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PRODUCTION OF RECOMBINANT PROTEINS AND MONOCLONAL ANTIBODIES – TECHNO-ECONOMICAL EVALUATION OF THE PRODUCTION METHODS

Thesis for the degree of Licentiate of Science in Technology submitted for inspection, Espoo, 27th of March, 2009.

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ABSTRACT OF LICENTIATE THESIS

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Abstract

Recombinant proteins and antibodies for therapeutic or diagnostic use can be produced in many host organisms (microbial, insect and mammalian cells), in different bioreactors (stirred-tank bioreactor, hollow fiber bioreactor and disposable bag bioreactors) and using various feeding strategies (batch, fed-batch or perfusion). The manufacturing cost (€/g) of the product depends on the characteristics of the production host (growth rate, productivity) and on the production method (cell density in the bioreactor phase and the overall yield). Most critical variables are the fermentation titer (g/l) and the total yield (%).

The manufacturing costs of production of recombinant proteins and monoclonal antibodies were analyzed for two applications. In the recombinant protein application the focus was on the effect of production host (*E. coli*, *P. pastoris* and *Drosophila* S2) and in the monoclonal antibody application in different bioreactor setups. The *E. coli* was found to be the lowest cost system in HIV-1 Nef-protein production. In Mab production, the hollow fiber bioreactor was found to have slightly lower manufacturing costs than the perfusion stirred-tank bioreactor. Also a crystallization method for recombinant HIV-1 Nef protein was developed.

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LISENSIAATINTUTKIMUKSEN TIIVISTELMÄ

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Rekombinanttiproteiinien ja vasta-aineiden tuotanto – tuotantomenetelmien teknillistaloudellinen vertailu

Tiivistelmä

Terapeuttisia tai diagnostiikassa käytettäviä rekombinanttiproteiineja ja vasta-aineita voidaan tuottaa useissa eri tuotto-organismeissa (mikrobi-, hyönteis- tai eläinsoluissa), erilaisissa bioreaktoreissa (sekoitusreaktorissa, onttokuitureaktorissa tai kertakäyttöisissä pussireaktoreissa) sekä käyttäen erilaisia tuotantomenetelmiä (panos, puolipanos tai jatkuvatoiminen kasvatus). Tuotantokustannukset (€/g) riippuvat tuotto-organismin ominaisuuksista kuten kasvunopeudesta ja tuottavuudesta sekä tuottomenetelmästä kuten kasvatusvaiheen solutiheydestä sekä kokonaissaannosta. Kriittisimpiä tekijöitä ovat bioreaktorivaiheen tuotto sekä kokonaissaanto.

Tässä työssä analysoitiin rekombinanttiproteiinin ja monoklonaalisen vasta-aineen pienen mittakaavan tuotannon taloudellisuutta. HIV-1 Nef-proteiinia tuotettiin erilaisissa tuotto-organismeissa (*E. coli*, *P. pastoris* and *Drosophila* S2) ja vasta-ainetta erilaisissa bioreaktoreissa. *E. coli* –bakteerin todettiin olevan edullisin tuotto-organismi HIV-1 Nef-proteiinin tuotannossa. Vasta-aineen tuotannossa, onttokuitureaktorin tuotantokustannukset olivat hieman matalemmat kuin sekoitusreaktorin. Työssä esitellään myös kiteytysmenetelmä rekombinantti HIV-1 Nef-proteiinille.

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PREFACE

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Espoo, March 27th, 2009

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ABBREVIATIONS

ALF	Air-lift bioreactor
AIID	Arthirits, inflammation and immune disorders
BHK	Baby hamster kidney
βOG	Beta octyl-glucopyranoside
CE	Cost of equipment
CHO	Chinese hamster ovary
CIP	Cleaning in place
COG	Cost of goods
DSP	Downstream processing
DTE	Dithioerythritol
ECS	Extracapillary space
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
Fab	Fragment antigen binding portion of an antibody
FCI	Fixed capital investment
FDA	U.S.A. food and drug administration
GMP	Good manufacturing practice
GST	Glutathione s-transferase
HFB	Hollow fiber bioreactor
HIC	Hydrophobic interactions chromatography
hIL-2	Human interleukin -2
HIV	Human immunodeficiency virus
HPAC	Heating, plumbing and air-conditioning
HVAC	Heating, ventilation and air-conditioning

Ig	Immunoglobulin
IPA	Isopropanol
IPTG	Isopropyl-ß-D-thiogalactopyranoside
ISBL	Inside battery limits
L	Lang's factor
Mab	Monoclonal antibody
MF	Microfiltration
Nef	Negative factor
NMR	Nuclear magnetic resonance
PEC	Purchased equipment cost
PEG	Polyethylene glycol
QCQA	Quality control and quality analysis
RIA	Radioimmunoassay
rtPA	Recombinant-tissue plasminogen activator
scFv	Single chain variable fragment
SIP	Sterilization in place
STR	Stirred-tank reactor
UF	Ultrafiltration
USP	Upstream processing

1 INTRODUCTION

Biopharmaceuticals are medical drugs – proteins, antibodies and nucleic acids that are produced using biotechnology and are used for therapeutic or *in vivo* diagnostic purposes. The approval for human use requires several years of clinical trials.

The first biopharmaceutical agent was insulin that was approved for human use in 1982. Today, over 160 biopharmaceutical agents are approved in the USA and/or EU and most biopharmaceuticals are cancer-related. Besides diabetes and cancer, the biopharmaceuticals have advanced the treatment of rheumatoid arthritis and different blood disorders (Walsh, 2006).

The markets of biopharmaceuticals have increased significantly over last years. Reasons for the rapidly increasing market value are naturally the increased number of products on the markets but also the fact that many of the therapeutic products are used in chronic diseases and in large quantities. As the new, efficient biopharmaceuticals are wanted to be available for more patients, increase in the manufacturing capacity is required.

The development and manufacturing of these biopharmaceuticals is expensive and as a result the patient's treatment costs may range from 10 000 € to even 100 000 € per year. The high costs of the biopharmaceuticals are partly explained by the long and expensive development time. The clinical trials take several years and still the drugs' failure rate is 80 %. Manufacturing costs are also high, as clean room facilities and expensive methods are required. The biopharmaceuticals may also require administration by healthcare professionals, which obviously increases the costs. As the number of biopharmaceuticals is increasing, also the health care budgets will grow and the authorities may set maximum prices to the product.

Because of the pressure to decrease the expenses, the focus is today in decreasing the manufacturing costs of the biopharmaceuticals. The manufacturing costs depend on the production host qualities, especially on expression levels and on the manufacturing process. The expression levels have already improved significantly, and now the processes must be evaluated more thoroughly. In biopharmaceutical

field, the decisions of the production method and process steps have to be made earlier than in the normal process development, as after clinical trials none or only few modifications can be made. Therefore, the techno-economical evaluations in early process development phase are necessary in finding the optimal production method.

In this thesis, different methods for producing therapeutic recombinant proteins and antibodies are reviewed and the manufacturing cost variables studied. Techno-economical comparisons of different production hosts in recombinant protein production and different production methods in monoclonal antibody production are presented.

LITERATURE PART

2 Production of biopharmaceuticals for therapeutic and diagnostic use

Typical biopharmaceutical products for therapeutic and diagnostic use are proteins produced in microbial, mammalian or insect cell cultures using different bioreactor setups. The purification procedure depends on the characteristics of the product.

2.1 Products

The biopharmaceuticals include a variety of protein products such as recombinant proteins and monoclonal antibody- or nucleic acid-based products and vaccines as presented in Table 1. Here the focus is on recombinant proteins and monoclonal antibody based products. The first approved biopharmaceuticals in the 80's and early 90's were proteins with unaltered sequences or murine antibodies. Nowadays engineered products have captured the markets. The engineered products mean for example engineered proteins (e.g., insulin and interferon), humanized antibodies or antibody fragments (Walsh, 2005).

Table 1. The different biopharmaceutical products (Walsh, 2006).

Recombinant proteins
Blood factors (e.g. Factor VIII)
Thrombolytic agents (e.g. tissue plasminogen activator)
Hormones (e.g. insulin, growth hormones)
Growth factors (e.g. erythropoietin)
Interferons (e.g. interferon-α)
Interleukin-based products
Monoclonal antibodies and antibody fragments
Vaccines
Nucleic-acid based products
Therapeutic enzymes

On therapeutic field, the five major markets are the treatments or diagnostics for cancer, diabetes, growth disturbances, hemophilia and hepatitis. The biggest single product type based on sales was in 2005 erythropoietin (EPO). The combined sales of EPO products exceeded $10 billion (Walsh, 2006). In diagnostic field, the recombinant proteins and antibodies are usually used for detection of certain viruses or diseases.

In 2006, approximately 162 biopharmaceutical products were approved in the USA and/or EU and recombinant proteins covered 70 % of these products (Figure 1). Monoclonal antibodies and antibody fragments together covered 18 % and vaccines 12 %.

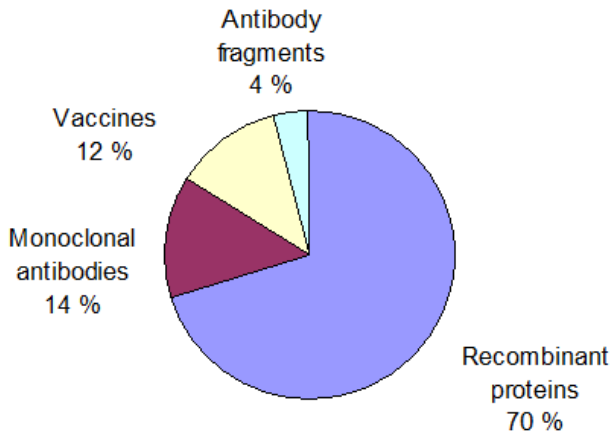

Figure 1. The biopharmaceuticals approved in the US or EU until 2006, total number or approved products 162 (Walsh, 2006).

In 2006 Walsh (2006) estimated that approximately 2 500 biopharmaceutical drugs were in the discovery phase, 900 in preclinical trials and 1 600 in clinical trials. Oncology was the most common indication target. Most products were either monoclonal antibodies or vaccines. Already in 2004 the Mab products were about 25 % of all biopharmaceutical products in clinical development (Yang *et al.*, 2004).

2.1.1 Biopharmaceutical markets

In 2004 the combined market of biopharmaceuticals was estimated to $44 billion (Lawrence, 2005). The markets are projected to reach $70 billion by the end of the decade (Pavlou and Reichert, 2004; Pavlou and Belsey, 2005). Sommerfeld and Strube (2005) evaluate, that the global growth rate of the biopharmaceutical markets is approximately 10 %, whereas the average growth rate of antibody-based products exceeds 30 % and of other recombinant proteins over 15 %. The monoclonal antibody markets are estimated to continue to grow 20 % per year at least until 2010 (Johansson *et al.*, 2007). As comparison, the traditional small-molecule pharmaceutical markets face approximately 4 % annual increase.

2.1.2 Recombinant proteins

Walsh (2006) has grouped therapeutic recombinant proteins in five different categories: recombinant blood factors (used in indications of hemophilia), recombinant thrombolytics and anticoagulants, recombinant hormones (e.g. insulin and growth hormones), recombinant growth factors (e.g. erythropoietin), recombinant interferons and interleukins (used for example in indications of hepatitis).

In therapeutic use the dose of a therapeutic protein e.g. erythropoietin or human growth hormone, is typically a few micrograms of protein (Aldington and Bonnerjea, 2007).

2.1.3 Antibodies

Antibodies (or immunoglobulins (Ig)) are proteins that are used by the immune system. They are built of two heavy protein chains and two light protein chains. Antibodies are categorized in five different isotypes based on their heavy chain type. The most typical immunoglobulin class is IgG (see Figure 2).

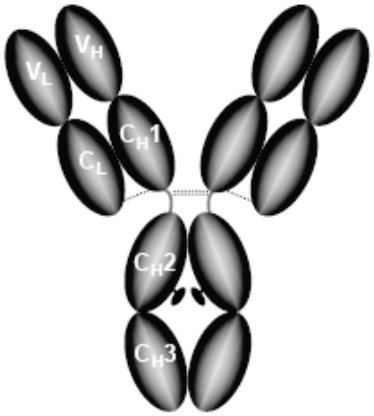

Figure 2. IgG full-length antibody contains the heavy chain (V_H, C_H1, C_H2 and C_H3) and light chain (V_L and C_L) domains. The different chains are linked with disulfide bonds that are marked as dotted lines (Chadd and Chamow, 2001).

The antigen binding sites, the variable regions are located at the tips of the Y-shaped protein (V_L / V_H). The constant domain is located at the other end of the macromolecule.

Recombinant antibodies are called monoclonal antibodies (Mab, mAb or moAb), because they are all identical and produced by a single clone of cells. The recombinant Mabs are grouped based on their protein origin (murine/human) (Werner, 2004):

- Murine (mouse) antibodies are 100% murine protein and therefore their therapeutic applications are limited (side-effects, short serum half-life, inability to trigger desired immune effects)
- Chimeric antibodies are genetically engineered and contain components of both murine and human proteins, approximately one third murine protein components. Can trigger allergic-like reactions.
- Humanized antibodies are genetically engineered, almost all human protein. Only 5 – 10% of protein is murine origin. Can still trigger minor allergic-like reactions.
- Fully human antibodies are from human cells or from genetically engineered murine. These have the lowest side-effect profile, and lower doses are needed as these are not eliminated as strange proteins in the body.

In 2006, 22 monoclonal antibody products were approved, and six of these were murine, five chimeric, nine humanized and two fully human (Walsh, 2006). The traditional method for production of Mabs is murine hybridoma cell cultivation. These products came in the market in the 80's. Now the studies are more directed into chimeric and fully human Mabs (Pavlou and Belsey, 2005).

Monoclonal antibodies are used as therapeutic agents (in gene therapy, in targeting objects, in neutralizing objects, in signaling or in cross-linking objects), in diagnostics, in immunoaffinity purification or as catalytic antibodies (also called abzymes or catmab's) in enzyme engineering (Roque *et al.*, 2004).

As therapeutic agents, the monoclonal antibodies are focused on oncology and arthirits, inflammation and immune disorders (AIID), but they are also used in treating poisoning or viral infections and have become important treatment in chronic conditions (e.g. human malignancies).

In diagnostic use, monoclonal antibodies are currently utilized in ELISA (Enzyme-Linked ImmunoSorbent Assay) and RIA (Radioimmunoassay), and as imaging and immunosensor agents.

Of the 22 whole monoclonal antibodies currently on the market, three are used in diagnostics or in indications of detection of cancer or carcinomas. Two of these Mab's are murine and one fully human antibody (Walsh, 2006). Most of the diagnostic antibodies are not on the approved list, as they *in vitro* products do not need as thorough approval procedure.

The need for large-scale monoclonal antibody processes is increased: they are used in chronic conditions and the doses needed are high, typical annual dose of a patient is 2 – 5 g (Sommerfeld and Strube, 2005). The high dosage is result of the relatively low potency (Birch and Racher, 2006; Chu and Robinson, 2001; Farid, 2006; Reichert *et al.*, 2005; Werner, 2004). The scale of antibody production depends on the field it is used: for research and toxicology studies the quantities needed are in µg or g range; for diagnostic applications of early-state clinical evaluations tens of grams or a few hundred grams; for late-stage clinical or for licensed products a few kilograms or hundreds of kilograms (Carson, 2005).

All approved therapeutic Mab's are produced in mammalian cell cultures, but (Simmons *et al*., 2002) have published a study where they presented whole antibody production in *E. coli* (aglycosylated IgG1). Mab production has also been studied in yeasts and in fungi *Aspergillus* and studies are focusing on the correct glycosylation by these hosts (Birch and Racher, 2006).

2.1.4 Antibody fragments

An antibody fragment (Fab) contains the antigen binding region of the antibody, and therefore maintains the biological activity of the parent immunoglobulin (Figure 3). In a single chain variable fragment (scFv) the variable regions of the heavy and light chains are fused together (Figure 4). The scFv is half the size of the Fab fragment, but it still retains the original specificity.

Until June 2006, six Fabs were approved in the US or within the EU (Walsh, 2006). Reichert *et al*. (2005) have listed the antibody based products in the pipeline and in 2005, there were three Fabs and three scFv's in phase III trials.

Figure 3. An antibody fragment (Fab) contains the the V_H, V_L, C_L and C_H1 domains of an antibody (Chadd and Chamow, 2001).

Figure 4. A scFv fragment contains only the antigen binding site (the V_H and V_L domains linked with a polypeptide linker) (Chadd and Chamow, 2001).

Fabs, Fab'2 fragments and scFvs can be produced using microbial expression systems, although all approved antibody-based products are so far produced in mammalian cell cultivations. Yeasts and insect cell perform some degree of glycosylation (although it differs from the glycosylation performed by mammalian cells), but in bacterial cells, only aglycosylated antibody fragment products can be produced (Birch and Racher, 2006; Jain and Kumar, 2008; Reichert *et al.*, 2005).

Fabs have been produced in *E. coli* and *P. pastoris* and these products are now in preclinical and clinical trials (Chadd and Chamow, 2001; Farid, 2007; Reichert *et al.*, 2005). scFv's have been produced in *E. coli*, *P. pastoris* and *Drosophila* insect cell lines (Andersen and Reilly, 2004; Holliger and Hudson, 2005; Johansson *et al.*, 2007).

2.2 Host systems

Most of the proteins and antibodies for therapeutic or diagnostic use are produced in either *E. coli*, *Saccharomyces cerevisiae*, or mammalian cell lines (Chinese hamster ovary (CHO), Baby hamster kidney (BHK) and hybridoma cells) (Chu and Robinson, 2001; Jana and Deb, 2005; Walsh, 2006). Between 2003 and 2006, 31 new biopharmaceuticals (proteins and antibody-based products) were approved, and nine of these were produced in *E. coli* and 17 in mammalian cell cultures (Walsh, 2006).

The host systems used in the production of approved therapeutic recombinant proteins (no monoclonal antibody- or nucleic acid based products) are shown in Figure 5.

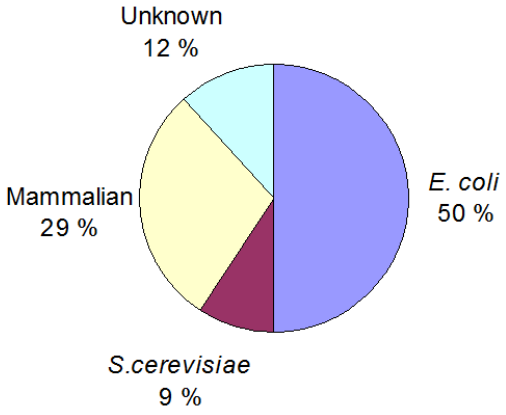

Figure 5. Host organism in production of approved (until june 2006) therapeutic proteins (not Mab-based or nucleic-acid based) (Walsh, 2006).

In case of monoclonal antibodies, the mammalian cell culture is the most used system. In 2006, all licensed antibodies were produced in mammalian cell systems. If the antibody based products are included into the scene presented in Figure 5, the mammalian cells are the most used system with share of 47 % (Walsh, 2006). Also most of the Mabs in clinical development were produced in mammalian cell systems (Birch and Racher, 2006).

2.2.1 Selecting the right production host

When choosing an expression host for production of a specific recombinant protein, one can essentially select from a multiplicity of different systems. The *Escherichia coli* bacterium is usually the starting point for any cloning and expression effort, because it has a variety of expression systems and is easy to cultivate. There is, however, no universal expression host system that would work optimally for all proteins. And even though the mammalian cell culture is more complex and expensive, it is often the only choice to produce large proteins that require extensive post-translational glycosylation. Therefore, when selecting the right production host, the glycosylation of the product must be the first criteria. Annual production requirements are often the second criteria. The higher the titer is, the lower the production volumes and therefore also the investment costs. The choice of the expression host also often determines the time-to-market, as the host development times vary significantly from host to host.

Each system has different advantages and disadvantages. In addition to the titer and the ability to perform post-translational modifications the practical issues to consider include means of induction and protease activity of the host. When aiming for industrial production, one has to consider a multitude of additional factors, such as the royalty burden of the host cell and vector, cost of raw materials, regulatory issues related to the host cell and vector (e.g. required containment level), harmful side-products (e.g. endotoxins, expression of tumor-associated proteins, baterial contaminants), reproducibility, up-scalability, and ease of host cell contamination.

Cha *et al.* (2005) have compared the properties of different recombinant expression systems (*E. coli*, *P. pastoris* and insect cell lines) in human interleukin-2 (hIL-2) production. The different production systems were compared in terms of the productivity and product qualities. Beside their own studies, they also presented a comparison of the different expression systems based on literature data (Table 2.).

Table 2. Comparison of recombinant expression systems (modified from Cha *et al.* (2005)).

	E. coli	**Yeast**	**Insect**	**Mammalian**
Growth rate	Very fast	Fast	Slow	Very slow
Expression yield (based on dry weight)	High (1-5 %)	High (>1 %)	Very high (30 %)	Very low (<1 %)
Productivity	Very high	High	High	Low
Media cost	Very low	Low	High	Very high
Culture techniques	Very easy	Easy	Difficult	Very difficult
Production cost	Very low	Low	High	Very high
Protein folding	Fair	Good	Very good	Very good
Simple glycosylation	No	Yes	Yes	Yes
Complex glycosylation	No	No	Yes	Yes
Secretion	Poor	Very good	Very good	Very good
Availability of genetic systems	Very good	Good	Fair	Fair
Pyrogen problem	Possible	No	No	No

As can be surmised, the costs of *E. coli* culture are very low and mammalian cell culture very high. But as stated earlier, *E. coli* cannot perform even simple glycosylation.

2.2.2 Escherichia coli - bacteria

Even though there's many alternatives in the production of biopharmaceuticals and other recombinant proteins, *E. coli* is still used most widely, because it offers a rapid and economical production possibility (Andersen and Krummen, 2002; Schmidt, 2004; Walsh, 2006).

Sarramegna *et al.* (2003) stated that the main advantages of *E. coli* are the low production cost, homogeneity of the recombinant protein and short generation time.

However, the tendency to form of inclusion bodies can be a problem in recombinant protein production, as the proteins should be refolded for adequate biological activity (Datar *et al*., 1993). *E. coli* also lacks the ability to perform post-translational modifications and there is no secretion mechanism for the efficient release of proteins into the culture medium (Jana and Deb, 2005).

If no post-translational modifications are required, the *E. coli* is a good choice. For example many recombinant hormones (e.g. insulin) and interferons are produced commercially in *E. coli* (Walsh, 2006).

2.2.3 Yeasts

Engineered *S. cerevisiae* is used in the production of at least 10 approved biopharmaceuticals. These include most notably some insulin products and growth hormones. In addition to these, most of the vaccines now available are produced in *S. cerevisiae*. Most important of these are the recombinant hepatitis B vaccines (Walsh, 2006).

No biopharmaceutical products have been produced in other yeast systems (data until 2006). *Pichia pastoris* would be an attractive host, because it can grow to high cell densities and is as easy to manipulate as *E. coli* but can also perform post-translational protein modifications (Cha *et al*., 2005). However, the yeast performed post-translational modifications differ from those performed by mammalian cells (Chiba and Jigami, 2007).

When compared with mammalian cell systems, yeasts have faster growth rate, and generally also produce higher amounts of the protein product. In terms of production titer, *P. pastoris* is one of the most productive expression hosts, with recombinant protein titers even up to 14 g/liter (Clare *et al.*, 1991). Additional advantage is the secretion of protein products into the growth medium, which usually makes the downstream processing easier (Cha *et al.*, 2005).

Unwanted glycosylation of the recombinant protein is a possible disadvantage of yeasts, but the degree of glycosylation depends on the strain as well as on the expression system used. Generally, the degree of glycosylation in *P. pastoris* is not as high as in *S. cerevisiae* (Schmidt, 2004; Schuster *et al.*, 2000).

2.2.4 Insect cells

Among the new systems, insect cells are gaining ground rapidly (Ikonomou *et al.*, 2003). In 2007, the first vaccine (Cervarix® by GlaxoSmithKline, UK) produced in insect cells using the baculovirussustem was approved within the EU, in Australia and in Philippines. Cervarix® is a vaccine against certain types of the human papillomavirus that causes cervical cancer (Anon., 2008b). There is also another vaccine with the same implication, but it is produced in *S. cerevisiae* (Walsh, 2006). To date, many recombinant proteins made using insect cell lines have already been approved for use in veterinary medicine (Walsh, 2003).

In comparison to mammalian cells, the ease of culture, high tolerance of osmolality and by-product concentrations, as well as higher expression levels are considered advantages of the insect cell systems (Ikonomou *et al.*, 2003). Insect cells are also able to carry out the post-translational modifications some microbial systems can not, although the modifications still differ from those performed by mammalian cell cultivations (Cha *et al.*, 2005).

2.2.5 Mammalian cells

Almost all recombinant proteins that are produced in mammalian cell cultivations are produced with CHO and BHK (Walsh, 2003). Other mammalian cell lines used in biopharmaceutical productions are murine myeloma cell lines and hybridoma cells (Chu *et al.*, 2005).

Mammalian cell cultivations are the only way to produce large biomolecules that require specific glycosylation. The disadvantages of mammalian cells are the slow growth rate and very low expression yield. These factors make the mammalian cell cultivation very expensive and the manufacturing cost is further increased by expensive media and difficult culture techniques (Cha *et al.*, 2005).

All antibody-based products are also produced in mammalian cell cultivations. According to Farid (2006) most monoclonal antibodies are using cultivating mammalian cell systems in batch or fed-batch mode in stirred tank bioreactor. Mabs are purified generally using filtration and chromatography (Farid, 2006; Sommerfeld and Strube, 2005).

In the last 15 years, the expression technologies and cultivation techniques (for example fed-batch and perfusion processes) have improved and resulted in significant improvements in antibody productivity of cell lines - even 100 fold (Birch and Racher, 2006). Now high cell line productivities (20 – 60 pg/cell/day) are relatively common and typical titers are in the range of 0.1 – 0.5 g/l. Also higher titers, even 1 – 5 g/l have been reported e.g. by Andersen and Krummen (2002), Birch and Racher (2006), Farid (2007) and Thiel (2004). With the improvements today the titers are expected to improve even to 10 – 15 g/l (Birch and Racher, 2006; Farid, 2007; Werner, 2005).

2.3 Bioreactors and production strategies

Bioproducts for therapeutic and diagnostic use can be produced in different types of bioreactors. The choice depends on e.g. the expression host and the production scale. Generally in large scale the stirred-tank bioreactor (STR) is the most desired option, as it is studied extensively and used in various systems for years. In 2001 over 70% of all therapeutic proteins and antibodies on the market were produced in STR (Chu and Robinson, 2001; Kretzmer, 2002).

The STR is probably the lowest cost alternative in large scale, where the costs are wanted to be decreased as low as possible. But in smaller scale, there are also other options for the bioreactor setup. Hollow fiber bioreactors are relatively common in small or medium scale commercial antibody processes. Most hybridoma cell lines are grown in air-lift or in hollow fibre bioreactors (HFB) (Chu and Robinson, 2001; Kretzmer, 2002).

Air-lift bioreactors (ALF) have been used mostly in production of monoclonal antibodies. In air-lifts, as also in STRs, both anchorage dependent and suspension cells can be grown. Air-lift reactors up to 5 m^3 have been used and even 10 m^3 air-lift bioreactors have been reported for Mab production in hybridoma cultures by Lonza Biologics (Birch and Racher, 2006; Jain and Kumar, 2008)

Perfusion technologies, such as hollow fibers and fixed-beds have several advantages: very high cell density, protection of the cells from the shear and high product concentrations. Disadvantages include limited mass transfer and difficulties in scale up, particularly of the hollow fiber system (Guardia and Hu, 2003). In fixed-bed bioreactors the cells are immobilized on microcarriers and kept in a reactor and the media is circulated through the microcarrier beads. Fixed-bed bioreactors are high cell density culture bioreactor likewise the HFB, and have been used for perfusion cultures of mammalian cells. Fixed-bed bioreactors are used in 5 – 30 l scale (Jain and Kumar, 2008).

The competition on the biopharma market is tough, and the time-to-market is often critical on the success of the product. As the cell line optimization is generally the most time demanding step, more emphasis is nowadays been placed on process

optimization and on the development of disposable bioreactors in order to shorten the time-to-market (Birch and Racher, 2006). The disposable bioreactors are a hot topic, but their size is limited so far to about 1 000 – 2 000 liter volume (Aldridge, 2005; Anon., 2008e).

The differences between stirred-tank and hollow fiber bioreactor in Mab production have been reviewed by Yang *et al.* (2004). Some of their data is shown in Table 3.

Table 3. Comparison of stirred-tank and hollow fiber methods for Mab production (modified from Yang *et al.*, 2004).

	Stirred-tank	Hollow fibre
Oxygenation	Mechanical agitation and gas sparging	Diffusion and medium re-circulation
Cell density (1/ml)	10^6 – 10^7 /ml	>10^8 / ml
Specific productivities	0.2 – 0.25 µg/10^6 cells/h batch mode 0.29 µg/10^6 cells/h, cell retention, spin filter	0.6 – 1.4 µg product /10^6 cells /h
Volumetric productivity	19.2 g/l/day, batch mode 110 mg/l/day, cell retention, spin filter	500 – 3500 mg/l/day (based on the extracapillary volumes of the reactor)
Cell damage by shear stress	High, caused by agitation and gas sparging	Low
Culture stability	High for continuous perfusion culture	Detoration because of membrane fouling, cell clogging and accumulation of dead cells
Scale-up potential	High	Low – limited by oxygen supply

As mentioned before, the production scale and yield generally define the bioreactor type. In the presentation by Griffiths (2003), the different bioreactor types are categorized by volume and cell density requirements (Figure 6).

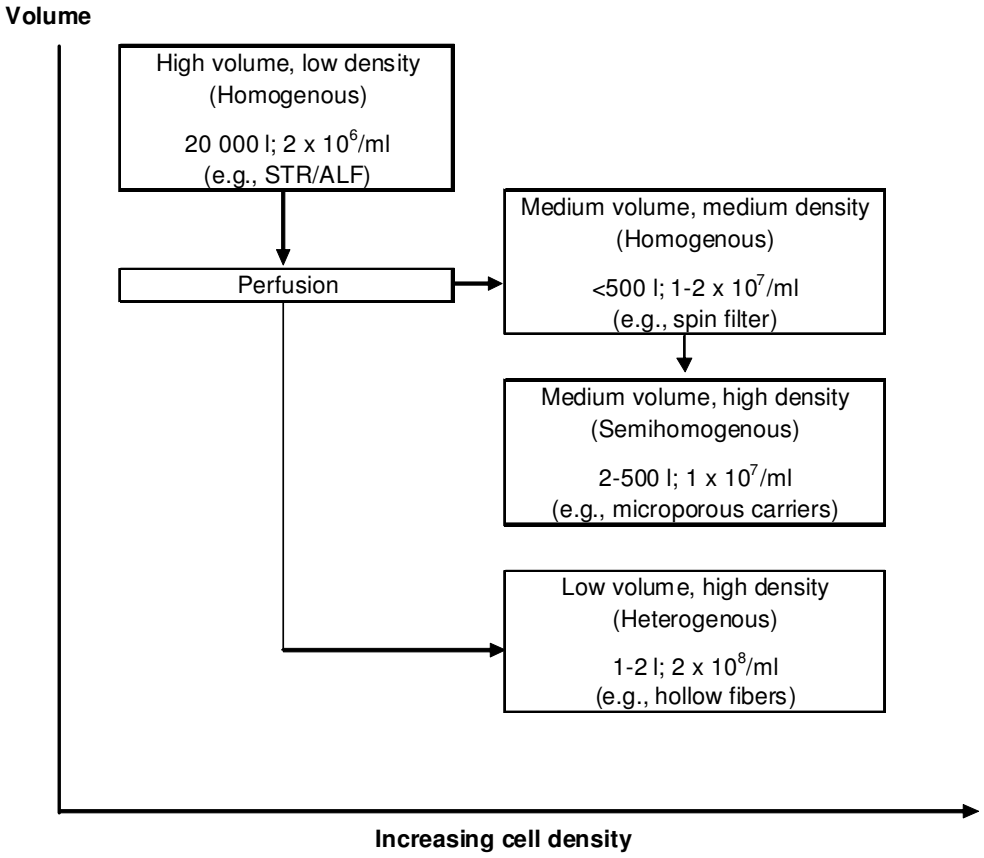

Figure 6. Bioreactor choice by scale (volume) and cell density requirements (Griffiths, 2003).

2.3.1 Batch, fed-batch or perfusion?

The bioreactors can be operated either in batch, fed-batch or in continuous perfusion mode. The different culture systems are presented in Figure 7. In batch system all nutrients are supplied in the beginning of the culture. The fed-batch is started at a low volume and the culture is later supplied with concentrated feed solution to maximum volume and no medium is removed. In chemostat the culture is constantly supplied with fresh medium and used medium and cells are removed simultaneously. In perfusion culture, fresh medium is supplied at the same rate than spent culture is withdrawn (biomass is returned or retained in the vessel).

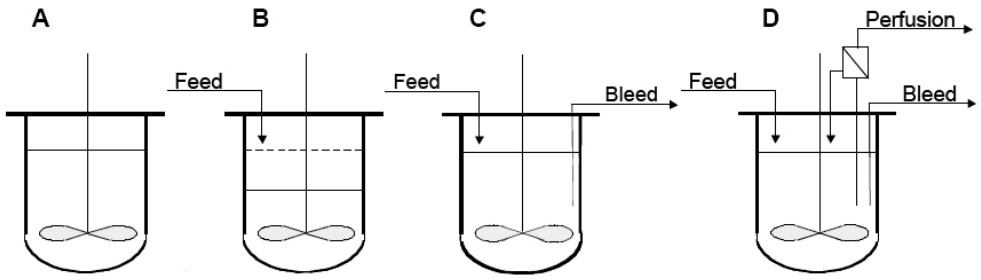

Figure 7. Schematic bioreactor overview of the different culture systems: (A) Batch, (B) Fed-batch, (C) Chemostat and (D) Perfusion system (Dalm, 2007).

In batch the cells grow until essential nutrients becomes limiting and the cells and product are harvested. The cell densities are generally about 5 x 10^6 cells/ml. In fed-batch process the culture time may be longer (even 10 – 15 days) as nutrients are added during the cultivation. Cell densities of 10^7 cells/ml are achieved. In perfusion the nutrients are constantly added and also possibly inhibiting products are removed. Cell densities are significantly higher on perfusion processes (even 2 – 4 x 10^7 cells/ml) and culture times can be 15 – 75 days (Dalm, 2007).

In the production of therapeutic products (approved until 1999), batch and fed-batch processes were used more and perfusion only with few products, whereas for diagnostic products, the perfusion was preferred either in STR or in HFB system (Farid, 2006). This might be because the therapeutic products are produced in larger scale than the diagnostic products and as Bibila and Robinson (1995) state, the fed-batch is simpler to operate than the perfusion in large scale and can still produce over ten times higher titers than the batch process. The perfusion systems are considered more difficult because of the continuous operation, requirement of a cell-retention device and the long run time. In small to medium scales, the perfusion is more efficient way of production than the fed-batch. The high cell concentration in perfusion process results also in high product titers: productivities that are even 10 times higher than in batch or fed-batch processes have been reported e.g. by Heine *et al.* (2000).

In the continuous perfusion bioreactor, very high cell densities and consequently very high product throughput is achieved by retaining the cells in the reactor. The cells are either prevented to leave the vessel or separated by external recycling

device (Birch, 2003). Various cell retention techniques include filters, settlers, centrifuges or hydrocyclones (Castilho and Medronho, 2002; Voisard *et al.*, 2003). The cell retention device may be positioned either inside or outside of the bioreactor vessel. At industrial scale the cell retention is usually done by centrifuges, settling devices or spin filters. The membranes and filters foul easily and therefore the rotated filters or baskets are widely used (Cartwright, 1994). The settlers were used up to 50 liter scale, the centrifuges up to 100 liter scale and the spin-filters up to 500 liter scale (Voisard *et al.*, 2003). In Figure 8, a typical spin-filter (or rotating filter) perfusion system is presented.

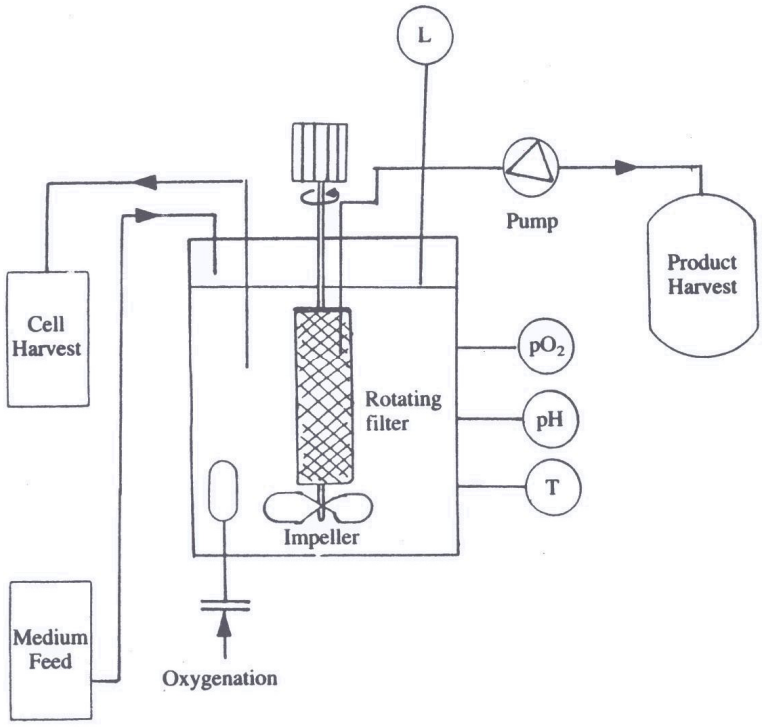

Figure 8. Perfusion culture by a spin filter arrangement (Cartwright, 1994).

According to Su (2000), the product quality is more consistent in perfusion system when compared to the batch and fed-batch processes. Small bioreactors and longer cultivation time also reduce the capital costs. The longer cultivation time means also fewer reactor turnovers, and therefore also less labor and energy costs (Castilho and Medronho, 2002).

2.3.2 Stirred-tank bioreactor

With microbial cultivations, the stirred-tank bioreactor is almost always the choice. The optimization and scale-up of the STR is straightforward until certain size limit. Microbial cells are grown with different feeding strategies: batch, fed-batch or continuous. Conventional stirred-tank bioreactor is presented in Figure 9.

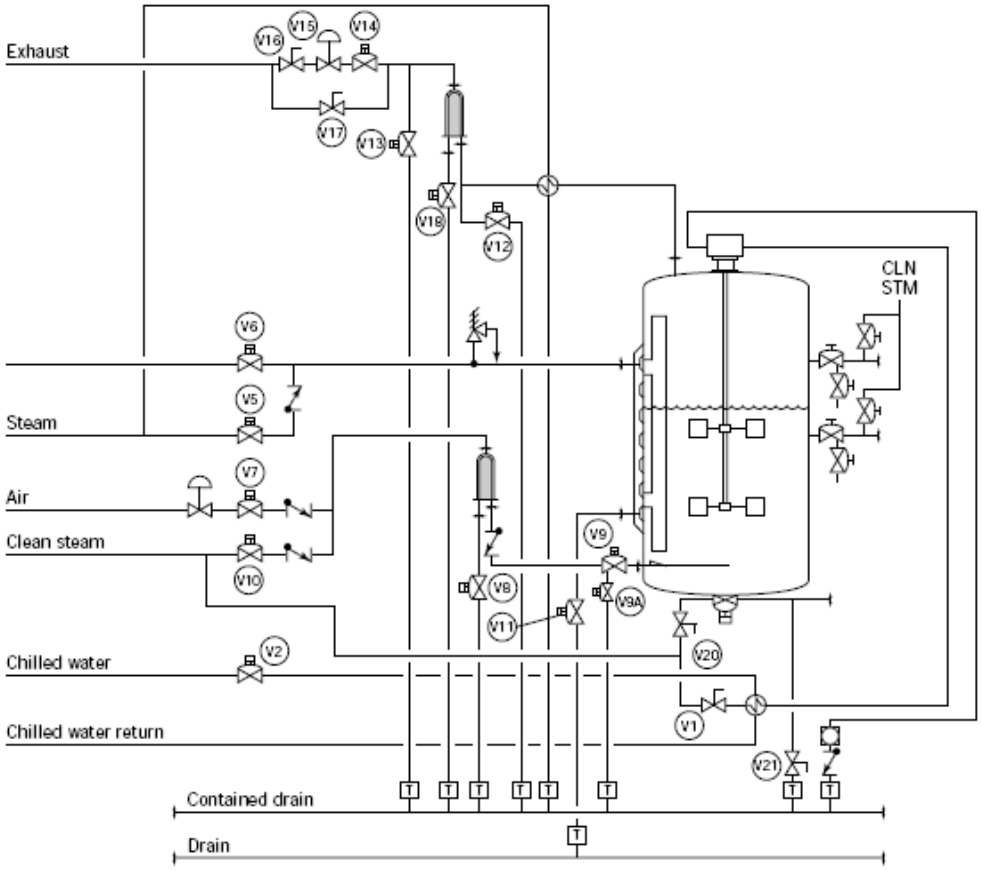

Figure 9. Schematic drawing of a typical jacket-cooled stirred-tank bioreactor and the piping (Charles and Wilson, 2003).

With the mammalian and insect cell cultures the STR technique is not quite as simple. As the cells are more shear sensitive than the microbes, the agitation and gas sparging can be a problem. Some cells are also so anchor-dependent that they cannot be adapted to grow in suspension. Microcarriers may sometimes enable also the anchor-dependent cell suspension culture, and when possible, the basic

suspension culture is used. With the most used cell lines these problems have been solved and they can be cultured in STR (Chu and Robinson, 2001).

As has been stated earlier, generally the biopharmaceuticals are produced in suspension cell cultures (STR) using batch or perfusion mode. The cell culture densities in stirred tank bioreactors are generally limited by oxygenation, because the agitation and gas sparging has to be kept low. In these cultivations, the viable cell count is usually quite low, only 10^6 cells/ml and Mab volumetric productivity is in the range of 20 – 70 mg/l/day. With better feeding strategies and/or perfusion with cell retention the cell density of 10^7/ml can be achieved and therefore also significant increase in Mab productivity (up to 150 mg/l/day) (Yang *et al.*, 2004).

2.3.3 Hollow fiber bioreactor

Hollow fiber bioreactors are perfusion bioreactors that can support extremely high cell densities (>10^9 cells/ml). The hollow fiber system gives a very large surface area for attaching cells and allows continuous removal of waste products and supply of nutrients (Griffiths, 2003). The hollow fiber bioreactor (Figure 10) consists of a series of capillary tubes, and the cells grow in the extracapillary space (ECS) and medium is circulated through the tubes i.e. in the intracapillary space (ICS). Ultrafiltration membrane is used frequently as a fiber material (Griffiths, 2003; Guardia and Hu, 2003). The product can be harvested from the extracapillary space through a sample port (Cartwright, 1994).

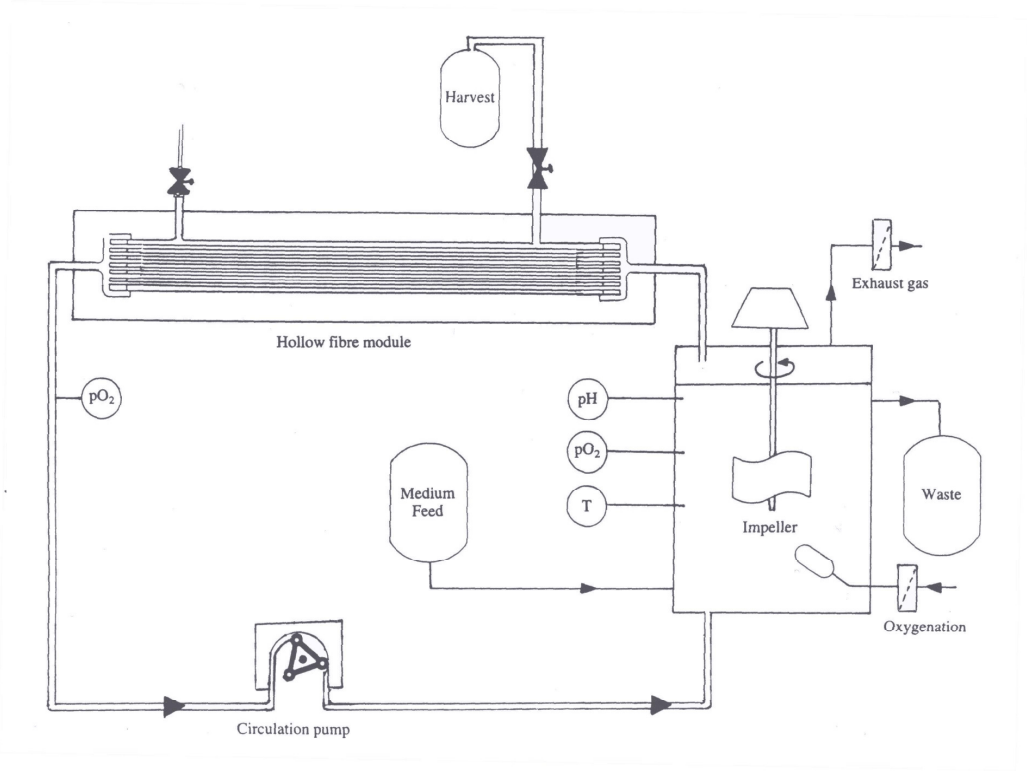

Figure 10. General arrangement of a hollow-fiber bioreactor, modified from Cartwright (1994).

Hollow fiber bioreactors have been studied extensively e.g. by Gramer and Britton (2002) and Valdés *et al.* (2001) and the productivity can easily be more than 20-fold that of a suspension culture (Yang *et al*., 2004). Big advantage of the immobilized hollow fiber reactor is that the product fluid is cell free and thus easily purified. When using protein-free media, the Mab levels can be more than 40 % of the total protein in the product flow (Castillo, 2003). According to Davis (2007), in comparison to STR the HFB equipment is smaller (fits to normal ceiling height room) and utilities required include only CO_2 and electricity whereas for the STR vast utility systems for O_2, N_2, compressed air, purified water, steam, drainage, CIP (clean in place) and SIP (sterilize in place) are required. As a result, for producing the same amount of product, the investment cost of the HFB is lower than that of the STR.

Most hollow fiber bioreactors are in use in small scale. The scale-up has proved to be difficult: oxygen concentration in the medium exit end of the system becomes the

limiting factor at larger scale. In order to ensure the adequate oxygen supply, the medium to the system is typically directed through an oxygenator. Another method for improving aeration is to apply silicone tubing inside the system. This method is, however, rarely used, because it makes the bioreactor more difficult to manufacture. Also the flow direction in the system can be changed periodically, and thus improve the cells oxygen supply in the exit end of the system (Guardia and Hu, 2003). The use of HFB is thus limited to small-to-medium scale. They also have relatively short operation life, because of the accumulation of dead cells and fouling of the membrane (Yang *et al.*, 2004).

However, when producing antibody products for research (laboratory scale, 10–100 mg) or for diagnostic purposes (100 mg to several grams of antibody product), the HFB is a viable and economical alternative (Griffiths, 2003; Jain and Kumar, 2008; Valdes *et al.*, 2001; Yazaki *et al.*, 2001). They are especially good in hybridoma cultures that are generally difficult to grow in bioreactor suspension culture, as these cells are sensitive to shear and bubble damage (Yang *et al.*, 2004).

2.3.4 Disposable bioreactors

“Disposables” mean single-used sterile bags, filters, of membranes, that are used in production or in product hold. Disposable bioreactors are sterile plastic bags that are inoculated, fed and aerated through plastic vents and that are generally mixed by keeping them on a swing.

Disposable bioreactors are already use in medium scale production, Wave bioreactor (Wave Biotech, USA) can be used in over 500 l productions (Weber *et al.*, 2002). Disposable stirred tank bioreactors (Hyclone and Baxter, USA) are for use for 250 l cultivations, maybe even 1000 l (Aldridge, 2005). XDR (Xcellerex, Marlborough, USA) single-use bioreactors are today available at 200 – 2000 l scale (Anon., 2008e). A figure of disposable Wave bioreactor is presented in Figure 11 (Singh, 1999).

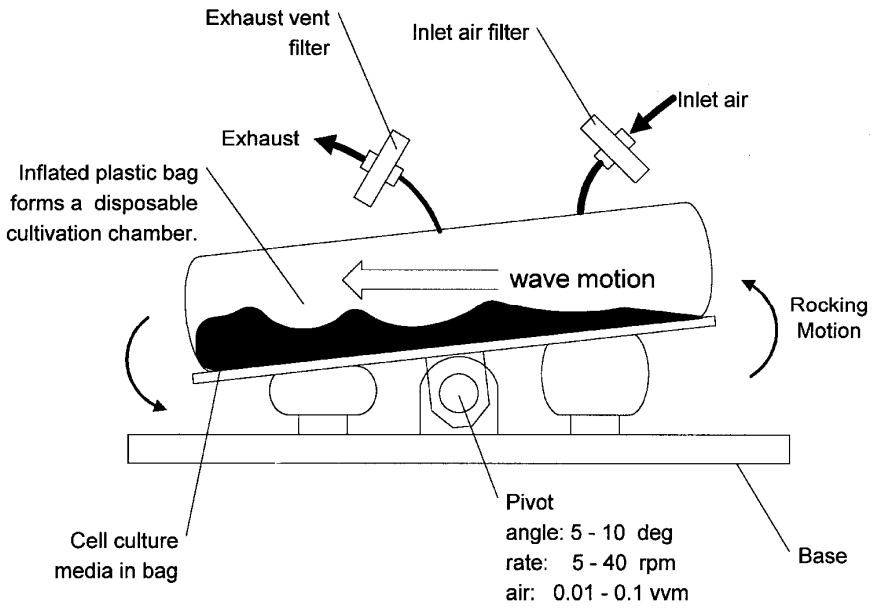

Figure 11. Disposable wave bioreactor (Singh, 1999).

Disposables are a good alternative in production of small quantities of diagnostic products or therapeutic products for clinical studies. Many studies (e.g. Fox (2005) and Jain and Kumar (2008)) have evaluated the arguments for and against of the use of disposable bag bioreactors. They are an attractive choice for a bioreactor, as the investment costs are lower than in traditional plants and the costs are spread over a plant's life (Farid *et al.*, 2005b).

The main advantage of the disposable bioreactors is that the cleaning and sterilizing issues are removed and that the investment costs are minimized (Farid *et al.*, 2005b). Other advantages are found in the reduced construction times and flexibility to modify the process configurations (Farid *et al.*, 2005a). The downtime and turnaround times are shortened because no cleaning is required and there is a lower risk for cross-contamination as new bags are used for each run. Most of the biopharmaceutical facilities are multipurpose-plants, where the potential cross contaminations are a great concern, and the lower contamination risk is really an advantage (Carson, 2005).

On the other hand, the main disadvantages are that the large-scale bags are unavailable and the operating costs can be increased significantly because of constant buying of new bags (Farid *et al.*, 2005b). The other disadvantages include

the scale-up complications, reliance on suppliers and the increased expenses as the operating costs and waste costs are increased as also more solid waste is created in the process. Also more warehouse storage space is needed (Carson, 2005; Farid *et al.*, 2005a; Fox, 2005).

Oxygen requirement is generally the limiting factor in larger scale, as the disposable bag reactors are usually operated through surface aeration. Singh (1999) studied, that the k_La values for disposable bag bioreactors (20 l and 200 l) were about 4 1/h, whereas they for similar size stirred tank bioreactors are usually around 50 1/h. The k_La values characterize the oxygen mass-transfer capability of a fermenter and with small k_La the oxygen delivery to the cells is limited. The oxygen mass-transfer can be improved for example using pure oxygen instead of air or increasing the air flow or impeller speed (Soderberg, 2002).

As the scale is limited, also the use of disposable bioreactors is limited. But if the titers grow as expected (even to 10 – 15 g/l, e.g. by Birch and Racher (2006), Farid, (2007) and Werner (2005)), the 2 000 l scale now available could be enough for commercial production.

2.4 Downstream processes

The purification of a biopharmaceutical can be divided into three steps: capture or separation, purification and final polishing. The separation means mechanical separation, such as cell removal by microfiltration or centrifugation.

In most cases chromatography is used in purification. The advantages of chromatography are the high selectivity and the gentle process. The most commonly used chromatographic techniques are affinity chromatography, ion exchange and hydrophobic interaction (HIC). Affinity chromatography is the most selective method, but unfortunately suitable affinity ligands are difficult to develop. Exceptions are the tagged proteins (i.e. GST or His(6) –tags) and monoclonal antibodies, that are commonly purified with glutathione sepharose, metal ion or

Protein A ligands, respectively (e.g. Low *et al.* (2007)). Another disadvantage is the high cost of the chromatographic resins.

Regardless of the disadvantages, the Mabs and Fabs are almost always purified with affinity chromatography. Two different kinds of ligands are mainly used, either antigens that use the antigen binding abilities or less specific ligands that binds to the constant domain (Fc fragment). The most used are Fc binding natural immunoglobulin-binding ligands Protein G and Protein A. The disadvantages with these proteins are the high cost and the limited binding capabilities to Fabs. Other Fc region binding ligands are for example the Protein A mimics (synthetic affinity ligands) and peptide ligands (Huse *et al.*, 2002; Low *et al.*, 2007; Roque *et al.*, 2004). The specific antigen-ligands offer more selective means for purification, but the antigens may not be easily achieved. So called cross-reactivity can sometimes allow the use of non-specific antigen for purification (Huse *et al.*, 2002).

Even though chromatography as a method is easily up-scaled, the capacity of the downstream processing may become the bottleneck process when the production rates or titers increase. The limiting factors of the chromatography are the minimum residence time of the desired component and the maximum fluid velocity of the liquid phase (Kelley, 2007; Sommerfeld and Strube, 2005).

The product must be free of viruses by FDA's demand and therefore the viral contaminations must be removed (Sommerfeld and Strube, 2005).

Sommerfeld and Strube (2005) have studied the downstream processes of several commercial monoclonal antibodies and have suggested a generic purification procedure (presented in Figure 12). After cell removal, the protein A chromatography is widely used as a capture step. Ion exchange chromatography is often used as a second chromatographic step as it removes possible DNA fragments and overflowed protein A. Additional chromatographic steps, such as HIC, are used if needed. With chromatographic methods, different membrane filtration techniques (micro, ultra and diafiltration) are used for cell/cell debris removal, concentration or buffer exchange. As a polishing step, diafiltration and sterile filtration is typically used

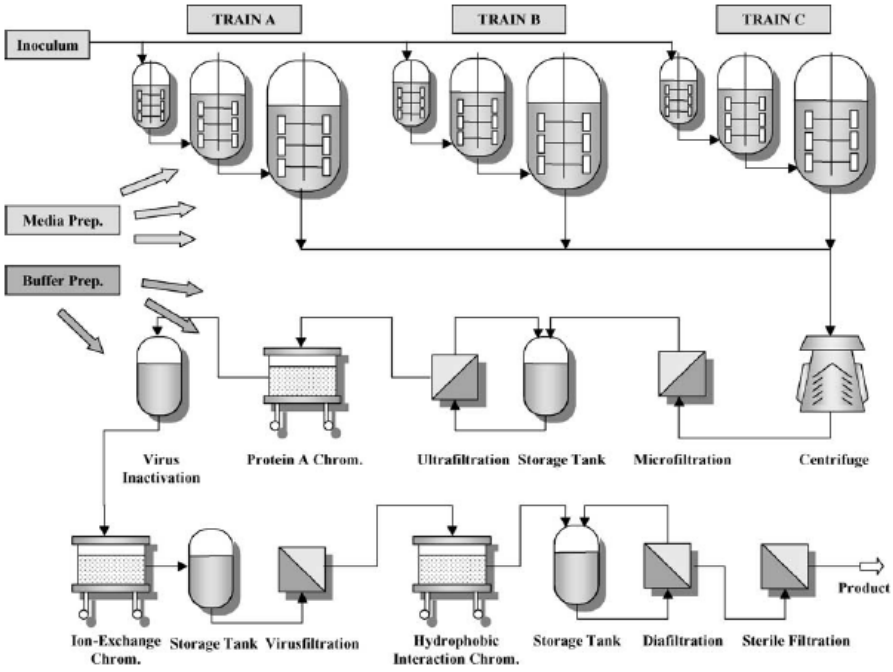

Figure 12. A generic purification process of a monoclonal antibody purification from Sommerfeld and Strube (2005).

Farid *et al.* (2007) states that the typical yield of antibody purification process is 60 – 80 %, depending on the number of steps.

2.5 Crystallization of proteins

The crystallization of proteins is used in three different applications: in protein crystallography, in protein purification and in protein formulation for drug delivery. The most common application is the small scale crystallization of a protein for structural analysis. As a purification method, the low-cost crystallization could for example replace the expensive chromatographic step, but crystallization is almost never used in biopharmaceutical purification (Kelley, 2007; Low *et al.*, 2007; Schmidt *et al.*, 2005).

The reason for the rare use of crystallization is the complexity of the crystallization of biological molecules. There is no universal method that would work to all proteins

or even groups of proteins. The method must always be searched through elaborate experimental work. Especially the monoclonal antibody crystallization is difficult because the molecules are large, flexible and highly glycosylated (Low *et al.*, 2007). But the advantages of the crystallization would be low cost, high selectivity, high purity and unlimited scale-up potentials.

In biopharmaceutical protein formulation, crystallization is used only for one product, insulin that is the only crystalline biopharmaceutical on the market so far. USA Food and Drug Administration (FDA) approved the first inhaled insulin product (Exubera, Pfizer, USA) in January 2006 (Anon., 2008c). Other crystalline products (e.g. α-interferon) are currently in clinical trials. The challenge here beside in developing a crystallization method is to get a constant particle size in the crystalline product.

3 Costs of the biopharmaceutical facility

The competition on biopharmaceutical product market is increasing constantly, especially on high-market-value therapeutic drug section. On this competition, the time-to-market period is outstandingly significant. Shorter time-to market periods can be achieved by using either disposable based technology instead of conventional stainless steel stirred tank bioreactors or buying the manufacturing from a contract manufacturer. Time-to-market period is also affected by the expression host selection.

But beside to shorter market times, the companies want also to reduce the manufacturing costs. Manufacturing costs now represent 20 – 25 % of annual sales of commercial monoclonal antibody products (Farid, 2007) but there is nevertheless pressure to reduce the manufacturing costs form thousands of dollars to hundreds of dollars (Chadd and Chamow, 2001; Farid *et al.*, 2005b). The prices (and resultant sales) will be reduced as the scales are increased and also the authorities may set maximum prices to the products (Sommerfeld and Strube, 2005).

Factors that are most critical on biopharmaceutical (antibodies and recombinant proteins) product manufacturing costs are the fermentation titer and the overall yield. The fermentation titer depends mainly on the host cell expression system, the genetic stability of the host cell or cell line and the cell density. The overall yield on the other hand is a result of the downstream process steps – of the number of the steps and the step yields. Total productivity is affected also by the scalability of the process, process robustness and success rates of the large scale fermentations (Farid, 2007; Werner, 2004).

Purification costs are significant in biopharmaceutical production. Purification costs are the highest in cases of inclusion body formation or in monoclonal antibody production. In therapeutic monoclonal antibody production (or antibody derivates), the downstream processing costs are 50 – 80 % of the total costs. Therefore, if the overall price of Mab and Fab products is wanted to be decreased, the efficient recovery of products must be the goal of the studies (Roque *et al.*, 2004).

Usually in bulk chemical production or even in bulk biotechnology product (industrial enzymes) the process development continues until the plant construction and even during the production stage. But in biopharmaceutical product manufacturing the process is inevitably defined in early stages of the project. This is because the process itself defines the product quality as no reliable chemical analyses are available for these macromolecules (Sommerfeld and Strube, 2005). Thus, after the pilot phase and product manufacturing to the first clinical trials, no modifications to the process (to equipment, unit operations, number of steps and process parameters) can be done. Therefore, the different options for the process and their performance and economy must be regarded well in before hand.

In this section, the studies regarding cost analysis are reviewed. All numbers are presented in Euros (€). The numbers that were presented in US dollars ($) in references are converted into Euros (€) with exchange rate 1€ = $1.5.

3.1 Investment costs

Investment costs (fixed capital investment, FCI) generally include the costs of purchased equipment, piping, instrumentation and utilities as well as the construction expenses for the buildings. In chemical engineering textbooks the fixed capital investment (FCI) is usually divided into direct and indirect costs as presented in Table 4 (Peters *et al.*, 2003). The typical FCI distribution used in Finland is given in Table 5 (Hurme, 2008). The distributions used in the U.S and in Finland have some differences. In the Finnish distribution there is no division into direct and indirect costs. The indirect costs are not presented in such a detailed way. Also the content of purchased equipment installation is not equivalent to the FCI distribution used in the U.S. Start-up costs are included in the Finnish distribution

Table 4. The distribution of the fixed capital investment cost (Peters *et al.*, 2003).

FCI = Direct costs + Indirect costs	
Direct costs	
1.	Purchased equipment
	• All equipment listed on a complete flowsheet • Freight charges
2.	Purchased equipment installation
	• Installation of all equipment, structural supports and equipment insulation and painting
3.	Instrumentation and control
	• Purchase, installation and calibration of instrumentation
4.	Piping
	• Pipes, hangers, valves and pipe insulation, installation
5.	Electrical systems
	• All electrical equipment (switches, motors, wires etc.) installed • Electrical materials and labor
6.	Buildings
	• Process and auxiliary (offices, warehouses, laboratories) buildings • Building services (plumbing and HVAC etc. systems)
7.	Yard improvements
	• Site development (site clearing, grading, roads, fences etc.)
8.	Service facilities
	• Utilities (steam, water, power, compressed air, fuel, waster disposal) • Facilities (water treatment, cooling towers, electric substation, fire protection) • Non-process equipment (office furniture and equipment, safety and medical equipment etc.) • Distribution and packaging (raw material and product storage and handling, product packaging equipment, loading stations)
9.	Land
	• Surveys and fees, property cost
Indirect costs	
1.	Engineering and supervision
	• All engineering tasks (e.g., process, design, general, cost eng.) and engineering supervision and inspection
2.	Legal expenses
	• Preparation and submission of forms required • Contract negotiations
3.	Construction expenses
	• Construction tools and equipment • Construction supervision, accounting, purchasing etc • Taxes, insurances, interest
4.	Contractor's fee
5.	Contingency

Table 5. The distribution of the fixed capital investment cost, Finnish system (Hurme, 2008). All categories include installation.

FCI	
1.	Purchased equipment (incl. freight charges and installation)
2.	Instrumentation and control
3.	Piping
4.	Electrical systems
5.	Steel structures
6.	Buildings (only process area) and yard improvements
7.	Plumbing and HVAC
8.	Insulation
9.	Painting
10.	Land
11.	Building site arrangements
12.	Engineering
13.	Start-up costs
14.	Contingency

Quite often in prefeasibility study phase the total FCI is calculated from the purchased equipment expenses using the Lang method (Lang, 1948), see *section 3.1.1*.

In case of conventional biopharmaceutical product facilities, largest purchased equipment expenses originate from the bioreactors. The choice of the bioreactor type (stirred-tank reactor, air-lift bioreactor, hollow fiber bioreactor or disposable bioreactors) is affected by many factors that are related to the characteristics of the host microbe or cell line (most important is the anchorage-dependency of the cell line) and to the scale of the production (e.g., Chu and Robinson, 2001; Wang *et al.*, 2005). The production scale is further affected by the demand of the product and by the titer. The different bioreactors and the factors influencing the bioreactor selection are discussed in more details in *section 2.3.*

3.1.1 Lang method

In the *Lang method* (Lang, 1948) the FCI of the manufacturing facility can be estimated with *Lang's factor* from the cost of the purchased equipment of the plant. The accuracy of this method is approximately 40 – 50 % and the method is best suited for prefeasibility study phase. In this method (Equation 1), the total cost of delivered equipment (*CE*) is multiplied by Lang's factor (*L*).

$$FCI = L \cdot CE \qquad (1.)$$

For greater accuracy, the Lang's factor *(L)* can be calculated from separate factors (f_i) for installation, piping, etc. as shown in Equation 2.

$$L = \sum_{i=1}^{j} f_i \qquad (2.)$$

For chemical engineering facilities, the Lang's factor generally is in the range of 3 – 5 depending on the process (Peters *et al.*, 2003; Sinnott, 1999). For biopharmaceutical facilities values in the range of 3.3 – 8.1 for Lang's factor have been suggested (Farid *et al.*, 2005b; Farid, 2007; Novais *et al.*, 2001). In their case study of Fab production in *E. coli* Novais *et al.* (2001) derived a Lang's factor of 8.1 for conventional STR based plant. They also presented correction factors for disposable bioreactors based plants (modified Lang method, see *section 3.1.2*). Datar *et al.* (1993) and Rouf *et al.* (2000) used a Lang's factor of 4.6 for a mammalian and bacterial cultivation facility.

One reason for higher Lang's factors in GMP facilities compared to bulk chemical manufacturing facilities is that in GMP facilities the HPAC/HVAC (heating, plumbing/ventilation and air-conditioning) costs are higher because of the higher containment level.

3.1.2 Modified Lang method

In the modified Lang method presented by Novais *et al.* (2001), the FCI of the disposable reactor based plant is calculated from the equipment cost of the conventional plant *(CE_{conv})* using a modified Lang's factor *(L_{disp})* as presented in Equation 3.

$$FCI_{disp} = L_{disp} \cdot CE_{conv} \qquad (3.)$$

The Lang's factor for the conventional reactor based system was calculated (Equation 4) from capital investment factors (f_i, where $i = 1…10$) representing various investment items (Table 6) and multiplied by contingency factor *(c)*.

$$L_{conv} = c \sum_{i=1}^{10} f_i \qquad (4.)$$

For modified Lang's factor *(L_{disp})* calculation the factors (f_i) were corrected by translation coefficients (f_i^*) (Equation 5).

$$L_{disp} = c \sum_{i=1}^{10} f_i f_i^* \qquad (5.)$$

For the capital investment factors and translation coefficients see Table 6. The case presented by Novais *et al.* (2001) was a small scale (300 l) *E. coli* Fab-fragment facility, and in disposables based facility also the bioreactors were disposable.

Also Sinclair and Monge (2005b) have presented similar comparison of Lang's factors for conventional and disposable based 1000 l mammalian cell processes (Table 6), but in their case study, the inoculum, seed cell and production cultures are grown in conventional bioreactors in both processes. The disposables were used mainly in solution preparation and handling, in downstream processing and in product hold.

However, although the translation coefficients and also the Lang's factors are different, in both cases the Lang's factor for disposable based process is approximately 40 % lower than the Lang's factor for conventional process.

Table 6. The Lang method capital investment factors and translation coefficients presented by Novais *et al.* (2001) and Sinclair and Monge (2005b).

	Convent. (f_i)	Disposable (f_i^*)	Convent. (f_i)	Disposable (f_i^*)
Reference	Novais *et al.* (2001)*		Sinclair and Monge (2005b)**	
Equipment (including utilities)	1.00	0.20	1.00	0.55
Pipe work and installation [a]	0.90	0.33	0.51	0.55
Process control	0.37	1.00	0.51	0.55
Instrumentation	0.60	0.66		
Electrical power	0.24	1.00	0.20	0.27
Building works	1.66	0.80	0.99	0.78
Detail engineering [b]	0.77	0.50	1.39	0.61
Construction and site management	0.40	0.75	--	--
Commissioning	0.07	1.00	--	--
Validation	1.06	0.50	0.65	0.46
Contingency factor	1.15	1.15	--	--
Lang's factor [c]	**8.13**	**4.73**	**5.25**	**3.08**

* Scale 300 l, *E. coli* cultivation, disposables used in every step

** Scale 1000 l, mammalian cell cultivation, disposables used only in solution preparations, downstream processing and product hold.

[a] In Sinclair and Monge (2005b) model only pipe work

[b] In Novais *et al.* (2001) model: detail engineering

[c] In Sinclair and Monge (2005b) model the Lang's factor is without contingency

3.1.3 Investment cost estimates for biopharmaceutical product facilities

Investment cost estimates for biopharmaceutical product facilities vary significantly depending on the production scale and concept type (conventional or disposables). The literature derived estimates are presented below and in Table 8.

The investment cost estimates for a GMP multiproduct monoclonal antibody facility (up to 46 000 m^2) are in the range of 4 700 € – 11 300 €/m^2 (referred by Farid (2007)). When these numbers are related to the facility bioreactor capacity, the costs are suggested to be in the range of 1 200 €/l – 2 800 €/l for bioreactor capacities in the range of 20 m^3 – 200 m^3 so that the average investment cost for a bioreactor capacity of 20 m^3 is 40 M€ and for a bioreactor capacity of 200 m^3 400

M€. However Farid (2007) emphasizes that these cost estimates must be read with caution, as it is not always explained what the presented FCI's above include (e.g. warehouses and utility systems). The data presented by Farid (2007) is shown in Table 7.

Table 7. Capital investment costs for antibody facilities using mammalian cell culture (Farid, 2007).

	Facility	Date facility completed	Capital investment M€	Production bioreactor capacity		
				number	Size m^3	total m^3
1	Genentech—Vacaville, CA, USA	2000	167	8	12	96
2	Imclone—Branchburg BB36, NJ, USA	2001	35	3	10	30
3	Biogen—LSM, RTP, NC, USA	2001	117	6	15	90
4	Boehringer ingelheim expansion—Biberach, Germany	2003	210	6	15	90
5	Lonza biologics expansion —Portsmouth, NH, USA	2004	138	3	20	60
6	Amgen—BioNext, West Greenwich, RI, USA	2005	333	9	20	180
7	Genentech NIMO** —Oceanside, CA, USA	2005	253	6	15	90
8	Imclone—Branchburg BB50, NJ, USA	2005	173	9	11	99
9	Biogen Idec—Hillerød, Denmark	2007*	233	6	15	90
10	Lonza biologics —Tuas, Singapore	2009*	167	4	20	80
11	Genentech expansion —Vacaville, CA, USA	2009*	400	8	25	200

* Expected completion date

** Originally built by Biogen Idec and sold to Genentech in 2005

The dependence of the FCI of the bioreactor capacity is studied by plotting the total production bioreactor capacity (m^3) and the total capital investment cost (M€) presented in Table 7 on a log-log graph (Figure 13). From the plot, it can be seen that the data presented by Farid (2007) is quite linear ($y = 1.9125x+10.304$; $R^2 = 0.85$).

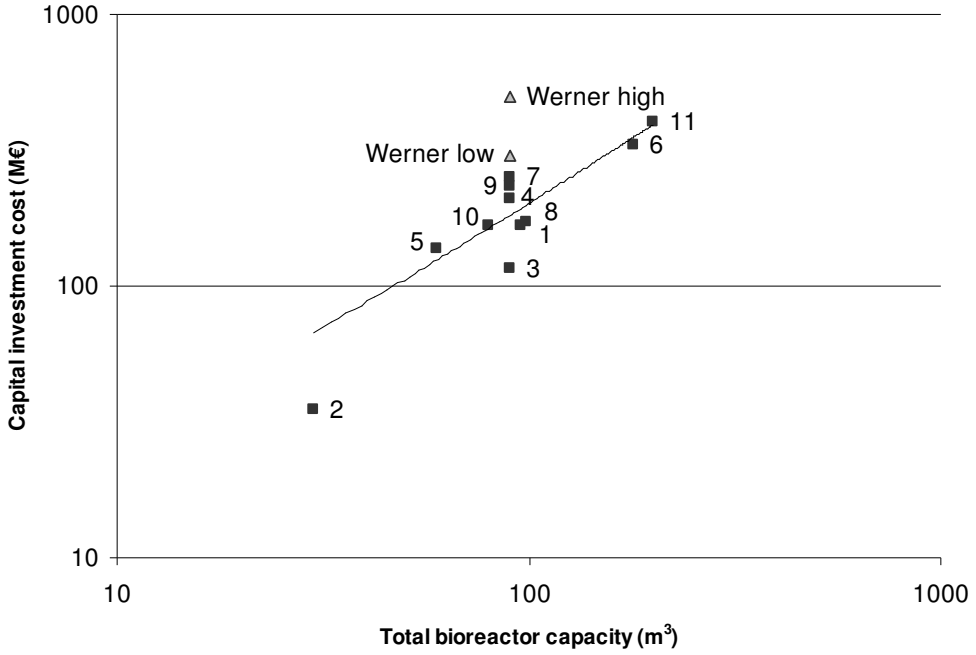

Figure 13. The dependence of the FCI (M€) on total bioreactor capacity (m^3) by Farid (2007) and Werner (2004). The linear line represents the data presented by Farid (2007).

According to Werner (2004) the total investment cost for a 6 x 15 m^3 (total capacity 90 m^3) mammalian cell culture bioreactor facility is in the range of 300 M€ – 500 M€ (3 300 € – 5 600 €/l). Here the cost estimate depends on the type of the investment: greenfield (i.e. a new facility investment where also all utilities must be build) or an extension to an existing plant. Also the data of Werner is plotted on the graph above (Figure 13). As can be seen on Figure 13, the cost estimates presented by Werner (2004) are higher than the costs presented by Farid (2007).

Novais *et al.* (2001) have presented investment costs for a conventional and a disposables based small scale (300 l) *E. coli* Fab facility. For conventional facility the FCI was 12.8 M€. For disposables based (using also disposable bioreactors) facility the FCI was calculated with a modified Lang method and the estimated FCI was 7.2 M€. Sinclair and Monge (2005b) have studied quite similar case in 1 000 l scale. In their study, the conventional process was similar to the one presented by Novais *et al.* (2001) but in their study conventional bioreactors were used also in the disposables based process. An earlier investment cost estimate for a recombinant

protein producing facility has been presented by Datar *et al.* (1993) for *E. coli* and mammalian cell (CHO) cultures.

The FCI estimates presented above are tabulated into Table 8.

Table 8. The literature derived investment cost estimates on different biopharmaceutical product facilities.

System	FCI (M€)	Bioreactor capacity (m^3)	FCI/ bioreactor capacity (M€/m^3)	Reference
Multiproduct mammalian cell Mab facility, traditional	40 – 400	20 – 200*	1.2 – 2.8	(Farid, 2007)
Mammalian cell culture, traditional	300 – 500	90**	3.3 – 5.6	(Werner, 2004)
E. coli, Fab facility, traditional STR	12.8	0.3	43.0	(Novais *et al.*, 2001)
E. coli, Fab facility, disposable-based (also bioreactors)	7.2	0.3	24.0	(Novais *et al.*, 2001)
Mammalian cell Mab multiproduct facility, traditional	38.5	1	38.5	(Sinclair and Monge, 2005b)
Mammalian cell Mab multiproduct facility, uses disposables in buffer and solution handling	22.6	1	22.6	(Sinclair and Monge, 2005b)
CHO, rtPA facility, traditional	40.9	14***	2.9	(Datar *et al.*, 1993)
E. coli, rtPA facility, traditional	259	17.3	15.0	(Datar *et al.*, 1993)

* varied number and sizes

** 6 x 15 m^3 bioreactors

*** In growth fermentation 4 x 7 m^3, in production fermentation 2 x 7 m^3 bioreactors

3.2 Operating and manufacturing costs

The costs of a (bio)chemical facility are divided into investment costs and **operating costs**. Operating costs include all the costs (materials, utilities, salaries, rent, laboratories, administrative etc.) that are originated from the manufacturing process. Direct or variable operating costs are directly related to the manufactured amount

(e.g. raw material expenses). The indirect operating costs or fixed overhead costs are independent of the manufactured amount (e.g. rent). The direct operating costs can be derived from the process flow diagram. The indirect costs are then derived as percentage either from the direct costs or from the FCI.

Manufacturing cost includes the operating costs and the depreciation of the investment.

3.2.1 Operating and manufacturing costs in U.S. literature

The terms operating, manufacturing or production costs are often used as synonyms in the U.S. literature (e.g. Peters *et al*., 2003). In some sources the manufacturing cost is called the cost of goods. Manufacturing cost takes into account the depreciation of the investment and in some cases also the financing costs (interest).

In Peters *et al.* (2003) the manufacturing cost is presented as in Equation 6. The general expenses are presented as in Equation 7 and the total product cost is the sum of manufacturing cost and general expenses (Equation 8).

$$\textit{Manufacturing cost} = \textit{variable production cost} + \textit{fixed charges} + \textit{plant overhead cost} \quad (6.)$$

$$\textit{General expenses} = \textit{administrative expenses} + \textit{distribution and marketing expenses} + R\&D \quad (7.)$$

$$\textit{Total product cost} = \textit{manufacturing cost} + \textit{general expenses} \quad (8.)$$

The operating cost breakdown presented in Table 9 is generally regarded as correct (Humphreys, 1991). Some authors however disagree whether distribution costs are indirect or general expenses.

Table 9. Components of the total product cost (Humphrey, 1991).

TOTAL PRODUCT COST:
1. OPERATING COSTS OR MANUFACTURING COST:
A. Direct production cost
1. Materials (raw materials, processing materials, utilities, etc.)
2. Labor (direct operating labor, operating supervision, etc.)
B. Indirect production costs
1. Plant overhead (administration, laboratory, etc.)
2. Depreciation
C. Contingencies
D. Distribution costs
2. GENERAL EXPENSES
A. Marketing or sales costs
B. Administrative expenses

3.2.2 Operating and manufacturing costs in Finnish practice

In the typical Finnish system the components of operating costs are calculated as presented in Table 10. The total operating cost is the sum of the variable and fixed costs.

Table 10. The operating cost estimation in Finnish system (Hurme, 2008).

OPERATING COST:
1. VARIABLE OPERATING COST
Raw materials
Utilities
Electricity
Fuel
Steam
Water (process and cooling)
Other utilities
Catalysts, solvents and operating supplies
Maintenance and repairs
Wastes
2. FIXED OPERATING COST
Salaries
Insurance
Rent
Administrative expenses
Marketing
Research and Development
Quality control
Safety and protection
Medical expenses

The main differences to the U.S. system is that the Finnish system does not include the depreciation or financing expenses into fixed operating costs, but calculates them separately in the manufacturing cost calculation (Equation 9).

The **manufacturing cost** in Finnish system is calculated from the operating cost (Table 10) and from the depreciation of the FCI as in Equation 9.

$$\textit{Manufacturing cost} = \textit{operating cost} + \textit{FCI depreciation} \qquad (9.)$$

3.2.3 Operating cost models for biopharmaceutical facilities

Several examples for fixed cost, plant overhead and general expense estimations for chemical facilities and traditional fermentations are found from the literature (e.g. Peters *et al.* (2003) and Sinnott (1999)). For GMP facilities fewer examples are found. In GMP biopharmaceutical facility for example the costs of HVAC, quality control and analysis (QCQA) as well as the regulatory costs are higher as compared to bulk chemical manufactory facilities (e.g. Farid *et al.* (2000)).

Farid *et al.* (2007) have presented a model for biopharmaceutical facility simulation and in their model the cost of goods are calculated as presented in Table 11. In the model also the FCI depreciation is included.

Table 11. Cost of good model presented by Farid *et al.* (2007)

Direct operating costs		Indirect costs	
raw materials	from flow sheet	maintenance	10 % of FCI
miscellaneous materials (e.g. safety clothing)	50 % of the raw materials	local taxes	2 % of FCI
utilities	from flow sheet	insurances	1 % of FCI
labor	from personnel	depreciation	FCI / depreciation period
supervisors	20 % of the labor costs	general utilities	300 \$/m^2 * facility size
QCQA	100 % of the labor		
management	100 % of the labor		

Generally, the costs of goods at commercial scale is in order of magnitude of 700 € per gram of concentrated biopharmaceutical therapeutic product (Farid, 2007), but there's increasing pressure to reduce the costs 10-fold or even 100-fold (Chadd and Chamow, 2001).

3.3 Manufacturing cost variables

The investment and operating costs depend on various production parameters. The most important are the host, titer, scale, yield, feeding strategy and bioreactor type. The parameters are correlated, for example the titer depends on the host. The titer has a major influence on bioreactor scale and therefore on investment and operating costs. The production scale and the also the host's characteristics influence the bioreactor type.

The investment and operating costs are quite easily calculated for different titer and yield scenarios for one host, but the comparison of different alternatives is more laborious if different hosts are used in the production as almost all parameters are changed. The bioreactor type may be different for example for microbial and mammalian cell cultures and also the purification procedure is probably different. Operating costs are unequal for different hosts as the host defines the medium complexity and cost and also different purification steps result in different costs.

The main possibility to decrease the costs of the upstream process lies in the increased titer. The titer can be increased first by increasing the specific production rate (g/g/h) by changing the conditions or by genetically engineering the host. Secondly the titer increases with higher cell concentration. Sommerfeld and Strube (2005) give theoretical maximum product concentrations that are 10 g/l for mammalian cell cultivations and 40 g/l for *E. coli*. The downstream costs can be decreased by reducing the number of purification steps and/or increasing the step yields.

3.3.1 The effect of titer, feeding strategy and scale on the costs

The ***titer*** has an enormous effect on investment and operating costs. Therefore increasing the titer has been the topic of many research groups and as a result the titers are now much higher than before (Birch and Racher, 2006; Farid, 2007; Werner, 2005). If simultaneously the yield is increased, the effect is even more positive. Werner (2004) calculated, that on 10 m^3 bioreactor scale and on 250 kg/a production rate with 10-fold increase in titer and simultaneously a 30 % increase in the overall yield, the number of bioreactors could be decreased from 31 to 2 and the resulting investment costs are decreased from 1067 M€ to 67 M€. At the same time the annual operating cost were decreased from 250 M€ to 43 M€.

The titer may be increased for example with optimized feeding strategy. Typically the processes now are fed-batch or perfusion operations. Carson (2005) states, that also the product quality is more constant when moving away from batch processes towards fed-batch and perfusion operations.

The effect of ***feeding strategy*** (fed-batch and perfusion) to the mammalian cell process economics has been evaluated e.g. by Lim *et al.* (2006), Farid (2007) and Werner (2004). In perfusion processes the productivities are higher and the investment costs lower as compared to the same numbers of fed-batch processes. The higher productivities are direct result of higher cell densities, as on perfusion process the cell densities are generally 10 or even 100 times higher than in fed-batch process (Bibila and Robinson, 1995) and therefore the bioreactor sizes are smaller. However, the operating costs were be same for both systems in Lim and co-worker's study (2006), as in perfusion culture the medium consumption was larger (2.6-fold) than in fed-batch operation. They also state, that the operational risks are higher (the probability of contamination of fouling can be even 10 %) on perfusion culture.

Generally, in larger ***scale*** (if larger bioreactors are used), the investment cost per capacity liter is lower. Therefore, the operating and especially the material costs rise to more dominating position. As Rouf *et al.* (2000) demonstrate in Figure 14, the manufacturing cost distribution is different on different scales.

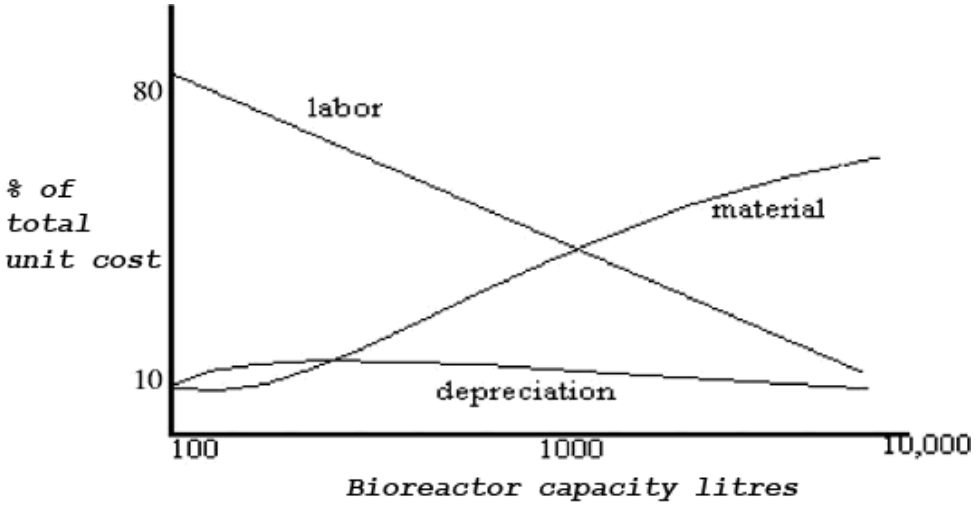

Figure 14. The manufacturing cost distribution depending on scale (Rouf *et al.*, 2000).

From Figure 14 it can be seen, that on smaller scale, the labor costs are in more significant position than in larger scale. The larger the scale the more significant are the material costs. The depreciation of the FCI is around 10 % of the manufacturing cost, independently on the scale.

Generally, the scale-up is done by implementing larger equipment, but it can be achieved also by multiplying the existing process. The scaling of a batch process by increasing the number of reactors instead of growing the size of a reactor has been studied by Rouf *et al.* (2000). They have compared the investment and operating costs of a single 6 m^3 bioreactor (case A) and of six 1 m^3 bioreactors (case B) for production of 11 kg rtPA (Table 12). The bioreactor cultivation and downstream processing times were same for both cases, as in single reactor case, larger downstream processing units were needed. In multireactor case, the modular approach allowed the downstream processing units to be scheduled better. As a result, in both cases, one set of downstream processing units was enough, but in single reactor case the equipment were larger. The total investment costs in the smaller scale were significantly (47 %) lower. The downstream processing costs were about 80 % of the total costs. The operating costs are more dependable of the mass of product and not so much of the bioreactor size. Therefore, although the operating costs of the multireactor case B were 24 % lower, they were not affected

as much by the bioreactor setup. In the operating costs of the multireactor case B, labor costs were twice the expenses of the single reactor case A. However, the increased need of SIP and CIP processes in the multireactor case were not included in the operating costs.

Table 12. The cost analysis of the single 6 m^3 bioreactor case compared to the six 1 m^3 bioreactors case (Rouf *et al.*, 2000).

Cost factor	Case A: 6 m^3 bioreactor	Case B: 6 x 1 m^3 bioreactors
Equipment cost	1.9 M€	1 M€
Bioreactors of PEC*	14 %	37 %
Chromatography columns of PEC*	52 %	33.3 %
FCI (Lang's factor 4.6)	8.7 M€	4.6 M€
Operating cost	13.5 M€	10.3 M€

*PEC = Purchased equipment costs

Rouf *et al.* (2000) stated that besides the economical benefits from size reduction of the downstream processing equipment, the multiple bioreactors have also other advantages: flexibility of operation, minimized risks of contaminations and ease of start up and inoculum preparation.

3.3.2 The effect of the equipment type on the costs

Most of the large scale biopharmaceutical facilities use stainless stirred tank bioreactors. They are straightforwardly scaled and easy to sterilize and handle. The STR can be used, if the production host cells are adapted to grow in suspension. Disposable bioreactors offer an inexpensive alternative for the stainless steel stirred tank bioreactors, but the scale of the disposable bioreactors is limited. According to Farid (2007), for inoculum cultivation or clinical material preparation, the STR bioreactors are more and more been replaced by disposable bioreactors. Now the disposables are limited in 500 l (Wave bioreactor, Wave Biotech, NJ, USA (Anon., 2008d)) or 200 – 2000 l (XDR, Xcellerex, Marlborough, MA, USA (Anon., 2008e)) scale.

Besides replacing the actual production bioreactors, the disposables are being used in traditional facility for buffer preparation and in downstream processing (Farid *et al.*, 2005b). Several comparative hypothetical studies of the costs of the conventional and disposable-based processes have been presented e.g. by Farid *et al.* (2005a), Farid *et al.* (2005b) and Novais *et al.* (2001) and these studies are discussed in the following.

The investment cost and manufacturing cost analysis for conventional and disposables-based ***E. coli* Fab-fragment facility (300 l)** has been compared by Novais *et al.* (2001). In their study, in the disposables based facility all possible unit processes were performed by using disposables. The bioreactors were disposable bioprocessing containers with plunging-jet design for mixing and all membranes were single-used. The investment cost calculations of the conventional process were based on actual purchased equipment data and the investment costs of the disposable-based process were calculated by using the Modified Lang method (*see section 3.1.2*). Resulting investment cost (Table 13) of a disposable-based facility was significantly lower (7.2 M€) than the cost of a conventional STR facility (12.8 M€).

Table 13. Investment costs of conventional and disposable-based small scale *E. coli* Fab facility (Novais *et al.*, 2001).

	Conventional process	**Disposable-based process**
Purchased equipment cost of conventional process (CE_{conv})	1.6 M€	
Langs' factor	8.13	
Modified Lang's factor		4.73
FCI	**12.8 M€**	**7.2 M€**

The manufacturing cost in the disposable-based facility (14.7 M€/year) was significantly higher than in the conventional facility (8.5 M€/year). This is mainly because the material expenses (Table 14) of the disposable-based facility were 16 times higher than of the conventional facility.

Table 14. The material cost estimated for 300 l conventional and disposable-based Fab processes (Novais *et al.*, 2001).

	Conventional process		Disposable-based process	
Material cost unit	**k€/year**	**% of total**	**k€/year**	**% of total**
Raw materials	24	33 %	92	8 %
Membranes	39	53 %	777	64 %
Matrices (IEX)	11	15 %	213	18 %
Other disposable equipment	0	0 %	127	11 %
Total material cost	**73**		**1209**	

In the material cost estimates presented on Table 14 the raw materials for disposable-based process were thought to be bought preprepared and sterile, and for the conventional process they were sterilized in-situ. The membranes and matrices were in conventional process used 20 times and only once in the disposable-based process. As a result, the material costs of the disposable-based process were 16.5 times the material costs of the conventional process.

Other manufacturing cost items are presented in Table 15. The percentages of the conventional process manufacturing cost breakdown are modified from the breakdown presented by Datar *et al.* (1993). The item “other” here includes costs such as patents and royalties, waste and indirect manufacturing expenses. To estimate the cost of disposables-based process based on the conventional process cost data, Novais *et al.* (2001) have generated correction factors (y’) shown in Table 15. The costs of the conventional process were multiplied by the correction factor to get the disposable-based process cost.

Table 15. The manufacturing cost breakdown of conventional and disposable-based small scale (300 l) Fab facility by Novais *et al.* (2001).

	Conventional process		Disposable-based process		
	% of total	**M€/year**	**Correct. fact. *y'***	**M€/year**	**% of total**
Labor costs	14 %	1.2	*1*	1.2	8 %
Materials	6 %	0.5	*16*	8.2	55 %
Utilities	14 %	1.2	*0.5*	0.6	4 %
Depreciation	19 %	1.6	*0.6*	1.0	6 %
Other	47 %	4.0	*1*	4.0	27 %
Total manufacturing cost		**8.5**		**15.0**	

In the study by Novais *et al.* (2001), the investment costs of the disposable-based process are approximately 60 % of the FCI of conventional process. However, the total manufacturing cost of the disposable-based process is almost twice that of the conventional process. This is mainly because of the extremely high material costs that result from single used matrices and membranes. In practice they would probably not be used only once, as the cost effect is so large.

The investment cost and manufacturing cost analysis for conventional and disposable-based **mammalian cell Mab facility (1000 l)** has been compared by Sinclair and Monge (2005b). The perfusion process time was 25.5 days and the product concentration 0.3 g/l and 2000 l of media containing the Mab would be recovered daily. The recovery and purification followed the standard Mab sequence: concentration, protein A chromatography, ultrafiltration, ion-exchange chromatography and viral inactivation (Sinclair and Monge, 2005a). The procedure is similar than the one described by Sommerfeld and Strube (2005).

The production was performed either using conventional methods or the disposables-based process using the disposables in buffer and media preparation as well as in product hold (hold vessels). It must be noted, that the bioreactors in both facilities were conventional. The process equipment costs for conventional facility were 7.3 M€ and for disposables-based facility 4.1 M€ (Table 16). In both facilities, the equipment for previral purification was the most expensive single operation. The most investment cost savings in disposable-based facility equipment costs came from the process utilities when the CIP and SIP requirements were

lessened and from the solution handling as disposables were used. Some equipment, such as autoclaves and washing machines could be totally removed from the concept facility.

The FCI (Table 16) was calculated from the process equipment costs with Lang's factors for installation, building, engineering and validation (*see section 3.1.2*). As the equipment costs were lower in disposable-based facility, also the FCI was significantly lower. Total savings in FCI were 41 %.

Table 16. The FCI of the conventional and disposable-based facility in Sinclair and Monge (2005b).

	Conventional (M€)	**Disposable-based (M€)**
Process equipment, total	7.3	4.1
Previral purification	*1.3*	*1.4*
Process utilities	*1.1*	*0.1*
Solution handling	*1.1*	*0.1*
Installation	8.9	4.5
Building	7.3	5.7
Engineering	10.2	6.2
Validation	4.8	2.2
Total	**38.5**	**22.6**

Sinclair and Monge (2005b) calculated also the manufacturing costs from labor, material, indirect material (from labor expenses), consumables (filter membranes, chromatography media, single-use systems), capital depreciation (15 % capital cost in eight years time) and waste management (waste water and plastics incineration). They estimated that the number of personnel could be reduced from 190 to 153 in the disposable-based facility, most of the reductions being from the quality control personnel. The expenses of the consumables was the only cost factor that was increased in the disposable-based facility compared to the conventional one. The manufacturing cost (€/g) in the disposable-based facility was 17 % lower than in the conventional facility.

Table 17. The manufacturing cost of the conventional and disposable-based facility (Sinclair and Monge, 2005b).

	Conventional		Disposable-based	
	(€/g)	% of total	(€/g)	% of total
Labor	129.1	37 %	99.3	35 %
Direct materials	39.7	12 %	36.8	13 %
Indirect materials	53.0	15 %	47.9	17 %
Consumables	26.3	8 %	49.2	17 %
Depreciation	96.2	28 %	54.0	19 %
Waste management	0.2	0 %	0.1	0 %
Total	**345**		**287**	

In the study by Sinclair and Monge (2005b), the investment costs of the disposable-based process are approximately 60 % of the FCI of conventional process. This is in accordance with Novais *et al.* (2001), although in the process by Novais *et al.* (2001) also the bioreactors were disposables. In the disposable-based process (Sinclair and Monge, 2005b), the direct material expenses are almost equal and the indirect material costs are even lower than in the conventional facility. Most manufacturing cost savings come from labor and depreciation expenses.

The investment cost and manufacturing cost analysis for conventional, hybrid and disposables-based **mammalian cell Mab facility (200 l)** has been compared by Farid *et al.* (2005a; 2005b). In their hypothetical case study they compared three different equipment setups for a mammalian cell culture pilot plant in a start-up biopharmaceutical company. The titers and yields were assumed to be equal in all setups:

A. A conventional pilot plant based on stainless steel equipment
B. A pilot plant utilizing only disposable equipment (disposable air-lift or bubble column)
C. A pilot plant utilizing stainless steel equipment for cell culture and otherwise disposables.

The process Farid *et al*. (2005b) considered contained inoculum, seed and product fermentations, clarification, concentration, and purifications with three-step chromatography and final filtration. The pilot plant was designed assuming a typical product titer of 0.4 g/L at the 200 l scale and assuming a 56 % purification yield

resulting 45 g of purified antibody as would be sufficient for phase I clinical trials. Pilot was thought to be used for the production of several different products. Media and buffers were assumed to arrive pre-made and pre-sterilized. In alternative B, no SIP and CIP procedures were needed. The campaign turnaround time in conventional option (A) was 4 days, otherwise only 1 day. In 48 weeks/year the facility can perform 6 campaigns using the option A, 8 using B and 7 using C.

The fixed capital investment was calculated by multiplying the total equipment cost by Lang's factor. Farid *et al.* (2005b) used Lang's factors of 7 for the conventional stainless steel option (A), 4 for totally disposables based option (B) and 5 for hybrid option (C). In options B and C they used the *Modified Lang method* (*see section 3.1.2*) that was first presented by Novais *et al.* (2001). The manufacturing cost in Farids *et al.* (2005b) calculations included the materials, utilities, labor, fixed overheads and depreciation of the equipment (10 years period).

The manufacturing costs (per gram of product) in the first year of operation were 30 % lower in option B and 19 % lower in a hybrid option C as compared to the conventional stainless steel option A (Figure 15). This is mostly because larger amount of product could be produced annually due to shorter campaign turnaround times and larger number of campaigns. Facility overheads and depreciation charges covered over 50 % of the manufacturing costs in all options. Naturally, due to lower investment costs, the depreciation costs in options B and C were significantly lower than in A. In options using the disposables, the labor and utilities costs were smaller than in conventional option because of lack of cleaning and sterilizing processes. On the other hand, in conventional option, the material costs were lowest, as the chromatography matrices were re-used.

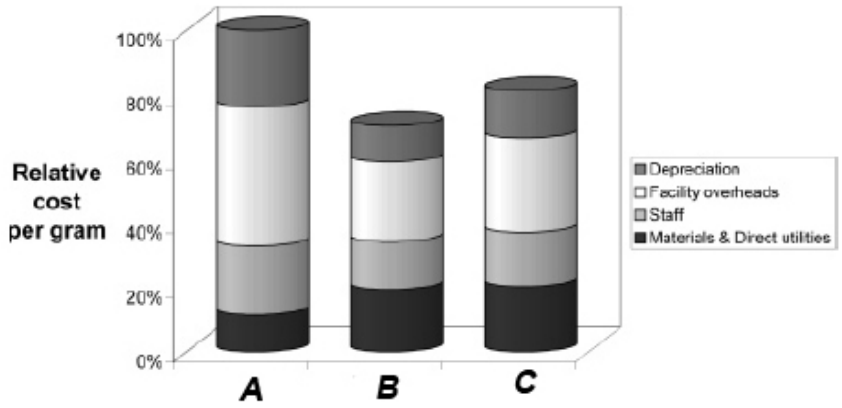

Figure 15. The manufacturing cost (per g of product) breakdown of different process options relative to the conventional option A (Farid *et al.*, 2005b).

The breakdown of the direct operation costs (per gram of product) on basis of different process steps (Figure 16) shows how significant the CIP and SIP expenses are in the conventional option (A). In disposable-using options B and C the need for cleaning and sterilizing operations are eliminated and therefore these expenses are reduced. On the other hand, it can be seen, how significant the chromatography matrix (mostly protein A) expenses are, if they are handled as single-use materials.

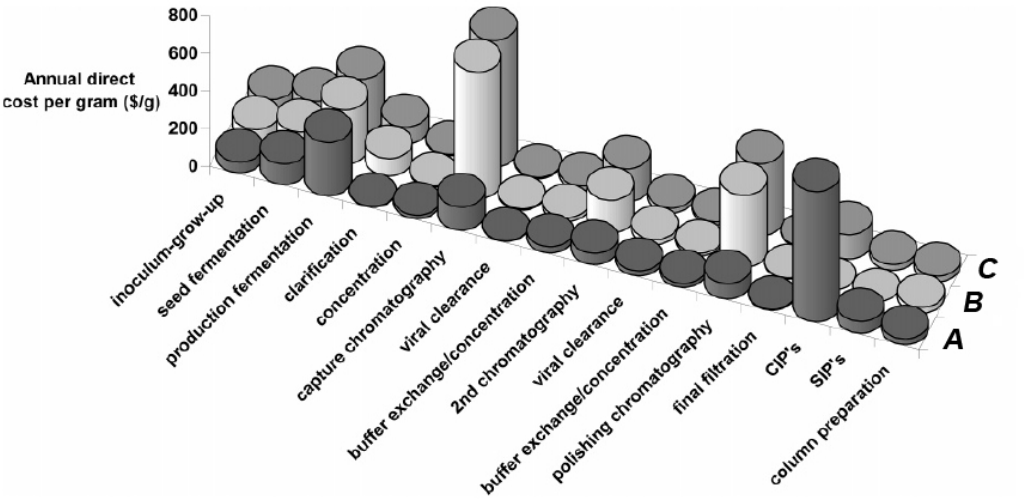

Figure 16. The manufacturing cost breakdown: different process steps (Farid *et al.*, 2005b).

Farid *et al.* (2005b) came to the conclusion, that the fermentation titer had the biggest impact on FCI and manufacturing costs. However, in their study it was assumed, that the titer was equal in all process options, and it is not yet possible to achieve the same titer in disposables as in STR.

Farid *et al.* (2005b) did not present exact FCI numbers, but from the Figure 15 it can be seen that the depreciation expenses are significantly lower in the process options using disposables. Also the total manufacturing cost in disposable-based processes is lower than in the conventional process, although the direct material costs are higher.

As a conclusion it is obvious, that in processes using disposables, the investment costs are significantly lower than in conventional processes. Both Novais *et al.* (2001) and Sinclair and Monge (2005b) estimated 40 % savings in total capital investment costs. Most likely also the total manufacturing costs in disposable-using processes are lower, although in the study presented by Novais *et al.* (2001) the manufacturing cost was almost double because of the extremely high material costs. The high material costs were thought to be because of single use chromatography matrices, but also in the study by Farid *et al.* (2005b) the matrices were used only once in disposable-based processes. But in Farid *et al.* (2005b) study the costs of CIP and SIP processes (utilities and labor) were assumed to be reduced more in the disposable using facilities than in the Novais *et al.* (2001) study. In Novais *et al.* (2001) study, the labor expenses were though to be the same in both facilities, but Farid *et al.* (2005b) and Sinclair and Monge (2005b) agree, that the labor expenses are significantly lower in disposables using facilities.

3.3.3 The effect of the host on the costs

The choosing of the production host was discussed earlier in more detail in *section 2.2 Host systems*. The selected host must fulfill two possibly contrary needs: it must be suited to produce the desired biopharmaceutical and it must be economical with high enough titer.

The host influences both the investment and operating costs dramatically, since almost the whole process is different if different hosts are used. This is the case in the study by Datar *et al*. (1993), where the production of a recombinant-tissue plasminogen activator (rtPA) in *E. coli* and in CHO cells was studied. In their case, the glycosylation was not essential for the biological activity of the product, so it could be produced also in *E. coli*. In both systems, the main purification was based on affinity and gel chromatography. The *E. coli* product however was produced in inclusion bodies and therefore needed to be refolded. Thus *E. coli* system included more unit operations (e.g. sulfonation, refolding tanks and more concentration steps). Consequently in CHO process there were five purification steps, as in *E. coli* system there were approximately sixteen purifications steps. The production parameters and investment costs of the study of Datar *et al*. (1993) are summarized in Table 18.

Table 18. The production parameters and economic indicators of the rtPA production by Datar *et al*. (1993).

	Mammalian (CHO)	**Bacterial (*E. coli*)**
Annual production rate	11 kg	11 kg
Annual number of batches	50	50
Fermentation mode	Batch, 5 – 7 days	Batch, 1 – 2 days
Fermentation titer	33.5 mg/l	460 mg/l
Number of purification steps	5	16
Overall yield	47 %	2.8 %
Batch volume	14 m^3 (2 x 7 m^3 bioreactors)	17.3 m^3 (one bioreactor)
Purchased equipment cost	7.4 M€	47.3 M€
FCI (Lang's factor 5.5)	**40.9 M€**	**259 M€**

The purchased equipment expenses of the mammalian cell process were about 16 % of those of the bacterial process. In mammalian system the bioreactors were the biggest equipment expense (54 % of equipment cost), but in bacterial system the most expensive equipment was the refolding tanks (75 % of equipment cost). The refolding was done in very low concentration (2.5 mg/l tPA) that resulted in ten 180 m^3 refolding tanks and also to 4 500 m^2 ultrafiltration membrane area.

The direct operating costs consisted of used materials, utilities, labor as well as the waste expenses and patent and royalty costs (Table 19). Fermentation and

recovery materials were 44 % of direct operating costs in CHO and 9 % in *E. coli*. On the contrary, the labor expenses were 9 % in CHO and 22 % in *E. coli*. This is in accordance with the general opinion that in mammalian cell processes the material expenses are high. However, generally the downstream processing expenses are higher than in the presented cost breakdown. In bacterial production, the material expenses are the only expenses that are lower than in the mammalian cell process.

Table 19. Direct operating cost of the rtPA production by Datar *et al*. (1993).

	Mammalian (CHO)		**Bacterial (E. coli)**	
Direct operating cost	**M€**	**%**	**M€**	**%**
Fermentation materials	15.6	33 %	0.8	1 %
Recovery materials	5.2	11 %	6.0	8 %
Utilities	8.0	17 %	15.1	20 %
Patents and royalties	8.0	17 %	15.1	20 %
Waste	4.7	10 %	12.0	16 %
Labor	4.3	9 %	16.6	22 %
Other	1.4	3 %	9.8	13 %
Total	**47**		**75**	

Total manufacturing costs included the direct operating costs, indirect operating costs, the depreciation costs and the general expenses. The direct operating costs were 60 % of total in CHO and 47 % in *E. coli*. The general expenses were about 30 % of the total manufacturing costs in both cases. Total manufacturing cost breakdown for both processes is presented in Table 20 (the numbers are not scaled from the 1992 prices).

Table 20. Manufacturing cost breakdown of the rtPA production by Datar *et al*. (1993).

	Mammalian (CHO)		Bacterial (*E. coli*)	
Manufacturing cost unit	**M€**	**%**	**M€**	**%**
Direct operating cost (DOC)	47	60 %	75	47 %
Fermentation materials, % of DOC	*15.6*	*33 %*	*0.8*	*1 %*
Recovery materials, % of DOC	*5.2*	*11 %*	*6.0*	*8 %*
Utilities, % of DOC	*8.0*	*17 %*	*15.1*	*20 %*
Patents and royalties, % of DOC	*8.0*	*17 %*	*15.1*	*20 %*
Waste, % of DOC	*4.7*	*10 %*	*12.0*	*16 %*
Labor, % of DOC	*4.3*	*9 %*	*16.6*	*22 %*
Other, % of DOC	*1.4*	*3 %*	*9.8*	*13 %*
Indirect operating costs	3	4 %	15	9 %
Depreciation	3	4 %	21	13 %
General expenses	25	32 %	50	31 %
Total manufacturing cost	**78**		**162**	

As it can be seen from the results of Datar *et al.* (1993), the host's effect on the process and investment and manufacturing costs is critical. Here the *E. coli* process is economically infeasible because of the formation of inclusion bodies and following very low refolding concentration (2.5 mg/l) and yield (20 %) that results in huge investments and low overall yield (2.8 %). However, Datar *et al.* (1993) states, that if the renaturation yield is increased from 20 % to 90 % (overall yield 15.4 %), the *E. coli* process is more profitable than CHO process.

3.3.4 The effect of the purification procedure to the costs

The purification process has a significant impact on overall manufacturing costs. Roque *et al*. (2004) and Rouf *et al.* (2000) state, that even 50 – 80 % of the manufacturing costs comes from downstream processes in production of Mabs and Mab derivates. As mentioned before, downstream processing costs can be decreased by reducing the number of steps and/or increasing the step yields. The dependence of the overall yield on the number of steps and on the step yield (Figure 17) was originally presented by Fish and Lilly (1984).

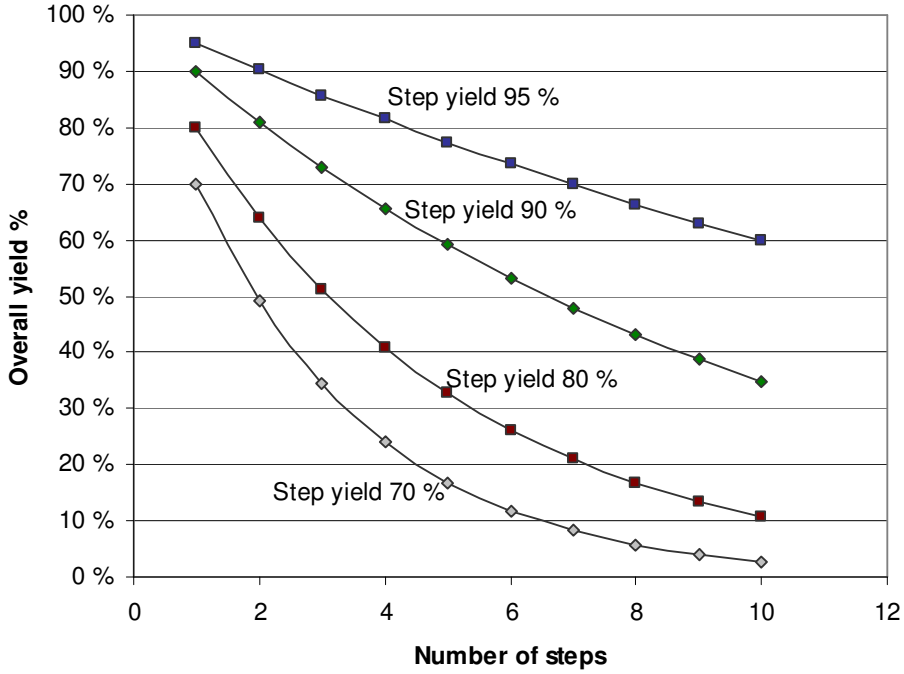

Figure 17. The overall yield dependance on the number of steps and on the step yield (modified from Fish and Lilly (1984)).

Sommerfeld and Strube (2005) calculated that if the annual fermentation titer is 94 kg and the step yield increases from 90 % to 95 % (total yield increases from 50 % to 70 %), the downstream processing manufacturing costs are reduced 22 %. In their calculations the manufacturing cost included the depreciation of equipment (10 years time period), raw materials, consumables such as membranes and resins, labor and waste treatment.

The significance of the downstream process costs on the total operating and manufacturing costs depends not only on number of steps and step yield but also on the scale and titer of the production. Harrison (2003) suggested that with annual capacity of 6.2 kg and titer of 0.1 g/l, the ratio of upstream to downstream costs was 46:54, but with annual capacity of 100 kg and titer of 0.5 g/l the same ratio was 20:80. Same trend is presented also in the calculations of Sommerfeld and Strube (2005): With titer of 0.1 g/l the ratio of upstream to downstream manufacturing costs was 55:45, with titer 0.3 g/l 46:54 and with titer 1 g/l 30:70. This is explained by the fact that with higher titers the upstream processing equipment and volumes are

smaller and therefore also the material costs and the depreciation of the upstream processing investment costs. But the downstream processing, mainly protein A chromatography, volumes are independent on the concentration and are dependent on the mass of the product. Thus, although the same amount of product could be produced in smaller volumes, the size of the downstream processing equipment is not changed as much.

Sommerfeld and Strube (2005) state that the affinity chromatography costs could be decreased either by increasing the resin binding capacity or by increasing the resin lifetime. Especially the resin lifetime has major effect on operating costs. The costs of membrane filtrations that are generally considered as inexpensive rise quite high according to Sommerfeld and Strube (2005). This is mostly because the filtrate flux are quite low resulting to long processing times, and the required membrane areas are high resulting also high amount of buffers used. The cost of filtration is approximately one third of that of chromatography.

3.4 Conclusions of the costs of the biopharmaceutical facility

The investment costs of biopharmaceutical facilities have recently been reviewed e.g. by Farid (2007) and manufacturing costs of recombinant protein production has been presented e.g. by Datar *et al.* (1993) and Rouf *et al.* (2000) and antibody production e.g. by Novais *et al.* (2001), Sinclair and Monge (2005b), Farid *et al.* (2005b) and Sommerfeld and Strube (2005).

The techno-economical analysis of different setups is relatively easy, if only titer and/or yield are changed and the process is otherwise kept the same. But if two different hosts are compared for commercial production, the comparison is more laborious as the processes may be completely different.

As the competition on biopharmaceutical markets tightens up and the time-to-market must be reduced, the disposable products come more and more interesting as the validation times are shortened. Based on the techno-economical comparisons found in literature, the use of disposables is also economically viable

alternative. In disposable-based processes, the investment costs are significantly lower and the material costs are typically higher than in conventional processes. Still, the total manufacturing cost is lower in disposable-based process, as cost of utilities, labor and QCQA are reduced.

The titers have increased significantly over years and now the process development focus is more on the downstream processes. The impact of the downstream processing costs on total manufacturing costs is high and most purification costs emerge from the chromatography steps (usually protein A). Still, no real options for chromatographic purification are available.

RESEARCH PART

4 Manufacturing costs of recombinant protein production in microbial and insect cell host systems

The manufacturing costs of recombinant protein production were analyzed by using HIV-1 Nef (negative factor) as a model protein. Nef protein was produced in three different host systems, *E. coli*, *Drosophila* S2 and *P. pastoris* and the manufacturing costs of strain/cell line development, production and purification were calculated based on the experimental work (Vermasvuori *et al*., 2009).

HIV-1 Nef is an approximately 27 kDa myristoylated protein of the primate immunodeficiency viruses (HIV and SIV). The protein is expressed in large amounts early in the virus replication cycle and it is essential for the progression of AIDS. The high importance of Nef for the viral life cycle and pathogenesis is clear (Arold and Baur, 2001; Deacon *et al*., 1995; Geyer and Peterlin, 2001). Because of its criticality in pathogenesis and development of AIDS, Nef is an attractive target for drug research as the molecules that could block the interaction sites of Nef could for example be used as therapeutic agents (Geyer and Peterlin, 2001). Nef is also used in clinical trials for analysis of the immunogenic response of other HIV vaccine candidates (e.g. DNA-based vaccines). The Nef protein encoding sequence has been included in some DNA-based vaccines (Blazevic *et al*., 2006; Krohn *et al*., 2005).

Techno-economical analysis of the three different host systems was based on the production and purification data presented for *E. coli* and *Drosophila* S2 in Vermasvuori *et al.* (2009) and for *P. pastoris* in Sirén *et al.* (2006). Only few similar cost analyses of the production of recombinant proteins in different host systems has been published previously. Datar *et al.* (1993) had studied the costs of recombinant therapeutic protein (rtPA) in *E. coli* and in mammalian system in large scale (*see section 3.3.3*).

Here the product was supposed to be produced for research or diagnostic use and the production goal was set at 100 mg of purified Nef protein (base case). The protein was supposed to be produced campaign-wise in one batch using a bioreactor of sufficient volume. As the titers of the hosts varied, the production expenses were calculated also for equal titers and for 10 l bioreactor scale (equal titer case).

4.1 Production and purification data of Nef protein production

The Nef protein was produced in three different host systems, *E. coli*, *Drosophila* S2 and *P. pastoris.* In *E. coli* the Nef was produced as GST-tagged protein and the GST-tag was in purification cleaved by enzymatic cleavage. In *P. pastoris* and in *Drosophila* S2 the protein was produced with a hexahistidine-tail (His(6)-Nef), and the tag was not removed from the purified product.

E. coli was cultured in fed-batch mode in 3.5 l stirred-tank bioreactor, and the production time was about one day. Approximately 30 g/l dry cell mass and 1125 mg/l Nef protein was achieved in the *E. coli* cultivation (Vermasvuori *et al*., 2009). *P. pastoris* was also cultured in fed-batch mode in 3.5 l stirred-tank bioreactor and the cultivation time was about 4 days. 140 g/l dry cell mass and 56 mg/l Nef protein was achieved in the *P. pastoris* cultivation (Sirén *et al*., 2006). *Drosophila* S2 insect cell line was cultured in batch mode in 5 l stirred-tank bioreactor and the cultivation time was about 4 days. Nef titer in *Drosophila* S2 culture was 6 mg/l (Vermasvuori *et al*., 2009).

The purification procedures (presented in Figure 18) were different for GST-Nef (intracellular) and His(6)-Nef (extracellular) products.

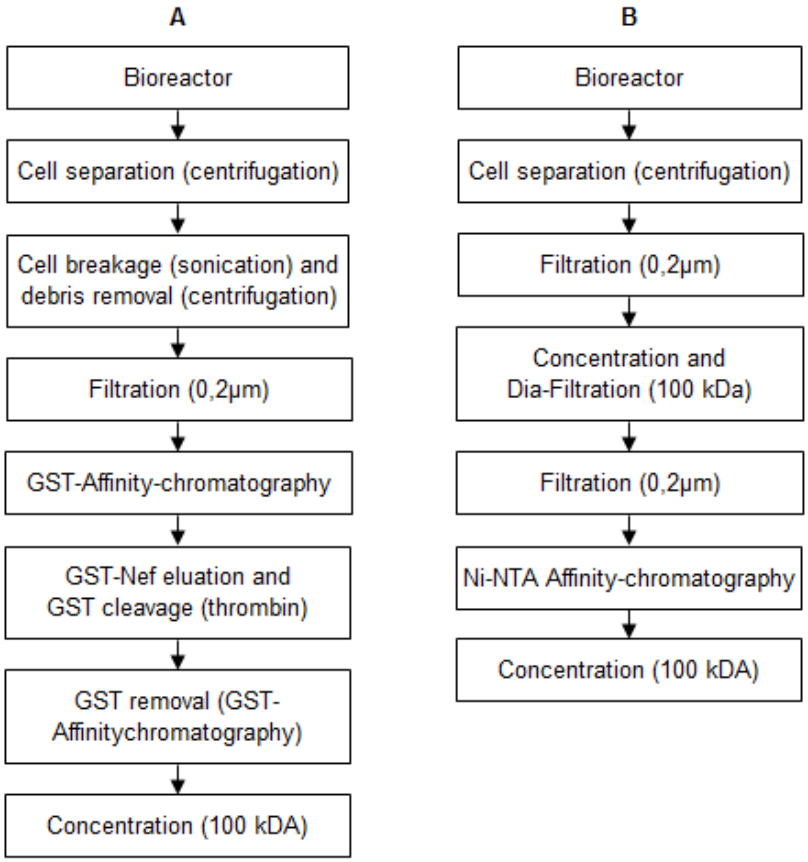

Figure 18. The production and purification procedures of recombinant Nef. Procedure A is for GST-Nef that was produced in *E. coli* and procedure B was used for His(6)Nef, produced in *P. pastoris* and in *Drosophila* S2

The total purification yields varied from 6 to 24 mass-%. The *E. coli* Nef purification yield was burdened by the fact that the Nef protein was not secreted to the growth medium (resulting in the necessity of yield-lowering cell disruption), inclusion body formation (as seen in gels after disruption; data not shown) and the necessity of a two-step chromatographic separation. In case of the extracellularly-producing systems (*P. pastoris* and *Drosophila* S2) additionally concentration by ultrafiltration was applied. Primary purification was performed by affinity chromatography. The *Drosophila* S2 Nef capture and purification was disturbed by other host cell produced histidine-rich proteins, which have affinity towards the Ni-NTA resin used. This can also be observed in a low purity achieved after the chromatographic step. Combining the total purification yields, the bioreactor productivities and the goal of

100 mg of protein in one batch resulted in calculated production volumes of 1.5 l (*E. coli*), 7.4 l (*P. pastoris*), and about 260 l (*Drosophila* S2).

The production and primary purification results are summarized in Table 21 (Vermasvuori *et al.*, 2009).

Table 21. Data of the production and primary purification of Nef protein produced in *E. coli*, *P. pastoris* and *Drosophila* S2 cells (Vermasvuori *et al.*, 2009).

	E. coli	***P. pastoris***	***Drosophila* S2**
Pre-culture time (h)	20	22	72
Mode of production culture	fed-batch	fed-batch	batch
Temperature (°C)	37	30/10	28
pH	7.5	5	6.2 – 6.5
DOT minimum (% of air saturation)	>30	>30	~ 50
Aeration, vvm (l/l/min)	2 – 2.5	1 – 2	N/A
Production culture time (h)	25	93	96
Final cell dry weight (dry g/l) or cell count (million cells/ml)	31	141	19
Final working volume (liter)	2.4	3.4	2.4
Nef concentration at end of culture (mg/l final volume)	1125	56	6
Nef volumetric productivity (mg/l/h)	44.3	0.6	0.1
Purification yield (% of Nef in the production broth)	6.1	24.1	6.5
Purity (% of the total protein mass in the product)	~ 90	~ 80	~ 40
N-terminal sequence	GSMGGKWS	(EA)EFMGGKWS	RSPWMGGKWS
Measured mass by Maldi-TOF MS (Da)	23104	24114	24414
Calculated mass (Da)	22870	23825/24025	24075
Required production volume for 100 mg, if one batch (l)	1.5	7.4	257

4.2 Manufacturing cost in the base case

In the base case, the production goal was set to 100 mg of purified Nef protein. The required batch volume was calculated from the titer and from the purification yield;

for *E. coli* the required batch volume was 1.5 l, for *P. pastoris* 7.4 l and for *Drosophila* S2 257 l (Table 21).

The techno-economical analysis of the manufacturing costs in Nef protein production was structurally divided into three phases: 1) strain/cell line construction, 2) bioreactor production, and 3) recovery and primary purification. The cost categories were materials, labor and rental expenses of the equipment. The direct operating costs (material and labor costs) were estimated based on the actual consumption in the research work (Vermasvuori *et al.*, 2009). The utilities (e.g. steam and electricity), indirect operating costs (i.e. maintenance, insurances, taxes and general utilities) and depreciation of equipment were assumed to be included in the equipment rental expenses.

Material expenses were calculated based on the actual consumption in laboratory research work. Materials consisted of genetic engineering tools, culture media (including gases), additives (e.g. pH control), chromatography resins, buffers and disposable equipment such as sterile filters.

Equipment costs were calculated making the assumption that for the one production batch the equipment would be rented from a contract manufacturing organization and the costs were thus estimated based on the time the equipment was needed. In the needed time, also the sterilization times were included. The rental prices varied from 3.5 €/h to 10 €/h. The rented equipment was assumed to be in larger scale than the equipment used in the described laboratory process (Vermasvuori *et al.*, 2009).

Cost of labor was calculated based on the actual working hours spent. As the working hours were divided into a longer period of time, the actual hours were added together and divided to *man days* so that one man day presents 8 hours work load. Man day (eight man hours) cost 429 €/day was used in calculations.

Fixed cost and cost of quality control were not considered in the calculations as these costs were assumed to be virtually equal for all three systems. Licensing costs and royalties (IPR costs) were not included either.

4.2.1 Strain/Cell line development

The *E. coli* Nef-protein producing strain was received from FIT Biotech Oyj Plc (Finland), but it was estimated that the development of the construct would have taken 80 – 160 man hours during 30 – 40 working days and the material costs of the genetic engineering tools used in the strain/cell line development phase were 400 €.

The development of the *P. pastoris* construct took 120 – 160 man hours in 30 – 40 working days and the material costs were 2 156 € (Sirén *et al*., 2006).

The development of the stable *Drosophila* S2 cell line took 120 – 240 man days in 45 – 60 working days and the material costs of genetic engineering tools used in the development were 1 361 € (Vermasvuori *et al*., 2009).

Table 22. The material and labor expenses of the strain/cell line development of Nef-hosts.

Strain/cell line development	***E. coli*** **(€)**	***P. pastoris*** **(€)**	***Drosophila* S2** **(€)**
Labor costs	3 214	7 499	9 641
Material costs	400	2 156	1 361
TOTAL	**3 614**	**9 655**	**11 002**

4.2.2 Production

The material cost of the production was scaled to production of 100 mg Nef protein from the actual consumption in the laboratory scale research work. The prices of the materials were taken from the Sigma-Aldrich (Sigma-Aldrich Inc., USA).

The equipment costs of the production was calculated based on the specific equipment rental price (€/h) and the time the equipment was assumed to be needed (e.g. the bioreactor that was used for the *E. coli* cultivation was needed for 25 hours cultivation time plus 4 hours for sterilization, loading and emptying).

The labor costs were calculated from the man days (eight man hours, 429 €/day) used in production, e.g. in the *E. coli* system, in production phase two man days were used.

4.2.2.1 Nef production in E. coli system

The materials consisted of inoculum and fermentation media, as well as of fed-batch substrate solution and of additived such as isopropyl-ß-D-thiogalactopyranoside (IPTG) used for induction and ammonium solution used in pH control. Trace element solution was added to media and feeding solution. Detailed production procedure data is presented by Vermasvuori *et al.* (2009). Equipment in production included inoculum equipment (shake flasks) and a bioreactor. For cleaning and sterilization, a rental price was given. In production, two man days were used. The production step manufacturing costs are presented in Table 23.

Table 23. The material, equipment rental and labor expenses of the production of Nef in 1.5 l *E. coli* cultivation.

	Expense €/ unit		Amount for 1.5 l		Total (€)
Materials					
Trace element solution (TES)	3.84	€/l			
Inoculum medium incl. TES	2.73	€/l	0.12	l	0.33
Fermentation medium incl. TES	2.72	€/l	1.08	l	2.94
Fed-batch feeding solution incl. TES	9.25	€/l	0.21	l	1.94
IPTG	40.00	€/g	0.35	g	14.00
25 % ammonium solution	14.50	€/l	0.04	l	0.58
					20
Equipment					
SIP/CIP/Autoclave	3.50	€/h	4	h	14
Inoculum equipment	4.50	€/h	20	h	90
Bioreactor (2l)	7.50	€/h	25	h	188
					292
Labor					
Labor	429	€/day	2	days	858
					858
TOTAL					**1 169**

The scaled material costs of the production in *E. coli* system were 20 €, the equipment rental costs were 292 € and the labor expenses 858 €. Total manufacturing cost of the production was about 1 200 k€. In production step, the materials were only 1.7 % of the manufacturing costs and labor expenses were 73 % of the costs. The most expensive cost item in materials was the IPTG that was used for inducing the protein production.

4.2.2.2 Nef production in P. pastoris system

The materials consisted of inoculum and fermentation media, as well as of substrate solutions for glycerol and methanol fed-batch phases and of ammonium solution used in pH control. Trace element solution was added to media and feeding solutions. Detailed production procedure data is presented in Sirén *et al.* (2006). Equipment in production included inoculum equipment (shake flasks) and a bioreactor. For cleaning and sterilization, a rental price was given. In production, 3.5 man days were used.

The material, equipment rental and labor expenses (i.e. production step manufacturing costs) are presented in Table 24.

Table 24. The material, equipment rental and labor expenses of the production of Nef in 7.4 l *P. pastoris* cultivation.

	Expense	€/ unit	Amount for 7.4 l		Total (€)
Materials					
Trace element solution (TES)	20.45	€/l	0.02		0.35
Inoculum medium	31.97	€/l	0.43	l	13.74
Fermentation medium	6.94	€/l	3.87	l	26.85
Fed-batch glyserol solution incl. TES	53.90	€/l	0.32	l	17.38
Fed-batch methanol solution incl. TES	29.30	€/l	1.50	l	44.08
25 % ammonium solution	14.50	€/l	0.34	l	4.99
					107
Equipment					
SIP/CIP/Autoclave	3.50	€/h	4	h	14
Inoculum equipment	4.50	€/h	22	h	99
Bioreactor (9l)	7.50	€/h	93	h	698
					811
Labor					
Labor	429	€/day	3.5	days	1502
					1 502
TOTAL					**2 419**

The scaled material costs of the production in *P. pastoris* system were 107 €, the equipment rental costs were 811 € and the labor expenses 1 502 €. Total manufacturing cost of the production was about 2 400 €. In production step, the materials were only 4.4 % of the manufacturing costs and the labor expenses covered 62 % of the costs. In the material costs, the methanol feeding solution was the most expensive cost item (the methanol price used in calculations was 29 €/l).

4.2.2.3 Nef production in Drosophila S2 system

The materials consisted of fermentation media and of oxygen that was used to enrich the inlet air. Detailed production procedure data is presented in Vermasvuori *et al.* (2009). Equipment in production included inoculum equipment (roller bottles) and a bioreactor. For cleaning and sterilization, a rental price was given. In production, 4.5 man days were used.

The material, equipment rental and labor expenses (i.e. production manufacturing costs) of production in 257 l *Drosophila* S2 system are presented in Table 25. The fermentation medium price was estimated from list price (40 €/l) assuming a bulk reduction.

Table 25. The material, equipment rental and labor expenses of the production of Nef in 257 l *Drosophila* S2 cultivation.

		Expense €/ unit		Amount for 257 l		Total (€)
Materials						
	Fermentation medium	30.00	€/l	257.00	l	7 710
	Oxygen	1.08	€/m^3	16.70	l/l/h	447
						8 155
Equipment						
	SIP/CIP/Autoclave	3.50	€/h	4	h	14
	Inoculum equipment	4.50	€/h	72	h	324
	Bioreactor (260l)	10.00	€/h	96	h	960
						1 298
Labor						
	Labor	429	€/day	4.5	days	1 931
						1 931
TOTAL						**11 384**

The scaled material costs of the production in *Drosophila* S2 system were 8155 €, the equipment rental costs 1298 € and the labor expenses 1931 €. Total manufacturing cost of the production was about 11 400 €. The materials were 72 % of the manufacturing costs, the labor equipment expenses 11 % and the labor costs covered 17 % of the costs. The scale in *Drosophila* S2 production was much larger than the scale in microbial cultivations, and also the cost breakdown is different. In smaller scales, the labor expenses were in more significant position than in this larger scale. The differences in the costs breakdown are also explained by the differences in the material expenses (13 €/l for *E. coli*, 14 €/l for *P. pastoris* and 32 €/l for *Drosophila* S2).

4.2.3 Purification

The material cost of the purification phase was scaled similarly as in the production phase to represent production of 100 mg Nef protein. The protein amount in beginning of the purification was for *E. coli* system 3 260 mg, for *P. pastoris* 417 mg and for *Drosophila* S2 1 540 mg.

The material prices were taken from Sigma and Amersham Biosciences (GE Healthcare/Amersham Biosciences, USA) catalogs. The consumable (clarification and sterile filters) prices were from Millipore (Millipore, USA) and Pall (Pall, USA). The equipment costs of the purification phase were calculated based on the specific equipment rental price (€/h) and on the time the equipment was assumed to be needed. The labor costs were calculated from the man days (429 €/day) used in purification. Detailed purification procedure data for *E. coli* and *Drosophila* S2 is presented in Vermasvuori *et al.* (2009) and for *P. pastoris* in Sirén *et al.* (2006).

4.2.3.1 Nef purification in E. coli system

The materials consisted of chromatography resins and buffers and of consumables such as sterile filters and concentration units. Equipment in purification included only laboratory scale pump for chromatography (price not given) and filtration and centrifugation devices. In purification, three man days were used. The material, equipment rental and labor expenses are presented in Table 26.

Table 26. The material, equipment rental and labor expenses of the purification phase of Nef-protein in 1.5 l *E. coli* cultivation.

	Expense €/ unit		Amount for 1.5 l		Total (€)
Materials					
Glutathion sepharose resin	200.00	€/10ml	15	ml	300.00
Glutathion elution buffer	8.44	€/g	0.30	g	2.53
Thrombin enzyme	0.17	€/u	150	u	24.75
Benzamide sepharose	8.96	€/ml	2.14	µl	0.02
0.2 µm filter	4.50	€/piece	1	piece	2.25
Amicon ultra-15 concentration unit	10.00	€/piece	4	pieces	40.00
					370
Equipment					
Filtration / Centrifugation device for 1.5 l cultivation	4.5	€/h	3.7	h	17
					17
Labor					
Labor	429	€/day	3	days	1 287
					1 287
TOTAL					**1 673**

The scaled material costs of the purification in *E. coli* system were 370 €, the equipment rental costs were 17 € and the labor expenses 1287 €. Total manufacturing cost of the purification phase was about 1 700 €. In purification step, the equipment expenses were only 1 % of the manufacturing costs and labor expenses covered 77 % of the costs. The most expensive cost item in materials was the glutathion sepharose resin that here was handled as single-use, but that in larger scale would be used several times.

4.2.3.2 Nef purification in P. pastoris system

The materials consisted of chromatography resins and buffers and of consumables such as concentration units. Equipment in purification included only laboratory scale pump for chromatography (price not given) and of filtration and centrifugation devices. In purification, two and half man days were used.

The scaled material costs of the purification in *P. pastoris* system were 196 €, the equipment rental costs were 43 € and the labor expenses 1 073 €. Total

manufacturing cost of the purification phase was about 1 300 €. The material, equipment rental and labor expenses are presented in Table 27.

Table 27. The material, equipment rental and labor expenses of the purification phase of Nef-protein in 7.4 l *P. pastoris* cultivation.

	Expense (€/unit)		Amount for 7.4 l		Total (€)
Materials					
Ni-NTA resin	14.45	€/ml	10.4	ml	150.56
Imidazole for elution buffer	0.29	€/g	17.0	g	4.97
Amicon ultra-15 concentration unit	10.00	€/piece	4	pieces	40.00
					196
Equipment					
Filtration / Centrifugation device for 7.4 l cultivation	4.50	€/h	9.5	h	43
					43
Labor					
Labor	429	€/day	2.5	days	1073
					1 073
TOTAL					**1 311**

In purification phase, the equipment expenses were only 3 % of the manufacturing costs and labor expenses covered 82 % of the costs. The most expensive cost item in materials was again the affinity chromatography resin that here was handled as single-use, but that in larger scale would be used several times.

4.2.3.3 Nef purification in Drosophila S2 system

The materials consisted of chromatography resins and buffers and of consumables such as concentration units. Equipment in purification included filtration and centrifugation devices. In purification, two and half man days were used.

The scaled material costs of the purification in *Drosophila* S2 system were 582 €, the equipment rental costs were 71 € and the labor expenses 1 073 €. Total manufacturing cost of the purification phase was about 1 700 €. The material, equipment rental and labor expenses are presented in Table 28.

Table 28. The material, equipment rental and labor expenses of the purification phase of Nef-protein in 257 l *Drosophila* S2 cultivation.

	Expense €/unit		Amount for 257 l		Total (€)
Materials					
Ni-NTA resin	14.45	€/ml	38.6	ml	557.24
Imidazole for elution buffer	0.29	€/g	17.01	g	4.97
Amicon ultra-15 concentration unit	10	€/piece	2	pieces	20.00
					582
Equipment					
Sterile Filtration / Centrifugation device for 250l	4.50	€/h	3.5	h	16
Concentration (4 m^3)	4.50	€/h	4	h	18
Concentration (0.1 m^3)	5.50	€/h	2	h	11
Diafiltration (0.1 m^3)	6.50	€/h	4	h	26
					71
Labor					
Labor	429	€/day	2.5	days	1 073
					1 073
TOTAL					**1 725**

In purification phase, the equipment expenses were only 4 % of the manufacturing costs and labor expenses covered 62 % of the costs. The most expensive cost item in materials was again the affinity chromatography resin that here was handled as single-use, but that in reality would be used several times. As the scale here is larger than in the microbial productions, the significance of the labor expenses is lower. Still, the labor expense is the most important cost item.

4.2.4 Total manufacturing cost

The costs of producing 100 mg Nef protein using the three expression host systems are summarized in Table 29.

Table 29. Manufacturing costs of producing 100 mg Nef protein using three different host systems

		E. coli (€)	*P. pastoris* (€)	*Drosophila* S2 (€)
Strain/cell line development				
	Labor costs	3 214	7 499	9 641
	Material costs	400	2 156	1 361
Production				
	Material costs	20	107	8 155
	Equipment costs	292	811	1 298
	Labor costs	858	1 502	1 931
Purification				
	Material costs	370	196	582
	Equipment costs	17	43	71
	Labor costs	1 287	1 073	1 073
TOTAL		**6 458**	**13 387**	**24 112**

Quite expectedly, the *E. coli* system was found to be the lowest cost system (manufacturing cost about 6 500 €), whilst the manufacturing costs of the *P. pastoris* and *Drosophila* S2 systems were about two (13 400 €) and almost four (24 100 €) times higher. Generally, the *P. pastoris* and *Drosophila* S2 systems were burdened by clearly longer strain/cell line construction phases compared to *E. coli* system. The *Drosophila* S2 system was also burdened by a very expensive production step, mainly due to a higher cost of raw materials (i.e. growth medium and pure oxygen gas) and a longer culture time (compared to *E. coli*). The cost distribution of the manufacturing cost in respect to the different steps is shown in Figure 19.

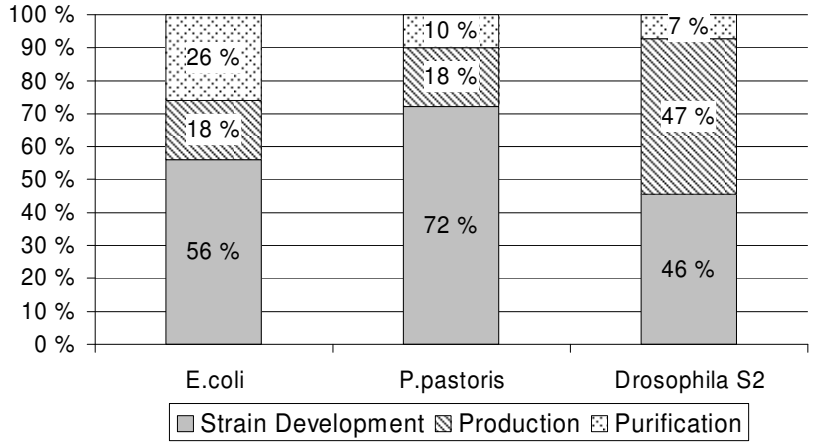

Figure 19. Cost distributions of producing 100 mg Nef protein using *E. coli*, *P. pastoris* and *Drosophila* S2 in respect to the different steps.

Comparing different cost factors (i.e. labor, material and equipment) of the manufacturing costs, labor costs were found to be in range of 80 % of the manufacturing costs for the microbial systems and about 50 % for the *Drosophila* S2 system (see Figure 20). Moreover, when produced commercially, the possible license payments and royalties related to the use of strains/cell lines and/or vectors must be added to the manufacturing costs.

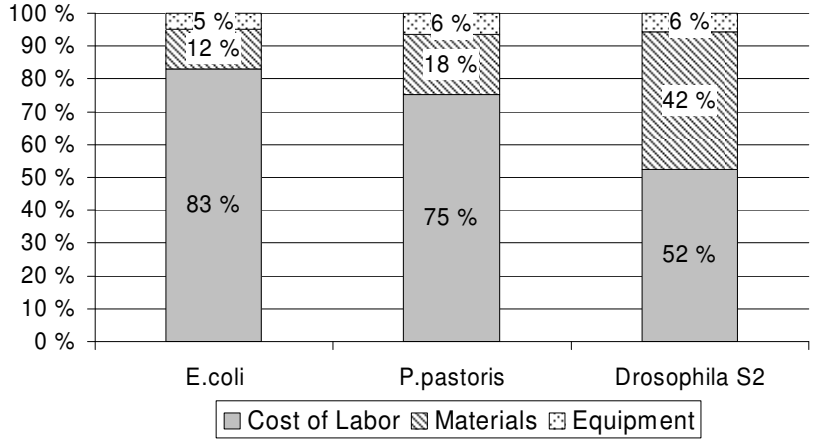

Figure 20. Cost distributions of producing 100 mg Nef protein using *E. coli*, *P. pastoris* and *Drosophila* S2 in respect to the different cost factors.

4.3 Manufacturing cost of the production step - the equal titer case

The manufacturing costs of the 100 mg Nef protein production were strongly dependent on the titers and yields of the practical experiments. If the systems were optimized, titers of the yeast and insect cell systems could surely be increased. Therefore the manufacturing cost of the production step was calculated assuming a constant titer and bioreactor working volume (10 liter). Material and equipment rental prices were re-calculated for this scale, but the labor costs were kept equal on both production volumes for all three production systems.

4.3.1 Nef production in E. coli system

For *E. coli* system, the production volume in original case was 1.5 l and in equal titer case 10 l. Both these scales are small/bench scale, and therefore the equipment and labor expenses were same in both scales. Also the material prices were same, only the amounts the materials were used were changed in this larger scale. The expenses are presented in Table 30.

Table 30. The material, equipment rental and labor expenses of the production of Nef in 10 l *E. coli* cultivation.

	Expense €/ unit		Amount for 10 l		Total (€)
Materials					
Trace element solution (TES)	3.84	€/l			
Inoculum medium incl. TES	2.73	€/l	0.83	l	2.26
Fermentation medium incl. TES	2.72	€/l	7.46	l	20.28
Fed-batch feeding solution incl. TES	9.25	€/l	1.45	l	13.41
IPTG	40.00	€/g	2.38	g	95.32
25 % ammonium solution	14.50	€/l	0.27	l	3.84
					135
Equipment					***292***
Labor					***858***
TOTAL					**1 285**

The total bioreactor step manufacturing costs was about 1 300 €. As the material expenses were the only one increased in the larger scale, also the material cost

share of the total manufacturing costs was increased. Still, the material expenses are only 10.5 % of the total manufacturing cost.

4.3.2 Nef production in P. pastoris system

Also in *P. pastoris* system, the equipment and labor expenses were same in both scales (7.5 and 10 l). Also the material prices were same, only the amounts the materials were used were changed in this slightly larger scale. The expenses are presented in Table 31.

Table 31. The material, equipment rental and labor expenses of the production of Nef in 10 l *P. pastoris* cultivation.

	Expense €/ unit	Amount for 10 l	Total (€)
Materials			
Trace element solution (TES)	20.45 €/l	0,02 l	0,47
Inoculum medium	31.97 €/l	0,58 l	18,57
Fermentation medium	6.94 €/l	5,23 l	36,28
Fed-batch glyserol solution incl. TES	53.90 €/l	0,44 l	23,48
Fed-batch methanol solution incl. TES	29.30 €/l	2,03 l	59,57
25 % ammonium solution	14.50 €/l	0,46 l	6,74
			145
Equipment			***811***
Labor			***1 502***
TOTAL			**2 419**

The total bioreactor step manufacturing costs was about 2 400 €. Similarly to the *E. coli* cultivation, also here the material expenses were the only one increased in the larger scale. Still, the material expenses are only 6 % of the total (compared to 4.4 % in 7.5 liter scale).

4.3.3 Nef production in Drosophila S2 system

The insect cell cultivation was downscaled from 257 l to 10 l for this equal titer case. Therefore the price of the medium was higher, but on the other hand, the rental

price of the bioreactor was lower. The labor expenses were equal in both scales. The expenses are presented in Table 32.

Table 32. The material, equipment rental and labor expenses of the production of Nef in 10 l *Drosophila* S2 cultivation.

		Expense €/ unit		Amount for 10 l		Total (€)
Materials						
	Fermentation medium	40.00	€/l	10.00	l	400
	Oxygen	1.08	€/m^3	16.70	l/l/h	17
						417
Equipment						
	SIP/CIP/Autoclave	3.5	€/h	4	h	14
	Inoculum equipment	4.5	€/h	72	h	324
	Bioreactor (10 l)	7.5	€/h	96	h	720
						1 058
Labor						***1 931***
TOTAL						**3 406**

The total bioreactor step manufacturing costs was about 3 400 €. In this smaller scale, the material expenses were 12 %, the equipment 31 % and the labor expenses 57 %. Therefore, the cost distribution of the production in this smaller scale resembles more the cost breakdown in microbial hosts where the labor expenses dominate the costs.

4.3.4 Production step manufacturing costs

The material costs of the production step were scaled to production volume of 10 liter. The scaled material costs of the *E. coli* system were 135 €, of the *P. pastoris* system 145 € and of the *Drosophila* S2 system 417 €. The equipment costs of the production and purification phases were calculated similarly as on the analysis above. For *E. coli*, the equipment rental time and prices were the same for 1.5 l and 10 l batch (292 €). Same applies to the *P. pastoris* system, were the volumes were 7.5 l and 10 l with equipment costs of 811 €. For *Drosophila* S2 system the bioreactor rental price was lower for the 10 l bioreactor as compared to the 258 l

bioreactor (equipment cost were decreased from 1 298 € to 1 058 €). The labor expenses were equal in both scales for all hosts.

Applying this assumption of the same titer and production volume, the manufacturing costs of the production (bioreactor) step for *E. coli*, *P. pastoris* and *Drosophila* S2 were about 1 300 €, 2 400 €, and 3 400 €, respectively. The manufacturing costs of 10 l cultivation for each host are presented in Table 33 and in Figure 21.

Table 33. Costs of production (bioreactor) step of Nef protein on a 10-liter bioreactor scale.

		E. coli **(€)**	***P. pastoris*** **(€)**	***Drosophila* S2** **(€)**
Production				
	Material costs	135	145	417
	Equipment costs	292	811	1058
	Labor costs	857	1500	1928
TOTAL		**1283**	**2455**	**3404**

The almost two-fold manufacturing cost difference between the bacterial and yeast system was mainly due to the difference in cultivation time (*E. coli* about one day and *P. pastoris* almost four days). This naturally reverberated in higher equipment rental costs and labor costs. The most expensive raw materials with the microbial systems were IPTG with the *E. coli* system (70 % of total material costs) and methanol with the *P. pastoris* system (41 % of total material costs). Ten liter *P. pastoris* cultivation consumes almost two liters of methanol.

The manufacturing cost difference between the yeast and insect cell systems was mainly due to the complex, more expensive growth medium needed for the *Drosophila* S2 cultivation and a slightly longer cultivation time (including the pre-culture).

On this scale, the labor cost was the most significant cost factor (57 – 67 % of manufacturing costs) in each system, whereas the influence of the material costs were very low (6 – 12 % of manufacturing costs) (Figure 21).

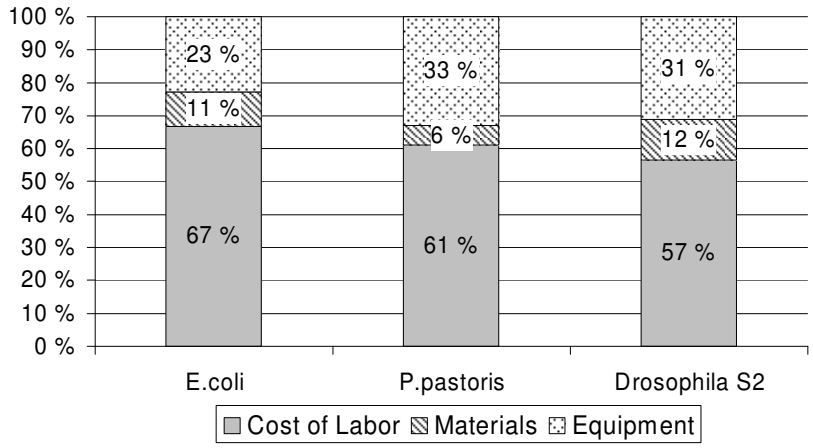

Figure 21. Cost distributions of the production cultivation (bioreactor) step using the *E. coli*, *P. pastoris* and *Drosophila* S2 host systems at a constant bioreactor working volume of 10 liters.

4.4 Conclusion

Recombinant proteins can be produced in a variety of different hosts, microbial, mammalian and insect cell systems. In cases where no post-translational modifications are required, also *E. coli* is a low cost alternative for production. Here three different hosts (*E. coli*, *P. pastoris* and *Drosophila* S2) were studied and the manufacturing costs of small scale (100 mg in one batch) production of a diagnostic recombinant protein (HIV-1 Nef) were compared.

As was to be expected, the microbial systems (*E. coli* and *P. pastoris*) had significantly lower total manufacturing costs than the insect cell system. Table 29, Figure 19 and Figure 20 show the manufacturing costs of 100 mg purified Nef-protein when the hosts had different titers and purification yields. The most significant difference between the two microbial system manufacturing costs was a result of a much longer strain construction time with the *P. pastoris* system (strain development costs of *E. coli* 3 600 € and of *P. pastoris* 6 900 €, Table 22). Omitting the strain construction costs, the microbial systems were cost-wise fairly comparable. In this case, the combined production and purification manufacturing costs for the *E. coli* and *P. pastoris* systems were about 2 800 € and 3 700 €, respectively.

The cell line construction expenses were even greater for the insect cell system (11 000 €, Table 22). The manufacturing costs for the *Drosophila* S2 system were also clearly higher compared to the microbial systems (combined production and purification 13 100 €). The higher manufacturing costs were a result of i) the lowest total Nef titer resulting in the need of a larger working volume, ii) the longest cultivation time, and iii) the need of a complex (expensive) growth medium.

On the scale of 100 mg purified protein and of bioreactor working volume less than 10 liters, the role of the material and equipment costs are relatively insignificant. In the microbial systems the labor costs accounted for 75 and 83 % of the total costs. In insect cell system the bioreactor working volume was notably larger (247 l) and also the cost distribution was slightly different, but the labor costs were still the major manufacturing cost (labor costs accounted for 52 % and material costs 42 %). For production on a bench/small pilot scale, this distribution of costs is fairly typical. In fact, the cost distribution as a function of scale presented by Rouf *et al.* (2000) is quite similar.

When comparing the three host systems assuming an equal titer and equal bioreactor working volume 10 l, the economical advantage of the microbial systems is still obvious (Table 33 and Figure 21) even though the share of the material costs for the *Drosophila* S2 system was now clearly reduced; labor costs 57 % and material costs 12 % of the manufacturing cost. However, here only the bioreactor step was considered. If also the capture and purification steps were included in this equal titer case, the downstream processing costs of *E. coli* and *P. pastoris* products would have increased, and of *Drosophila* S2 product would have decreased as compared to the base case.

From a technical perspective the three systems have different advantages and disadvantages. The *E. coli* system had the lowest costs of the studied systems. This was mainly due to fast growth rate and high Nef volumetric productivity. Generally, the *E. coli* system is easy to scale-up and it is highly reproducible. Similar opinions have previously been presented e.g. by Andersen and Krummen (2002), Schmidt (2004) and Walsh (2006). Disadvantages of the *E. coli* system include side-product production (e.g. endotoxins and proteases), intracellular production of recombinant proteins and formation of inclusion bodies. When producing more complex proteins

the lack of a post-translational machinery limits the use of *E. coli* (Jana and Deb, 2005; Sarramegna *et al*., 2003).

The specific productivity of *P. pastoris* is usually rather low. This, however, is often compensated by the high cell densities achieved in *P. pastoris* cultivations (Cha *et al*., 2005). The *P. pastoris* production protocols are straight-forward resulting in good reproducibilities. Scale-up is as easy as with *E. coli,* except for the fact that methanol is needed in high quantities and this can require some additional technical solutions. General disadvantages of the *P. pastoris* system include slow growth, significant strain and vector kits costs, and production of host cell proteases. When moving to commercial production, the license and royalty costs in regard to the strains and vectors must also be considered.

The insect cell systems are gaining popularity, especially for the production of complex (therapeutic) proteins (Ikonomou *et al*., 2003; Walsh, 2003). In comparison to mammalian cells, the ease of culture and higher expression levels are considered advantages of the insect cell systems. On the other hand, when compared to microbial cells, the insect cells grow very slow and the production levels are usually mediocre or low. When planning commercial use of the *Drosophila* S2, the used vectors result in additional IPR costs.

5 Manufacturing cost of monoclonal antibody production in stirred tank and hollow fiber processes

The manufacturing cost in production of a diagnostic monoclonal antibody in different bioreactor was analyzed. The equipment used in the analysis were stirred tank bioreactor (STR) equipped with a spin filter and a hollow fiber bioreactor (HFB). Both were operated as perfusion process.

The performances of the STR and the HFB in monoclonal antibody production have been reviewed e.g. by Yang *et al.* (2004) but they did not include economical analysis in their study. Analyses of the manufacturing costs based on the bioreactor type used have been previously presented for conventional STR and for disposable bioreactors (e.g. Farid *et al.*, 2005a, 2005b; Novais *et al.*, 2001, 2005b), but no cost analyses of the STR versus HFB have been published. The STR is generally used in large scale, as it is easy to scale and operate, but in smaller scale, as in case of diagnostic or research monoclonal antibodies, the HFB is also quite frequent (Griffiths, 2003; Jain and Kumar, 2008; Valdes *et al.*, 2001; Yazaki *et al.*, 2001). The HFB is especially good alternative for hybridoma cultures (Yang *et al.*, 2004).

In this manufacturing cost comparison the scale used was 85 g of unpurified Mab that was to be produced in 60 days. Purification yield was assumed to be 55 % resulting to 47 g of purified Mab. According to Farid *et al.* (2005b), 45 g of Mab is sufficient to Phase I clinical trials. The purification procedure used for both production methods was the same, and it was assumed to be as described in e.g. Farid *et al.* (2005b) and Sommerfeld and Strube (2005).

The manufacturing costs of the production phase were calculated from investment and operating costs. The costs of the purification phase are estimated from the cost distribution (cost ratio of upstream and downstream processes) presented by Sommerfeld and Strube (2005).

5.1 Monoclonal antibody production data

5.1.1 Production phase

The antibody was intended to be produced either in stainless steel STR bioreactor with a spin-filter or in several HF cartridges (Figure 22). In the STR the cells are grown outside the filter and product is harvested from inside the filter. In the HFB the cells are grown in the intracapillary space and the product is harvested from the extracapillary scape, outside the membrane tubes.

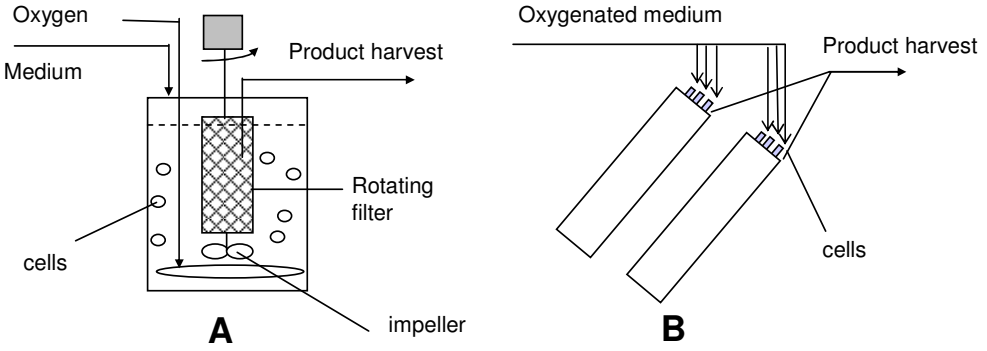

Figure 22. A Schematic picture of the processes used A) a stirred-tank bioreactor with a spin filter B) hollow fiber bioreactor.

The production goal was set to 46 g in 60 days. With purification yield of 55 % the fermentation titer was set to 85 g. The total annual production time is assumed to include five 60 days periods, i.e. the total produced Mab amount is 425 g that results to 234 g of purified Mab.

The FiberCell Systems Inc. (USA) hollow fiber cartridge 4300-C2018 High MWCO (Anon., 2008a) is supposed to be used in this hypothetical study. Hollow fiber bioreactor is operated first week as perfusion culture, where the fresh medium is supplied to the cartridge and used medium is withdrawn, but no actual harvest is made. After the week's growth period, the cells are grown to concentration of 10^8 cells/ml. In continuous cultivation, the cartridges extracapillary space (ECS) is

harvested every two days and also some cells are removed within the harvest. The harvest volume is 70 ml/cartridge (same as the cartridges ECS volume) and 400 mg Mab is assumed to be harvested. Harvest protocol is the same as used in FiberCell Systems Inc. (USA) products (Anon., 2008a)

According to Davis (2007), the HFB culture normally runs from four to six months, and the culture ends because either the cells may lose the productivity or the flowpath becomes partially blocked. Both of these happen usually because of the accumulation of dead cells and cell debris in the cartridge. In ProstaScint production the HFB hybridoma culture lasted 60 days and in Humaspect production the human cell line culture in HFB lasted 10 weeks (Farid, 2006).

The literature derived parameters of the perfusion (by spin filter device) cultures of hybridoma cells are presented in Table 34 (Castilho and Medronho, 2002).

Table 34. Performances of hybridoma cultivations in STR with spin filters (from Castilho and Medronho, 2002)

Cell line	Product	Reactor volume (l)	Max perfusion rate (1/d)	Cultivation time (d)	Max viable cell conc. (10^6 cells/ml)	Reference
Hybridoma	IgG	1.2	2.0	37	15	(Heine *et al.*, 2000)
Hybridoma and myeloma	IgM and IgG	7 to 500	1.2	30	>10	(Deo *et al.*, 1996)
Hybridoma	--	175	0.5	At least 8	2.8	(Yabannavar *et al.*, 1994)

The perfusion process is generally first cultivated 8 – 10 days in batch mode for growing the cells to production concentration (Deo *et al.*, 1996). After this, fresh medium is fed to the reactor with perfusion rate ranging from 0.5 to 2 reactor volumes (RV) per day. Antibody is collected from the used medium continuously.

The performance of the HFB system was estimated from FiberCell Systems Inc. (USA) product overview (Anon., 2008a) and from the protocol presented by Davis (2007). The cell concentrations presented by FiberCell Systems Inc. (Anon., 2008a) and a specific productivity of 20 pg/cell/day (Andersen and Krummen, 2002) was used in calculations. Same specific productivity is used also in STR calculations, although in some cases, higher specific productivities can be achieved in HFB

compared to STR (Yang *et al.*, 2004). The performance of the STR system and of the hybridoma cell line was estimated from Deo *et al.,* (1996). The performance data (estimated and calculated) is presented in Table 35.

Table 35. Assumptions made in the STR and HFB production

	STR with spin filter	HFB (data per one cartridge)
Cartridge ECS volume	--	70 ml
Inoculum	10^5 cells/ml	10^8 cells
Final cell concentration	10^7 cells/ml	10^8 cells/ml or 10^{10} cells/cartridge
Specific productivity	20 pg/cell/day	20 pg/cell/day
Productivity	0.2 mg/ml/day	400 mg/every 2nd day
Medium consumption	1 RV/first week, then 1.2 RV/day	3.75 l/first week, then 2 l/day
Harvest volume	1.2 RV/day	70 ml/every 2nd day
Harvest concentration	0.17 mg/ml	5.7 mg/ml
Culture runtime	29 days [a]	60 days [b]

[a] includes a 8 days batch period for cell growth, no harvest in this period

[b] includes a 7 days period for cell growth where no harvest is performed

Based on the cultivation data presented in Table 35 and on the goal of 85 g of antibody in 60 days, the size of the STR and the number of HF cartridges were calculated. As the STR perfusion run was assumed to last 29 days (plus one day for bioreactor cleaning and sterilizing), two runs were needed. The used equipment and consumables in the antibody production phase are summarized in Table 36. In STR option, shake flasks and a 10 l stainless steel bioreactor equipped with internal spin-filter was used. In HFB option disposable bioreactor was used for inoculum and for production, eight 1.2 m^2 hollow fiber cartridges (e.g. from Fibercell Systems, Inc, USA) were used.

Table 36. The equipment and consumables used in antibody production phase.

Task	Equipment		Consumables	
	STR	HFB	STR	HFB
Inoculum cultivation	Shake flasks	---	---	Disposable bioreactor
Production cultivation	10 l stirred-tank bioreactor (equipped with a spin-filter)	---	---	8 x 1.2 m^2 HFB cartridges

5.1.2 Capture and purification phase

The purification is performed as described in Sommerfeld and Strube (2005) and in Farid *et al.* (2005b): clarification, concentration, capture chromatography (protein A), virus inactivation, buffer exchange, ion exchange chromatography, viral clearance, buffer exchange, polishing chromatography (HIC) and final filtration. The same purification procedure is used for both production methods. The schematic figure of the generic purification is presented in Figure 12 in *section 2.4*.

5.2 Manufacturing cost

For comparing the production methods, the manufacturing costs of the product for both systems were calculated. The manufacturing cost consists of the direct operating costs, indirect operating costs and depreciation of the investment cost.

5.2.1 Investment costs of the upstream processing

For this size of production, no new production site is to be built. Both production options are most likely easily fitted to an existing research center or production facility and also no actual extension is needed. The equipment is still assumed to be bought and installed and also the heating, ventilation and air-conditioning (HVAC) issues are updated for therapeutics production. For STR option, also the CIP and SIP connections are assumed to be built.

The major equipment capital costs of upstream production for the spin filter equipped STR option are based on the equipment costs presented by Farid (2005b) and Bailey (2009) and presented in Table 37.

Table 37. The equipment capital costs of the upstream production for STR option.

Equipment	Cost	Reference
Shake flasks for inoculum (10 pieces)	200 €	Farid (2005b)
10 l bioreactor and spin-filter	75 000 €	Bailey (2009)
Total equipment cost	**75 200 €**	

The FCI of the upstream processing of the STR option was calculated from the total equipment cost presented in Table 37 with Lang's factor 6 resulting to FCI of 451 k€. Farid *et al.*, (2005b) used Lang's factor 7 in 200 l bioreactor scale, but here the bioreactor is significantly smaller and therefore e.g. the costs of the equipment installation are lower. Instrumentation and control, electrical systems, validation and utility (SIP and CIP) costs are same on both scales.

For the HFB option, the FCI was calculated from the STR option equipment cost by a modified Lang method (*see section 3.1.2* for more details) with a Lang's factor of 3.6. The resulting investment cost 271 k€ is 40 % lower as compared to the FCI of the conventional process as calculated by Novais *et al.*, (2001) and Sinclair and Monge (2005b).

Table 38. The fixed capital investment costs of the STR and HFB option (equipment cost used was 75.2 k€).

	Lang's factor	FCI
STR with spin filter	6	451 200 €
HFB	3.6	270 720 €

5.2.2 Operating costs of the upstream processing

The direct operating costs were calculated from the raw material (medium and consumables) and labor expenses. For miscellaneous material (e.g. safety clothing etc.) a value of 50 % of the direct raw material costs was used (Farid *et al.*, 2007). Utilities included oxygen for inlet air enrichment, CIP water and detergents and

steam for sterilization. The oxygen consumption was calculated from the cell specific oxygen uptake rate 0.5 pmol/cell/h (Henzler and Kauling, 1993) and from the cell density and culture time. Labor costs were calculated assuming two employees: Both production equipment are highly instrumented and easily operated and because of long cultivation times, few bioreactor turnovers are needed. QCQA cost was assumed to be equal to one person's annual salary. Management cost was assumed to be 30 % of one person's salary. Direct operating cost calculations are presented in Table 39. The material and utilities costs are calculated as costs per batch and as costs per year (5 HFB cultures and 10 STR batches are cultured annually).

Table 39. Upstream direct operating cost calculations.

			HFB			STR	
	€/unit	**usage**	**total (€)**	**total (%)**	**usage**	**total (€)**	**total (%)**
Raw materials							
Medium [1]	6 €/l	880 l	5 280		265 l	1 591	
Consumables							
Roller Bottles	25 €/pc	6 pcs	150				
HF cartridges	800 €/pc	8 pcs	6 400				
Miscellaneous [2]			2 640			795	
Utilities							
Oxygen	10 €/m^3	906 l	9		789 l	8	
CIP	1.3 €/l				40 l	53	
Steam	0.1 €/kg				10 kg	1	
Total / batch			14 479			2 448	
Total raw materials [3]			72 395	31		24 476	13
Labor (monthly)	4000 €	2	96 000	42	2	96 000	52
QCQA [4]			48 000	21		48 000	26
Management [5]			14 400	6		14 400	8
TOTAL DIRECT			**230 795**			**182 876**	

[1] Medium is supposed to be bought pre-made and sterile.

[2] Miscellaneous is calculated as 50 % of medium costs

[3] Total 5 HFB batches and 10 STR batches

[4] QCQA is calculated as one person's annual salary

[5] Management is calculated as 30 % of one person's annual salary

Direct upstream operating cost for the HFB process is 231 k€ and for the STR process 183 k€. Labor expenses cover 42 % of the direct operating costs of the HFB upstream process and 52 % of the STR upstream process. The combined labor, QCQA and management costs cover as much as 69 % of the direct operating costs of the HFB upstream process and even 87 % of the STR upstream process.

In indirect cost estimation, maintenance, depreciation of the investment, insurances and general utilities (i.e. electricity, heating etc) are included. The annual maintenance cost was estimated to be 8 000 €. The insurances are assumed to be approximately 4 % of the personnel salary. A depreciation period of 5 years and interest rate 15 % was used. The general utilities are calculated as 135 €/m^2 (Farid *et al.*, 2007) using floor area 30 m^2. The indirect cost calculations are presented in Table 40.

Table 40. Indirect operating cost calculation

	HFB	STR
Maintenance (estimated)	8 000 €	8 000 €
Insurances	3 840 €	3 840 €
Depreciation	80 760 €	134 600 €
General utilities	4 050 €	4 050 €
TOTAL INDIRECT COST	**96 650 €**	**150 490 €**

Total indirect costs for HFB option are 97 k€ and for STR option 150 k€. The investment depreciation covers 84 % of the indirect operating costs of the HFB upstream process and 89 % of the indirect operating costs of the STR upstream process.

5.2.3 Manufacturing cost for the upstream processing

The total manufacturing cost for the upstream HFB process was 327 k€ and for the upstream STR process 333 k€. Although the investment cost of the STR was almost double that of the HFB process, the higher material costs (73 k€ versus 24 k€) made the STR only slightly more expensive as the HFB process. In STR system manufacturing cost, the depreciation (40 % of the manufacturing cost) and the labor expenses (29 %) were the largest cost items. Also in the HFB system, the largest

cost items were labor expenses (29 %) and depreciation expenses (25 %), but the material expenses were also significant (22 %). The manufacturing cost distribution of the systems is presented in Figure 23.

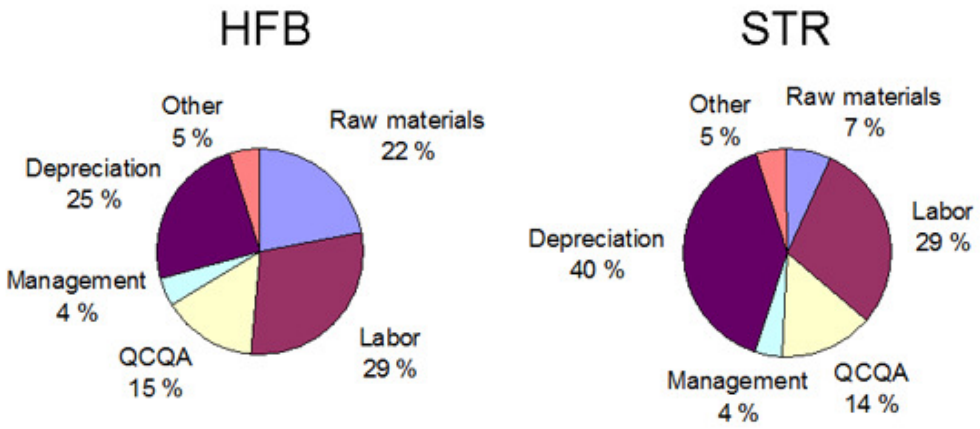

Figure 23. The USP manufacturing cost distribution of the STR and HFB processes. Other includes the costs of maintenance, insurances and general utilities.

Manufacturing cost per gram of product was calculated by dividing the total manufacturing by the produced amount of purified Mab, 234 g. The upstream manufacturing cost per gram of product was 1.4 k€/g for both options.

5.2.4 Manufacturing cost for the downstream processing

As has been stated before (*see section 3.3.4*), the cost of the downstream processing (DSP) does not depend as much on the fermentation titer as the upstream processing (USP) cost, since most expenses come from the chromatography columns, that are designed on mass basis and not on concentration basis. However, typical downstream process involves also numerous filtration processes that are designed on volume (and concentration) basis. The difference in the harvest volume between STR and HFB products is significant (STR daily harvest volume 12 l; HFB 70 ml every second day) and therefore the investment and operating costs of the HFB downstream system will be lower.

Based on the product mass, the downstream manufacturing costs for both HFB and STR processes should be almost equal. Sommerfeld and Strube (2005) have presented a ratio of the upstream processing costs versus downstream processing costs as a function of product titer for monoclonal antibody production, but this applies only if the processes are otherwise equal.

For titer of 0.17 g/l (the titer in the STR process), the ratio of USP and DSP costs is 52 % to 48 % (Sommerfeld and Strube, 2005). Therefore it is safe to estimate, that the downstream process costs for the STR process are 308 k€. Although from the presentation of Sommerfeld and Strube (2005) it could be assumed that the downstream processing costs of the HFB product are higher than the purification process costs of the STR product, most likely the opposite is true. As the STR product is in lower concentration, it needs an additional concentration step in the purification process as compared to the purification process of the HFB product and as a result, the expenses are then higher.

5.2.5 Total manufacturing cost

The total manufacturing cost of the STR process is 641 k€, or 2.7 k€/g and if the same downstream processing cost is applied to the HFB process, the total manufacturing cost is 631 k€, or 2.7 k€/g (Table 41).

Table 41. The total manufacturing cost of the Mab production in HFB and in STR processes.

	HFB	STR
manufacturing cost of USP	327 k€	333 k€
DSP cost of the STR process (USP:DSP ratio 52:48)		308 k€
Total manufacturing cost	**635 k€**	**641 k€**
Total manufacturing cost per gram	**2.7 k€/g**	**2.7 k€/g**

However, as stated before, the savings in the filtration investment and operation costs due to much lower harvest volumes will lower the downstream processing manufacturing cost of the HFB option and result lower total manufacturing cost.

5.3 Conclusion

Monoclonal antibodies can be produced in a variety of processes. The processes include different equipment types (e.g. stirred-tank bioreactor, hollow fiber bioreactor or disposable bag bioreactors) and several different product modes (batch, fed-batch and perfusion). These are discussed in more details in *section 2.3.* In large scale, the stirred-tank bioreactor is usually the choice, also in monoclonal antibody production. In smaller scale, as in production of monoclonal antibodies for diagnostic of research use, also other equipment types are applicable. The perfusion process is the most efficient way to produce proteins, as higher cell concentrations can be achieved. In large scale, the fed-batch is still used more, as it is easier to operate and the contamination risk is smaller due to shorted culture period.

In this study the perfusion stirred-tank bioreactor and hollow fiber bioreactor were compared techno-economically. In the scale of producing annually 234 g of purified Mab, the hollow fiber bioreactor and the stirred-tank bioreactor (equipped with a spin filter) are cost-wise comparable. Both bioreactors were run as perfusion processes, the HFB 60 days and the STR 30 days.

The investment cost of the STR option is 1.7 times that of the HFB option, but the operating costs of the HFB option were higher and as a result, the upstream manufacturing costs are almost equal (HFB 327 k€, STR 333 k€). The result is in agreement with the general opinion, that in scale up to several grams, the HFB is viable and economical alternative (Griffiths, 2003; Jain and Kumar, 2008; Valdes *et al.,* 2001; Yazaki *et al.,* 2001).

In the cost analysis, the direct operating costs (raw materials, consumables, utilities and labor expenses) covered 70 % of the upstream manufacturing costs in the HFB process and 55 % in the STR process. This is typical for a small scale process, where the investments are quite small.

Labor costs alone accounted for 29 % of the upstream manufacturing costs in both processes. Investment depreciation covered 25 % of the upstream manufacturing cost in the HFB process and 40 % in the STR process.

The Mab purification costs were assumed to be the same for both processes, as same purification procedure was used and the purified amount of raw protein was the same. With this assumption, the downstream processing costs were calculated as 308 k€ and the total manufacturing costs were equal, 2.7 k€/g. However, the product concentrations and harvest volumes varied significantly, and this will likely reduce significantly the filtration expenses of the HFB process. The most expensive downstream processing unit is the affinity chromatography that is designed on product mass basis and the harvest volume variation will not have as large effect on these costs. The downstream manufacturing costs of HFB will probably be lower than estimated here.

From technical perspective, the STR requires more cleaning and sterilization processes whereas the HF cartridges are operated as disposables. Both are operated automatically, but as the STR process runtime is only half that of the HFB process it needs more reactor turnovers. A disadvantage of the HFB process is larger contamination risk as the culture time is much longer. A possible disadvantage with the STR is the shear sensitivity of the hybridoma cells. Although many hybridomas are cultures in suspension, the specific production rate may be less than in HFB (Yang *et al.,* 2004).

6 Crystallization of recombinant protein as a purification method

Most recombinant proteins and especially monoclonal antibodies are purified using chromatographic methods. Affinity chromatography is used because of its high selectivity but the disadvantages include the low flow rate and high expenses (Kelley, 2007; Low *et al.*, 2007; Sommerfeld and Strube, 2005). Especially as the titers have increased, the significance of the chromatographic purification cost in the manufacturing cost is revealed. Even 50 – 80 % of the total costs come from the downstream processing (Roque *et al.*, 2004; Rouf *et al.*, 2000; Sommerfeld and Strube, 2005) and typically the chromatography steps are the most expensive (Farid *et al.*, 2005b).

Alternatives for the expensive chromatographic purification have been searched, and among others, crystallization has been proposed e.g. by Kelley (2007), Low *et al.* (2007) and Schmidt *et al.* (2005). The advantages of the crystallization include high selectivity and low costs. The disadvantage is that no universal method is available, but the crystallization method must always be searched through elaborate experimental work.

In this study we have developed a crystallization method for HIV-1 Nef protein that is typically purified by affinity chromatography. Nef proteins were crystallized for two different purposes: First, to create crystals big enough for x-ray structure studies and second, for studying the possibility to purify Nef (and possibly other GST-tagged proteins) by means of crystallization.

Many research groups have tried to crystallize the full-length Nef-protein, but have not succeeded (e.g. Franken *et al.*, 1997; Lee *et al.*, 1996). The Nef-protein core domain (HIV-1 NL4-3, residues 54-205) has been crystallized together with the Fyn tyrosine kinase SH3 domain (Lee *et al.*, 1996) and again (HIV-1 LAI, residues 58-206, NefΔ1,57) alone and with Fyn SH3 domain (Franken *et al.*, 1997).

As the full-length Nef has not been crystallized, also the structure has not been solved as whole. However, the structure has been constructed from the X-ray structure of the folded core domain and from the NMR-structures of the flexible

anchor domain. The Nef structure is presented in Figure 24 (Arold and Baur, 2001). The proteolytically cleaved core domain has been crystallized successfully unliganded (Franken *et al.*, 1997) and in complex with the Fyn SH3 domain (Franken *et al.*, 1997; Lee *et al.*, 1996).

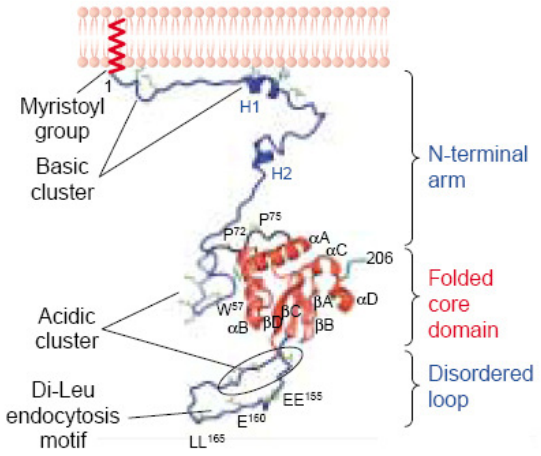

Figure 24. The structure of Nef-protein (Arold and Baur, 2001).

6.1 Nef crystallization screening conditions and results

Nef-proteins were produced in *E. coli* and purified as described in Vermasvuori *et al.* (2009)

Crystallization screens were performed in room temperature using the hanging-drop vapor-diffusion method in 24-well Lindbro plates (ICN, USA). The crystallization conditions were optimized by changing the protein and precipitant concentrations. Polyethylene glycol (PEG) 3350 (Sigma-Aldrich, Germany) and isopropanol (Rathburn, UK) were used as precipitants with Na-K phosphate buffer. The Na-K phosphate buffer was prepared by adding 4 M K_2HPO_4 to 4 M NaH_2PO_4 to desired pH at room temperature.

The crystals grew from droplets containing 10 – 15 mg/ml protein, 10 mM Na-K Phosphate buffer (pH 6.9), 1 – 8% w/v PEG 3350 and 7 – 13 % v/v isopropanol

(IPA), against a reservoir solution containing 2 – 15 % w/v PEG 3350, 15 – 25 % v/v isopropanol and 20 mM Na-K-phosphate buffer (pH 7). Crystals were shaped as rods or needles (Figure 25), and were maximum 200 µm long. Only in few cases, the crystals were alone, and generally grew as star-like clusters (Figure 26).

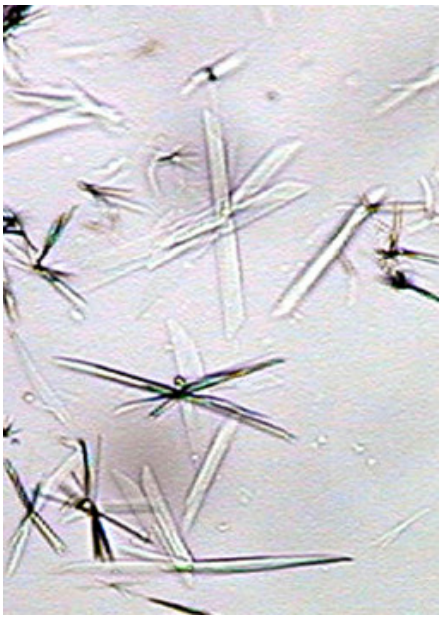

Figure 25. The rod-shaped Nef crystals (length ~ 200 µm). Crystallization conditions: protein concentration 15 mg/ml, PEG 3350 5 %, IPA 20 %, Na-K-phosphate buffer (pH 7.0) 20 mM, RT 3 days.

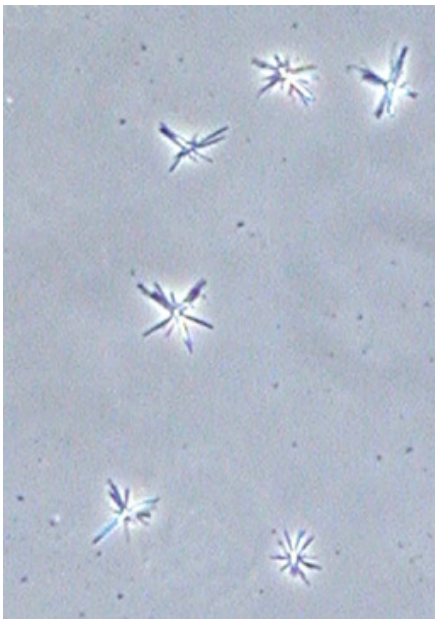

Figure 26. The star-like Nef crystal clusters (diameter ~ 50 µm). Crystallization conditions: protein concentration 10 mg/ml, PEG 3350 10 %, IPA 20 %, Na-K-phosphate buffer (pH 6.9) 20 mM, RT 2 days.

The crystallization conditions were screened further with different additives (Beta octyl-glucopyranoside (βOG), Dithioerythritol (DTE) and metal ions Ca, Mg, Li and Zn) but they did not improve the crystal quality. Micro-seeding was used in attempts to produce bigger crystals, but it did not help in producing bigger crystals.

Small amount of crystals were gathered up from hanging-drop droplets and dissolved in NA-K phosphate buffer (pH 7). Then the solution was tested with SDS-PAGE and the proteins were identified was Nef.

6.2 Batch crystallization

Generally it is thought, that the batch crystallization by direct mixing of additives occurs at about 60 – 80 % of the concentration of the precipitant required in a hanging drop experiment (Chayen, 1998; Rayment, 2002). For example, if the hanging-drop conditions are (reservoir solution) 10 % w/v PEG, 20 % v/v IPA and 20 mM Na-K-phosphate buffer (pH 6.9), the same proteins stock will probably crystallize when mixed 1:1 with a solution that contains 6 – 8 % w/v PEG, 12 – 16 % v/v IPA and 12 – 16 mM Na-K-phosphate buffer (pH 6.9).

Batch crystallization was attempted with three different setups. First, the batch crystallization was performed in hanging-drop droplet against an empty reservoir, secondly in droplets against a reservoir containing 1:1 crystallization solution and water, and thirdly 1:1 of protein solution and crystallization solution in 1.5 ml eppendorf tube. In all setups, the protein stock was mixed 1:1 with crystallization solution.

In first setup the crystallization solution was too strong (Na-K phosphate buffer 20 or 50 mM, PEG 3350 10 – 50 % and IPA 20 – 50 %) and in most droplets amorphous precipitate was formed in 24 hours. In the only droplet to form plain crystals the protein stock was mixed 1:1 with a solution containing 50 mM Na-K phosphate buffer, 10 % PEG 3350 and 20 % IPA.

In second setup, small crystals were formed in 48 hours in droplets were the crystallization solution used contained Na-K phosphate buffer 20 mM, PEG 8 – 20 % and IPA 20 %. Bigger crystals (without amorphous precipitation) grew from a droplet where the protein stock was mixed 1:1 with a solution containing 50 mM Na-K phosphate buffer, 10 % PEG 3350 and 20 % IPA.

In third setup the same solution that produced crystals in second setup was mixed into a 1.5 ml eppendorf tube. Protein stock was mixed 1:1 with a solution containing 50 mM Na-K phosphate buffer, 10 % PEG 3350 and 20 % IPA. No crystals or amorphous precipitation was seen within 2 weeks.

6.3 GST-Nef crystallization

A GST fusion protein that contains the DNA binding domain (16 – 115) of DREF has been crystallized by Kuge *et al.* (1997). They also suggested, that as the structure of a GST-molecule is known, the structure of a GST-fusion protein would be easier to solve than the structure of the target protein alone. Zhan *et al.* (2001) says, that the GST-fusion proteins could be crystallized easier using a standard method that they call the GST-driven crystallization. They summarize the crystallization of three GST-fusion proteins, and found out, that in every case, 30 – 60 % PEG (molecular weight 3350 or 4000) was used as a precipitant. The peptides crystallized as fusion proteins were only 5 – 42 residues in length.

Here, the crystallization of un-cleaved GST-Nef fusion protein was attempted with few different PEG and isopropanol conditions (PEG 3350 10 – 20%, IPA 10 – 20%, 0 or 20 mM buff (pH 6.9), but no crystals were observed. Later in SDS-PAGE it was seen, that the protein stock had been fragmented.

6.4 Conclusion

The crystallization method for HIV-1 Nef protein was developed, but the protein crystallized only in small scale hanging-drop experiments and not in batch experiments (1.5 ml eppendorf tupe). Also the crystallization of the uncleaved GST-Nef proteins did not succeed, probably because of the fragmented protein stock.

7 CONCLUSIONS

Recombinant proteins and antibodies for pharmaceutical or diagnostic use can be produced in many different host organisms (microbial, insect and mammalian cells), in a variety of different bioreactors (e.g. stirred-tank bioreactor, hollow fiber bioreactor and disposable bag bioreactors) and using different feeding strategies (batch, fed-batch or perfusion). The manufacturing cost (€/g) of the product depends on the characteristics of the production host (growth rate, productivity) and on the production method (cell density of the bioreactor phase and the overall yield). Most critical variables are the fermentation titer and the overall yield.

The fermentation titers have already increased significantly over last years and now the focus is on upstream and downstream processing methods. The economical comparison of different processing methods is straightforward, if only titer and/or yield are changed and the process is otherwise kept the same. But if two different hosts are compared for commercial production, the comparison is more difficult as the processes may be completely different. In this study, the production of recombinant HIV-1 Nef protein was produced in three different host systems (*E. coli*, *P. pastoris* and *Drosophila* S2) and the processes were analyzed techno-economically. The bacterial host was found to be the lowest cost system with a manufacturing cost of 100 mg of Nef about 6 460 €. The manufacturing cost of the *P. pastoris* and *Drosophila* S2 systems were about two and almost four times higher. The low cost of the *E. coli* system is mostly due to the fast growth rate and high titer. Therefore, if no post-translational modifications are required, the bacterium is usually the choice.

The equipment type and feeding strategy affect the titer and manufacturing costs significantly. In this study, the production of monoclonal antibody in perfusion stirred-tank bioreactor and in hollow fiber bioreactor was economically compared. In scale 234 g of Mab per year, the investment cost of the STR system was 451 k€ and of the HFB system 271 k€. The upstream manufacturing costs were almost equal, USP manufacturing cost of the STR was 333 k€ and of the HFB 327 k€. If the scale is increased, the stirred-tank bioreactor would probably be more economically viable, because of the higher direct operating costs of the HFB

system. The downstream processing manufacturing cost of the HFB is lower due to smaller volumes and higher concentrations and therefore the total manufacturing cost of the HFB is lower.

Downstream processing costs are significant in biopharmaceutical production, even 50 – 80 % of the total costs. Most expenses emerge from the expensive chromatography steps. Still, the typical purification process uses several chromatographic methods. Expanded and simulated moving beds, membrane chromatography, precipitation and crystallization have been presented as alternatives for affinity chromatography. The use of crystallization is limited because no universal crystallization method is available, but the method and crystallization conditions must be searched for each protein separately. In this study the crystallization method for HIV-1 Nef was developed. The crystallization occurred in hanging-drops in room temperature and pH 7 with PEG 3350 and isopropanol as precipitants. Obtained crystals shaped as needles or rods.

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