities and therefore the appearing of significant error of the results in the Plackett Burman designs (Table 5.4-2), the standard deviation based on five replicates of each experiment was evaluated. (Castro and others 1992)
The specific growth rate μ_{max} was calculated from doubling time, which was provided and computed by the Cellscreen® software. The inoculation area had to be at least >5% for each wells, otherwise the lag phase was lasted longer or no cell growth was observed.
The calculated standard deviation (Table 5.4-3) for Plackett Burman Design 2 and 3 (Table 5.4-2) was very satisfied and validated the decision to formulate the new medium formulations.

5.4.3 Amino acids consumption of CHO K1 in T175 Flasks

Table 5.4-4: Amino Acid Consumption.

Amino acid	Consumption [%]
serine	97.46
aspartic acid	93.95
glutamic acid	79.80
leucine	70.25
isoleucine	61.14
phenylalanine	59.28
valine	55.33
methionine	54.69
threonine	54.15
tyrosine	53.60
cysteine	47.46
lysine	45.35
histidine	41.85
proline	33.33
arginine	21.90
glycine	18.27

The high level of amino acid concentrations in the Plackett Burman Design was estimated by the comparison of literature value and the consumption of amino acids by CHO K1 (Table 5.4-4). Amino acid consumption was determined in T175 in DMEM/F12 (Coon´s modified); 1% dFCS and STE. When 100 % confluence was reached in a T175flask - a medium change was done and sampling started, each day a sample was taken and analysed by Waters Alliance HPLC. The characteristics of amino acid usage by CHO K1 are shown in Appendix 9.11. The run was terminated after four days, because under standard culture conditions and medium change would be carried out. The amino acid consumption is shown in the table 5.4-4. As an example high consumption of Aspartic acid and Serine was observed (Appendix, Figure 9.11-1). The consumption rate was very important for estimating the concentration range for Plackett Burman Design. The new medium formulation should have the capacity to provide sufficient quantities of amino acids for not undergoing a level of 50% after 4 days. This was taken as rule.

5.4.4 Plackett Burman design applied on CHO K1 producing EGFR Fragment

A wide range of media additives were considered for the development of a low serum formulation for CHO K1 cell line. The Plackett Burman Design was used similar to Designs from chapter 5.4.1. CSIRO medium (supplemented with 0.5% dFCS and STE) was used as basal formulation and the effect on cell growth of amino acids, vitamins, growth factors and trace elements were screened in 24 experiments based on the orthogonal matrix (Appendix, Table 9.4-3) according to Table 9.5-2 (Appendix).

Table 5.4-5: Results of Plackett Burman Matrix based on Matrix (Table 9.4-3; Appendix). Dummies were used for error calculation with equation 3.6.

	Effect	t-value	Significance [%]
Alanine	0.0254	5.27	91.9
Asparagine	0.0049	1.03	77.3
Aspartic acid	-0.027	-5.70	
Glutamic acid	0.0142	2.94	86.7
Dummy1	0.001	0.20	62.6
Glycine	-0.012	-2.6	
Isoleucin	-0.019	-4.00	
Leucin	0.019	3.94	89.3
Methionine	0.0135	2.79	86.2
Dummy2	-0.014	-2.80	
Phenylalanine	0.0025	0.51	71.0
Proline	0.0133	2.76	86.1
Serine	-0.01	-2.10	
Biotin	-0.016	-3.40	
Hydrocortisone	0.05	10.4	98.0
Hypoxanthine	0.0132	2.73	86.0
Linoleic acid	0.0115	2.38	84.8
long R^3 IGF	-0.112	-23.0	
Putresciene	0.0056	1.15	78.3
BSA	-0.122	-25.0	
Pluronic (F68)	0.0046	0.96	76.6
Trace elements	0.0251	5.21	91.8
Dummy3	0.0043	0.89	76.0
V_E	$2.33 \cdot 10^{-05}$		
$S.E._{eff}$	0.0048		

Six replicates were used to draw conclusions in a confident way. Doubling times were calculated by the Cellscreen® system and μ_{max} values were used to calculate the single effects of various components. Generally dummy variables had low effect which means low

statistical errors, however relatively high significance of the dummies might indicate interaction between variables.

Positive and negative effects of the individual components on cell growth were calculated. The new media formulation is based on the results of components with positive effects were identified. Variables which had negative effect on the cell growth were not added to the basal medium. The positive group consists of: alanine, glutamic acid, leucine, methionine, proline, hydrocortisone, hypoxanthine, linoleic acid and trace elements.

Table 9.5-2 (Appendix) shows the concentration of the compounds used for the new media formulation for the CHO K1 cell line.

5.4.5 Medium performance of SF1 - 4 and BD CHO Medium

Four media formulations were developed using the Plackett Burman Design. The performance of these formulations was tested in this experiment. The screenings were conducted using 96 well plates.

Table 5.4-6: Shows the contents of the developed medium formulations based on DMEM/F12 (Coon´s modified) supplemented with GS-supplements. The contents of BD CHO Medium are unknown.

		Developed with CHO Lec8		Developed with CHO K1		Purchased BD CHO Medium	
	Concentration	**SF1**	**SF2**	**SF3**	**SF4**	**BD CHO STE**	**BD CHO STE N**
Hypoxanthine	10 *mg/l*	•	•	•	•		
Hydrocortisone	10 *mg/l*		•	•	•		
Long R³ IGF	25 *ng/ml*	•	•				
Linoleic acid	0.902 *mg/l*	•	•	•	•		
STE	1 x	•	•	•	•		
Trace	1 x			•	•		
Alanine	37.9 *mg/l*			•	•		
Glutamic acid	189.7 *mg/l*			•	•		
Leucine	95.55 *mg/l*			•	•		
Methionine	39.48 *mg/l*			•	•		
Proline	64.25 *mg/l*			•	•		
Serine	82.1 *mg/l*				•		
Aspartic acid	46.7 *mg/l*				•		
Nucleosides	1 x	•	•	•	•		•

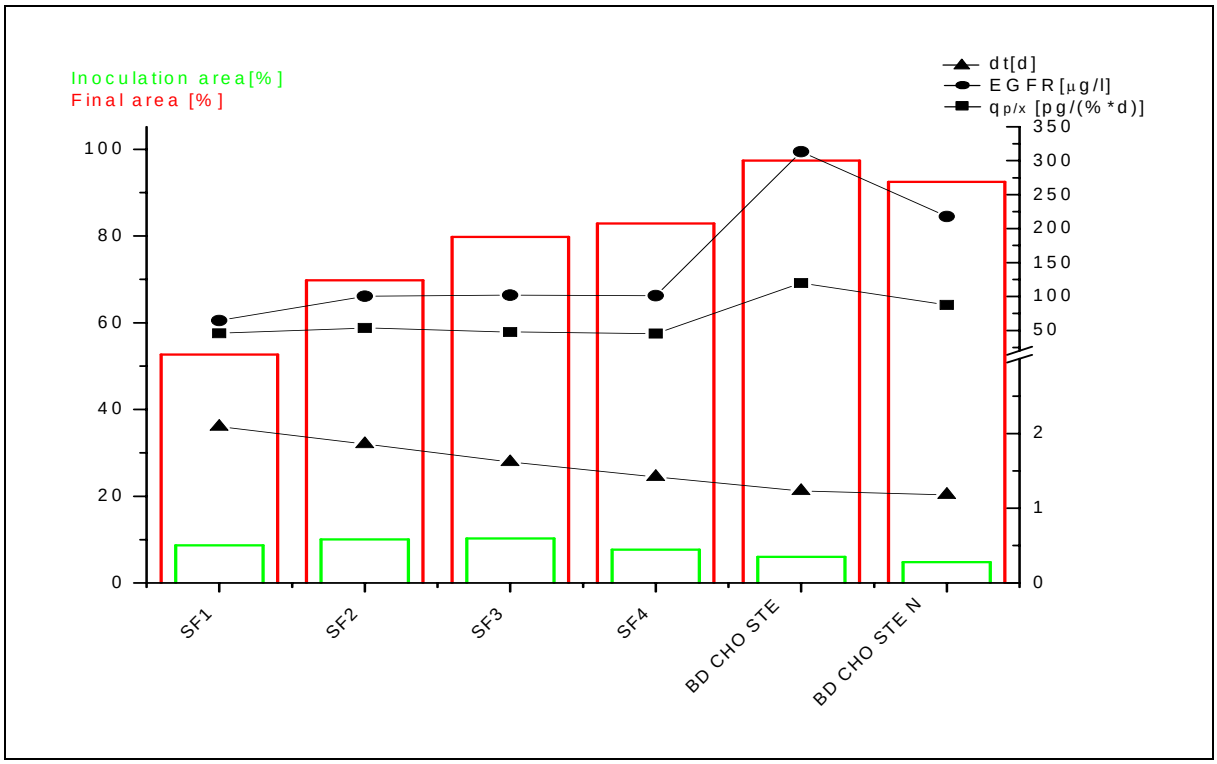

Figure 5.4-1: Performance of the four developed medium formulation SF 1-4 and BD CHO Medium and BD CHO Medium supplemented with Nucleosides at 0.5% dFCS.

The four media formulations are shown own in Figure 5.4-1. SF1 and SF2 were developed for Lec8 line. SF3 and SF 4 were developed for CHO K1. Each development step provided an improvement in growth with CHO K1 producing EGFR fragment. Productivity was not improved by any of the self developed medium formulation.

Table 5.4-7: Show the summarization of data shown in Figure 5.4-1.

	dt [*d*]	[%]	$q_{p/x}$[*pg/(%·d)*]	[%]	EGF [*µg/l*]	[%]
SF1	2.09	177.12	45.58	38.09	64.52	20.60
SF2	1.86	157.63	53.51	44.71	100.40	32.05
SF3	1.62	137.29	47.49	39.68	101.85	32.52
SF4	1.42	120.34	45.48	38.00	101.31	32.34
BD CHO STE	1.23	104.24	**119.68**	***100.00***	**313.25**	***100.00***
BD CHO STE N	***1.18***	***100.00***	87.64	73.23	217.84	69.54

The best performance in productivity was observed with BD CHO STE. However, based on the doubling time, the commercial BD CHO Medium supplemented with Nucleosides performed best. Slightly improved growths were achieved using SF 1–4 medium, but no significant improvement on productivities were accomplished. The average specific production rates are about 1.5 fold in the SF 1-4 media formulation compared to value achieved using BD CHO Medium.

5.4.6 Performance of SF1- 4 and BD CHO Medium T75 flask with CHO K1

Using productivity as response parameter in for the screening experiments when growth rates are different is misleading. 100% confluence of the growth area was reached at different times and thus the stationary production phase of CHO cells.

To avoid this error, an experiment was conducted in T75 flasks. After 100% confluence area each T flasks were achieved, medium was changed-over and the productivity of the CHO K1 line in different media was determined.

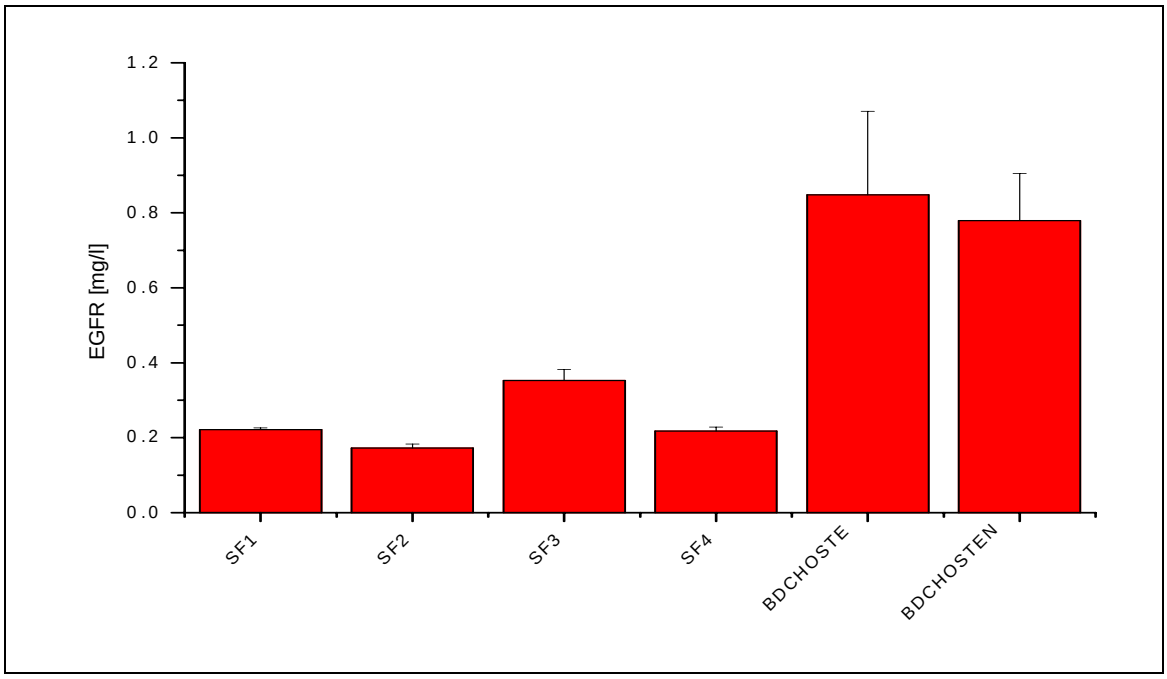

Figure 5.4-2: Shows the comparison of productivity with CHO K1 in T75 Flask and different medium formulations.

The highest product yield was achieved in BD CHO supplemented with STE. Nucleosides addition had a negative effect on the productivity at about 10%. The highest product yield observed using own developed medium was 60% less compared to BD CHO Medium.

Table 5.4-8: Shows cell parameter of CHO K1 recorded utilizing HiRes Cedex® (Innovatis,Germany) after at least two passages in different medium conditions.

	Medium					
	SF1	**SF2**	**SF3**	**SF4**	**BD CHO**	**BD CHO N**
Viability [%]	99.30	99.80	99.70	99.90	99.50	99.80
TCD [10^5 *cells/ml*]	20.41	16.42	11.58	14.11	10.11	8.65
VCD [10^5 *cells/ml*]	20.27	16.39	11.55	14.11	10.06	8.63
Compactness [-]	1.32	1.33	1.33	1.32	1.31	1.32
Diameter[μm]	11.75	11.48	11.05	11.21	12.69	12.73
Aggr. Rate [%]	12.40	11.10	11.40	17.80	19.80	18.30
STDEV [10^5 *cells/ml*]	9.46	9.87	9.15	17.68	9.83	8.03

None of the medium formulation had effect on cell viability or morphology, the cell viability was under each condition on a very high level. The final cell density greatly varied from flask to flask, therefore computing of the specific product yield was omitted. Compactness, the ratio between circumference and cell area can be used as an indicator regarding status of the cell culture, as suboptimal culture conditions (Innovatis 2002). For example, results in a decrease diameter while the compactness is still the same, the diameter of the CHO K1 cells ranged from 11.05 - 12 μm (Table 5.4-8).

5.4.7 Growth and Production promoting effects of insulin with CHO K1

The improved medium formulations SF1-4 were developed without insulin, because of the nature of the product (truncated IR). The product is to be purified by insulin affinity chromatography thus excess insulin interferes with the purification process. Insulin's growth and productivity promoting property are well known. The influence of insulin was investigated in this experiment.

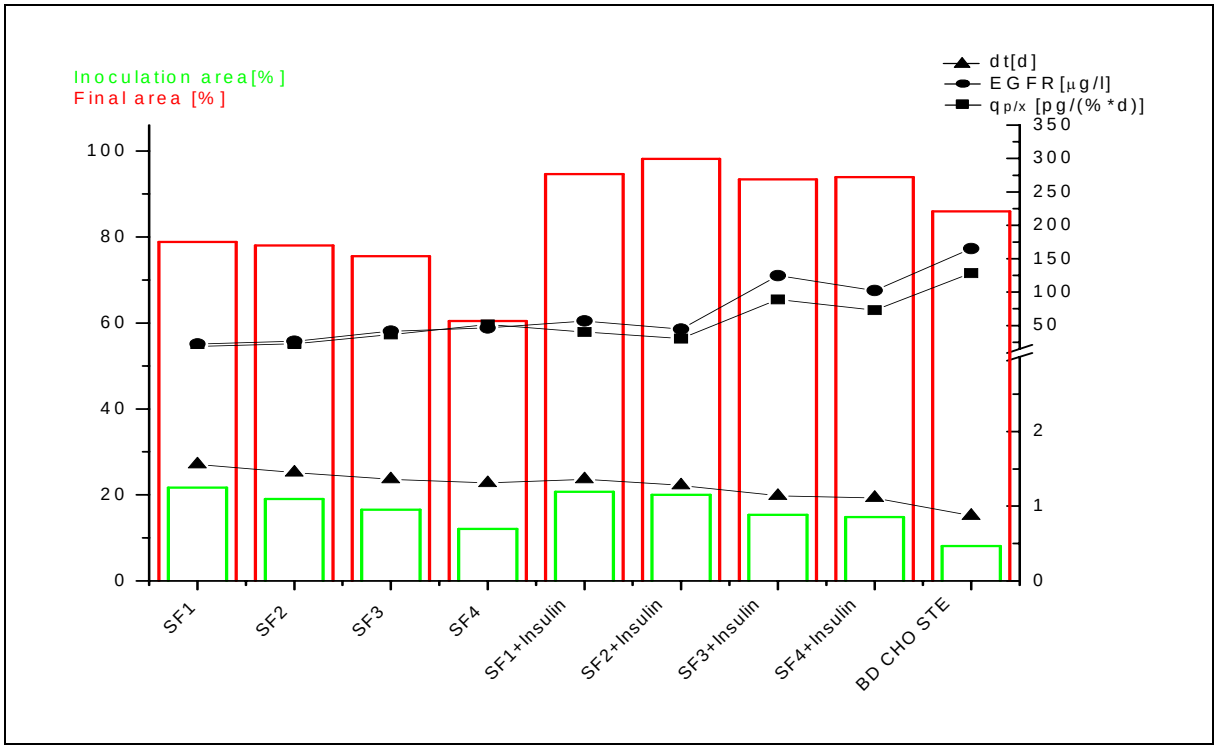

Figure 5.4-3: Shows the four developed medium formulations SF1-4 in their origin formulation and supplemented with Insulin vs. BD CHO Medium.

Most commercially available serum free medium formulations contain insulin, as a growth factor. Hence insulin was omitted during the development of low serum medium formulations. BD CHO Medium was purchased from BD Medium and as commercial medium; it could be assumed that insulin is used for the formulation. During this screening, the doubling time decreased around 10 - 13% by adding insulin to the newly developed medium formulations. The doubling time achieved in BD CHO Medium was very low, almost 45% less compared with best performing SF3 and SF4 media. The productivity was increased by adding insulin in all medium formulations (SF1-4), while the highest product yield of EGFR was still observed in BD CHO medium. The positive impact of insulin addition on productivity for SF3 and SF4 media was significant.

5.5 Effects of MSX, Ammonium and Sodium Butyrate

The following experiments were conducted to investigate the influence of non medium supplements which are not essential for cell growth. However these substances are effecting cell proliferation and productivity.

5.5.1 Influence of MSX on Productivity

This experiment was conducted to investigate the influence of MSX on cell growth and productivity. Glutamine Synthetase expression system is used in both cell lines CHO Lec8 and CHO K1. Glutamine Synthetase catalyses the production of glutamine from glutamate and ammonia. A specific and irreversible inhibition of endogenous Glutamine Synthetase can be achieved by MSX (methionine sulphoximine) at a concentration of 10-100 μM. A higher MSX level might results a higher amplification rate (Wurm 2004) (Lonza Biologics 2006).

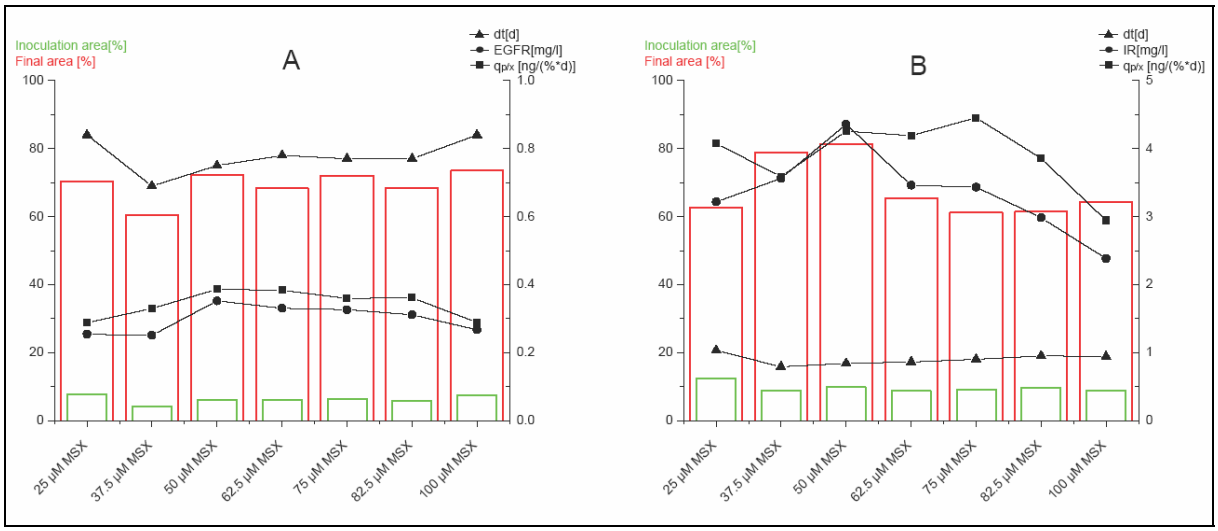

Figure 5.5-1: Impact of MSX on growth and productivity with CHO K1 (left chart) and Lec8 (right chart).

The CHO K1 producing EGF receptor fragment yield at about 14 fold less product compared to CHO Lec8. An unstable transfection of the CHO K1 cell line could be a possibility for the difference. The first productivity peaks were observed for both cell lines at a MSX concentration of 50 μM. During the experiment the growth was not significantly effected by higher MSX levels. However, a long term proliferation study was not conducted.

At 50 μM MSX the yield from the Lec8 cell line was increased at about 33% without affecting the growth. The same MSX level produced a 40% higher yield for the CHO K1 line. After obtaining the results (Figure 5.5-1) a screening was conducted to compare the new medium formulations SF1 and SF2, as well as in-house medium and BD CHO Medium. The screening was carried out at 0.5% dFCS, which was 0.5% less than screening one (Figure 5.5-1). (Backup cells were successfully cultured at 0.5% dFCS)

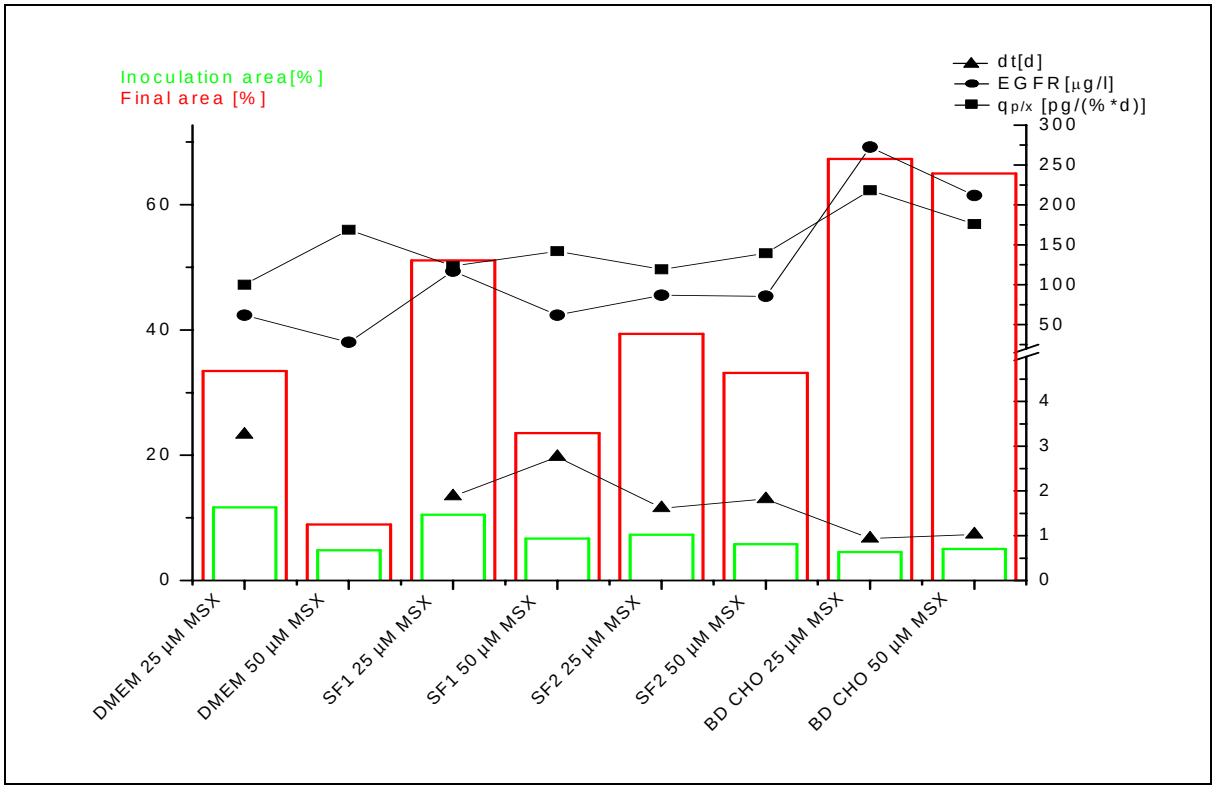

Figure 5.5-2: Different medium formulations with normal MSX (25 μM) level and increased MSX (50 μM) level.

New formulations called SF1 and SF2 were developed for CHO Lec8. The culture of Lec8 died and further screenings has to be conducted with CHO K1 cell line. 25 μM MSX represents the standard for this screening. The best performance still observed with BD CHO Medium. Higher MSX concentration of (50μM) was not affected the growth significantly. Even at lower serum level, doubling time was not significantly changed in BD CHO Medium. The comparison of the formulations SF1 and SF2 in Figure 5.5-2 is shown a different in the growth. SF2 contains additionally Hydrocortisone (Table 5.4-6) and the growth in SF2 under higher MSX levels is not affected as much as in SF1. The results from the first screening (Figure 5.5-1) were not confirmed. No significant improvement of production was achieved by increasing the MSX concentration.

5.5.2 Influence of Sodium Butyrate on IR production

Sodium butyrate (NaBu) enhanced production was observed in the past. In this experiment Sodium Butyric Acid was tested in a concentration range of 0 -10 *mM* (Sung and Lee 2005), in a 96-well-plate.

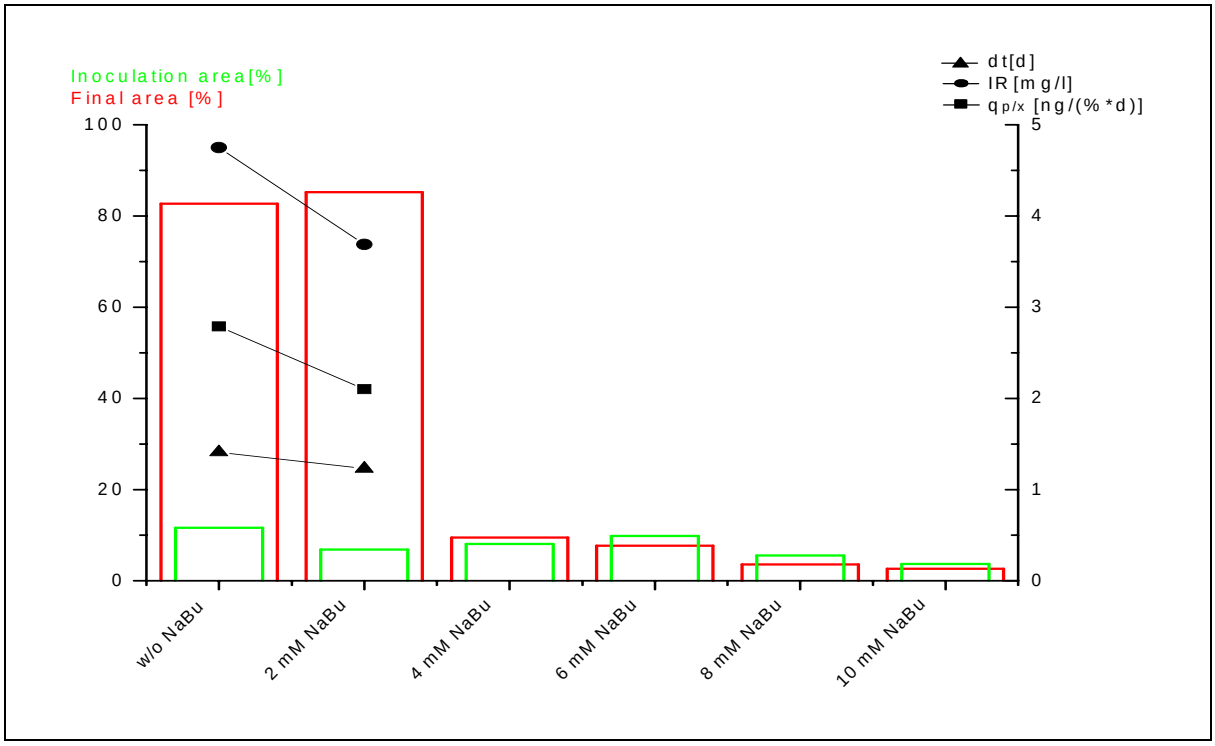

Figure 5.5-3: Influence of Sodium butyrate on cell proliferation and productivity of CHO Lec8.

Standard formulation (w/o NaBu) is represented by the first column Figure 5.5-3. It seems Sodium butyrate level above 2 *mM* has a negative effect of the cell growth. 2 *mM* of Sodium butyrate was started with 63% of the standard inoculation area, doubling time dropped at about 27% compared with the standard, which could be a result of a lower seeding cell density. Productivity of truncated IR was 3.7 *mg/l* and consequently lowers than the standard yield. This experiment did not confirm that utilizing Sodium butyrate may enhance productivity. Experiment was repeated as enhanced productivity by adding Sodium butyrate to the culture is widely known. During the second screening a lower range (0-2 *mM*) of Sodium butyrate should be tested.

5.5.3 Influence of Ammonia on the growth and productivity of the Lec8 line

It is well known that one of the most important inhibitory substances accumulating in cell culture is ammonia. The possible use of peptone as serum substitute in serum free or low serum medium formulations makes an ammonium study crucially important as peptones are blends of peptides and digested peptides with high level of amino acids. Ammonium could have positive or negative effect on cell proliferation and productivity depends on concentrations (Chen and Harcum 2005;Hansen and Emborg 1994).

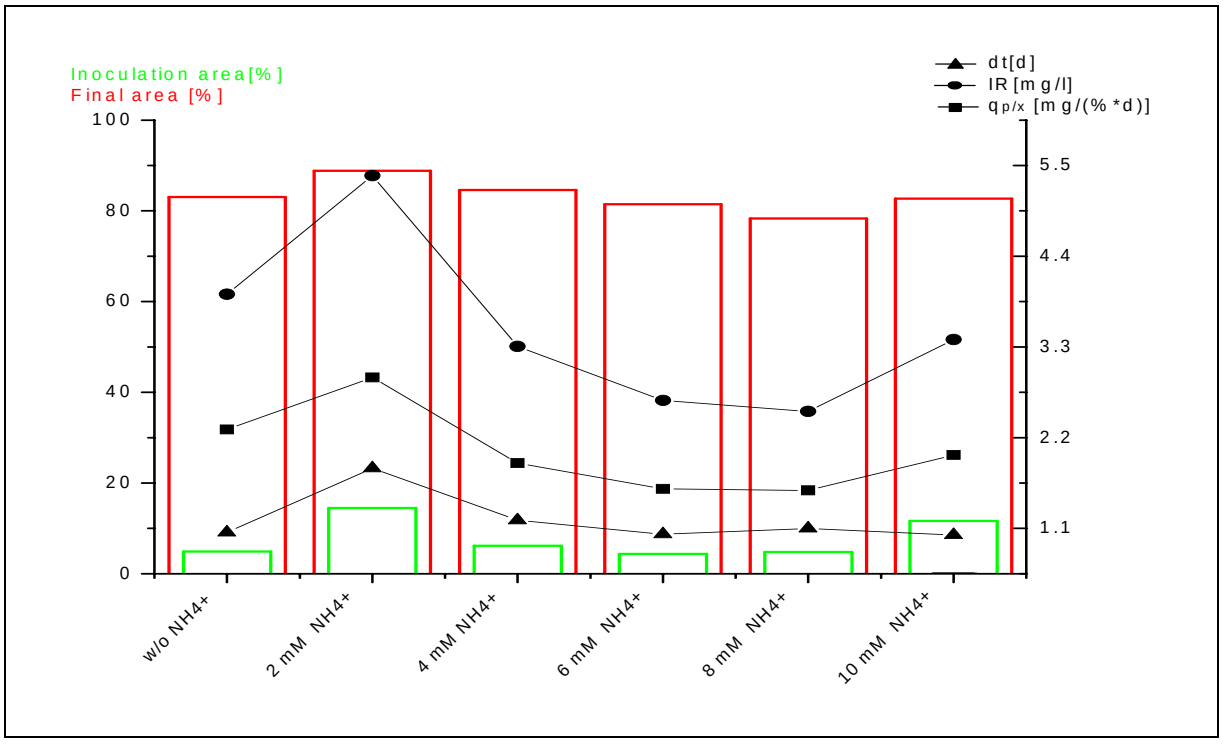

Figure 5.5-4: Influence of Ammonia on cell growth and productivity with CHO Lec8.

It was determined that ammonium level above 2 *mM* has no negative effect on cell growth. The doubling time was constant except at 2 *mM* it was increased by 83 %. A significant influence of ammonium above 2 *mM* was not observed. At a level of 2 *mM* NH_4^+ a higher productivity and lower growth was founded. The difference of productivity and growth might be explained by the higher inoculation area. Even at 10 *mM* ammonium started with a higher seeding cell density. The product yield for the standard (w/o NH_4^+) was 4 *mg/l*.

5.6 Purification Results of truncated IR

Figure 5.6-1 represents a typical pattern of truncated IR detected by UV/Vis after the ion exchange column. Each peak represents the expressed truncated IR with different glycosylation pattern. The peaks are shown in Figure 5.6-1 and they are divided by black lines in three fractions.

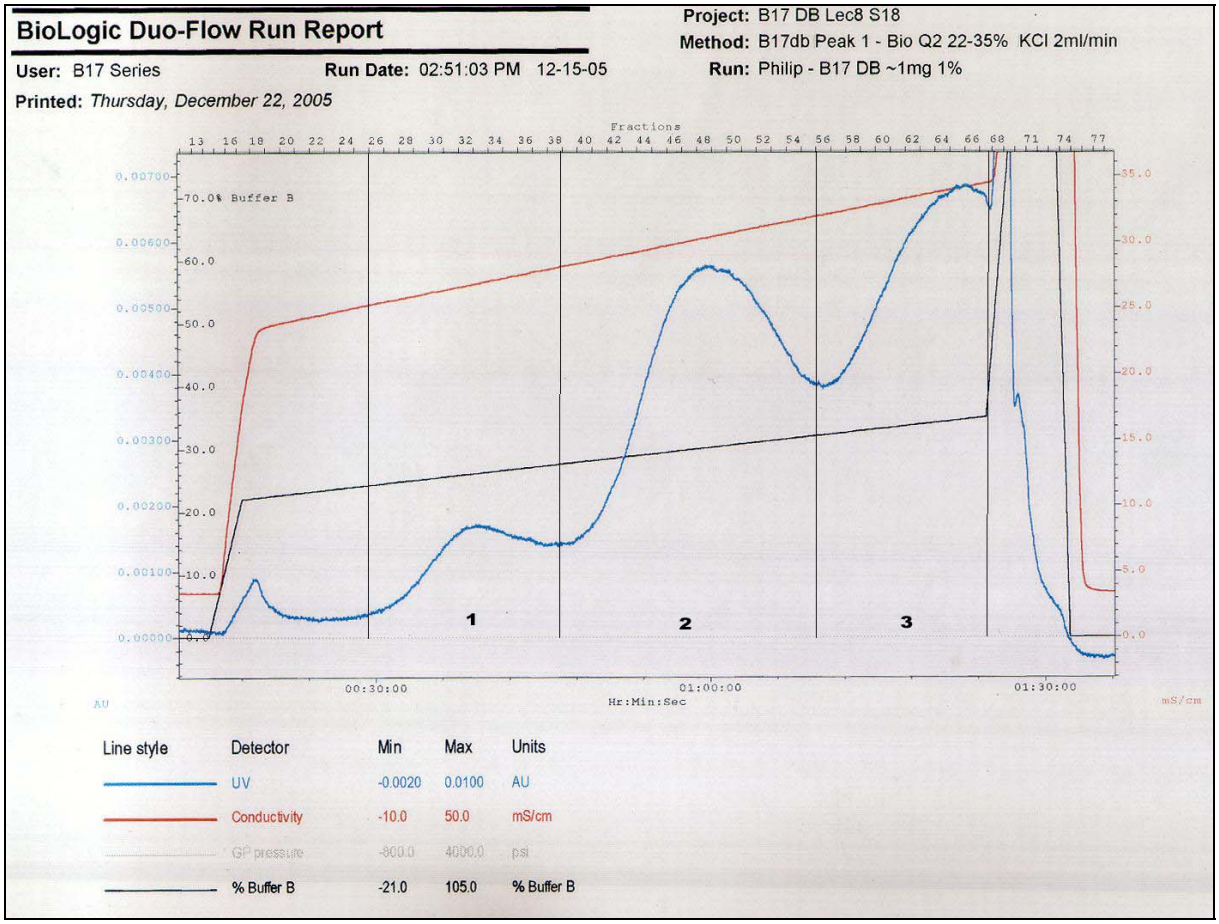

Figure 5.6-1: Truncated IR with different glycosylation pattern detected by UV/Vis after Ion Exchange column. This pattern was achieved by using DMEM/F12 (Coon´s modified) supplemented with STE and 1%dFCS in a Spinner Basket® with Fibra-Cel® Support.

Three different medium formulations were taken to investigate their impact on the product quality. The results achieved under anchorage depended and serum free conditions with BD CHO Medium in T175 Flask and a suspension culture with in-house medium supplemented with 4% dFCS and 0.1% Pluronic (F68) are shown in Appendix 9.6.

Table 5.6-1: Shows the location of the not shown purification results achieved with different conditions than the shown condition.

Purification Results (Condition)	Location
Suspension Culture in DMEM/F12, 4% dFCS, 0.1% Pluronic	Figure 9.6-1; Appendix
BD CHO Medium (sf)	Figure 9.6-2; Appendix

Table 5.6-2: Shows the area of the different peaks of purified truncated IR resulted after ion exchange chromatography.

	IR[*mg*]	**[*mm²*]**				**[%]**		
Medium		entire area	Peak1	Peak2	Peak3	Peak1	Peak2	Peak3
1% dFCS	1	7694	810	3587	3297	10.53	46.62	42.85
4% dFCS	1	4507	254	1450	2803	5.64	32.17	62.19
BD CHO (sf)	1.3	4044	161	1306	2577	3.98	32.29	63.72

The peak area was determined by utilizing an area calculation tool from adobe acrobat 6.0 Professional. The determined areas in square millimetre of Peak 1-3 were bounded by lines as the fractions would be selected for further investigations. The highest percentage (10%) of peak1 was achieved with in-house medium supplemented with 1% dFCS, GS-supplements and STE (Table 5.6-2, Figure 5.6-1). Under this condition Peak 2 and Peak 3 were nearly balanced with 46.62 and 42.85 percent based on the entire product yield of truncated IR produced by CHO Lec8.

The suspension culture of CHO Lec8 in CSIRO medium supplemented with 4% dFCS, GS-supplements and 0.1% Pluronic (F68) (Table 5.6-2, Figure 9.6-1) and CHO Lec8 in BD CHO Medium (serum free) (Table 5.6-2; Figure 9.6-2;) are shown almost the same pattern of the peak1-3.

In case of 1% dFCS and 4% dFCS the same amount (1mg) of cut IR was abandoned on the ion exchange column. The pattern detected after the ion exchange column differs between both conditions, while the desired peak 1 with the lowest sugar residue increased at a low serum concentration and the ratio of peak 2 and 3 is almost 1:1 exist at higher serum concentration of 4% dFCS a ratio of peak 2 and 3 of 1:2. A resulted yield of truncated IR (peak1) with the CHO Lec8 suspension culture was 5.6%, which is the same percentage compared to 10% dFCS in DMEM/F12 (Coon´s modified) and GS- supplements.

6 Discussion

6.1 Adaptation of Lec8 cells into suspension conditions

Two different approaches were employed to adapt the anchorage depended Lec8 cells to suspension culture. (1)Adaptation work started from serum containing medium (DMEM/F12 (Coon´s modified)) and (2) from serum free culture (BD CHO Medium). Growth of Lec8 cells was achieved in DMEM/F12 (Coon´s modified) media supplemented with GSS, 4% dFCS and 0.1% Pluronic (F68) with a shaker speed of 100 *rpm.* Growth in suspension mode was never achieved using BD CHO medium (serum free) initially.

The adaptation process into suspension conditions may be considered as a selection process, rather than an adaptation process. During the adaptation of cells into serum free conditions, the culture always had high cell viability, while the adaptation into a suspension mode had shown a low viability. For instance, on day 3.5 (Figure 5.3-3), after the first medium replacement, cell growth was observed, even though the cell viability or cell density was not high.

Adapting a fibroblast cell line into suspension condition is time consuming which might be shortened by starting with a low shaker speed (30-50 *rpm*). The idea is to decrease the alteration of initial cell environment. During the adaptation into suspension mode the shaker speed should be low at start and increased to maintain of high cell viability and higher cell density in future experiments, while higher shaker speed (100-120 *rpm*) recommended (Kuchibhatla J. 2005)

6.2 Peptone screening for Lec8

Three peptones and their blends (four mixtures) of the peptones were tested to check their effect on production. Different concentrations of peptones and their blends were added to the basic medium formulation (DMEM/F12 + GSS + STE + 1% dFCS). Peptones in general are blends of peptides and in their initial constitution they are not rich in free amino acids as determined by amino acid analyses. The concentration of free amino acids in a culture medium supplemented with protein peptones may differ during the growth phase. This is due to the cleavage activity of proteases released primarily at the end of the cell growth phase when the cell viability decreases (Schlaeger 1996).

The effects of peptones on growth and productivity are more significant without serum supplementation (Schlaeger 1996) (Kuchibhatla J. 2005).

However, growing Lec8 cells under the serum-free conditions was accompanied by significant changes in morphology and the inhibition of cell growth, even by utilizing peptones. Because Lec8 cells could not be adapted into serum free conditions, during peptone screening the media (DMEM/F12) had to be supplemented with 1% dFCS as described above. Serum level was reduced from 10% dFCS until a 1% serum level was achieved. This low level did not trigger cell death in Lec8 cells. Data from screening experiments do not show a correlation between cell growth and productivity (see: Appendix 9.3). This was true for all peptones.

A considerable decrease in cell growth was found when higher concentrations of the peptones were used similar as published by Schlaeger (Schlaeger 1996).

One exception was the Blend2 (Appendix, Figure 9.3-5) when higher productivity was observed at a concentration of 5-8 *mg/l*. Productivity levelled by using peptones is a very important fact, because it means that the cell culture is not as much effected by the changing the concentrations of peptones.

A great variation in productivity and growth is given by most of the Peptones especially by using TC Yeastolate. Under the conditions described above the use of peptones is not beneficial, in terms of growth and production (see: Figure 9.3-3 for TCY; Figure 9.3-4 for Blend 1 and Figure 9.3-6 for Blend 3).

Consequently, the use of peptones and their blends in serum supplemented medium is not very questionable. Still, many suppliers are using peptones in their media formulations as low cost supplement.

Considering that during these screening experiments the in-house media containing 1% serum was used it is recommended to repeat the Peptone screening with the self developed and more enriched medium formulations SF3 and SF4 with insulin addition but in serum free condition. Peptones as low-cost medium supplements will have significant positive impact when serum free conditions are used (Kuchibhatla J. 2005;Schlaeger 1996).

6.3 Purification results of the IR fragment (Lec8)

The truncated insulin receptor expressed in Lec8 cells is used in crystallisation studies for further structural studies. Therefore, it is important to keep uniform product quality. Three peaks, representing three different varieties of the receptor fragment were observed. These peaks can only be detected after the last purification step. It was important to understand which environmental factors are affecting the ratio between the peaks in order to be able to control the process. It is widely recognized that the alteration of media composition also has an impact on the product quality (Table 5.6-2). Changes in pH, ammonium, and the presence of serum have been demonstrated to have significant effects on the product quality (Lipscomb; Palomares; Hernandez; Ramirez, and Kompala 2005).

Using serum free BD CHO media has not shown a shift of the product quality. Therefore it is not, the serum which effects the ratio of these peaks. Comparing the ratios of protein obtained from suspension culture (4% dFCS) of Lec8 or from serum free culture in BD CHO no significant difference was found.

However, a significant alteration of the product quality was observed by low serum concentrations. Insulin might have an effect on the ratio as insulin is a ligand to the product (IR) (Figure 5.6-1, Appendix Figure 9.6-1 and 9.6-2). This assumption might explain the higher level of peak 1 in low serum or low insulin concentrations (Table 5.6-1, Figure 5.6-1).

Serum free media formulations contain insulin while dialyzed foetal calf serum (10 *kDa* cut-off) should not contain single insulin as its molecular weight is ≈ 6 *kDa*. However insulin might form hexamers in the presence of Zinc which is stable and can interact with the receptor (Smith; Swenson; Dodson; Dodson, and Reynolds 1984). Insulin is most likely a key supplement for this specific cell line and product.

6.4 Plackett Burman Design (CHO K1)

To be able to identify media additives with positive effects on cell proliferation experiments utilizing the Plackett Burmann Design were conducted. Amino acid concentration levels were selected on their uptake in a T175 flask (see: 5.4.3 Amino Acid Consumption) and observations published in the literature. (Castro and others 1992)(Kim and others 1999;Lee and others 1999).

Adaptation into low serum conditions was not affected by a lack of amino acids because the dialyzed foetal calf serum contains negligible levels of free amino acids. This was confirmed by the HPLC analysis.

The effects of the amino acids for the major pathways were tested independently as the metabolism of one may be influenced by the levels of others (see: 2.3.3 Amino Acid Catabolism) (Castro and others 1992). It is a common procedure to put amino acids in groups according to their metabolic relationships (Castro and others 1992) (Ganne and Mignot 1991). The same degradation product can be derived from different amino acids (see: 2.3.3 Amino Acid Catabolism). Only single amino acids were screened in this Plackett Burman application, whereas at least one amino acid of each sub group was present in the screening. This is because the in-house medium (DMEM/F12 (Coon´smodified) + GSS) was already quite an enriched formulation.

High concentrations of amino acids can affect the cell growth. This fact was described by Eagle and was considered by choosing the high level of amino acids in the Plackett Burman application (EAGLE 1955). Despite the positive effects of most of the amino acids, some amino acids inhibited cell growth: aspartic acid, glycine, isoleucine and serine were identified as inhibition at higher concentration levels. Some other additives had negative effects on the cell growth such as biotin, long R^3 IGF and BSA see table 5.4-5.

CHO Cells are known to use serine extensively during their cultivation (Castro and others 1992) this was confirmed during the T flask experiment (Table 5.4-4, Appendix Figure 9.11-1): 97% of Serine was consumed within two days in a T175 flask. Experiment using the Plackett Burman Design was performed in 96 well plates which contains ≈ 0.6 *ml* medium per cm^2 compared to a T175 Flask contains ≈ 0.29 *ml* medium per cm^2 growth area. This means that in a 96 well plate almost twice as much media available compared to a T175 flask and thus altering the results. Consequently, serine was added to SF4 due its determined

consumption in a T175 flask and the new medium was developed for the use large scale with a lower volume of media per *cm²*.
Other amino acids, such as alanine, glutamic acid, leucine and methionine confirmed growth promoted effects and therefore included into the new medium formulation (Table 5.4-6). In addition to the above mentioned amino acids, aspartic acid was added to medium formulation SF4, based on its high consumption level (Table 5.4-4, Appendix Figure 9.11-1).

Cells require the addition of glycine, purine and thymidine to cope with either missing folate or elevated intracellular DHFR activity. Purine might be replaced by hypoxanthine or adenine, for cells lacking the DHFR gene (Gandor; Leist; Fiechter, and Asselbergs 1995). While the CHO K1 cell line used in the experiment has no DHFR gene deficiency as the GS expression system was chosen for protein production. However, hypoxanthine was confirmed as a positive factor by a significance of 86% (Table 5.4-5). Hypoxanthine as purine derivate has growth promoting effects, because the purine de-novo synthesis limits the growth rate (Kondo; Yamaoka; Honda; Miwa; Katashima; Moritani; Yoshimoto; Hayashi, and Itakura 2000).

CHO K1 cells are proline auxotrophs (Kao and Puck 1967) (Austin and Winkler 1988). A higher concentration of proline is used to prepare in the present media formulation, only 33% of proline was consumed by CHO K1 after four days in basal medium (Table 5.4-4, Appendix Figure 9.11-1) as observed during T175 flask experiments. However another 10 *mg/l* of proline was added to the present media which already had concentration of 44.25 *mg/l* proline. Higher proline concentration might have a further growth promoting effect, without being essential for cell metabolism.

Hydrocortisone is used in many commercialized serum free medium formulations (Darling D.C. and Morgan S.J. 1994). It could promote cell attachment and cell proliferation (Freshney 1999). Hydrocortisone had a positive effect with a significance of almost 100% (Table 5.3-5).

Linoleic acid is one of the fatty acids that constitute the basic lipid building blocks for cell growth (Kim and others 1998). Its growth promoting effects has been reported and was confirmed in with > 80% in this Plackett Burmann Design (Kovar and Franek 1986).

One major difference when the in-house media and commercial media formulations are compared is the lack of trace elements in the in-house media formulation. Thus, a trace element solution was prepared and included into the Plackett Burman Design. Observations (Table 5.4-5) confirmed the positive effect of the trace element solution. Trace elements were found essential by several researchers (Castro and others 1992) (Ahuja and others 2004).(Ganne and Mignot 1991) Trace elements are needed supplements in media formulations even with lower purification grade (Table 5.4-5).

The following components were not added to any medium formulation, because their significance level was determined as below 80%. However, these results have to be considered when Plackett Burman Design will be repeated or can be taken for new medium formulation based on a lower significance level:

Phenylalanine and asparagine were identified as growth promoting amino acids. However these were not chosen for the new medium formulation, because the significance was less than 80%.

The polyamines spermidine, spermine and its precursor putrescine are required for cell growth and proliferation (Bottenstein and Sato 1979).(Ray and others 1999) The reduction of serum levels results a rapid decrease of the putrescine transport rate into human fibroblasts. However adding insulin might reverse this process (Bottenstein and Sato 1979). Experimental data (Table 5.4-5) suggest that, the addition of putrescine is not significant above 80% and therefore was not included when the new media was formulated.

Pluronic (F68) as surfactant protects the cells not only from shear forces during the aeration and agitation but also it may promote growth as described in the literature (Sato and Hendrick V. 2002) (Kim and others 1998).

In conclusion, the Plackett Burman method enabled the development of a low serum medium formulation for the EGFR producing CHO K1 cell line. The Plackett Burman Design should be the method of choice (chapter 2.4.2) considering the large number of variables and limited resources and time. This technique which reduces the numbers of experiments while provides all the necessary information regarding the significance of the chosen variables. The screenings were repeated twice and with three replicates each. Due to some contradicting results it should be repeated in the in the future.

6.5 Impact of Ammonium in media formulations

Ammonium in cell culture is a by-product of the cellular metabolism or results of chemical degradation of glutamine in the medium. High level of ammonium has a inhibitory effects on the cell growth (Yang and Butler 2000). Peptones might be a significant source of ammonium in the current medium so peptones as a serum substitute for serum requires an investigation (Heidemann R. and Zhang C. 2000). Ammonium as a degradation product released from peptones has negative impact on cell growth and product yield. Uptake rates of glucose, glutamine and alanine as consequently production rate of lactate, increase progressively (Yang and Butler 2000). (Hansen and Emborg 1994)Glutamate is oxidatively deaminated yielding ammonia as a by-product (Voet D. and others 1998).

Ammonium formation has to be investigated, because its impact on cell metabolism. Ammonia concentration was measured during the experimental work with peptones in T flasks after 4 days, where no media was changed. The highest determined ammonium accumulation was < 0.1 *mM* , which is low (Art. 91315, Riedel-de Haen).

The growth of CHO Lec8 was not affected by high ammonium concentrations, apart from 2 *mM* ammonium. Inadvertent in certain wells a higher inoculation cell density was achieved, which induced an increasing of the doubling time. However, the growth area in the wells at 2 *mM* was covered first with cells. A stationary and production phase for this condition was reached earlier compared to the residue of tested conditions. Therefore a higher productivity at 2 *mM* NH_4^+ was occurred. In summary there was no observation of a significant effect of ammonium on cell growth and productivity.

6.6 Amino acid contents in culture media

After investigating the positive effects of certain amino acids an HPLC assay on BD CHO Medium, in-house, - and the improved formulation (SF4) was conducted. SF4 was formulated based on results of the Plackett Burman Design experiments. The comparison of amino acid concentrations in BD CHO and SF4 (Table 6.6-1), suggested that higher level of amino acids are not affecting the proliferation of the cells. This was published by Eagle and accepted by other authors (EAGLE 1955). Certain amino acids in BD CHO Medium, in most of the cases, has several times higher concentrations compared to SF4. The required amino acid concentrations were calculated based on the amino acid consumption (Table 5.3-4, Appendix Figure 9.11-1) to make sure that SF4 medium will contain sufficient amount of amino acids. Sufficient levels were assumed when the final amino acid concentrations are at least 50% of the initial concentration. This rule was applied to calculate the high level of amino acids in a culture medium and compared to concentration published in reports (Schroder and others 2004).(Castro and others 1992;Kim and others 1999) The in-house developed medium performance was below the performance of BD CHO medium which suggest that higher level of amino acids should be used for further Plackett Burman Design.

Table 6.6-1: Amino acid concentration in Cell Culture Media.

Amino acids	BD CHO [*mg/l*]	DMEM/F12 +GSS [*mg/l*]	SF4 [*mg/l*]
alanine	34.5	13.23	37.4
arginine	1197.29	173.59	174.01
aspartic acid	89.62	19.77	46.3
cystein	98.64	24.23	23.42
glutamic acid	118.44	94.7	188.3
glycine	62.33	19.71	19.76
histidine	91.76	26.58	26.97
isoleucine	253.82	41.63	41.68
leucine	309.04	49.58	95.5
lysine	278.67	67.1	66.7
methionine	91.16	18.39	39.8
phenylalanine	155.45	30.07	29.61
proline	72.5	49.1	64.35
serine	152.33	29.95	82.53
threonine	210.26	59.64	60.4
tyrosine	157.64	32.03	32.91
tyrosine	180.62	43.27	42.76

6.7 Comparing of Performance of in-house developed medium formulations and BD CHO Medium

The majority of screenings were done by using cells directly from cell cultures maintained using DMEM/F12 supplemented with GSS, STE and 1 % dFCS. Normally mammalian cells need adaptation – several generations – for accustom to new media conditions. However for screening it is not necessary to maintain the new condition for longer periods.

BD CHO Medium performed better, cell recovery experiments when short term storage at -80°C and subsequent culturing was conducted. Each of these trials with Lec8 in serum free conditions utilizing BD CHO Medium succeeded. However, similar trials using CSIRO Medium conditions often failed. The cell viability increased until >80% and after a few days the culture died. This pattern was observed at repeated trials. There is no explanation at this stage for this phenomenon.

BD CHO Medium most likely contains insulin. This proprietary information was not declared by the manufacturer. The growth and production promoting effects of insulin was extraordinary. Results are shown in Table 6.7-1:

Table 6.7-1: Determined growth and production parameter of different medium formulations.

	EGFR [µg/ml]	[%]	$q_{p/x}$[pg/(% · d)]	[%]	Doubling time [d]	[%]
SF1	22.10	13.37	18.74	14.58	1.56	179.31
SF2	26.60	16.09	22.80	17.73	1.45	166.67
SF3	41.30	24.99	36.57	28.45	1.36	156.32
SF4	46.49	28.13	51.42	40.00	1.31	150.57
SF1+Insulin	56.88	34.42	40.19	31.26	1.36	156.32
SF2+Insulin	44.37	26.85	30.23	23.51	1.28	147.13
SF3+Insulin	124.46	75.31	89.10	69.31	1.14	131.03
SF4+Insulin	102.38	61.95	72.91	56.71	1.11	127.59
BD CHO STE	**165.27**	**100.00**	**128.56**	**100.00**	**0.87**	**100**

The addition of insulin was not essential as the culture survived (see: SF1-4 with out insulin). Nevertheless, insulin contribution to medium performance is high. Improvements in production and growth were achieved. The best performing formulation (SF3) was still 30% behind the BD CHO Medium when specific productivities are compared. The doubling rate was also only 27% higher compared to BD CHO media.

Both screenings (Table 5.4-8 in T75 and Table 5.4-7 in 96 well plates) showed a constant level of productivity for the four conditions SF1-4. The Plackett Burman design suggested a negative impact of aspartic acid and serine. However based on their high uptake by CHO K1,

these amino acids were added to the medium formulation SF4 (Table 5.4-6). A logical result would be a higher doubling time of SF4 compared to SF3, only growth promoting variables are contained in SF3 (Table 5.4-6). Doubling time of SF4 was increased compared to SF3 in the screening (Table 5.4-7; Figure 5.4-1) and it is the same level in Figure 5.4-3 and Table 6.7-1.However the difference of the doubling time may be the result of a difference of the inoculation area. While the Plackett Burman method is a very powerful tool for process optimisation. Nevertheless, replication including randomization of experimental settings is needed.

6.8 Cellscreen® System Innovatis

All experiments were made in at least three replicates. During these experiments the Cellscreen® systems performed with high accuracy and generated data with high reliability. However, to fully utilise the capability of the Cellscreen® appropriate experiment design is needed. Some of the issues were considered and discussed below:

6.8.1 Problems with small scale work

It is very important to establish procedure for the inoculation of 96 well plates. The affects of the dilution rate has to be kept at a low level. A dilution rate of less than 5% was chosen. The inoculation volume was 5-10 µl for a working volume per chamber of 200 µl. The most important issue utilizing the Cellscreen® system was the even dispersion of cells in the chamber and over the whole plate. Differences in inoculation concentration or inoculation area between the chambers may cause different growth pattern even under the same conditions.

6.8.2 Cell Aggregation using Trypsin Inhibitor

Under certain conditions the trypsin inhibitor (Sigma) supports cell aggregation, which can not be avoided. The inoculation regarding its small was volume with partly aggregated cells 96 well plates is not recommendable. The impact of cell number per volume was significant. An aggregate of cells was able to cover 30% of the well area. Many experiments had to be postponed. If the cells were not dispersed evenly over the area of the surface growth

inhibition due cell contact appeared locally in the chamber (Freshney 1999). These assumption suggest that the interaction between trypsin (or versene) and trypsin inhibitor had to be investigated. There is might be a better protocol, which does not induce cell aggregation. Cell aggregation had never appeared when serum was used to neutralize trypsin. A cell aggregation was not induced by higher concentrations of Mg^{2+} and Ca^{2+} as PBS-buffer used for the experiments was Mg^{2+} and Ca^{2+} ion free.

6.8.3 First measurement of 96 well plates with Cellscreen®

The so called adherent module of the Cellscreen® was used for proliferation studies with CHO K1 and its mutant Lec8. The area covered by cells (confluent area) was measured by the Cellscreen® System to calculate the doubling time and draw growth curves. After inoculation the morphology of the cells differs in diameter and area. The confluence area is the parameter for compiling growth curves of the chambers.

Usually the cells were seeded on the 96 well plate in their mid log phase, this should avoid or minimize the lag phase. If the plate was measured almost directly after inoculation, when the cells were already settled, but still in a circular shape, the inoculation area was smaller compared to the same cell with a flat and wide shape. The resulted doubling time was decreased. During the last experiments, this observation was considered and the plates were inoculated in the morning and the first measurement was taken in the afternoon to avoid error in growth rate estimation. Starting the first measurements ≈ 8h after inoculation is sufficient to avoid errors.

The above mentioned variations, the difference of inoculation cell density, size and shape of cells at inoculation, are causing experimental errors. To estimate and minimize the effect of these variations, the inoculation area was checked randomly after starting every experiment. The so called PA-result view (proliferation result view, Software module Cellscreen®) was used to determine the differences in the inoculation area.

In order to confirm observations, it is first important to study the generated growth curve showing deviations in inoculation area and a randomly check of the dispersion of the cells in the chamber. This work is time consuming. A plate overview showing the inoculation areas and the deviation inside a compiled group would be very handy and user friendly.

6.8.4 Error bars in Result view

Proliferation studies are the main purpose for using proliferation adherent (PA) and proliferation suspension (PS) Modules (Software modules for proliferation studies, Cellscreen®) and every scientific study requires replication of the experiments. The present version of the results viewer PA and PS Module are only providing growth curves without error bars.

The growth of chambers representing the same experiment and pooled in a compiled group were shown as a single experiments. Using the replicates of each experiment for statistical computing of experiment deviation would make more reliable and more results were more confident.

7 Recommendation for future work

7.1 Medium optimisation

Cellscreen® System (Innovatis,Germany) makes media development possible in a time and cost effective way. The accuracy and screening speed of the system offer a new window of opportunity for rapid statistical investigations. CellScreen® combined with the Plackett Burman design creates a very powerful experimental tool should be used in the future.

Experiments using Plackett Burman design should be repeated with higher amino acid concentrations as the better performing BD media has higher amino acid levels compared to in-house developed media formulations.

A robust cell line such as CHO K1 is essential for medium optimization. The glycosylation mutant Lec8 and its specific requirements for culture conditions make the optimisation work very difficult. Results regarding the mutant CHO lines should be a good starting point for future work when cell lines with similar expression systems should be optimized. One hopes that using the same host cell (CHO) and expression system, cell metabolisms will be comparable regardless the different nature of products.

Most of commercial medium has significant higher amino acid concentrations compared to CSIRO media. Interactions between variables could not be detected by Plackett Burman application; however interaction can be exposed by a Full Fractional Design. A significant higher number of screening experiments has to be conducted, but knowledge of interaction between amino acids and other variables can contribute to more efficient medium formulation. The development and evaluation of statistical designs should be supported by utilizing statistical software for example: Minitab, Statsoft or SPSS. These softwares provide a broad spectrum of statistical applications, are widely used for process development and user forums are available in the web.

The positive effect of insulin on production and cell growth was confirmed. Long R^3 IGF was surprisingly not identified as a growth promoter during these experiments which is unexpected as long R^3 IGF is widely known as a growth factor. The bioactivity and stability of long R^3 IGF was not tested during the experiments so it is possible that its activity was low.

As long R^3 IGF could be a cost saving alternative for insulin as growth promoter its role and potential effect should be clarified in the future.

After the finalising of the new media formulation for serum free conditions a new screening of peptones and their blends in a concentration range up to 10 *g/l* is recommended. An estimated time frame for a well developed medium formulation under consideration of growth and production promoting effects utilizing the Cellscreen® would take 6 month.

The response surface method (see: Appendix 9.12 for details) is the suggested method using growth and productivity as response parameter.The final outcome of this investigation should be an optimal ratio between the different amino acid concentration, which provides a greater understanding of their pathways, interactions and significance.
The second variable should be a vitamin and fatty acid solution. The screening of the 12 vitamins (used in the cell culture medium) in a Full Fractional Design can be difficult, while some of them are involved in the same type of reaction, it is still difficult to combine them in to groups. A simple PB Design should be used to screen vitamins and fatty acids might give information about their significance

I think the suggested strategy is more effective compared to a very simplified Plackett Burman Design. As the new strategy are offers several advantages:

- The knowledge about interactions and their possible conclusions about catabolic pathways for amino acids.
- The outcome is a defined ratio of concentrations pooled in two variables. This allows the screening in different concentrations and enables to discover the optimal concentrations of Amino Acids, Vitamins and organic compounds.
- After conducting a full factorial design, the number a variables will be reduced to two. It seems the simple Plackett Burman Design does not offer any real advantages as it requires a labor intensive fine-tuning of each significant medium supplement.

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9 Appendix

9.1 DMEM/F12 (Coon´s modified) including GSS

Table 9.1-1: DMEM (Coon´s modified) Medium formulation with GS-supplements; without Glutamine and Phenolred.

Amino Acids	[*mg/l*]	Vitamins and Organic Compounds	[*mg/l*]
Alanine*	17.9	Ascorbic acid (Vitamin C)	7.5
Arginine	253	Biotin	0.035
Asparagin*	108.5	Choline Cloride (Vitamin B4)	8.98
Aspartic acid*	26.7	D-Calcium pantothenate	2.24
Cysteine,HCl, H2O	35.12	DL-Alpha-Lipoic Acid	0.105
Cystine, 2HCl	31.29	Folic acid	2.65
Glutamic acid*	109.7	I-Inositol	12.6
Glycin*	30.1	Linolic acid	0.042
Histedine,HCl,H_2O	41.98	Niacinamide	2.02
Isoleucin	56.47	Putrescine dihydrochlorite	0.161
Leucin	65.55	Pyridoxal hydrochloride	2
Lysine	109.5	Pyridoxine hydrochloride	0.031
Methionine	19.48	Riboflavin	0.219
Phenyalanie	37.98	Thiamine hypochloride	2.17
Proline*	54.25	Vitamin B12 (cyanocobalamin)	0.68
Serine*	42.1	D-Glucose	3151
Threonine	59.45	Sodium Pyruvate	110
Thryptophan	10.02	**Inorganic components**	
Tyrosine	59.68	Calciucium Chloride,Anhydrous	151
Valine	58.7	Magnesium Chloride,anhydrous	24.83
Trace		Manesium Sulfate,anhydrogenous	58.43
Cupric Sulfate,5·H_20	0.001	Potassium Chloride	342
Ferric Nitrate, 9·H_2O	0.05	Potassium Phosphate, Monobasic,anhydrous	29.5
Ferric Sulfate,7·H_2O	0.4	Sodium Phosphate, Dibasic, anyhdrous	44.49
Zink Sulfate, 7·H_2O	0.072	Sodium Phosphate, Monobasic,H_2O,ACS	62.5
Nucleosides		HEPES	3574.5
Adenosine*	7	Sodium bicarbonate	2150
Guanosine*	7	Sodium Chloride	6975
Cystidine *	7	* GS-Supplements	
Uridine *	7		
Thymidine*	3.6		

9.2 Buffers and Solutions

Ligand- binding buffer:
100 mM HEPES
100 mM NaCl
0.05 % (w/v) Tween 20
2 µM Diethylenetriamine-pentaacidic acid (DTPA), (Fluka, Switzerland)
- adjust to pH 8.0

3 % (w/v) skim milk/TBST block solution:
3 g skim milk powder in 100 ml TBST
- keep solution at 4 °C

2 % (w/v) Natrium azide:
2 g sodium azide in 100 ml ddH_2O

Trypsin/Versene reagent:
100 ml 10 x Trypsin- EDTA 1:250 (pH 7.0) (MultiCell™, USA)
900 ml 1 x PBS
- sterilize solution by sterile filtration

1 x TBST buffer :
for TBST add 0.05 % (w/v) Tween 20 (Polyoxyethylene sorbitane monolaureate)
- for 2 L use 200 ml 10 x TBS and 1 ml Tween 20.

1 x TBSA buffer :
for TBSA add 0.02 % (w/v) sodium azide

1 x Tris- buffered saline (TBS) buffer
3 g (0.25 mM) Tris base
8 g (0.15 M) NaCl
0.2 g (2.6 mM) KCl
- add ddH_2O to 1000 ml total volume
- adjust to pH 7.4 with HCl

1 x Phosphate buffered Saline (PBS)

4g	NaCl
0.1g	KCl
0.7g	Na_2HPO_4
0.1g	KH_2PO_4

Adjust to pH 7.4 with HCl add 500 ml dH_2O to finale Autoklave

9.3 Peptone charts

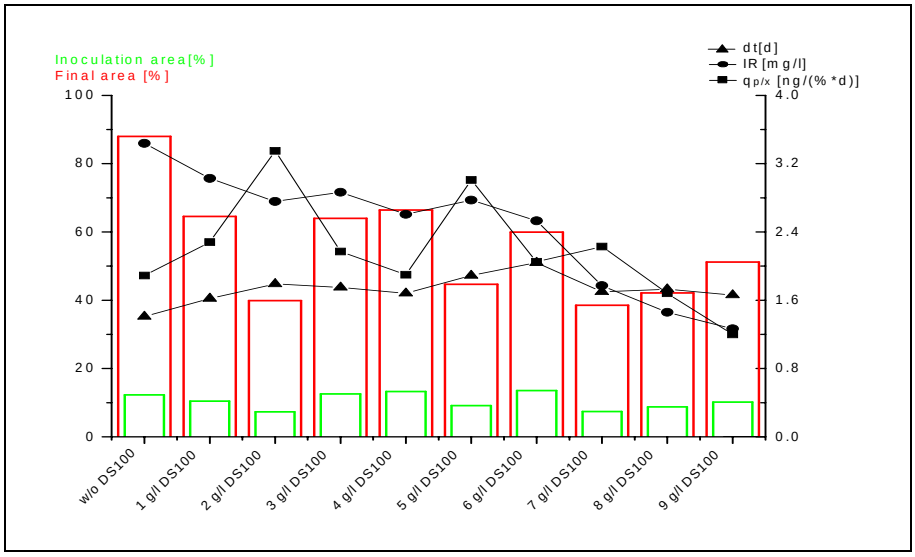

Figure 9.3-1: Difco™ Springer DS 100 Soy Peptone UF screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. By an increased DS100 concentration the product yield was dropped simultaneously. The lowest doubling time was found at the standard (w/o DS100) and the growth was negative effected, but two peaks of specific product yield were determined at 2 and 4 *g/l* of DS100. Peak

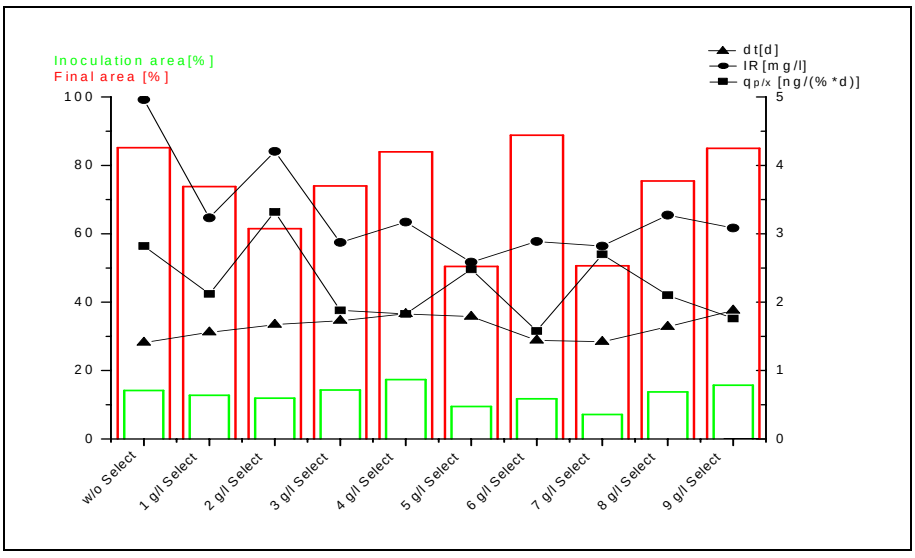

Figure 9.3-2: Difco Select Phytone™ Peptone UF screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. The production of truncated IR has not been dropped much. Over the entire concentration range a nearly constant amount of product was produced. At 2 *g/l* the $q_{p/x}$ is higher compared with the specific productivity of the standard, but not significantly.

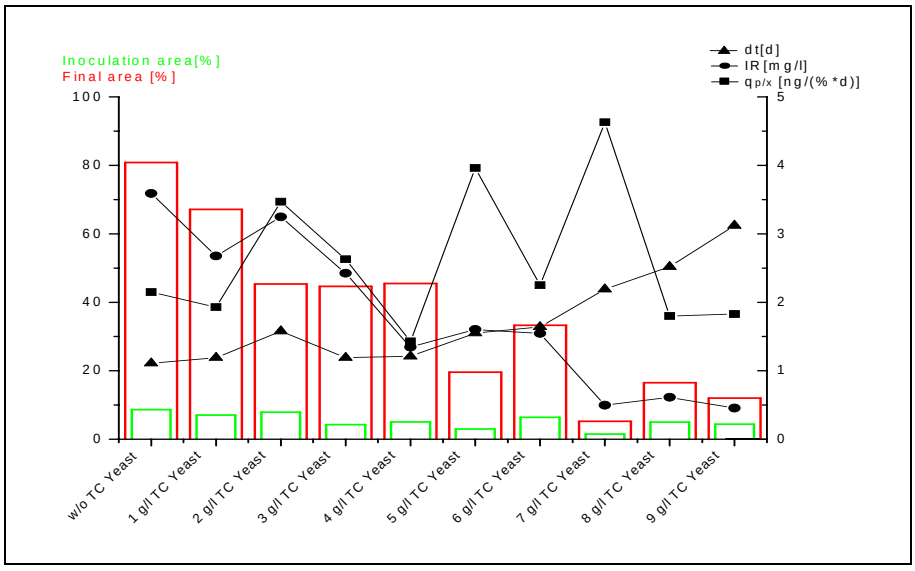

Figure 9.3-3: TC Yeastolate UF screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. The growth was high affected particularly in high concentrations of TC Yeastolate, the doubling time was increased up to 2.8 fold of the standard time. The production was dropped till 4 *g/l* from there a small plateau was found. An interesting peak of the specific product yield and a quite high amount of product were found at also 2 *g/l*.

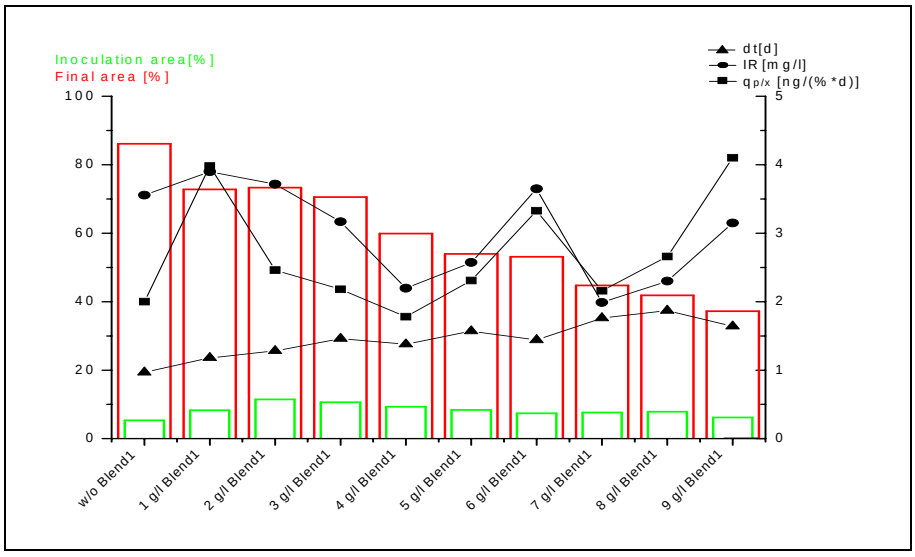

Figure 9.3-4: Blend1 (consist to one third of each Peptone) screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. It seems that growth is affected by TCY , the shape of the whole histogram looks similar compared to TCY. The second peak of the specific product yield was switched from 5 *g/l* (see: DS100, Select, TCY) to 6 *g/l*. Even at concentration of 1 *g/l* the quantity of the product is slightly higher and the specific product yield was raised two times

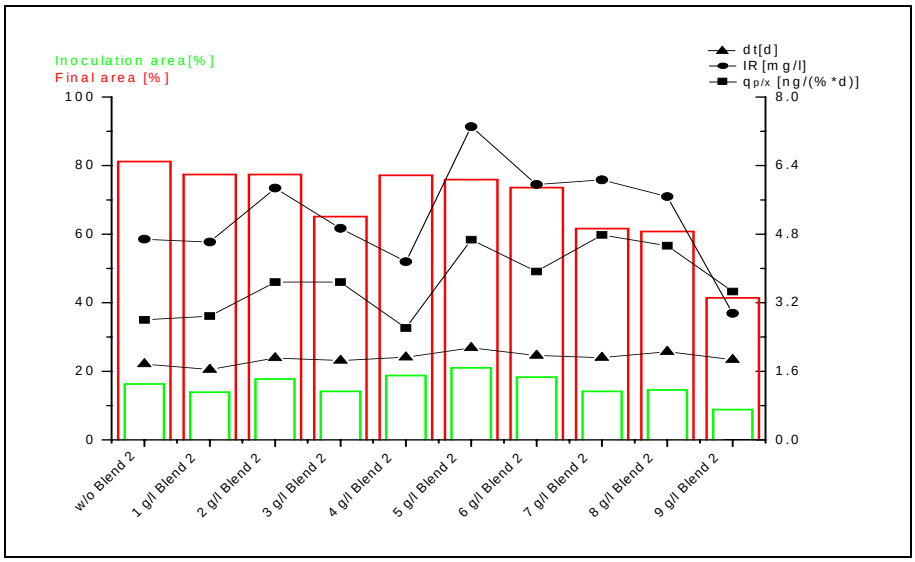

Figure 9.3-5: Blend2 (1:1 of Select,TCY) screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. The comparison of histogram shapes from Select and TCY present the result that Select Phytone was interspersed more in this blend. Over the entire concentration range the doubling time was not effected much, a constant growth was found. A very high product yield was found in a concentration range of 5-7 *g/l* of Blend2. Up to 7 *mg/l* cut IR by utilizing *5 g/l* of Blend2.

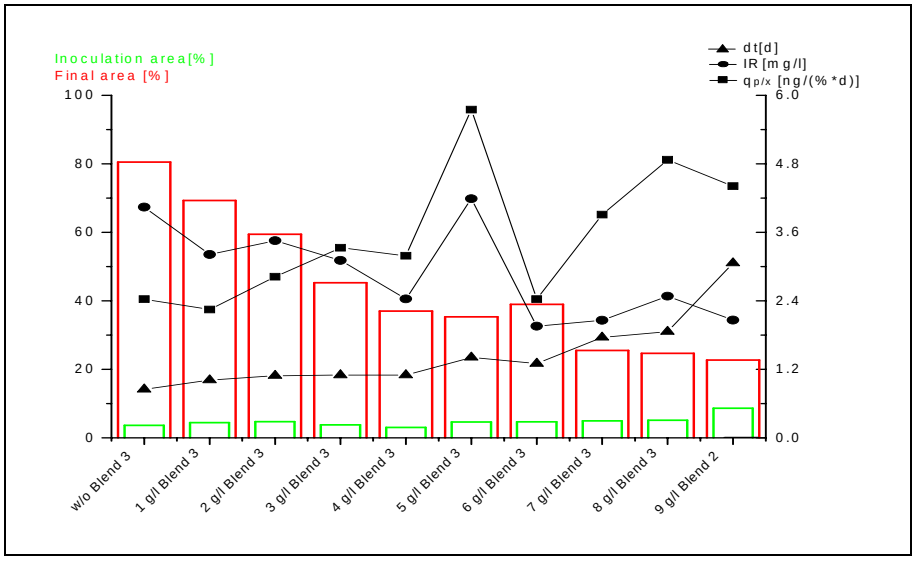

Figure 9.3-6: Blend3 (1:1 DS100, TCY) screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. Blend3 was most effected by TCY, the shape of the histogram compared to utilizing pure TCY. The significant peak at 5 *g/l* of Blend3 for promoting productivity and the growing of doubling time was founded as a unique reaction of CHO Lec8 by using of TCY. Apart from the productivity peak at 5 *g/l* – blend3 affected production and growth of cells.

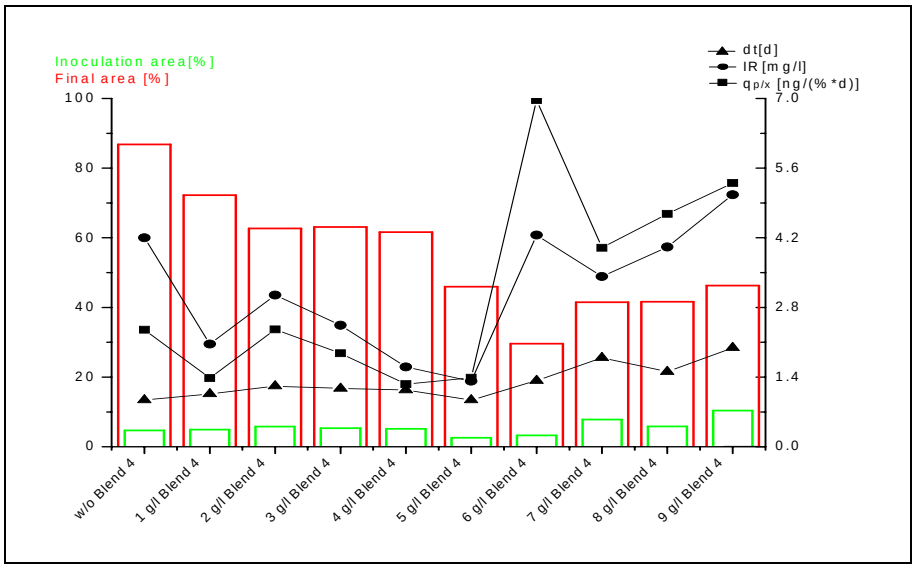

Figure 9.3-7: Blend4 (1:1 DS100, Select) screening with CHO Lec8 in DMEM/F12 (Coon´s modified) supplemented with 1% dFCS and STE. Doubling time was increased at about 2 fold at 9 *g/l*, but more on a constant level. Blend4 and Blend2 have the same significantly high plateau of productivity at about 6-7 *g/l*, in both Blends Select Phytone was used. The main peak is at 6 *g/l* at this concentration high production was found in Blend1.

9.4 Placket Burman Matrixes

Table 9.4-1: Plackett Burman Matrix used for Design 1.

Experiments	Biotin	Hydrocortisone	Hypoxanthine	Dummy	linoleic acid	Putrescine	Transferrin
1	-	-	-	-	-	-	-
2	-	-	+	-	+	+	+
3	+	-	-	+	-	+	+
4	+	+	-	-	+	-	+
5	+	+	+	-	-	+	-
6	-	+	+	+	-	-	+
7	+	-	+	+	+	-	-
8	-	+	-	+	+	+	-

Table 9.4-2: Plackett Burman Matrix used for Design 2 and 3.

	Biotin	Hydrocortisone	Hypoxanthine	Long R3 IGF	Linoleic acid	Putrescine	Transferrin	TRACE	Dummy1	Dummy2	Dummy3
1	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	-	-	-	+	-	+	-	+
3	+	+	+	+	-	-	-	+	-	+	-
4	-	+	+	+	+	-	-	-	+	-	+
5	+	-	+	+	+	+	-	-	-	+	-
6	-	+	-	+	+	+	+	-	-	-	+
7	+	-	+	-	+	+	+	+	-	-	-
8	-	+	-	+	-	+	+	+	+	-	-
9	-	-	+	-	+	-	+	+	+	+	-
10	-	-	-	+	-	+	-	+	+	+	+
11	+	-	-	-	+	-	+	-	+	+	+
12	+	+	-	-	-	+	-	+	-	+	+

Table 9.4-3: Plackett Burman Matrix used for CHO K1.

Experiment	Alanine	Asparagine	Aspartic acid	Dummy	Glutamic acid	Glycine	Isoleucin	Leucin	Methionine	Dummy	Phenylalanine	Proline	Serine	Biotin	Hydrocortisone	Hypoxanthine	Linoleic acid	long R IGF	Putresciene	BSA	Pluronic (F68)	Trace	Dummy
1*	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-
2	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-
3	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-
4	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-
5	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+
6	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-
7	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+
8	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-
9	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-
10	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+
11	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+
12	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-
13	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-
14	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+
15	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+
16	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-
17	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+
18	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-
19	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+
20	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+
21	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+
22	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+
23	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

9.5 High and Low level Plackett Burman

Table 9.5-1: Used concentrations in high and low level for Plackett Burman application (chapter 5.4.1).

Additive	Low Concentration [*mg/l*]	High Concentration [mg/l]	Add [*mg/l*]
Biotin	0.035	0.535	0.5
Hydrocortisone	0	10	10
Hypoxanthine	0	10	10
Long R^3 IGF	0	$25 \cdot 10^{-3}$	$25 \cdot 10^{-3}$
Linoleic acid	0.042	0.945	0.903
Putrescine	0.161	1.161	1
Transferrin	0	5	5
Trace elements			
Cupric Sulfate · $5H_2O$	$1.0 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$
Ferric Sulfate · 7 H_2O	0.4	0.9	0.5
Patassium Nitrate	0	0.09	0.09
Zink Chloride	0.072	0.672	0.6

Table 9.5-2: Used concentrations in high and low level for Plackett Burman application (chapter 5.4.4).

Variable	Low level [*mg/l*]	High level [*mg/l*]	Add [*mg/l*]
Alanine	17.9	37.9	20
Asparagine*	108.5	188.5	80
Aspartic acid*	26.7	46.7	20
Glutamic acid*	109.7	189.7	80
Glycine*	30.1	40.1	10
Isoleucine	56.47	76.47	20
Leucine	65.55	95.55	30
Methionine	19.48	39.48	20
Phenylalanine	37.98	57.98	20
Proline*	54.25	64.25	10
Serine*	42.1	82.1	40
Biotin	0.035	0.535	0.5
Hydrocortisone	0	10	10
Hypoxanthine	0	10	10
Linoleic acid	0.042	0.1323	0.0903
long R^3 IGF	0	0.000025	$2.50 \cdot 10^{-05}$
Putrescine	0.161	1.161	1
BSA	0	1000	1000
Pluronic (F68)	0	0.1	0.1
TRACE	0	1 x	1 x
*GS-Supplements			
TRACE 1 x			
Cupric Sulfate · $5H_2O$	$1.0 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$
Ferric Sulfate · 7 H_2O	0.4	0.9	0.5
Patassium Nitrate	0	0.09	0.09
Zink Chloride	0.072	0.672	0.6
Cupric Sulfate · $5H_2O$	$1.0 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$

9.6 Purification results

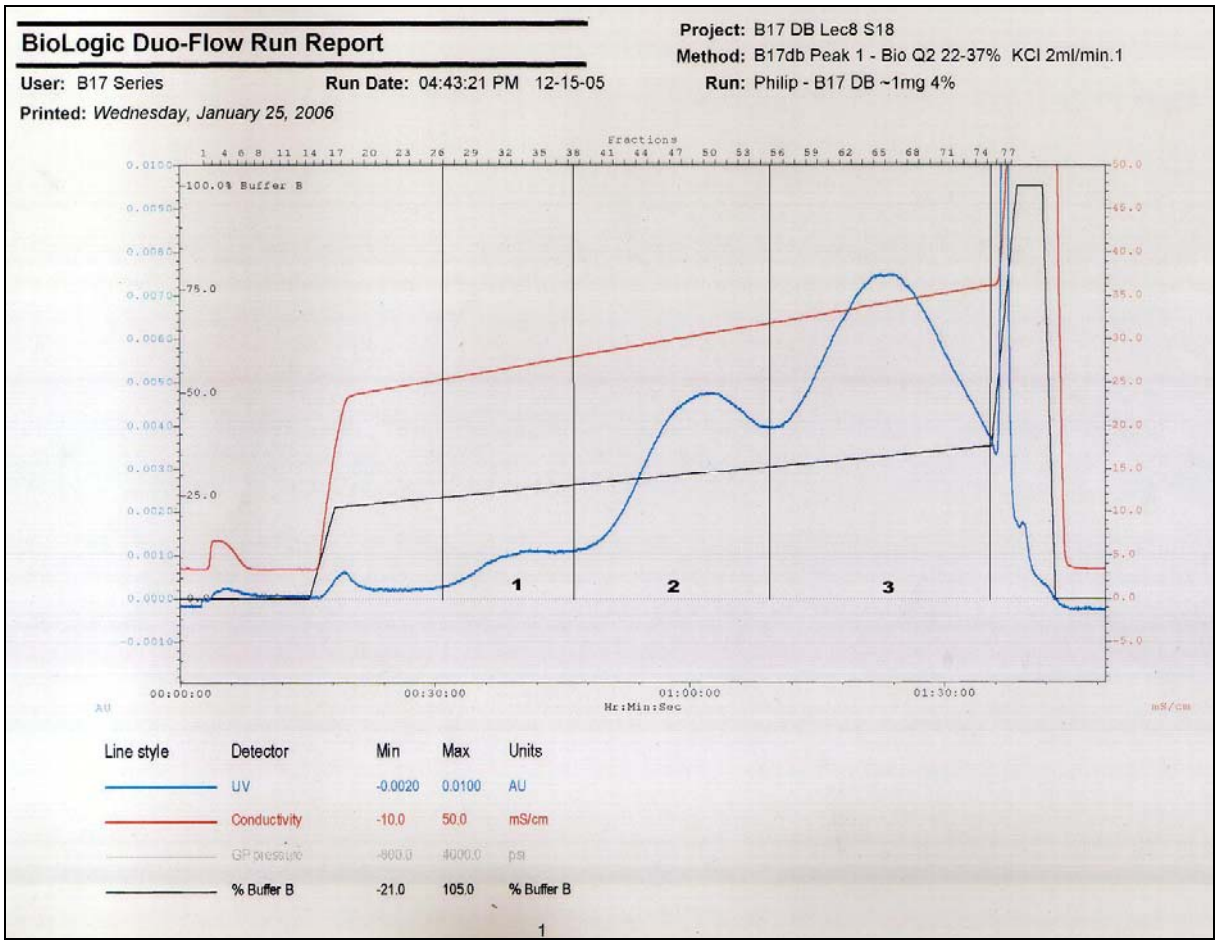

Figure 9.6-1: Truncated IR with different glycosylation pattern detected by UV/Vis detector after Ion Exchange column. This pattern was achieved by using DMEM/F12 (Coon´s modified) supplemented with 4% dFCS and 0.1% Pluronic (F68) in a Spinner Flask (Coring, 1L).

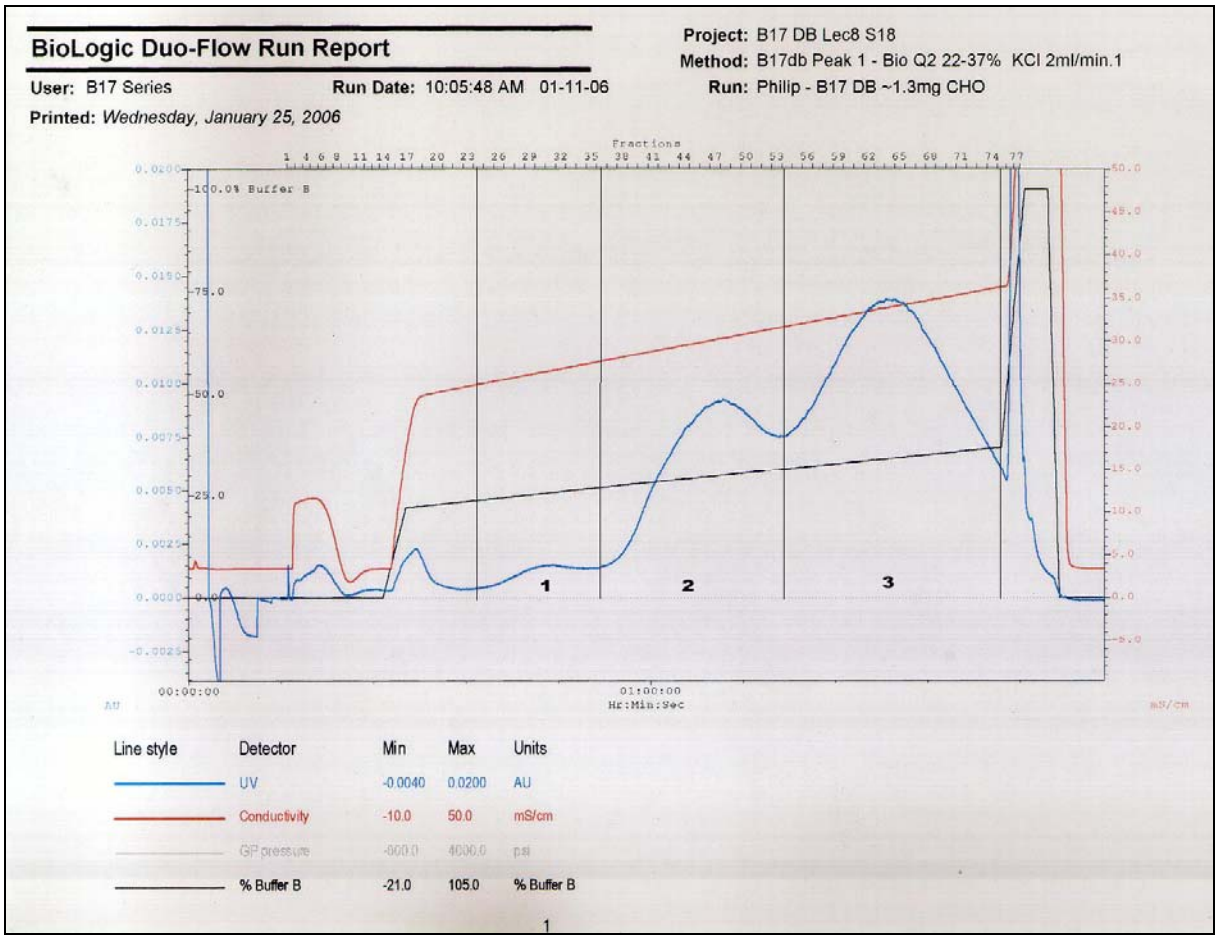

Figure 9.6-2: Truncated IR with different glycosylation pattern detected by UV/Vis detector after Ion Exchange column. This pattern was achieved by using BD CHO Medium serum free supplemented with 1 fold STE in T175 Flasks.

9.7 Calculation of parameters for BD CHO performance in T25 Flasks

The calculation of μ_{max} was conducted by two approaches:

1. $$\mu_{max} = \frac{\ln(\frac{x_2}{x_1})}{t_2 - t_1}\left[\frac{1}{d}\right] = \frac{\ln(\frac{0.571 \cdot 10^6 \frac{cells}{ml}}{0.212 \cdot 10^6 \frac{cells}{ml}})}{(5.87d - 3.03d)} = \underline{\underline{0.348 \frac{1}{d}}}$$

2.

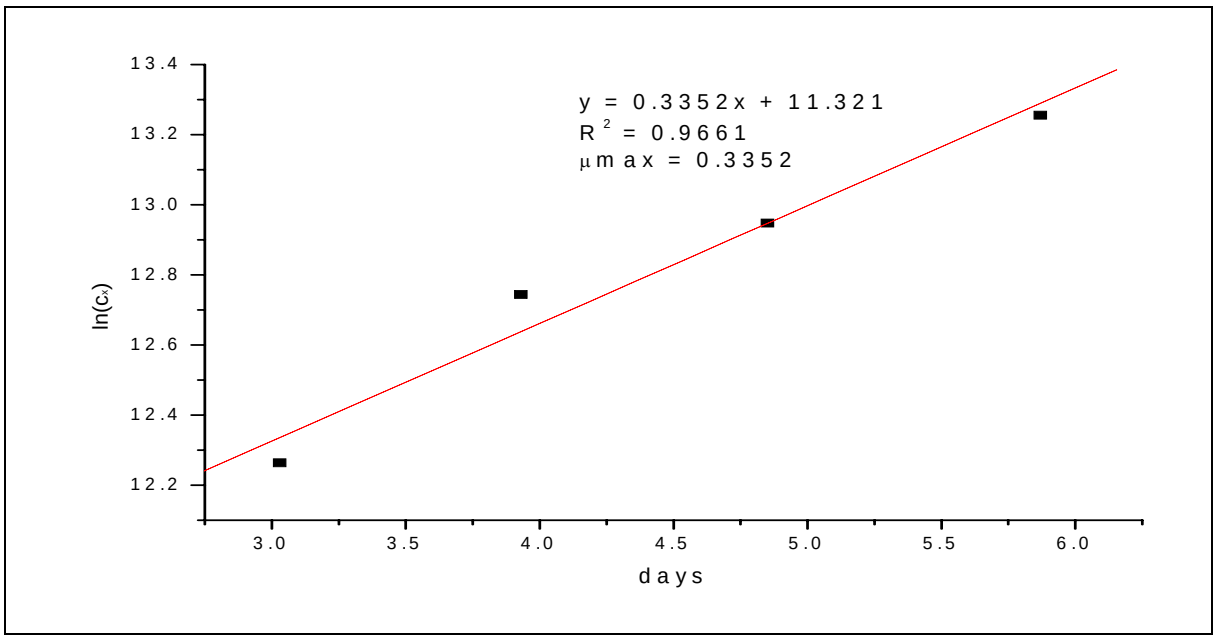

Figure 9.7-1: A linear regression was done with $\ln(c_x) = f(t)$. µmax is the slope of the regressions line.

The μ_{max} value shown in Table 5.1-1 is the average of the results obtained from the two approaches above. $\mu_{max} = 0.341\ [d^{-1}]$ and $dt = \frac{\ln(2)}{\mu\left[d^{-1}\right]} = \frac{\ln(2)}{0.341\left[d^{-1}\right]} = \underline{\underline{2.033d}}$

The specific production rate was computed according the following calculation and shown in Table 5.1-1:

$$q_{p/x} = \frac{c_p\left[\frac{mg}{l}\right] \cdot vol._{T25}\left[l\right]}{c_x\left[10^{6} cells\right] \cdot t\left[d\right]} = \frac{4.64\left[\frac{mg}{l}\right] \cdot 10 \cdot 10^{-3}\left[l\right]}{4.2\left[x10^{6}\ cells\right] \cdot 4.85\left[d\right]} = \underline{\underline{2.28 \frac{\mu g}{10^{6} cells \cdot d}}}$$

9.8 Calculation of parameters for suspension culture

The calculation of μ_{max} was conducted by two approaches:

1. $$\mu_{\max} = \frac{\ln(\frac{x_2}{x_1})}{t_2 - t_1}\left[\frac{1}{d}\right] = \frac{\ln(\frac{1.467 \cdot 10^{6} \frac{cells}{ml}}{0.299 \cdot 10^{6} \frac{cells}{ml}})}{(2.62d - 0d)} = \underline{\underline{0.607 \frac{1}{d}}}$$

2.

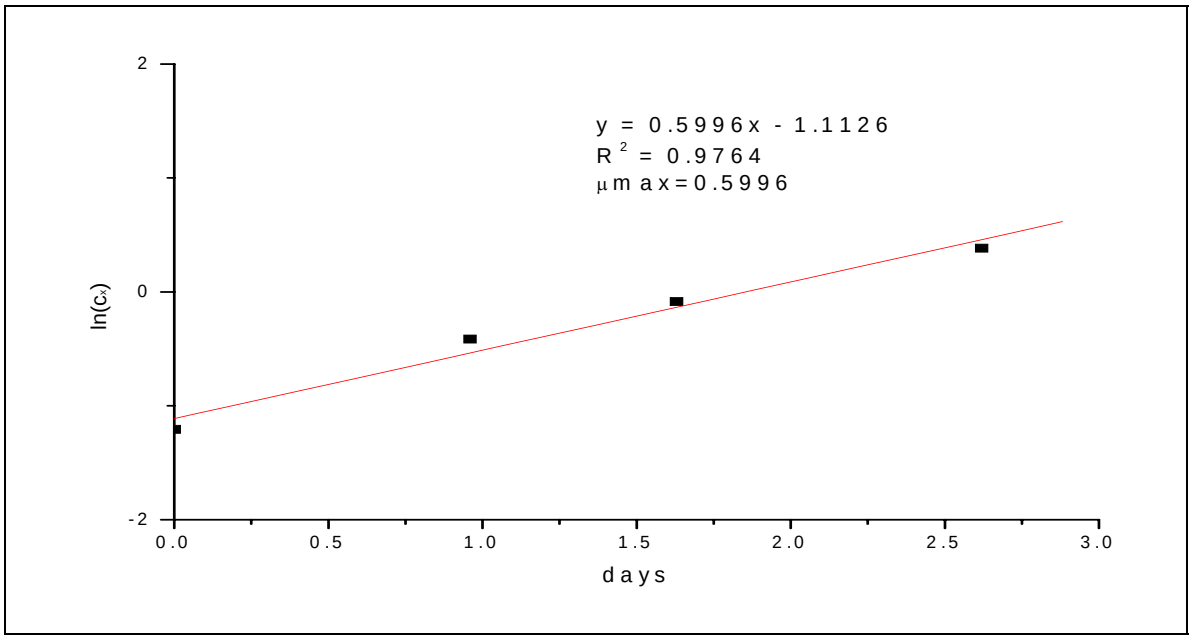

Figure 9.8-1: A linear regression was done with ln(c_x) = f(t). µmax is the slope of the regressions line.

The μ_{max} value shown in Table 5.3-2 is the average of the results obtained from the two approaches above. μ_{max} = 0.603 [d^{-1}] and $dt = \frac{\ln(2)}{\mu\left[d^{-1}\right]} = \frac{\ln(2)}{0.603\left[d^{-1}\right]} = \underline{1.149d}$

The specific production rate was computed according the following calculation and shown in Table 5.3-2:

$$q_{p/x} = \frac{c_p\left[\frac{mg}{l}\right] \cdot vol._{shaker}\left[l\right]}{c_x\left[10^{\ 6} cells\right] \cdot t\left[d\right]} = \frac{6.64\left[\frac{mg}{l}\right] \cdot 50 \cdot 10^{-3}\left[l\right]}{77.5\left[x10^{6}\ cells\right] \cdot 2.62\left[d\right]} = \underline{\underline{1.635 \frac{\mu g}{10^6 cells \cdot d}}}$$

9.9 Correlation between confluence area and cell number

The Cellscreen® provides growth parameter based on detecting the area covered with cells as function of the time. With the Cellscreen® parameter it possible to calculate the specific production rate in mass per percentage growth area and time. A coefficient to calculate the cell numbers based on the covered growth area in percent and cell number. Unfortunately no experiment was conducted to calculate this coefficient in a confident way. The only range of time, when the data could be obtained for this purpose is after seeding the cells, when the cells have not been divided. Fibroblast cell lines have shown a circular shape after seeding the cells in the cell culture vessel. It has been taken approximately 6-8 hours or more until the cell are attached to the cell surface and switched their shape from a circular into a flat and wide one. The first measurement of the plate was not recorded and it is not accurate to estimate the time of the first measurement after seeding the cells. An experiment should be designed to show the increasing of the covered area without growth after seeding the cells.
The following calculation run trough the theoretical procedure based on the screening of the Plackett Burman Desgin with CHO K1 cell line:

The average of the seeding area from 96 well plate (Plackett Burman Design with CHO K1) was calculated with $4.88\% \pm 0.54\%$.

The seeding cell density was $2 \cdot 10^4$ cells/ml multiplied by the working volume per chamber:

$$2 \cdot 10^4 \frac{cells}{ml} * 0.2 \frac{ml}{well} = \underline{\underline{4000 \frac{cells}{well}}} = \underline{\underline{0.04 \cdot \frac{10^5 cells}{well}}}$$

The cell concentration per well divided by the covered ceding area per well to obtain the desired coefficient:

$$\frac{0.04 \cdot \frac{10^5 cells}{well}}{4.88 \frac{\%}{well}} = \underline{\underline{8.197 \cdot 10^{-3} \frac{10^5 cells}{\%}}} = \underline{\underline{0.008197 \frac{10^5 cells}{\%}}}$$

The theoretical final cell number per well was calculated by multiplying the coefficient with 100% covered area of the well:

$$0.008197 \frac{10^5 cells}{\%} \cdot 100 \frac{\%}{well} = \underline{\underline{0.8197 \frac{10^5 cells}{well}}} = \underline{\underline{81970 \frac{cells}{well}}}$$

The theoretical final cell number per cm^2 was calculated by dividing the cell number per well with area per well:

$$81970 \frac{cells}{well} \cdot \frac{well}{0.33 cm^2} = \underline{\underline{2.484 \frac{10^5 cells}{cm^2}}}$$

Conclusion:

First of all the calculation can be done very fast and the accuracy is given by the Cellscreen® and even with an inculcation volume of 10 μl. The plates were measured >24 h after seeding the cells, but no point of time was recorded. Because of no specific experiment was conducted to evaluate this coefficient, the specific production rate was calculated with growth area instead of cell numbers, which is unfortunate, because a direct comparison with the culture conditions in a 96 well plate and a larger scale work such as T75 and bigger vessels is not possible.

9.10 Calculation of specific production rate used in charts

The specific production rate obtained from screenings in a 96 well plate was calculated based on the growth area in percent, because of the discussed error in 9.9.

The covered area with cells was provided by the Cellscreen® and exported in an Excel sheet. The provided area was shown as thousandth part, e.g. 100% area was represented by 0.1.The area was multiplied by 1000 to obtain the growth area in percent. The Cellscreen® calculates the culture time in hours; this value was multiplied by 1d per 24h to get the culture time days. The following calculation is representing for all the charts were the data was obtained from a screening in a 96 well plate and is the value of the standard in the ammonium investigation (Figure 5.5-4) conducted with CHO Lec8:

$$q_{p/x} = \frac{c_p\left[\frac{mg}{l}\right] \cdot vol._{chamber}\left[l\right]}{A\left[\%\right] \cdot t\left[d\right]} = \frac{3.94\left[\frac{mg}{l}\right] \cdot 0.2 \cdot 10^{-3}\left[l\right]}{83.03\left[\%\right] \cdot 4.125\left[d\right]} = \underline{\underline{2.3 \frac{ng}{\% \cdot d}}}$$

This calculation represents the specific production rate obtained from a screening with CHO K1 producing a fraction of the protein amount compared to Lec8. The example was taken from the condition BD CHO supplemented with STE (Figure 5.4-3):

$$q_{p/x} = \frac{c_p\left[\frac{\mu g}{l}\right] \cdot vol._{chamber}\left[l\right]}{A\left[\%\right] \cdot t\left[d\right]} = \frac{165.27\left[\frac{\mu g}{l}\right] \cdot 0.2 \cdot 10^{-3}\left[l\right]}{85.99\left[\%\right] \cdot 2.99\left[d\right]} = \underline{\underline{1.286 \cdot 10^{-4} \frac{\mu g}{\% \cdot d}}} = \underline{\underline{128.6 \frac{pg}{\% \cdot d}}}$$

9.11 Amino acid consumption

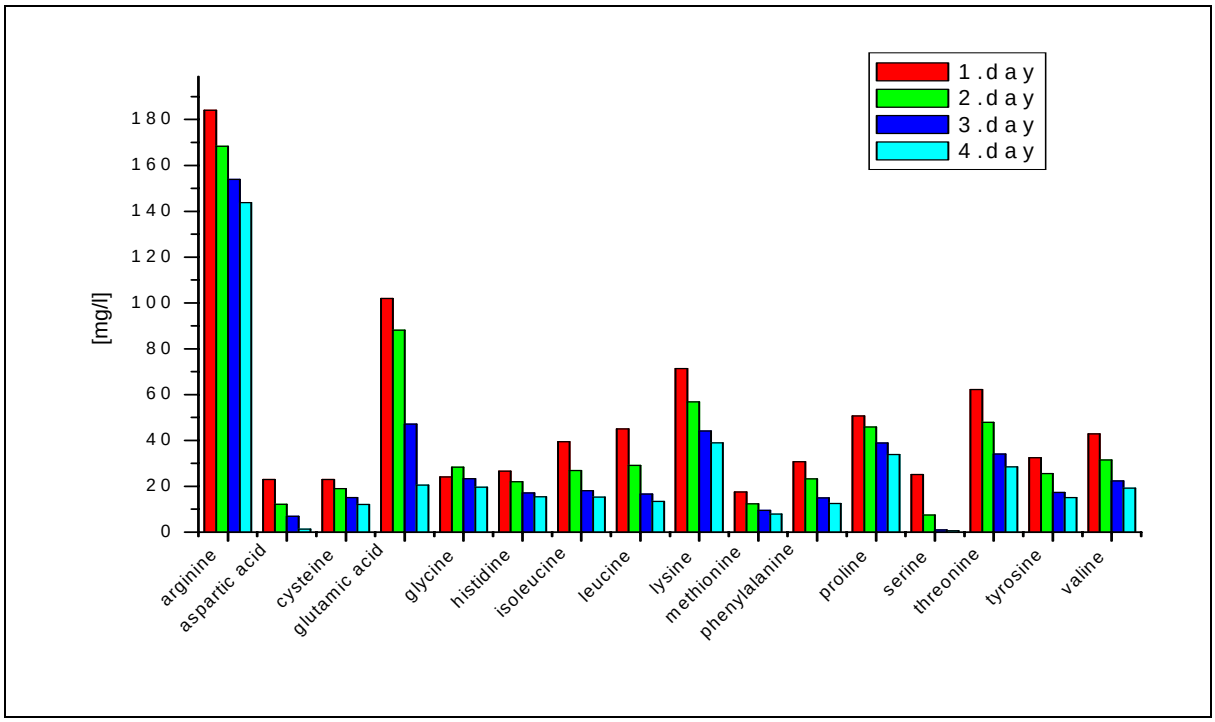

Figure 9.11-1: shows the characteristics of amino acid consumption by CHO K1 cells in a T175 flask. The area of the flasks was to 100% covered with cells. The experiment lasts 4 days.

9.12 Example for Full Factorial Design

A Full factorial design for the degradation pathway of the amino acids arginine, glutamate, glutamine histidine and proline to α-ketglutarate (chapter 2.3.3.3) and each amino represents a factor screened in two levels with the growth rate μ [*1/d*] as response parameter can be conducted in 16 experiments. Glutamine could be omitted as variable, because using GS expression system. In total 72 experiments are essential to obtain the needed results from 20 amino acid pooled in groups according their metabolic pathways. The results provide information about possible interaction and importance of the each variable. For instance, two amino acids are interacting in a growth promoting manner, these amino acids can be pooled as one variable for further screenings or an in-depth analysis could be conducted to expose the optimal ratio between the variables.

The finally outcome of this investigation should be pattern of amino acid concentration under consideration of their pathways, interactions and significance.

The screening of the 12 vitamins (used in the cell culture medium) in a Full Fractional Design can be difficult. Some of them acting in the same type of reaction, but it is difficult to combine them in groups. A simple Plackett Burman Desgin applied on organic medium compuounds (vitamins and fatty acids) can give information about significance of any variable. Only growth promoting variables can be combined in one vitamin solution as additive.

After a labour intensive screenings the range of possible variables is narrowed down to two main groups. Amino acids are pooled according their interactions and significance in one variable. The same should be done with the vitamins and organic compounds. It is believed that the ratio of amino acid, vitamins and organic compounds is an important issue in medium optimisation. The two variables can be screened in different concentration by using a multiple of the stock solution. In Figure 7.1-1 in shown an example for a possible screening with the Amino Acid and Vitamin solutions.

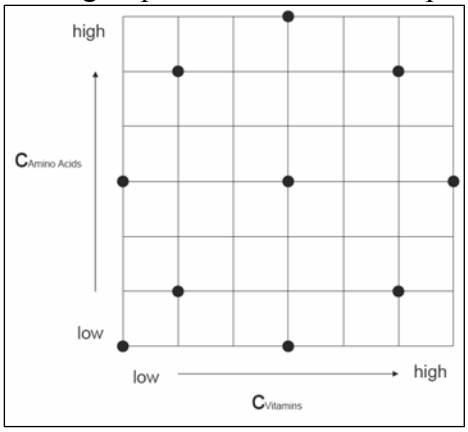

Figure 9.12-1: Central Composite Design with two compounds; An Amino Acid and a Vitamin solution based on results of a Plackett Burman and Fully Fractional Design.

10 Abbreviations

BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
cAMP	cyclic Adenosinmonophosphate
CHO	chinese hamster ovary
Cys	cystein
dFCS	dialyzed fetal calf serum
DH	dehydrogenase
DMEM	dulbecco’s modified eagle’s medium
DS100	Difco™ Springer DS 100 Soy Peptone UF
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Eu	europium
F12	Ham's F12 medium
FBS	foetal bovine serum
GSS	glutamine synthetase supplements
IGF-1R	insulin-like growth factor -1 receptor
IR	Insulin receptor
kDa	kilo Dalton
L1	large region 1
L2	large region 2
LBB	ligand binding buffer
MSX	methionine sulphoximine
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered
PLP	Pyridoxal-phosphate
rCHO	recobinant chinese hamster ovary
SAM	S-adenosylhomocyteine
Select	Difco Select Phytone™ Peptone UF
sf	serum free
SITE	Selenium, Insulin,Transferrin and Ethanolamine
STE	Selenium, Transferrin and Ethanolamine
TAE	tris-acetate, EDTA
TBS	tris-buffered saline
TBSA	tris-buffered saline azide
TBST	tris-buffered saline Tween 20
TCY	TC Yeastolate
UDP	uridine diphosphat
UGT	uridinediphosphate-glucuronosyl transferase

EU	endotoxin unit
kg	kilogram
g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
l	litre
ml	millilitre
µl	microlitre
µm	micrometer
mOsm	milli Osmolarity