Platform Technology for the Production of Therapeutic Antibody Fragments in *Escherichia coli*

Evaluation of *Escherichia coli* as host system for the production of scFv- and Fab fragments in biopharmaceutical industry

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Abstract

Latest advances in antibody- engineering and production have led to a broad variety of novel antibody therapeutics now entering the biopharmaceutical market. However, remarkable costs for patients and the insurance system caused by the high dosage and the vast field of application have to be considered. Antibody fragments have been established as promising alternatives to conventional antibody therapeutics in recent years. Owing to their reduced size they can be produced easily and therefore less costly in microbial host systems. This work describes the establishment of a manufacturing platform to gain generic knowledge about the intracellular expression of these formats in *E. coli*. The hosts *E. coli* Strain B and Strain A were evaluated for expressing the single-chain-fragment (scFv) of the mAbs H6, B1, and A4. Furthermore, *E. coli* Strain A was investigated for expressing the individual chains of antigen-binding-fragment (Fab) of mAb A4.

At 35°C, fermentations with both strains expressing H6- and A4 scFv yielded volumetric concentrations from 5.9 g/L to 8.4 g/L. However, volumetric B1 scFv concentrations were only 3.1 g/L in both strains. In a next step, the influence of expression temperature on intracellular B1 scFv yield and solubility in both strains was investigated. Lowering the expression temperature to 28°C decreased the volumetric concentration of B1 scFv to 1.5 g/L for Strain B and 2.1 g/L for Strain A. Increasing the expression temperature to 42°C also decreased the volumetric concentration to 1.8 g/L for Strain B and 2.0 g/L for Strain A. No soluble fraction of B1 scFv could be detected at any expression temperature. Subsequently, the influence of the linker peptide on intracellular B1 scFv yield and solubility was investigated in *E. coli* Strain A. Four different linker peptides were introduced into B1 scFv replacing the original $(Gly_4Ser)_3$ linker. B1 scFv comprising E-linker, L205, or LLB18 yielded comparable volumetric concentrations (3.2 g/L, 4.0 g/L and 3.8 g/L). B1 scFv comprising L218 yielded notably more (8.9 g/L). None of the four linkers led to increased intracellular B1 scFv solubility.

A further aim of this study was to evaluate the feasibility of intracellular expression of Fab fragments in *E. coli* Strain A. The individual chains of the heterodimeric A4 Fab served as model proteins. The light chain yielded 14.2 g/L and the heavy chain 11.7 g/L. Therefore, intracellular expression of the individual A4 Fab chains proved to be a promising expression strategy.

Zusammenfassung

Die jüngsten Fortschritte in der Konstruktion und Produktion von Antikörpern führten zur Etablierung einer Reihe neuer Antikörper-Therapeutika auf dem biopharmazeutischen Markt. Durch die vielfältigen Anwendungsbereiche und hohen Dosierungen entstehen allerdings erhebliche Kosten für Patienten und des Gesundheitssystem. Antikörperfragmente konnten sich in den letzten Jahren als vielversprechende Alternativen zu den konventionellen Antikörper-Therapeutika etablieren. Wegen ihrer geringen Größe ist es möglich diese Formate einfach und kostengünstig in mikrobiellen Wirtssystemen herzustellen. Diese Arbeit beschreibt den Aufbau einer Produktionsplattform um generisches Wissen über die intrazelluläre Expression dieser Fragmente in *E. coli* zu erlangen. Dazu wurde das Potential der *E. coli* Stämme B und A zur intrazellulären Expression der single-chain Fragmente (scFv) der mAbs H6, B1, und A4 untersucht. Außerdem wurde versucht die einzelnen Ketten des antigen-binding Fragments (Fab) von A4 getrennt voneinander in *E. coli* Stamm A zu exprimieren.

Volumetrische Konzentrationen von H6- und A4 scFv, die in beiden Stämmen bei 35°C exprimiert wurden, ergaben zwischen 5,9 g/L und 8,4 g/L. Die volumetrischen Konzentrationen von B1 scFv betrugen hingegen nur 3,1 g/L in beiden Stämmen. In einem nächsten Schritt wurde der Einfluss der Expressionstemperatur auf die intrazelluläre Ausbeute und Löslichkeit von B1 scFv in beiden Stämmen untersucht. Ein Absenken der Expressionstemperatur auf 28°C verringerte die volumetrische Konzentration von B1 scFv auf 1,5 g/L in Stamm B und 2,1 g/L in Stamm A. Das Erhöhen der Expressionstemperatur auf 42°C verringerte die volumetrische Produktivität auf 1,8 g/L in Stamm B und 2,0 g/L in Stamm A. Bei keiner Expressionstemperatur konnte eine lösliche Fraktion von B1 scFv nachgewiesen werden. Anschließend wurde der Einfluss des Linker Peptids auf die intrazelluläre B1 scFv Ausbeute und Löslichkeit in *E. coli* Stamm A untersucht. Vier verschiedene Linker Peptide wurden in B1 scFv eingefügt, die den $(Gly_4Ser)_3$ Linker ersetzten. Die volumetrische Konzentrationen von B1 scFv die den E-Linker, L205 oder LLB18 beinhalteten waren ähnlich (3,2 g/L, 4,0 g/L und 3,8 g/L). B1 scFv das den L218 linker enthielt ergab einen viel höheren Wert (8,9 g/L). Keiner der neu eingeführten Linker bewirkte eine erhöhte intrazelluläre Löslichkeit von B1 scFv.

Ein weiteres Ziel dieser Arbeit war die Machbarkeit der intrazellulären Expression von Fab Fragmenten in *E. coli* Stamm A zu beurteilen. Die einzelnen Ketten des A4 Fab Heterodimers wurden als Modellproteine verwendet. Die leichte Kette ergab 14,2 g/L und die schwere Kette 11,7 g/L. Daher erwies sich die intrazelluläre Expression der einzelnen A4 Fab Ketten als vielversprechende Expressionsstrategie.

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1. Aim of the Study

Monoclonal antibodies and their fragments are promising therapeutic formats with a significant high market potential. The use of microbial host systems for the production of antibody fragments is commonly assumed as major benefit for an inexpensive manufacture compared to mammalian expression systems and full length antibodies.
First attempts were made to set up platform technologies for scFv and Fab fragments. Expression systems of choice were *E. coli* for intracellular- and periplasmic expression and *P. pastoris* for secretion to the culture supernatant.

The major aim of this study was the intracellular expression of single chain antibody fragments as inclusion bodies in *E. coli*.
The influence of different strains, codon usage, linker sequence, and cultivation temperature on product yield and inclusion body purity was investigated. Moreover, fermentation temperature and different linker sequences were examined as influencing factors on intracellular scFv solubility.

A further aim of this study was to evaluate the feasibility of intracellular expression of Fab fragments in *E. coli*.

2. Introduction

In vertebrates, antibodies represent the key defense of the humoral immune response. Antibodies recognize pathogens (antigens) such as microorganisms, larger parasites, viruses, and bacterial toxins highly specific. Antibodies are glycoproteins and belong to the immunoglobulin family (Ig) which comprises five different immunoglobulin classes: IgA, IgD, IgE, IgG, and IgM. IgG is the main serum antibody and is mostly used as a therapeutic agent (Joosten *et al.*, 2003). Antibodies are naturally produced by B-lymphocytes. Since the hybridoma technology was introduced (Kohler and Milstein, 1975), it was possible to immortalize B-lymphocytes which allowed the production of sufficient amounts of monoclonal antibodies (mAbs) with defined binding specificity.

The majority of applications of recombinant antibodies are in the field of diagnosis and therapy in human medicine. Because of their properties, antibodies are the ideal cancer targeting reagent (Hazra *et al.*, 1995; von Mehren and Weiner, 1996; Colcher *et al.*, 1998; Hudson, 1999). Consequently, therapeutic mAbs directed against breast cancer cells (Herceptin™) are already available on the market. Additionally, mAbs are applied in other therapeutic fields e.g. as rattlesnake antidote (CroFab™) or to avoid transplant rejections (Zenapax™) (Hudson and Souriau, 2003). Applications outside research and medicine e.g. consumer applications can also be considered. Antibodies in shampoos to prevent dandruff or in toothpaste to protect against tooth decay caused by caries (Frenken *et al.*, 1998) are examples. In 2005, 18 monoclonal antibodies were on the market and more than 100 were in clinical trials. It is expected that engineered antibodies will account for >30% of all revenues in the biotechnological market in 2008 (Holliger and Hudson, 2005). The global market is estimated to grow from about US$ 10 billion in 2006 (Pavlou and Belsey, 2005) to US$ 30 billion in 2010 (Evans and Das, 2005).

Antibody therapies usually require high doses (>1 g per patient per year). As the anticipated treatments will involve large number of patients, the consequence is a multi-tons production demand per year. Because of their complex structures, manufacturing of full length antibodies in state of the art production processes currently employs hybridomas and mammalian cell cultures such as CHO-cells (Gasser and Mattanovich, 2006). Limitating factors for the use of

these production systems are a strict patent-situation, viral contamination issues, limited production capacity and high costs (Dorai *et al.*, 1994; Jost *et al.*, 1994). Antibody production costs in mammalian cell culture are estimated to range between 300 and 3,000 US$ / g product (Farid, 2007).

A full-length antibody is required in some cases if recruitment of cytotoxic effector-functions or higher serum half-life mediated by the Fc domain are needed (Batra *et al.*, 2002; Joosten *et al.*, 2003). In other cases, properties such as rapid tissue penetration and rapid blood- and kidney clearance rates are favorable. These characteristics are best fulfilled by antibody fragments (Yokota *et al.*, 1992). The production of antibodies and their fragments for preclinical and clinical trials has been evaluated in different expression systems such as mammalian cells, insect cells, plants, transgenic animals, filamentous fungi, yeasts, and bacteria (Hudson and Souriau, 2003; Chambers, 2005). Small, nonglycosylated antibody fragments such as scFv and Fab fragments, diabodies, and V domains can be easily produced in microbial expression systems (Hudson and Souriau, 2003; Joosten *et al.*, 2003). A major advantage of microbial expression is the quick generation of expression strains as well as fast and cheap cultivation. In addition, yields ranging from few mg/L up to several g/L were previously reported (Harrison and Keshavarz-Moore, 1996; Fischer *et al.*, 1999; Damasceno *et al.*, 2004). Simple fermentation conditions, rapid genetic manipulation, ease of scale-up, low costs for fermentations, and no concerns about human pathogenic viruses favor the expression of antibody fragments in *E. coli* (Arbabi-Ghahroudi *et al.*, 2005).

In the last ten years, the number of applications for biopharmaceuticals has steadily increased with 15 new approvals in 2003 (Werner, 2004). At the same time, a pipeline of 270 new biopharmaceuticals was under clinical evaluation with 101 clinical studies in the field of oncology (Werner, 2004). In 47 of those clinical studies, mAbs were the new biological entities investigated (Werner, 2004). Therefore, avoiding shortages in mAb production in biopharmaceutical industry is essential to stay competitive. This is of particular importance during pre-clinical and early clinical phase. Rapid choice of the most suitable production host and its application in simple and standardized processes which yield high titers are essential. By implementing a platform technology it is possible to meet those demands by providing standardized processes which do not require one-by-one process-optimization.

2.1 Therapeutic antibody formats

Most antibody fragment formats are based on human immunoglobulin G (IgG). IgGs are heterodimeric molecules and consist of four polypeptide chains. They have a molecular mass of approximately 150 kDa. Structurally, IgGs include two identical light chains (25 kDa) and two identical heavy chains (125 kDa). The heavy and light chains are connected by disulfide bridges and non-covalent bonds. The disulfide bridges are formed correctly during secretion in the endoplasmic reticulum in eukaryotes. However, these disulfide bridges cannot be formed in the prokaryotic cytosol owing to its reducing milieu (Padlan, 1994). The chains are further structured in constant and variable domains. The light and heavy chain consists of one N-terminal variable domain (V_L and V_H) and one or three constant domains (C_L, C_H1, C_H2 and C_H3) (**Figure 2-1**):

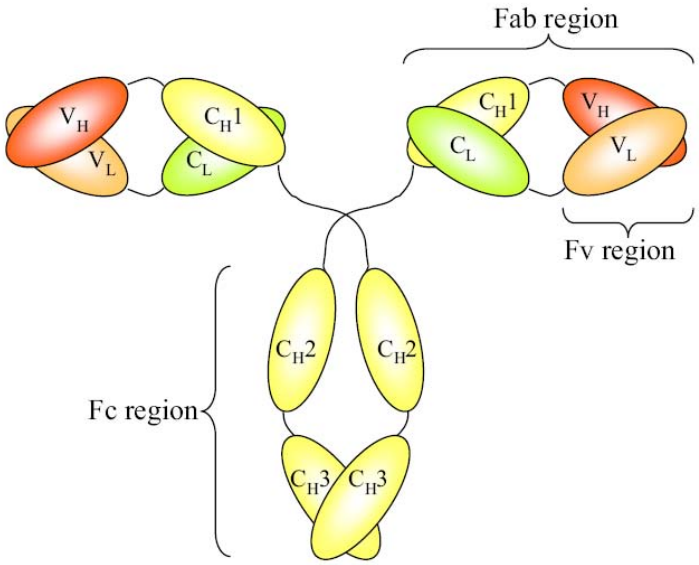

Figure 2-1: Schematic structure of a conventional IgG antibody (Joosten *et al.*, 2003)

The variable domains build the antigen-binding-site (Joosten *et al.*, 2003; Holliger and Hudson, 2005). Variable domains comprise four conserved framework regions (FR) and three hypervariable complementarity determining regions (CDRs). The CDRs of V_L and V_H significantly vary in sequence and therefore determine the specificity of the antibody. The Fc regions (fragment crystallizable regions) interact with the complement system and Fc receptors on cell surfaces. For many biotechnological applications, the Fc region is not essential. This has been considered for the construction of many antibody fragments such as

single-chain fragments (scFv) and antigen-binding fragments (Fab). The nomenclature of antibody fragments is based on the domains that are present in the fragments (**Figure 2-2**).

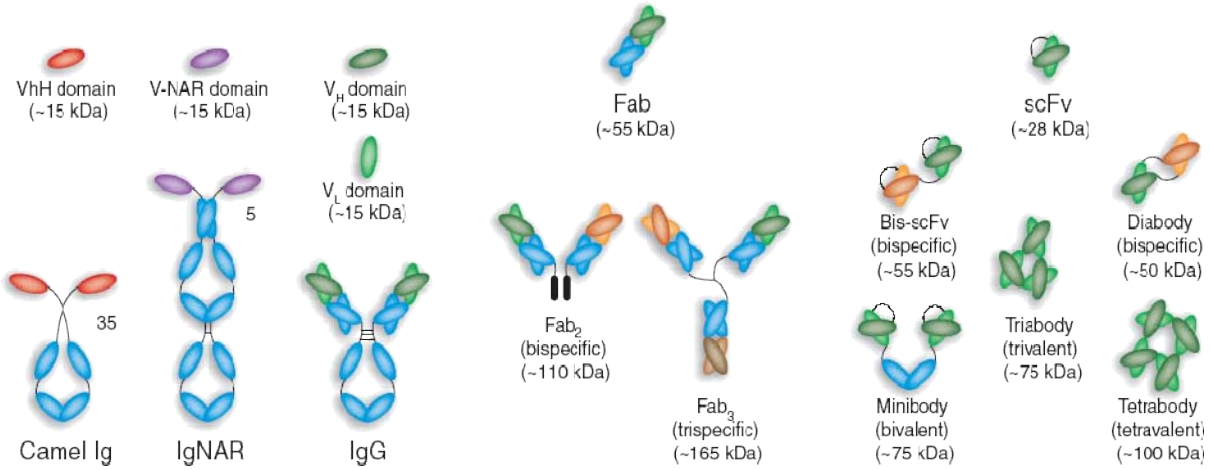

Figure 2-2: Multitude of antibody fragment formats (Holliger and Hudson, 2005)

Genetic improvements resulted in novel antibody fragments with new characteristics such as enhanced specificity, therapeutic efficacy, and stability (Schmitz *et al.*, 2000). Overall, two trends could be recognized in the current development of novel formats: firstly, the reduction of size, and secondly, the improvement of the antibody properties. An example for size reduction is the camelide heavy-chain antibody (V_{HH}) which has a molecular weight of 15 kDa (Joosten *et al.*, 2003). An example for enhancing antibodies' properties is the Fusion protein technology: functional proteins are fused to antigen-binding domains. In antibody-enzyme prodrug therapy (AEPDT) conjugations of antigen-binding domains to enzymes are used for *in situ* conversion of a prodrug to its active form. By arming antibodies with drugs, toxins and radionuclides, potent therapeutic cancer agents were generated (Senter and Springer, 2001).

2.1.1 Single-chain fragment (scFv)

A single-chain fragment (scFv) has a molecular weight of about 30 kDa and consists of the V_H and the V_L domain, which are fused by a short and flexible peptide linker (also see **Chapter 2.2.5**) (Huston *et al.*, 1988). Either V_L or V_H can be the NH_2-terminal domain resulting in V_H-linker-V_L or V_L-linker-V_H orientation (Huston *et al.*, 1988) (**Figure 2-3**). Desplancq *et al.* reported that significantly better binding affinities were observed with the V_L-linker-V_H construction independent from linker length (Desplancq *et al.*, 1994). This observation suggests that structural features of the V_H-linker-V_L construction interfere with antigen binding.

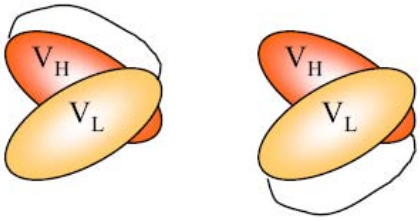

Figure 2-3: Structure of single-chain fragment with V_H-linker-V_L or V_L-linker-V_H orientation (Joosten *et al.*, 2003)

When reducing the length of the linker peptide the formation of diabodies (60 kDa), triabodies (90 kDa) and tetrabodies (120 kDa) were previously observed (Dolezal *et al.*, 2003). Diabodies have two functional antigen-binding domains that can be the same (bivalent diabodies) or specific for distinct antigens (bispecific diabodies) (Joosten *et al.*, 2003). Multivalent Fv formation is indirectly proportional to linker length (Essig *et al.*, 1993). Bispecific antibodies are used for example for the recruitment of novel effector functions (e.g. cytotoxic T cells) to the target cells making them a powerful tool in medicine (Joosten *et al.*, 2003).

2.1.2 Antigen-binding fragment (Fab)

The antigen-binding fragment (Fab) is a heterodimeric and monovalent antibody fragment. With a molecular weight of about 50 kDa it is approximately double the size of the corresponding scFv fragment and consists of the N-terminal variable and constant domain of heavy (V_H and C_H1) and light (V_L and C_L) chain (**Figure 2-4**).

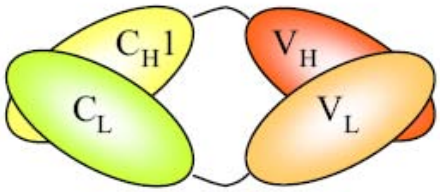

Figure 2-4: Structure of antigen-binding fragment (Joosten *et al.*, 2003)

For the assembly of the two polypeptide chains, the formation of at least one interchain-disulfide bond is required. This disulfide bond has a major contribution to interchain-binding energy and therefore to Fab stability. In fact, Fab fragments show enhanced stability when being compared to the corresponding scFv fragments which lack interchain-disulfide bonds. Additionally, Röthlisberger and coworkers proved that constant domains strongly contribute to antibody stability – even without disulfide-linkage (Röthlisberger *et al.*, 2005).

2.1.3 Therapeutic monoclonal antibody H6

The monoclonal antibody H6 (mAb/H6) belongs to a group of anti-idiotypic antibodies directed against HIV1-neutralizing antibodies (Kunert *et al.*, 2002). Monoclonal Ab/H6 mimics the natural epitope – a sequence of 6 amino acids (ELDKWA-motif) – of the HIV1-neutralizing antibody 2F5. The ELDKWA-motif is found on the HIV1-envelope protein gp41 (Muster *et al.*, 1993). During viral infection, the sequence usually maintains conserved, but is cryptic to the human immune system. In competitive ELISA experiments, it was demonstrated that H6 antibodies significantly diminished the binding capability of the HIV1-neutralizing antibody 2F5 to a synthetic ELDKWA peptide as well as to HIV1-envelope protein. Immunization experiments could further reveal a specific and neutralizing immune reaction in mice (Kunert *et al.*, 2002).

For these reasons, H6 is a promising vaccine candidate against human HIV1-virus and an interesting model protein.

2.1.4 Therapeutic monoclonal antibody B1

The monoclonal antibody B1 (mAb/B1) is a humanized antibody with a short half life directed against the 'fibroblast activation protein alpha' (FAPα). The invasive growth of epithelial cancers is associated with the induction of FAPα, a cell surface molecule of reactive stromal fibroblasts (Garin-Chesa *et al.*, 1990). The FAPα antigen is selectively expressed in the stroma of a range of epithelial carcinomas, independent of location and histological type. Therefore, a FAPα-targeting concept has been developed for imaging, diagnosis and treatment of epithelial cancers. FAPα is not only expressed in the stroma of epithelial carcinomas but also in the stroma of solid tumors, sarcomas, and leukemias.

Therapeutic agents such as radioisotopes, toxins, and enzymes can be conjugated to the monoclonal Ab/B1. The preferred therapeutic agents are β-emitting radioisotopes which achieve localized irradiation and destruction of malignant tumor cells. The most preferred radioisotope is ^{131}Iodine. Thereby, a highly specific tool for targeting therapeutic agents to the FAPα antigen is provided. (WO 02/083171).

2.1.5 Therapeutic monoclonal antibody A4

The monoclonal antibody A4 (mAb/A4) is a humanized version of the murine monoclonal antibody VFF-18. For mAb/A4 construction, the complementary determining regions (CDRs) of VFF-18 were grafted in a complete human framework including the constant regions. The mAb/A4 has a favorable biodistribution and tumor uptake *in vivo*. Monoclonal Ab/A4 is directed against a sequence of 11 amino acids (WFGNRWHEGYR-motif) located in the domain v6 of the human CD44 protein (CD44v6). CD44 is a protein which is expressed in several different isoforms on the surface of a wide variety of cell types. The smallest isoform – standard CD44 (CD44s) – is thought to mediate cell attachment to extracellular matrix components. It may also transmit a co-stimulus in lymphocyte and monocyte activation. In

contrast, expression of splice variants of CD44 which contain the domain v6 (CD44v6) in the extracellular region is restricted to a certain group of epithelia. CD44v6 has been shown to be a tumor-associated antigen with a favorable expression pattern in human tumors and normal tissues. Its physiological role is not yet fully understood, however (Heider *et al.*, 1995; Heider *et al.*, 1996). It has been utilized to antibody-based diagnostic and therapeutic approaches, especially in radioimmunotherapy of tumors (Stroomer *et al.*, 2000; Verel *et al.*, 2002).

Monoclonal Ab/A4 can be covalently linked to the cytotoxic antibiotic maytansinoid via a disulfide linkage to form an antibody conjugate. Therefore, local concentrations of the antibiotic can be elevated by targeted delivery to the tumor (WO 02/094325).

2.2 Protein expression in *E. coli*

Escherichia coli represents the most frequently used prokaryotic expression system for mAbs and especially their fragments (recently reviewed by Arbabi-Ghahroudi *et al.*, 2005). Large numbers of proteins have successfully been produced in *E. coli* (Baneyx, 1999) among them several antibody fragments (Donovan *et al.*, 2000). The advantage of this system is the vast knowledge of its genetic manipulation and cultivation. Besides, *E. coli* requires simple and inexpensive media for rapid growth and it can easily be cultured in fermenters for large scale production of target proteins (Joosten *et al.*, 2003). Furthermore, high-level expression under control of inducible promoters such as the T7 promoter is feasible (Studier *et al.*, 1990). In shake-flask cultures, general production yields are low (several mg/L). However, in fermentation processes several g/L can be obtained (Harrison and Keshavarz-Moore, 1996).

Overall, there are two possibilities of recombinant protein production in *E. coli*: Firstly, recombinant proteins can be secreted into the culture medium or to the oxidizing milieu of the periplasmic space. Secondly, they can accumulate in the reducing milieu of the cytoplasm where they usually form insoluble high-density aggregates called inclusion bodies (IBs) (Skerra, 1993). Both ways of expression provide several advantages and disadvantages: The translocation to the periplasmic space assures proper folding and disulfide bond formation, less host-cell protein in the starting material for purification (Jonasson *et al.*, 2002) and an improved authenticity of the N-terminus of the target protein (Makrides, 1996). Translocation

to the culture medium provides the least level of proteolysis (Nakamura *et al.*, 1992) as well as improved protein folding and least host-cell protein in the starting material for purification (Makrides, 1996). However, secretion into the medium or periplasmic space is often accompanied with cell lysis and subsequent product loss (Plückthun, 1994). Cytoplasmic expression is mainly preferable because of its high expression yields. Disadvantages are demands of laborious and cost-intensive *in vitro* refolding (denaturation and renaturation) and purification steps (Rudolph and Lilie, 1996). This can lead to a final yield of only a small percentage of the protein that was initially present in the inclusion bodies. However, in some cases product constitutes up to 30% of total cellular protein when being expressed as IB which can countervail the time-consuming and expensive refolding efforts. Cytoplasmic expression of antibody fragments in *E. coli* is particularly difficult when the expressed domains require disulfide linkage (Martineau *et al.*, 1998). Visintin *et al.* demonstrated that antibody fragments which were expressed in the cytoplasm do not form disulfide bonds and were therefore significantly less stable (Visintin *et al.*, 1999). In some cases, disulfide bonds were formed despite of the reducing conditions in the cytosol (Miele *et al.*, 1990). An interesting alternative is the production of soluble and functional scFv by *E. coli* in the cytosol. This was achieved by improving cytosolic disulfide bond formation activity, employing mutants and overexpression of disulfide-bond isomerase (Jurado *et al.*, 2002). Another option is the utilization of the reduction-deficient *E. coli* strain FA113. The strain is deficient for the genes of the thioredoxin- and the glutathione reductase which lead to oxidizing conditions in the bacterial cytosol. High levels of functional Fab using FA113 as expression system could be achieved (Venturi *et al.*, 2002). A review about soluble expression of recombinant proteins in the cytoplasm of *E. coli* was recently published by Sorensen and Mortensen (Sorensen and Mortensen, 2005).

2.2.1 Inclusion body (IB) formation

In the majority of cases, cytoplasmic overproduction of recombinant proteins in *E. coli* results in the formation of inclusion bodies (IBs) which correlates to several parameters: Charge average, turn-forming residue fraction, hydrophilicity, the amino acid sequence, and the total number of residues of the protein (Wilkinson and Harrison, 1991). IB formation is also influenced by alteration of cultivation parameters such as lower temperatures (Cabilly, 1989;

Shirano and Shibata, 1990; Schein, 1993), osmotic stress (Blackwell and Horgan, 1991), or different pH (Makrides, 1996). In terms of recombinant protein production, IB formation mostly occurs in response to the cultivation parameters, the expression-level, the redox-potential and the chaperone availability (Carrio and Villaverde, 2001).

IBs are inactive, densely-packaged aggregates of pure and homogeneous target protein (Singh *et al.*, 2005). IB formation is a result of specific aggregation between folding intermediates of the recombinant protein molecules (Speed *et al.*, 1996). Therefore, target proteins in IBs already exist at an intermediate stage of the protein folding pathway with a considerable amount of native-like secondary structure (Bowden *et al.*, 1991; Przybycien *et al.*, 1994; Speed *et al.*, 1996). If recombinant proteins from IBs can be solubilized without disturbing its existing native-like secondary structure, the overall yield of the bioactive target proteins from IBs can be significantly higher (Oberg *et al.*, 1994). This fact is especially interesting for downstream issues, where one wants to obtain as much bioactive target proteins from IBs as possible. This can be achieved by optimizing pH and the use of different solubilizing agents in the presence of low concentrations of denaturants.

2.2.2 Influence of host strains on protein expression

Heterologous protein expression in *E. coli* can be associated with several problems concerning expression itself as well as downstream relevant issues (Terpe, 2006): **(i)** Target protein may or may not form inclusion bodies affecting the purification process. **(ii)** It might not be folded correctly leading to the loss of activity. **(iii)** It may be degraded by host proteases during cell disruption. **(iv)** It may not be expressed at all. **(v)** It can lead to growth inhibition or cell death. All theses issues directly influence final product yield. Problems regarding growth inhibition or cell death caused by target protein expression are usually approached by optimizing promoter systems (lower basal expression; tightly controlled promoter systems) or cultivation conditions (lower temperature; lower inducer concentrations).

Otherwise, numerous genetically engineered strains have been developed to overcome other problems: E.g. inclusion body formation of cytosolic expressed membrane proteins is avoided by using *E. coli* strains C41(DE3) or C43(DE3) (Miroux and Walker, 1996). In these two

strains, heterologous protein expression is accompanied by the proliferation of intracellular membranes causing the absence of inclusion bodies (Arechaga *et al.*, 2000).

In other cases incorrect protein folding and subsequent inclusion body formation can be prevented by applying thioredoxin reductase and glutaredoxin deficient strains. The *E. coli* Origami strains (Novagen, Germany) lack the *trxB* and *gor* genes encoding the enzymes which reduce cysteines in the cytoplasm. The reductases therefore prevent disulfide bond depending folding in the cytoplasm (Sorensen and Mortensen, 2005). By fusing the target protein to thioredoxin in strains lacking the *trxB* gene, disulfide bond formation and folding of the target protein can be increased (Stewart *et al.*, 1998). Disulfide bond formation can further be enhanced by overexpressing the periplasmic foldase DsbC in the cytoplasm (Bessette *et al.*, 1999).

In case of intracellular or periplasmic target protein expression, cell disruption has to be applied to obtain the desired protein. During cell disruption, *E. coli* strains such as Strain A release proteases into cell debris that are capable of protein degradation. Consequently, this can lead to lower product yields. The B strain *E. coli* Strain B lacks the *lon* protease and the *ompT* outer membrane protease providing the advantage of less target protein degradation during purification (Campbell *et al.*, 1978).

Differences in the codon usage between the target protein's strain of origin and the *E. coli* expression host can attenuate or even inhibit the translation of a recombinant protein. This is caused by the demand for tRNAs recognising non-host-codons during translation. These tRNAs are rare or lacking in the expression host (Goldman *et al.*, 1995; Kane, 1995). The *E. coli* Rosetta strains (Novagen, Germany) supply rare tRNAs for AGG, AGA, AUA, CUA, CCC, and GGA codons on a plasmid, enhancing the expression of the mentioned codons. For further information of the influence of codon usage on protein expression see **Chapter 2.2.3.**

2.2.3 Influence of codon usage on protein expression

It is well-known that all amino acids except for methionine and tryptophan are encoded by more than one codon. Each organism has its own bias in the use of the 61 available amino acid codons (Terpe, 2006). As described by Makrides, following observations for codon usage

patterns in *E. coli were* made (Makrides, 1996): **(i)** There is a bias for one or two codons for almost all degenerate codon families. **(ii)** Certain codons are most frequently used by all different genes irrespective of the abundance of the protein; for example, CCG is the preferred triplet encoding proline. **(iii)** Highly expressed genes exhibit a greater degree of codon bias than do poorly expressed ones. **(iv)** The frequency of use of synonymous codons usually reflects the abundance of their cognate tRNAs. Consequently, when rarely used codons for heterologous gene expression in *E. coli* are applied, poor expression rates may result. In addition, the codon context of specific genes not only affects the quantity but also the quality of protein levels (Makrides, 1996).

Several strategies to overcome poor expression rates have been investigated: in theory, lowering the cultivation temperature and therefore the growth rate as well as changing media composition might shift the codon usage bias enough to avoid codon-usage based expression problems (Terpe, 2006). However, Dong *et al.* reported that different growth rates do not affect levels of most tRNA isoacceptors corresponding to rare codons (Dong *et al.*, 1996). Other studies showed that heterologous protein expression in *E. coli* can be enhanced by replacing low-usage codons with high-usage codons (Buell *et al.*, 1985; Hernan *et al.*, 1992) or by coexpressing ‘rare’ tRNA genes in the host (Brinkmann *et al.*, 1989; Del, Jr. *et al.*, 1995). *E. coli* strains such as the Rosetta strains (Novagen, Germany; see **Chapter 2.2.2**) supply sufficient ‘rare’ tRNAs and therefore significantly enhance heterologous protein expression when low-usage codons are applied.

2.2.4 Influence of temperature on protein expression

Low cultivation temperatures generally decrease growth rates and protein synthesis in *E. coli*. This affects recombinant protein expression as well which is usually lower at low cultivation temperatures compared to standard cultivation temperatures at 37°C. A sudden decrease in cultivation temperature inhibits replication, transcription, and translation (Shaw and Ingraham, 1967). The expression efficiency of promoters routinely used in vectors for recombinant protein expression are negatively affected as well by a sudden temperature decrease (Vasina and Baneyx, 1996). Similar transcriptional effects have been observed by

applying weak promoters or partially induced strong promoters resulting in higher amounts of soluble protein (Weickert *et al.*, 1996).

The aggregation reaction is generally favored at higher temperatures as it is determined by strongly temperature dependent hydrophobic interactions (Kiefhaber *et al.*, 1991). Increasing the expression temperature therefore favors the formation of the insoluble protein fraction (IBs). It is well known that increasing the cultivation temperature increases growth rates as well as protein synthesis rates.

Reducing the cultivation temperature in order to limit the *in vivo* aggregation of recombinant proteins is routinely applied (Schein, 1989). This strategy has been persued to improve the solubility of many different proteins such as human interferon α-2, subtilisin E, ricin A chain, bacterial luciferase, Fab fragments, β-lactamase, rice lipoxygenase L-2, soybean lypoxigenase L-1, kanamycin nuclotidyltransferase, and rabbit muscle glycogen phosphorylase (Vasina and Baneyx, 1997).

By lowering the cultivation temperature, heat shock proteases that are induced under overexpression conditions are partially eliminated (Chesshyre and Hipkiss, 1989). In addition, lowering the cultivation temperature to 30°C increases the expression level and activity of a number of *E. coli* chaperones which assist correct protein folding (Mogk *et al.*, 2002; Ferrer *et al.*, 2003). Within the *E. coli* cytoplasm, oxidation occurs post-translationally and is favored at low temperatures (Schneider *et al.*, 1997). Consequently, disulfide-bonded recombinant proteins can accumulate in the cytoplasm of *E. coli trxB* mutants that lack thioredoxin reductase, a protein responsible for the reduction of oxidized thioredoxins (Aslund and Beckwith, 1999).

2.2.5 Influence of linker sequence on protein expression

In a scFv fragment a short, hydrophilic, and flexible peptide linker bridges the approximately 3.5 nm to 4.0 nm gap between the C and N termini of the V_H and the V_L domains (Huston *et al.*, 1991; Raag and Whitlow, 1995; Joosten *et al.*, 2003). Considering the 0.38 nm distance between adjacent peptide bonds, a linker should comprise a minimum of 10 amino acids (Huston *et al.*, 1991). Linkers of about 15 amino acids in length are commonly applied in

order to avoid conformational strain caused by too short linkages as well as avoiding steric interference with the variable domains resulting from an excessively long peptide (Huston *et al.*, 1991; Joosten *et al.*, 2003).

Linker length influences several properties: **(i)** scFv fragments comprising linkers shorter than 15 amino acids tend to aggregate (Whitlow *et al.*, 1993; Desplancq *et al.*, 1994; Raag and Whitlow, 1995). **(ii)** Additionally, scFv fragments comprising linkers with less than 15 residues tend to form dimers (diabodies), trimers (triabodies), and higher oligomers (see **Figure 2-2** and **Chapter 2.1.1**) (Desplancq *et al.*, 1994; Atwell *et al.*, 1999). **(iii)** Excessively long linkers with 47, 51, and 59 residues show increasing precipitation characteristics possibly caused by scFv fragment cross-folding (Robinson and Sauer, 1998). **(iv)** As determined by urea denaturation studies thermodynamic stability of scFv fragments comprising linkers from 9 to 19 residues increases with increasing linker length reaching a maximum at 19 residues (Robinson and Sauer, 1998). Linkers with more than 19 residues show decreased thermodynamic stability (Robinson and Sauer, 1998). **(v)** Linker length does not significantly influence the binding affinity of a scFv fragment causing a certain degree of flexibility when choosing the linker's length (Condra *et al.*, 1990). However, within a 12- to 18-residue linker range, scFvs with longer linkers tend to have higher binding affinities for their respective antigen (Whitlow *et al.*, 1993).

The solubility of scFv fragments varies to a great extend with altering linker lengths and sequences (Tang *et al.*, 1996). An overabundance of polar residues within the linker sequence favors scFv fragments' solubility and avoids intercalation within or between variable domains during scFv fragment folding (Huston *et al.*, 1991). Studies with scFv fragments comprising different linkers show that expression levels (Tang *et al.*, 1996) as well as folding properties are mainly influenced by the domains and not by the linkers (Hennecke *et al.*, 1998). Therefore, the linker peptide is considered as a 'passive connector' (Hennecke *et al.*, 1998).

The influence of following linkers on product yield, inclusion body quality, and intracellular scFv fragment solubility was investigated in this work (amino acid sequences are listed in **Table 3-2**):

2.2.5.1 $(Gly_4Ser)_3$ Linker

The serine residues add hydrophilicity to the linker (Huston *et al.*, 1991) whereas the glycine residues account for linker flexibility (Ramachandran *et al.*, 1963; Ramachandran *et al.*, 1966). The $(Gly_4Ser)_3$ linker has been used most frequently (Huston *et al.*, 1993) and has proven to yield scFv fragments with binding affinities comparable to the parental antibody (Tai *et al.*, 1990; Glockshuber *et al.*, 1990; Skerra *et al.*, 1991).

2.2.5.2 Extended Linker (E-Linker)

There are indications that the linker sequence does not have to compare with the fidelity of a scFv fragment binding site in order to produce functional scFv fragments (Huston *et al.*, 1991). Therefore, some flexibility exists in linker design (see **Chapter 2.2.5**). A linker with 20 amino acids was chosen comprising four repetitions of a slightly varied Gly_4Ser-motive. It was called ‘Extended Linker’ (E-Linker).

2.2.5.3 Linker 218 (L218)

To enhance linker solubility additional amino acids with charged residues such as glutamic acid or lysine were interspersed into serine and glycine rich linkers (Huston *et al.*, 1988; Bird *et al.*, 1988; Whitlow *et al.*, 1993). It is well known that most proteases cannot cleave peptide bonds at the N-terminus of proline. Therefore, an additional proline was introduced into the newly designed linker to increase proteolytic stability (Whitlow *et al.*, 1993). This new linker was named L218 and showed reduced aggregation, improved protease resistance, higher antigen binding affinity, and higher tumor uptake rates than other linkers (Whitlow *et al.*, 1993).

2.2.5.4 Linker 205 (L205)

Contrary to rather flexible linker sequences of glycine and serine rich linkers, Pantoliano *et al.* used a 25 residues long linker containing several repeats of the pentapeptide sequence Asp-

Asp-X-Lys-Lys (Pantoliano *et al.*, 1991). This sequence introduces a helical structure into the linker with possible stabilizing effects (Pantoliano *et al.*, 1991; Whitlow *et al.*, 1993). It contains an overabundance of relatively hydrophilic residues (Pantoliano *et al.*, 1991). The linker was named L205.

2.2.5.5 LLB18

In contrast to the $(Gly_4Ser)_3$ linker, Hennecke *et al.* constructed non-repetitive linkers which contained genes less susceptible to deletions in PCR-based gene assembly and directed evolution experiments (Hennecke *et al.*, 1998). One of these linkers was the LLB18 which is 4 residues longer than the $(Gly_4Ser)_3$ linker favoring monomer-formation (Desplancq *et al.*, 1994). To assist correct scFv fragment folding it has a type I β-turn encoded by the sequence Ser-Pro-Asn-Gly at the beginning (Wilmot and Thornton, 1988; Hennecke *et al.*, 1998) and a type II' β-turn encoded by the sequence Ser-Gly-Ser-Gln at the end (Mattos *et al.*, 1994; Hennecke *et al.*, 1998). Additionally, it has several charged and polar residues between the β-turns favoring solubility (Hennecke *et al.*, 1998).

3. Materials and Methods

Shake-flask cultures were performed in orbital shakers (INFORS® Multitron II, Switzerland).

3.1 Bacterial strains

Expression strains are listed in **Table 3-1**. All strains and vectors were purchased from Novagen (Germany).

Table 3-1: Overview of used *E. coli* host strains

Host strain	Type	Expression system	Genotype
Strain B	B strain	T7	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$
Strain A	K12 strain	T7	F- recA1 hsdR(rK12- mK12+) (DE3) (Rif R)

3.2 Expression vectors

All genes were synthesized by GENEART AG (Germany) and delivered in pGA4 vectors. As resistance marker the pGA4 vectors contained the amp^r gene conferring ampicillin resistance. The gene of interest was provided with *Nde*I and *Sal*I restriction sites, in order to clone it in frame with the start codon. Original codon usage may be the reason for poor protein expression of antibody fragments in *E. coli* (Buell *et al.*, 1985; Hernan *et al.*, 1992). Therefore, only codon optimized gene sequences were employed. For expressing antibody fragments, IPTG inducible pET30a vectors comprising the T7 expression system (Studier *et al.*, 1990) were used. As a resistance marker the pET30a vectors contained the kan^r gene conferring kanamycin resistance.

3.2.1 Cloning

For amplification, plasmids were chemically transformed in *E. coli*. Subsequently, the cells were grown in LB medium containing the appropriate antibiotic. Plasmid purification was performed using Qiagen® Plasmid Midi Kits (QIAGEN, Germany). Subcloning from pGA4 to pET30a vectors was done using standard cloning procedures, illustrated in **Figure 3-1**.

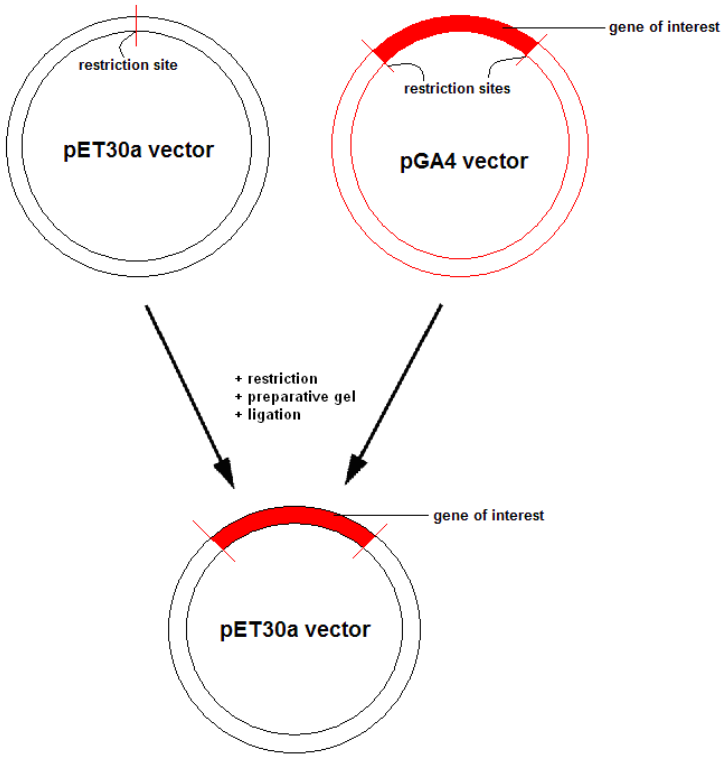

Figure 3-1: Subcloning of gene of interest from pGA4 to pET30a vectors

3.3 Model proteins

For in silico analysis of genes and proteins, the software Vector NTI® (Invitrogen, USA) was used. Gene synthesis and codon optimization was done by GENEART AG (Germany) using Gene Optimizer™ software. Model proteins were derived from mAb B1 and A4 (Boehringer Ingelheim Pharma, Germany) and mAb H6 (Polymun Scientific, Austria). B1, A4, and H6 were expressed as scFv fragments. Additionally, A4 was expressed as Fab fragment.

The scFv fragments were constructed in V_L-linker-V_H orientation. For all scFvs, the standard $(Gly_4Ser)_3$ linker was used. Moreover, different linkers (**Table 3-2**) were inserted into the existing B1 scFv fragment in order to investigate the linker peptides' influence on cytoplasmic scFv solubility. **Table 3-3** gives an overview of all used scFv fragments.

Table 3-2: Overview of linkers used for B1 scFv fragment

Linker	Amino acid sequence	Source	Approx. Mr [kDa]	Theoretical pI
$(Gly_4Ser)_3$	GGGGS GGGGS GGGGS	(Huston *et al.*, 1988)	0.96	5.52
E-Linker	GGGGS GGGGS GGGGS SGGGS	extended motive of $(Gly_4Ser)_3$	1.31	5.52
L205	SSADD AKKDA AKKDD AKKDD AKKDG	(Pantoliano *et al.*, 1991)	2.62	5.97
L218	GSTSG SGKPG SGEGS TKG	(Whitlow *et al.*, 1993)	1.54	8.59
LLB18	SPNGA SHSSS ASQTG SASGS Q	(Hennecke *et al.*, 1998)	1.89	6.46

Table 3-3: Physicochemical properties of scFv fragments

Molecule	Linker	Expression host	Approx. Mr [kDa]	Theoretical pI
B1 scFv	$(Gly_4Ser)_3$	*E.coli* Strain B and Strain A	27.60	9.06
A4 scFv			24.88	8.35
H6 scFv			26.01	4.99
B1 scFv	E-Linker	Strain A	27.86	9.06
	L205		29.17	8.97
	L218		28.08	9.14
	LLB18		28.44	9.06

The heavy and light chains of A4 Fab fragment were expressed separately from each other in *E. coli* Strain A (**Table 3-4**).

Table 3-4: Physicochemical properties of A4 Fab fragment

Molecule	Chain of Fab	Approx. Mr [kDa]	Theoretical pI
A4_Fab_LC_bacMiG	LC	23.14	5.74
A4_Fab_HC_bacMiG	HC	23.54	9.05

3.4 Preparation, purification, and modification of DNA

3.4.1 Preparation of plasmid DNA

Plasmid preparations usually comprise three steps:

- plasmid amplification by bacterial growth
- harvest and lysis of bacteria
- purification of plasmid DNA

Bacterial lysis can be accomplished by mechanical, thermal, or alkaline lysis. An appropriate lysis method is chosen depending on plasmid size, utilized bacterial strain, and the method of plasmid purification. In this work alkaline lysis according to (Birnboim and Doly, 1979) was utilized. The plasmid DNA was purified by applying potassium acetate precipitation and ion exchange chromatography,

3.4.1.1 Miniprep

A miniprep is a molecular biological method to extract plasmid DNA (pDNA) from bacteria. For miniprep, the QIAprep® Spin Miniprep Kit (QIAGEN, Germany) was used (up to 20 µg pDNA).

Table 3-5: Reagents used for miniprep

Reagent	Composition
Buffer P1 (resuspension buffer)	50 mM Tris Cl, pH 8.0
	10 mM EDTA
	100 µg/mL RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer N3 (neutralizing buffer)	Contains guanidine hydrochloride and acetic acid
	Puffer composition not published
Buffer PE (washing buffer)	Contains ethanol
	Puffer composition not published
Molecular biology grade water	–

Procedure

Bacteria were grown on LB agar plates containing the appropriate antibiotic. After incubation over night at 37°C cells were homogeneously resuspended in 1.5 mL reaction tubes containing 250 µL buffer P1 and LyseBlue. 250 µL buffer P2 were added and the reaction tubes were inverted carefully until the suspensions turned blue. Afterwards, 350 µL of buffer N3 were added and the reaction tubes were inverted until the suspensions turned colorless. The suspensions were centrifuged for 10 min at 13.000 rpm and room temperature. The supernatants were immediately applied to QIAprep spin columns plugged into a 2 mL reaction tube. The suspensions were centrifuged for 1 min at 13.000 rpm and room temperature and flow-through was discarded. To wash the columns 0.75 mL buffer PE were applied on the columns and centrifuged at the same conditions. To dry the columns, they were centrifuged again at the same conditions. Afterwards, the columns were placed in sterile 1.5 mL reaction tubes. The DNA was eluted with 30-50 µL molecular biology grade water (Eppendorf, Germany) and eluted by gravity flow. Afterwards, the columns were centrifuged for 1 min. The eluate contained the purified plasmid DNA.

3.4.1.2 Midiprep

A midiprep is a molecular biological method to extract pDNA from bacteria. For midiprep, the Qiagen® Plasmid Midi Kit (QIAGEN, Germany) was used (up to 100 µg pDNA).

Table 3-6: Reagents used for midiprep

Reagent	Composition
Buffer P1 (resuspension buffer)	50 mM Tris Cl, pH 8.0
	10 mM EDTA
	100 µg/mL RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)
Buffer P3 (neutralizing buffer)	3.0 M potassium acetate; pH 5.5
Buffer QBT (equilibration buffer)	750 mM NaCl
	50 mM MOPS, pH 7.0
	15% isopropanol (v/v)
	0.15% Triton® X-100 (v/v)
Buffer QC	1.0 M NaCl

(washing buffer)	50 mM MOPS, pH 7.0
	15% isopropanol (v/v)
Buffer QC (elution buffer)	1.25 M NaCl
	50 mM Tris Cl, pH 8.5
	15% isopropanol (v/v)
100% isopropanol	–
70% ethanol (v/v)	–
Molecular biology grade water	–

Procedure

Bacterial culture, harvest, lysis, and lysate clearing

Bacteria were grown in 1 L shaking flasks in 100 mL of LB-medium containing the appropriate antibiotic. After incubation over night at 37°C the cell suspensions were centrifuged at 6000 rpm and 4°C for 15 min. The bacterial pellets were resuspended in 4 mL buffer P1 containing LyseBlue. Afterwards, 4 mL buffer P2 were added and the reaction tubes were inverted carefully until the suspensions turned blue. The suspensions were incubated at room temperature for 5 min. Then, 4 mL of buffer P3 were added and the reaction tubes were inverted until the suspensions turned colorless. The reaction tubes were incubated on ice for 15 min and centrifuged for 45 min at 10.000 rpm and 4°C.

Binding and washing of plasmid DNA on QIAGEN-tips

For equilibration of QIAGEN-tips 100, 4 mL of buffer QBT were applied on the tips and the columns were allowed to empty by gravity flow. The discharges were discarded. The supernatants from centrifugation were applied on the QIAGEN-tips and allowed to enter the resins by gravity flow. The discharges were discarded. The tips were washed with 2 x 10 mL buffer QC. Buffer QC was allowed to move through the QIAGEN-tips by gravity flow and the discharges were discarded.

Eluting, precipitating, washing, and redissolving of plasmid DNA

Plasmid DNA was eluted with 5 mL buffer QF into clean 15 mL vessels and precipitated by adding 3.5 mL room tempered isopropanol (Merck, Germany). The suspensions were mixed and centrifuged at 10.000 rpm and 4°C for 30 min. Supernatants were decanted carefully and

the pellets were washed with 2 mL room tempered 70% ethanol (Merck, Germany). The suspensions were centrifuged at 10.000 rpm and 4°C for 10 min. Supernatants were discarded carefully and the pellets were air dried at 37°C for 15 min. The pellets were resuspended in 20-100 µL molecular biology grade water (Eppendorf, Germany).

3.4.2 Purification of plasmid DNA

3.4.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) is a method to separate DNA or RNA molecules by their size. This is achieved by the principle that the negatively charged DNA moves in electric fields towards the cathode. Consequently, molecules migrate through the agarose pores and can be separated by their size. Shorter molecules migrate faster than longer ones. 1% gels are routinely used for DNA molecules in a range from 100 bp to 20 kbp. Increasing the agarose concentration of a gel increases the resolution and therefore molecules with similar size can be separated. For visualization in gels, fluorescent dyes, such as ethidium bromide or SYBR Gold that interact with the double stranded DNA can be used. For analytical purposes standard agarose was used. For preparative AGE agarose with a lower melting point was used. The lower melting point facilitates DNA extraction after AGE. For details of electrophoresis conditions see **Table 3-10**. In order to perform agarose gel electrophoresis, Sub-Cell® GT Agarose Gel Electrophoresis System (Bio-Rad, USA) was utilized.

Table 3-7: Reagents used for agarose gel electrophoresis

Reagent	Composition
10x TBE buffer	108.0 g/L Tris(hydroxymethyl)aminomethan (Merck, Germany)
	55.0 g/L boric acid (Merck, Germany)
	9.3 g/L EDTA (Serva, Germany)
1x TBE buffer	Dilution of 10x TBE buffer 1:10
Agarose (Biozym scientific GmbH, Germany)	For size determination of DNA fragments and restriction control, standard agarose was used (Biozym LE agarose). For preparative gels, low melting point agarose was used (Biozym plaque agarose).
DEPC-treated water (Fermentas)	Diethylpyrocarbonate (DEPC) inactivates Rnase enzymes from water by covalent modifications of histidin residues.
SYBR® gold	Nucleic acid gel stain (Invitrogen, USA)
Bromphenol blue	0.05 g bromphenol blue (Merck, Germany)
	50 mM EDTA (Serva, Germany)

	25.0 g Glycerin (Merck, Germany)
	Addition of TBE buffer to a final volume of 50 mL
Direct load™ PCR 100 bp low ladder (Sigma, USA)	–
DNA ladder, 1 KB (Sigma, USA)	–

Procedure

Gel preparation

Mini gels were prepared for up to 8 samples, regular gels were prepared for up to 15 samples. For further details see **Table 3-8**.

Table 3-8: Gel preparation for agarose gel electrophoresis

	Mini gel	**Regular gel**
Wells per comb	8	15
1x TBE buffer [mL]	50	200
Agarose [g]	0.4	1.6
SYBR® Gold [µL]	5	20

0.8% (w/v) of agarose were weighed in a shaking flask and filled up with 1x TBE buffer. The suspension was mixed thoroughly by panning the shaking flask and heating in a microwave (Inno-Wave, Siemens, Germany) at 900 W until the agarose was completely dissolved. Afterwards, the solution was cooled down to about 55°C. At this temperature the appropriate amount of SYBR® Gold was added to the solution and mixed thoroughly. A comb was inserted into a gel tray and the gel tray was placed into a gel caster. Afterwards, the prepared agarose solution was casted into the tray until the gel had a thickness of 5-7 mm. Air bubbles in the gel were removed with a pipette tip. The gel could polymerize for 30-40 min. Then the comb was carefully removed from the polymerized gel and the gel was placed in the electrophoresis unit. The unit was filled up with 1x TBE buffer until the gel was completely covered with buffer.

Sample preparation

The samples were diluted with DEPC-treated water so that the amount of DNA loaded per well was about 40-100 ng. Afterwards, samples were mixed with bromphenol blue according to **Table 3-9**.

Table 3-9: Sample preparation for agarose gel electrophoresis

	Mini gel	**Regular gel**
Bromphenol blue added to samples [µL]	4	6
Sample loaded on gel [µL]	20	30
Bromphenol blue added to DNA Ladder [µL]	1	1

The solutions were mixed thoroughly and shortly centrifuged. DNA ladders were used for size determination. **Figure 3-2** shows the sizes of the DNA ladders' fragments.

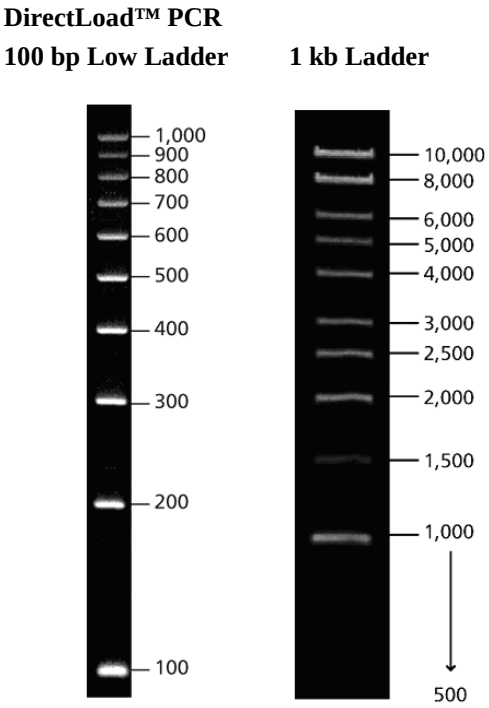

Figure 3-2: DNA ladders (SIGMA, USA) used for size determination in AGE

Sample loading and electrophoresis

As listed in **Table 3-10** the appropriate amounts of DNA ladder and samples were loaded on the gel close to the cathode. Finally, the lid of the electrophoresis unit was placed onto the unit

with correct anode-cathode orientation so that the DNA could migrate towards the anode. Afterwards, electrophoresis was carried out with the conditions listed in **Table 3-10**.

Table 3-10: Sample loading and electrophoresis conditions

	Mini gel	**Regular gel**
DNA Ladder loaded per well [µL]	6	6
Samples loaded per well [µL]	10-20	20-50
Electrophoresis conditions	60 V, 60-100 min	60 V, 100-180 min

Analysis

After electrophoresis the gels were transferred to the UV-transilluminator. The gels were analyzed with the Gel Documentation System (Gel Doc System) from Bio-Rad (California, USA). The software used for analysis was Quantity One, version 4.6.1 build 055 from Bio-Rad, USA.

Gel extraction

Gel extraction is a technique used to isolate a desired DNA fragment from an agarose gel after AGE. During PCR or DNA-restriction proteins and unwanted DNA fragments are present in the reaction mixture. In order to separate this junk from the specific DNA fragment AGE and subsequent gel extraction is applied. After extraction, the isolated DNA fragment can be used for further experiments. Gel extraction involves four basic steps:

- identifying the fragments of interest
- isolating the corresponding bands
- isolating the DNA from those bands
- removing the accompanying salts and stain

For all gel extractions Qiagen® QIAquick® Gel Extraction Kit (Qiagen®, Germany) was used containing all necessary materials unless stated otherwise. More information to buffer composition can be found in the kits' manual. All steps were carried out according to the QIAquick Gel Extraction Microcentrifuge Protocol.

Excision of DNA fragments from agarose gels and dissolving the agarose gels

The desired DNA fragments were localized in the gels applying the UV-transilluminator. The fragments were excised using a scalpel and placed in reaction tubes. The gel slices were weighed and 3 volumes of buffer QG were added to 1 volume of gel (100 mg ~ 100 µL). The reaction tubes were incubated at 50°C until the gel was completely dissolved but no longer than 10 min. Every 2 minutes the reaction tubes were shortly vortexed.

Binding of DNA to silica membrane and washing of DNA

1 gel volume of isopropanol (Merck, Germany) was added to the reaction tubes and aliquots were applied to QIAquick columns and centrifuged for 1 min at 10.000 rpm and room temperature. The flow-through was discarded. For washing, 0.75 mL buffer PE were applied onto the columns. The columns were centrifuged for 1 min, 10.000 rpm and room temperature. The flow-through was discarded. The columns were centrifuged for another minute at 10.000 rpm and room temperature and the flow-through was discarded again.

Elution of DNA

The columns were placed into clean 1.5 mL reaction tubes. 50 µL molecular biology grade water (Eppendorf, Germany) were applied to the center of the columns and incubated for 5 min. The columns were centrifuged for 1 min, 10.000 rpm and room temperature. The flow-through contained the extracted DNA.

3.4.3 Modification of plasmid DNA

3.4.3.1 Restriction of DNA

Restriction endonuclease II cuts double-stranded DNA by recognizing short and specific nucleotide sequences in the target DNA. They are commonly used for excising small fragments for subsequent cloning purposes or generation of restriction maps. Enzymes were purchased from Fermentas (*Nde*I and *Sal*I) and New England Biolabs (*Btg*I and *Pst*I).

Procedure

The restriction mixtures contained following components:

100 ng - 2 µg DNA
2 µL 10x buffer (O or NEBuffer)
0.4-2 µL *Nde*I, *Sal*I, *Pst*I, *Btg*I
0.2 µL 100x purified BSA (only for New England Biolabs chemicals)
filled up to 20 µL with molecular biology grade water

The mixtures were incubated for 1-4 hours at 37°C mixing on a thermomixer (300 rpm). To determine, if the entire DNA was digested, samples were taken every hour during restriction. Restriction was controlled by AGE. If needed, preparative gels and subsequent extraction was performed.

3.4.3.2 Ligation

In ligation an enzyme forms covalent phosphodiester bonds between the 3'-hydroxyl end of one nucleotide with the 5'-phosphate end of another. Therefore, two separate DNA fragments can be linked together. T4 DNA ligase was used from Fermentas was used.

Procedure

In order to ligate DNA fragments into a vector, the mass-ratio of insert to vector was 3:1. 2 µL of 10x T4 ligation buffer and 1 µL of T4 DNA ligase were added to the mixture and filled up with molecular biology grade water to 20 µL. The mixture was incubated for 4 h at 20°C.

3.5 Transformation of DNA into *E. coli*

In order to introduce foreign DNA into bacterial cells in principal two methods exist: physical and chemical. The physical method is called electroporation and utilizes high-voltage pulses (several kV) which are applied for several msec to a mixture of competent cells and plasmid DNA. Competent cells are derived from thorough washing in order to remove all medium salts to avoid short-circuits. Electroporation is usually carried out at 0°C to avoid heat damage to the cells. During electroporation, it is assumed that transient pores are formed in bacterial cell membranes which enable foreign DNA to enter the cells (Neumann and Rosenheck, 1972). Electroporation is more efficient than chemical transformation (Neumann *et al.*, 1982). In this work, chemical transformation using $CaCl_2$ was applied which is based on observations by Mandel and Higa (Mandel and Higa, 1970). It utilizes the divalent Ca^{2+} ion for generating chemically competent cells. Presumably, the ions increase the cell membrane's permeability by forming pores therein. Moreover, the ions assist the passage of DNA through the hydrophobic cell membrane by masking the negative charge of the DNA. An ice-chilled mixture of competent cells and plasmid DNA is exposed to 42°C for about 45 seconds and immediately incubated on ice again (heat shock). This heat shock results in a thermal current which assists the incorporation of the plasmid DNA into the cells.

Procedure

Preparation of chemically competent cells

After thawing untransformed *E. coli* for 30-60 min, 250 mL shaking flasks with 30 mL SLB medium were inoculated with 100 µL of cells. The cultures were grown at 37°C and 300 rpm (INFORS® Multitron II, Switzerland), until they reached an OD_{550} of 0.3. The cultures were centrifuged for 15 min at 4°C and 4000 rpm (Eppendorf® Centrifuge 5810R, Germany). The pellets were carefully resuspended in 20 mL of 0.15 M NaCl. The cultures were centrifuged for 15 min at 4°C and 4000 rpm. The pellets were carefully resuspended in 20 mL hypotonic $CaCl_2$ [11 g/L $CaCl_2$*$2H_2O$, 1 g/L $MgCl_2$*$6H_2O$, 12.1 g/L tris(hydroxymethyl)aminomethane adjusted to pH = 7.6 with 25% HCl]. The suspensions were incubated on ice for 20-30 min and centrifuged again for 10 min at 4°C and 2000 rpm. The pellets were carefully resuspended

in 2 mL hypotonic $CaCl_2$ + 15% glycerol (87%). Aliquots of 150 µL of the chemically competent cells were stored at -80°C.

Chemical transformation

100 µL chemically competent cells and 1-5 µL plasmid solution (10 ng/µL) were mixed in prechilled 1.5 mL reaction tubes on ice. The mixtures were incubated on ice for 10 min. For heat shock, the reaction tubes were incubated at 42°C for 45 sec (Eppendorf® Thermomixer comfort, Germany). Then the cells were incubated for 5 min on ice. The cells were transferred to 900 µL SOC Medium and incubated at 37°C for 20-30 min.

100 µL from this suspension were used to make 10-fold dilutions in SOC medium. If necessary, 100-fold dilutions were made. 200 µL from all three dilutions were used for plating on selective agar plates containing the appropriate antibiotic. Afterwards, the undiluted suspension was centrifuged and the pellet was resuspended in 200 µL of supernatant. The suspension was plated on selective agar plates containing the appropriate antibiotic. The agar plates were incubated at 37°C over night (Incubator: RENGGLI® Biocenter 2001, Switzerland).

3.6 Clone screening and cell banks

After transformation, the transformant yielding highest product concentration was selected by clone screening. This best producer was used for establishing a parent culture. Subsequently, master cell banks were established in order to guarantee sufficient supply of starting material with the same identity, purity, and usability. **Figure 3-3** shows the procedure for the generation of a master cell bank:

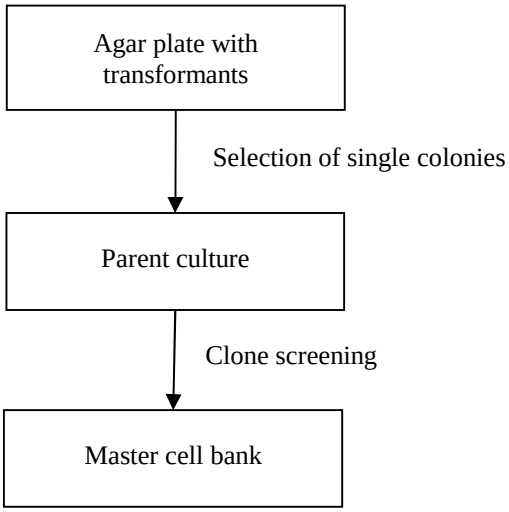

Figure 3-3: Establishing of a master cell bank

3.6.1 Parent culture

50 mL reaction tubes were filled with 15 mL SLB-Medium and 15 mg/L kanamycin sulfate. After transformation (see **Chapter 3.5**) transformants were randomly picked with an inoculating loop from selective agar plates and transferred into the reaction tubes. The tubes were cultivated at 37°C and 300 rpm until the suspensions reached an OD_{550} of 0.2-0.5. 3 mL glycerol (87%) were added resulting in a final glycerol content of 14.5%. Aliquots of 1.2 mL parent cultures were stored at -80°C.

3.6.2 Clone screening

Pre cultures were grown in 250 mL shaking flasks with 50 mL SLB medium and 50 µg/mL kanamycin sulfate. 100 µL of the desired parent culture were added and grown over night at 37°C and 300 rpm. Finally, the OD_{550} was measured.

Main cultures were performed in 1 L shaking flasks with 250 mL SLB medium and 50 µg/mL kanamycin sulfate. Pre cultures were inoculated to main cultures to a final OD_{550} of 0.1. Main cultures were cultivated at 37°C and 300 rpm until an OD_{550} of 1-1.5 was achieved. Cultures were induced with IPTG resulting in a final inducer concentration of 1 mM in the shaking

flasks. The main cultures were grown for additional four hours. Product samples were filled in reaction tubes according to **Equation 3-1**. The samples were centrifuged at 4°C and 13200 rpm for 15 min (Eppendorf® Centrifuge 5415R, Germany), the supernatant was discarded and the pellet was refrigerated at -20°C.

$$\frac{10}{OD550} = \underline{yyy}[ml]\ of\ main\ culture\ for\ product\ samples$$

Equation 3-1

For product quantification SDS-PAGE with densitometric quantification was performed (see **Chapter 3.8.2**). The parent culture with maximum product yield was chosen for producing master cell banks.

3.6.3 Master cell bank

1 L shaking flasks with 200 mL SLB medium, 50 µg/mL kanamycin sulfate, and 500 µL parent cultures were incubated at 37°C and 300 rpm until the OD_{550} reached 1.0-1.5. 40 mL glycerol (87%) were added resulting in a final glycerol content of 14.5%. Aliquots of 1.2 mL of the master cell banks were stored at -80°C.

3.7 Cultivation

3.7.1 Media

Unless stated otherwise all chemicals were purchased from Merck (Germany), SERVA (Germany), Sigma-Aldrich (USA), and Amresco (USA). All solutions and media were prepared using fully desalted HQ water. For fermentations *E. coli* T7 high cell density medium was used (Striedner *et al.*, 2003). Pre cultures were performed in shake-flasks using SLB-Medium (**Table 3-11**).

The batch medium components were dissolved in 9/10 of the required final volume and autoclaved at 121°C for 30 min. Components 5-9 (sterile additions) of pre culture- and batch

medium were dissolved in 1/10 of the required final volume and sterile-filtered through a membrane filter (Filter units MF75^{TM} Series, NALGENE). Feed medium components were dissolved in hot water and were filled up to the required final volume and sterile filtered through a 0.22 µm Millipak 100 filter (Millipore, Germany).

SLB-medium

Table 3-11: SLB-medium

	Component	Amount
1	Bacto yeast extract	5 g/L
2	Soy peptone	10 g/L
3	NaCl	10 g/L

Pre culture medium

Table 3-12: T7 preculture medium

	Component	Amount
1	KH_2PO_4	3 g/L
2	K_2HPO_4	4.58 g/L
3	Sodium citrate*$2H_20$	0.833 g/L
4	Trace-element solution	0.167 mL/L
5	Glucose (anhydrous)	10 g/L
6	$MgSO_4$*$7H_2O$	0.33 g/L
7	$(NH_4)_2SO_4$	1.5 g/L
8	NH_4Cl	1.23 g/L
9	$CaCl_2$*$2H_2O$ (presolved)	0.067 g/L
10	Bacto yeast extract	0.50 g/L

Batch medium

Table 3-13: T7 batch medium

	Component	Amount	
1	KH_2PO_4	3 g/L	
2	K_2HPO_4	4.58 g/L	
3	Sodium citrate*$2H_20$	0.833 g/L	
4	T7 trace-element solution	0.167 mL/L	
5	Glucose (anhydrous)	10 g/L	**Sterile additions**
6	$MgSO_4$*$7H_2O$	0.33 g/L	
7	$(NH_4)_2SO_4$	1.5 g/L	
8	NH_4Cl	1.23 g/L	
9	$CaCl_2$*$2H_2O$ (presolved)	0.067 g/L	

For fermentations with complex medium, 10 g/L bacto yeast extract and 20 g/L soy peptone were added to the batch medium (**Table 3-13**) and autoclaved.

Feed medium

Table 3-14: T7 Feed medium

	Component	Amount
1	KH_2PO_4	3 g/L
2	K_2HPO_4	4.58 g/L
3	Sodium citrate*$2H_20$	41.67 g/L
4	Trace-element solution	8.334 mL/L
5	$MgSO_4$*$7H_2O$	16.67 g/L
6	$CaCl_2$*$2H_2O$ (presolved)	3.33 g/L
7	Glucose (anhydrous)	500 g/L

For fermentations with complex medium, 10 g/L bacto yeast extract and 20 g/L soy peptone were added to the feed medium and sterile-filtered through a 0.22 µm Millipak 100 filter (Millipore, Germany).

Trace elements solutions

Table 3-15: Trace elements solution for T7 medium

	Component	Amount
1	$FeSO_4$*$7H_2O$	40 g/L
2	$MnSO_4$*H_2O	10 g/L
3	$AlCl_3$*$6H_2O$	10 g/L
4	$CoCl_2$*$6H_2O$	4 g/L
5	$ZnSO_4$*$7H_2O$	2 g/L
6	Na-molybdat*$2H_2O$	2 g/L
8	$CuCl_2$*$2H_2O$	1 g/L
7	Boric acid	0.5 g/L
9	85% phosphoric acid	173 g/L

Other solutions

Kanamycin sulfate stock solution (1000x)

50 mg/mL Kanamycin sulfate

Isopropyl β-D-1-thiogalactopyranoside stock solutions

100 mM for clone screening

1 M for fermentations

3.7.2 Fermentation systems

For this master thesis, shake-flasks as well as 5 L and 20 L stirred tank reactors were used.

3.7.2.1 Pre cultures

Pre cultures were grown in shake-flasks. Cultivation was done in an orbital shaker (INFORS® Multitron II, Switzerland) at 300 rpm and the desired temperature.

3.7.2.2 5 L and 20 L fermenters

Two different designs of stirred tank bioreactors were applied. Both were sterilisable in place.

- One had a gross volume of 6.9 L and a net volume of 5 L (Sartorius AG, Germany; **Figure 3-4**);
- One had a gross volume of 30 L and an approximate net volume of 20 L (New MBR, Switzerland; **Figure 3-5**)

All reactors were equipped with built-in controllers for pH, temperature, agitation, aeration, pressure, and feed pumps. The parameters mentioned above could be set and controlled using a process control system (Sartorius AG, Germany). All liquid inlet flows were quantified gravimetrically using balances (Mettler-Toledo, Switzerland). Air and

Figure 3-4: 5 L fermenter

oxygen inlet flow were controlled using Mass Flow Meters (Bronkhorst, NL). Oxygen and carbon dioxide concentration in the exhaust gas could be additionally monitored by an exhaust gas measurement module (LumaSense INNOVA, DK).

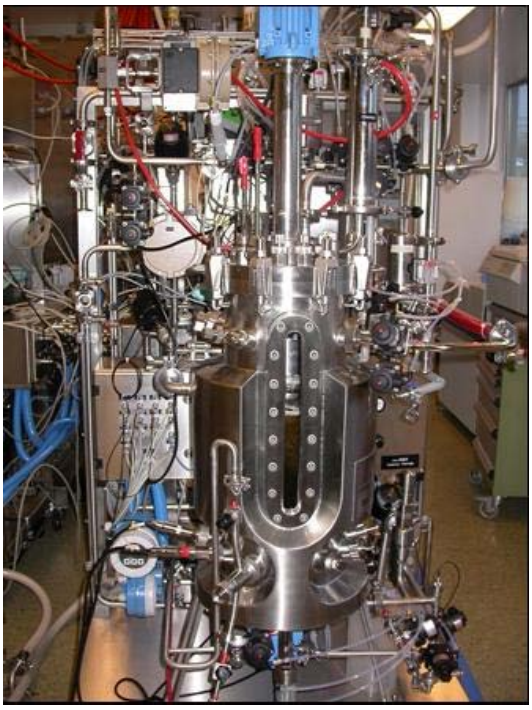

Figure 3-5: 20 L fermenter

The bioreactors were equipped with probes for pH, pO_2 (both from Mettler-Toledo, Switzerland) and temperature (Jumo, Austria). pH was controlled by adding 3 M H_3PO_4 and 25% NH_4OH to the fermentation broth. The dissolved oxygen level (pO_2, DO) was controlled in cascade with the stirrer speed from 400 rpm to 1000 rpm. Aeration rates were 1 vvm air. When the stirrer speed reached its maximum value, the further increase in oxygen consumption was met by addition of pure oxygen to the air supply. Temperature was measured using a Pt-100 electrode and was maintained at the desired temperature by a cooling / heating cycle. Additionally, foam formation was monitored by level electrodes (Sartorius AG, Germany). If foam formation occurred, antifoam agent PPG 2000 (Dow Chemical, USA) was added. All vessels were connected to the fermenter with plastic tubing and sterile needles that were pierced through fermenter port diaphragms. Feed medium, acid, base, and antifoam were added by peristaltic pumps (ISMATEC, Switzerland) into the fermenter.

3.7.3 Fermentation conditions

Standard operating parameters are listed in **Table 3-16**. The standard fermentation temperature was 35°C.

Table 3-16: Standard operating parameters

Parameter	*E. coli*
Temperature [°C]	35
Pressure [bar]	1
pH [-]	6.5 +/- 0.2

Dissolved oxygen [%]	20
Air inlet flow [vvm] (referred to the final fermentation volume)	1

3.7.4 Sampling

Throughout fermentations samples were taken in order to monitor growth, product-, and by-product formation:

3.7.4.1 Microscopy

Cultures were monitored employing a Nikon Eclipse E 6000 microscope equipped with a Nikon Digital Net Camera.

3.7.4.2 Optical density (OD_{550})

Optical density was measured at a wavelength of 550 nm (OD_{550}) with a spectral photometer (Spectronic© Genesis 5). In order to achieve an absorbance in the linear range between 0.2 and 0.6, the samples were diluted with OD-buffer (20.7 g/L $Na_2HPO_4*12H_2O$, 5.7 g/L KH_2PO_4, 11.6 g/L NaCl).

3.7.4.3 Dry cell weight (DCW)

For DCW determination, 10 mL of fermentation broth were centrifuged in 50 mL reaction tubes at 4000 rpm for 15 min (Eppendorf® centrifuge 5810R, Germany). For washing, the pellets were resuspended in deionized water and centrifuged again at the same conditions. The supernatants were discarded and the pellets were resuspended again in deionized water. Cell suspensions were dried to a constant weight in an automatic drier (HG 55 Halogen Moisture Analyzer, Mettler Toledo, Switzerland).

3.7.4.4 Wet cell weight (WCW)

The 50 mL reaction tubes for DCW determination were weighed empty. After washing and centrifugation, the supernatants were discarded and the tubes were weighed again. The differences of empty and full 50 mL reaction tubes were considered as DCW.

3.7.4.5 Glucose

Glucose concentration was measured via biochemistry analyzer YSI 2700 Select Glucose-analyzer (Kreienbaum, Germany) from 0.22 µm filtered supernatants.

3.8 Analytics

Unless stated otherwise all chemicals were purchased from Merck (Germany), SERVA (Germany) and Sigma-Aldrich (USA).

3.8.1 DNA quantification

DNA concentration in samples was measured utilizing the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA) together with the software NanoDrop ND-1000 3.3 (Coleman Technologies Inc., USA). Samples were measured in a continuous spectrum at wavelengths between 220-750 nm. Sample volumes had to be approximately 1 µL and DNA concentrations could vary in a range from 2 ng/µL to 3700 ng/µL.

3.8.2 SDS-PAGE

Proteins have ionizable side chains and are therefore able to migrate in an electrical field. If proteins have similar charges but different molecular masses they feature different charge-to-mass ratios. The different charge-to-mass ratios of proteins are the basis for native PAGE.

In order to separate proteins by their size the molecules are completely denatured with a mixture of the anionic detergent sodium dodecyl sulfate (SDS) and a reducing agent such as β-mercaptoethanol. SDS is an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures. Approximately 1.4 g SDS binds per 1.0 g protein. Therefore, a homogenously negative charge is applied to the proteins resulting in a constant mass-to-charge ratio of all proteins to be analyzed. Addition of a reducing agent such as β-mercaptoethanol denatures proteins by reducing disulfide linkages. This is the basis for the electrophoretic separation of proteins by their size (SDS-PAGE).

The optical density of stained samples was measured by laser densitometry using Personal Laser Densitometer SI (Amersham Bioscience, UK) and the software PDSI Scanner Control v5.03 (Amersham Bioscience, UK). The quantification was accomplished by computer-aided comparison of band intensities relative to known standards using TotalLab TL 120 (Nonlinear Dynamics®, UK).

Before SDS-PAGE can be carried out, samples undergo an intensive preparation procedure. Each preparation procedure represents a potential source of error. Therefore attention must be paid to the reliability of measurement results which can vary up to +/- 20%.

Table 3-17: Reagents used for SDS-PAGE

reagent	composition
Standards	ProtE scFv (BI RCV)
	B1 scFv (BI RCV)
	A4 scFv (BI RCV)
	H6 scFv (BI RCV)
	B1 scFv E-Linker (BI RCV)
	B1 scFv L205 (BI RCV)
	B1 scFv LLB18 (BI RCV)
Molecular weight standard Mark 12 (see **Figure 3-6**)	–
Sample buffer	NuPAGE 4x LDS sample buffer (Invitrogen, USA)
4x reducing sample buffer ($4x\ LDS_{red}$)	NuPAGE 4x LDS sample buffer
	10% β-Mercaptoethanol (Sigma Aldrich, USA)
1x reducing sample buffer ($1x\ LDS_{red}$)	NuPAGE 4x LDS sample buffer r
	diluted 1:4 with deionized water
	2.5% β-Mercaptoethanol
1x NuPAGE MES SDS running buffer	NuPAGE 20x MES SDS Running buffer (Invitrogen, USA)
	diluted 1:20 with deionized water

1x NuPAGE MOPS SDS running buffer	NuPAGE 20x MES SDS Running buffer (Invitrogen, USA)
	diluted 1:20 with deionized water

Procedure

Cell suspensions were centrifuged for 10 min at 13200 rpm (Eppendorf® Centrifuge 5415R, Germany). The remaining pellets were investigated for product contents. To break up bacterial cell walls, ultrasonic disintegration or enzymatic cell wall disruption utilizing BugBuster® reagent was applied.

Mark 12 (**Figure 3-6**) and Novex® sharp protein standard (both: Invitrogen, USA) as well as PageRuler™ Prestained Protein Ladder (Fermentas) were used as molecular weight standards. They were diluted with 1x LDSred to approximately 80 µg/mL and treated alike the samples as listed in **Table 3-18** from step 4 on. Step 6 was not carried out with the standards.

Electrophoresis conditions

SDS-PAGE was accomplished using NuPAGE Novex 4-12%, 10%, and 12% Bis-Tris gels, 1 mm thick, with 10, 12, 15, and 20 wells (Invitrogen, USA). Running conditions were 200 V and 45 minutes with MES buffer and 200 V and 55 min with MOPS buffer. Proteins were detected by Coomassie- or silver staining or were transferred from polyacrylamide gels to nitrocellulose membranes via western blot.

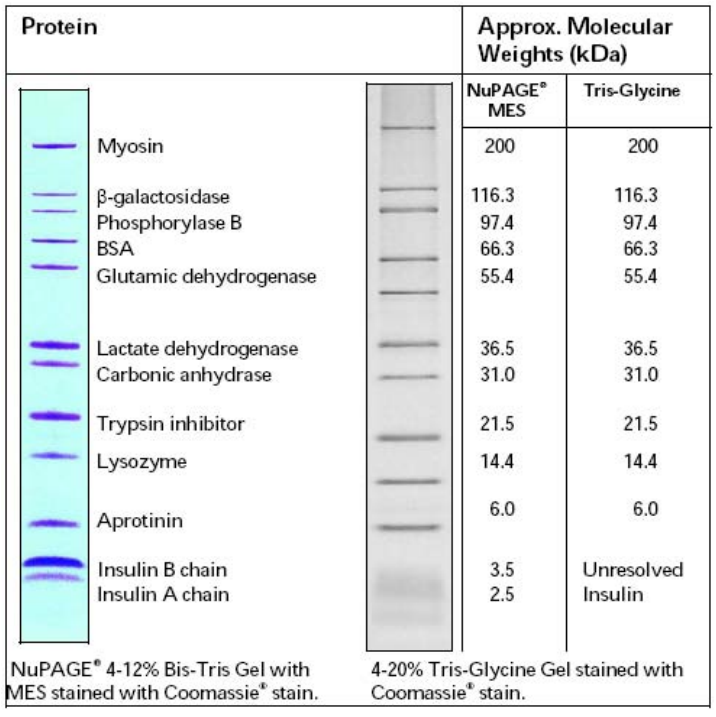

Figure 3-6: Approximate molecular weights of the protein bands from the molecular weight standard Mark 12 (Invitrogen)

3.8.2.1 Ultrasonic disintegration

Ultrasonic disintegration disrupts the cell wall by cavitation. Cavitation is a phenomenon of vapor bubble formation in a liquid in a region where the fluid pressure falls below the vapor pressure of the liquid. Inter alia, cavitation is a result of pressure fluctuations in a fluid caused by ultrasonic waves. The resulting vapor bubbles in the fluid are forced to oscillate in size or shape because of the energy input via ultrasonic waves. This causes pressure- and temperature-peaks which cause cell wall disruption. Ultrasonic disintegration of cells is not quantitative: about 2% of cells are not disrupted. **Table 3-18** gives an overview on how samples were treated for ultrasonic disintegration for subsequent SDS-PAGE.

Table 3-18: Ultrasonic disintegration of samples

Step Number	Procedure	Dilution	Comment
1	+ 800 µL 1x LDS_{red}	-	-
2	1 min ultrasonic disintegration, 5 cycles, 30% power	-	Bandelin Sonoplus HD and UW 2070 with MS72-probe (Bandelin, Germany)
3	15 min room temperature, 600 rpm	-	Eppendorf® Thermomixer comfort (Germany)
4	15 min 50°C, 600 rpm	-	Eppendorf® Thermomixer comfort (Germany)
5	3 min 13200 rpm	-	Eppendorf® Centrifuge 5415R, (Germany)
6	dilution of supernatant with 1x LDS_{red}	1:4	-
7	loading of samples on gels for SDS-PAGE	-	-

3.8.2.2 Enzymatic disintegration

Enzymatic disintegration of *E. coli's* cell wall using BugBuster® reagent was used for quantitative cell wall disruption. It was applied to determine the soluble and insoluble protein fraction. BugBuster® reagent utilizes a detergent mix supplied as a 1x Tris-buffered liquid that denatures the cell wall. Addition of rLysozyme™ Solution hydrolyzes *N*-acetylmuramide linkages in the cell wall and therefore enhances the extraction efficiency. Adding Benzonase® Nuclease results in degradation of chromosomal DNA and therefore reduction of viscosity.

Table 3-19: Reagents use for enzymatic disintegration of *E. coli*

reagent	composition
BugBuster® master mix	BugBuster® protein extraction reagent (Novagen, Germany)
	0.2% Lysonase™ Bioprocessing Reagent – mixture of rLysozyme™ and Benzonase® Nuclease (Novagen, Germany)
	0.1% Benzonase® Nuclease (Novagen, Germany)
4x reducing sample buffer (4x LDS_{red})	NuPAGE 4x LDS sample buffer (Invitrogen, USA)
	10% β-Mercaptoethanol (Sigma Aldrich, USA)
4x reducing sample buffer (1x LDS_{red})	NuPAGE 4x LDS sample buffer (Invitrogen, USA)
	diluted 1:4 with deionized water
	2.5% β-Mercaptoethanol (Sigma Aldrich, USA)

Procedure

Cell pellets were obtained from 10/OD samples of cell suspensions (**Equation 3-1**) with subsequent centrifugation at 4°C and 13200 rpm for 15 min (Eppendorf® Centrifuge 5415R, Germany). The supernatant was discarded and 400 µL BugBuster® master mix were added to the cell pellets. The samples were vortexed until the pellets were resuspended and incubated at room temperature, 600 rpm, 20 min.

The suspensions were vortexed again and then centrifuged at 13400 rpm and room temperature for 10 min. The supernatants were collected in new reaction tubes and considered as the soluble protein fraction. The pellets remained in the original reaction tubes and were considered as the insoluble protein fraction.

Soluble fraction

The supernatants were vortexed again and centrifuged at 13400 rpm and room temperature for 3 min. ¼ of the supernatants' volumes 4x LDS_{red} was added resulting in 1x LDS_{red} in the mixture. The mixture was vortexed and incubated at room temperature and 600 rpm for 15 min. The samples were incubated at 50°C and 600 rpm for 15 min, vortexed, and centrifuged for 3 min at room temperature and 13400 rpm. Finally, the samples were loaded on gels for SDS-PAGE.

Insoluble fraction

In order to minimize soluble protein in the insoluble fraction, 1 mL deionized water was added to the pellets. The reaction tubes were vigorously vortexed until the pellets were resuspended in the deionized water. The samples were centrifuged at 13400 rpm and room temperature for 10 min. The supernatants were discarded and 600 µL 1x LDS_{red} were added. Afterwards, the samples were treated as listed in **Table 3-18** beginning with step 2.

3.8.2.3 Staining methods

To visualize protein bands on a gel after SDS-PAGE, the gel is treated with staining solutions. In this work, Coomassie staining and silver staining was used. Coomassie staining is a quantitative and qualitative staining method. Silver staining is only a qualitative staining method with the potential advantage of lower detection limits, however.

Coomassie staining

Coomassie staining is a method to detect proteins after SDS-PAGE with a detection limit of about 0.1 µg of protein. The amount of Coomassie bound per protein depends upon the number of alkaline residues available in a protein where Coomassie binds stoichiometrically. Proteins with the same size may differ by the number of alkaline residues present in the polypeptide chain. Therefore, attention must be paid when comparing bands of different proteins after staining. This fact must also be considered when quantifying a target protein on a gel: different quantities of a standard protein solution are loaded on the gel. After electrophoresis, the bands of the standard protein solution are stained. Subsequently, a calibration curve can be calculated in order to quantify the target protein. The protein in the standard protein solution should be composed of the same kinds of amino acids as the target protein in order to guarantee a similar number of alkaline residues per protein.

Table 3-20: Reagents used for coomassie staining

reagent	composition
Coomassie brilliant blue R-250 staining solution	1 g Coomassie Brilliant Blue R-250 (Bio-Rad, USA)
	100 mL glacial acetic acid

	450 mL methanol
	450 mL deionized water
Destaining solution	100 mL glacial acetic acid
	300 mL ethanol
	600 mL deionized water
Fixing solution I	16 mL glycerin 87%
	200 mL ethanol
	600 mL deionized water

Procedure

Staining

The gels were stained with Coomassie Brilliant Blue R-250 staining solution for about one hour and destained for 2x ½ hour with destaining solution at room temperature and shaking (150 rpm). Afterwards, the gels were placed in deionized water for 30 min at room temperature and shaking (150 rpm).

Scanning, conservation, and quantification

The gels were scanned using the Personal Laser Densitometer SI (Amersham Bioscience, UK) and the software PDSI Scanner Control v5.03 (Amersham Bioscience, UK). The pixel size for gel scanning was 100 micron, the digital resolution was 12 bits. After scanning, the gels were placed in fixing solution I for 30 min at room temperature and shaking (150 rpm). For gel-conservation, the gels were laid between two precut cellophane sheets (Amersham Bioscience, UK) and were air-dried.

For quantification and estimation of the lane purity and molecular weight, the software TotalLab TL 120 (Nonlinear Dynamics®, UK) was used. The product purity within one lane was estimated by relating the product band to the bands of residual host cell proteins in one lane.

Silver staining

Silver staining is a method to detect proteins after SDS-PAGE with a detection limit between 0.1–1 ng of protein. It is a non quantitative but only a qualitative staining method. After electrophoresis, gels are treated with glutaraldehyde which binds unspecifically to proteins. Afterwards, the gels are treated with silver nitrate which binds to glutaraldehyde. In a last step, the silver ions are reduced to metallic silver with formaldehyde in alkaline pH resulting in blackish stained protein bands.

For silver staining SilverXpress® Silver Staining Kit (Invitrogen, USA) was used. More information to reagent composition can be found in the kits manual.

Table 3-21: Reagents used for silver staining

reagent	composition
Fixing solution II	90 mL deionized water
	100 mL methanol
	20 mL glacial acetic acid
Sensitizing solution	105 mL deionized water
	100 mL methanol
	5 mL Sensitizer – contains glutaraldehyde (Invitrogen)
Staining solution	5 mL Stainer A – contains silver nitrate(Invitrogen)
	5 mL Stainer B – contains ammonium hydroxide and sodium hydroxide (Invitrogen)
	90 mL deionized water
Developing solution	5 mL Developer – contains formaldehyde and citric acid (Invitrogen)
	95 mL deionized water
Stopping solution	5 mL Stopper – contains citric acid (Invitrogen)
Fixing solution I	16 mL glycerin 87%
	200 mL ethanol
	600 mL deionized water

Procedure

Staining

After SDS-PAGE, gels were rinsed with deionized water and fixed in the fixing solution II for 10 min. The gels were sensitized 2x 30 min in the sensitizing solution, washed with deionized water 2x 10 min, and stained for 15 min in the staining solution. Then, they were washed with

deionized water 2x 5 min and developed in the developing solution until protein bands were clearly visible. The gels were treated with stopping solution for 10 min to stop the developing reaction. Finally, the gels were washed 3x 10 min with deionized water.

Scanning and conservation

See Coomassie staining (**Chapter 3.8.2.3**).

3.8.2.4 Determination of product purity

Product purity was determined from samples derived from fermentation-end. Two different purity types were determined:

- Lane purity [%]: subsequent to SDS-PAGE and staining, gels were scanned applying the software TotalLab TL 120 (Nonlinear Dynamics®, UK). The product purity within one lane ('lane purity') was determined by relating the intensity of the product band to the intensities of residual host cell protein bands.
- IB purity [mg/g IB]: IBs were prepared from cell lysates by centrifugation and subsequent purification. Afterwards the IBs were solubilized. The IB purity was assessed by determining the mass fraction of the recombinant protein in relation to the whole IB.

Especially the lane purity of a SDS-PAGE gel is very error-prone. The main error sources are host cell proteins with similar molecular weights as the recombinant protein. During SDS-PAGE, those host cell proteins migrate the same distance on the gel as the recombinant protein. Therefore, they cannot be distinguished from one another. These host cell proteins can subsequently falsify the amount of recombinant protein and therefore values for lane purity as well. Nevertheless, the lane purity usually gives a good first impression of the success of recombinant protein expression: if the lane purity is high it is quite possible that sufficient amounts of recombinant proteins were produced and that IB purity is high as well.

The IB purity is more accurate than the lane purity: before the IB purity is determined, host cell proteins and all other cell debris are removed. Impurities mainly result from residual host cell proteins that aggregated as IBs as well or from insufficient removal of host cell proteins

and cell debris. Overall yield of the active, refolded, and purified biopharmaceutical protein greatly depends upon yields in downstream processing. If the IBs are already very impure, more effort has to be made for their purification. Each purification step is laborious and negatively affects the overall yield. Therefore, high IB purities are favorable.

3.8.3 Western blot

Western blot is an immunostaining method by which one specific protein can be specifically detected among many others. First, proteins are separated from each other by SDS-PAGE. Afterwards they are transferred to a membrane (blotting) which is usually composed of nitrocellulose, nylon, or Polyvinylidene fluoride. On the membrane, proteins can easily be detected by antibodies which are specific to the target protein (antigen). The antigen-antibody complex can be visualized by a secondary antibody specific to the primary antibody. The secondary antibody is usually linked to a reporter enzyme such as alkaline phosphatase. In a subsequent chemical reaction the alkaline phosphatase catalyzes the conversion of substrates to a colored precipitate which can be used for protein detection.

Table 3-22: Reagents used for western blots

reagent	**composition**
1x transfer buffer (Invitrogen, USA)	20% methanol
Tween20 (Kolb Distribution Ltd., CH)	–
Blocking solution	1x PBST (Invitrogen, USA)
	0.1% Tween20
	excess of blotting grade blocker non-fat dry milk (Bio-Rad, USA)
1st antibody solution	anti-$(Gly_4Ser)_3$-linker IgG, developed in mouse (BOKU, Austria)
	diluted 1:1000 in 1x PBST
	0.1% Tween20
	1% of blotting grade blocker non-fat dry milk (Bio-Rad, USA)
2nd antibody solution	anti-mouse IgG, conjugated with alkaline phosphatase (Sigma, USA)
	diluted 1:1000 in 1x PBST
	0.1% Tween20
	1% of blotting grade blocker non-fat dry milk (Bio-Rad, USA)
Washing solution	1x PBST
	0.1% Tween20

Staining solution (AP conjugate substrate kit, Bio Rad, USA)	1x staining buffer
	1% 5-Bromo-4-chloro-3-indolyl phosphate (BCIP)
	1% Nitro blue tetrazolium chloride (NBT)

Procedure

Sample preparation, blotting, and membrane blocking

Before blotting a SDS-PAGE was carried out. The equipment for blotting was derived from Invitrogen, USA. After SDS-PAGE the gels were rinsed with deionized water and placed on a nitrocellulose membrane. Synthetic sponges and Whatmanpapers were soaked with transfer buffer. Gels, membranes, sponges and Whatmanpapers were stacked in the blotting chamber (Invitrogen, USA), the chamber was filled up with transfer buffer, and blotting was started. Blotting conditions were 50 V and 45 min. After blotting, the membranes were blocked with blocking solution over night at 4°C.

Immunostaining

For subsequent steps in membrane preparation see **Table 3-23**. Solutions for immunostaining were discarded after each step.

Table 3-23: Immunostaining

Step Number	Procedure
1	rinsed with deionized water
2	1 h at 150 rpm and room temperature (RT) in 1st antibody solution
3	3x 10 min at 150 rpm and RT in washing solution
4	1 h at 150 rpm and RT in 2nd antibody solution
5	3x 10 min at 150 rpm and RT in washing solution
6	staining in staining solution at RT until bands can be seen well
7	3x 10 min at 150 rpm and RT in deionized water
8	air-dry membrane

4. Results and Discussion

4.1 Bacterial strains

In this work the *E. coli* strains Strain B and Strain A were used. Both strains are routinely applied for industrial processes. Strain A is a K-strain and is used as host for cloning and expression (Studier *et al.*, 1990). Moreover, it reportedly yielded high specific production rates. The B-strain Strain B provides the potential advantage of lacking the *lon* and *ompT* outer membrane protease which are capable of protein degradation during purification (Studier *et al.*, 1990). Strain B is easier to cultivate because high glucose concentration results in less acetate formation than in Strain A (Shiloach *et al.*, 1996). Both strains contain the lysogen bacteriophage DE3 – a λ derivative – providing the T7 RNA polymerase controlled by the *lac*UV5 promoter (Studier *et al.*, 1990).

4.2 Expression vectors and model proteins

As described in **Chapter 3.2** IPTG inducible pET30a vectors comprising the T7 promoter (Studier *et al.*, 1990) were used for heterologous protein expression. An overview of applied model proteins is listed in **Table 4-1**. For a detailed overview of applied antibody fragments see **Table 3-3** and **Table 3-4**.

Table 4-1: Overview of cytosolic expression in *E. coli*

Model protein	scFv fragment	Fab fragment
H6	P	–
B1	P	–
A4	P	P

In previous works from Wagner, 2008 the H6 scFv fragment was expressed in the cytosol and periplasm of *E. coli* Strain B and Strain A. Wagner reported low expression yields possibly resulting from a non-optimized *E. coli* codon usage (also see Buell *et al.*, 1985; Hernan *et al.*, 1992). In the present work, only codons optimized for *E. coli were* applied for all model

proteins. Additionally, B1- and A4 scFv fragments were used as model proteins to extend the technology platform on more molecules.

Fab fragments are heterodimeric proteins. The V_H-C_H1 and the V_L-C_L are linked by a disulfide bond. Disulfide bonds cannot be formed in the reducing environment of *E. coli's* cytosol. Therefore, intracellular expression of heterodimeric proteins is not state-of-the-art in *E. coli* (Plückthun and Skerra, 1989). In the present work the feasibility of separate and intracellular expression of Fab fragment's light- and heavy chain in *E. coli* was evaluated. For a first attempt, the A4 Fab fragment was used as model protein.

4.2.1 Subcloning and transformation

The genes of following antibody fragments were provided in pGA4 vectors by GENEART AG (Germany) and had to be subcloned into pET30a vectors:

- B1 scFv comprising E-Linker, L205, L218, and LLB18 (see **Table 3-3**)
- A4_Fab_LC_bacMiG (see **Table 3-4**)
- A4_Fab_HC_bacMiG (see **Table 3-4**)

After Subcloning, pET30a vectors containing the correct insert were identified by restriction screening. **Table 4-2** gives an overview of utilized restriction enzymes and calculated sizes of restriction fragments. After positive restriction screening, vectors were chemically transformed into *E. coli* Strain A. Best producers were selected by clone screening in shake flask cultures and used for cell bank establishing.

Table 4-2: Overview of restriction screening: subcloning

antibody fragment	BtgI	PstI	sizes of restriction fragments
B1 scFv E-Linker	P	P	3399, 1636, 525, 360, 60, and 57 bp
B1 scFv L205	P	P	3399, 1636, 525, 375, 60, and 57 bp
B1 scFv L218	P	P	3399, 1636, 525, 354, 60, and 57 bp
B1 scFv LLB18	P	P	3399, 1636, 525, 363, 60, and 57 bp
A4_Fab_LC_bacMiG	–	P	5255 and 650 bp
A4_Fab_HC_bacMiG	–	P	5255 and 668 bp

The genes of following antibody fragments were already provided in pET30a vectors from previous works (also see **Table 3-3**):

- H6 scFv comprising the $(Gly_4Ser)_3$ linker
- B1 scFv comprising the $(Gly_4Ser)_3$ linker
- A4 scFv comprising the $(Gly_4Ser)_3$ linker

The vectors were chemically transformed into *E. coli* Strain B and Strain A. Best producers were selected by clone screening in shake flask cultures and used for cell bank establishment.

4.3 Expression of scFv fragments in *E. coli*

Yields in the gram per liter range were reported by employing *E. coli* for cytosolic scFv fragment expression as IBs (Martineau *et al.*, 1998). After cytosolic expression IBs usually require *in vitro* refolding steps in order to obtain active proteins.

4.3.1 Expression of different scFv fragments at 35°C

As a first attempt, the influence of different *E. coli* strains on scFv fragments' yield and IB quality was investigated. **Table 4-3** gives an overview of model proteins and important expression features:

Table 4-3: Expression of different scFv fragments at 35°C

scFv fragment	Linker peptide	Cultivation temperature	Expression host
H6	$(Gly_4Ser)_3$	35°C	*E. coli* Strain B and Strain A
A4			
B1			

The fermentations were carried out in 20 L scale according to standard operating parameters (**Table 3-16**). A standard fed batch protocol was applied:

Table 4-4: Standard fed batch protocol for the expression of different scFv fragments at 35°C, 20 L scale

PRE CULTURE	Medium	**Table 3-12**	
	Inoculum	*E. coli* Strain B or Strain A + pET30a comprising H6 scFv, A4 scFv, or B1 scFv	
	Inoculation ratio	1:1000 (inoculum from MCB)	
BATCH	Medium	**Table 3-13**	
	Inoculation ratio	150 mL pre culture (OD_{550} between 1.0 and 3.0) were inoculated to 13 L batch medium	
FED BATCH	Feed Medium	**Table 3-14**	
	Exponential feed	$\mu = 0.15\ h^{-1}$	for 16.5 h
	Constant feed, induction	47.5 mL/h*L batch (617.8 mL/h; ρ=1.22)	for 5 h

Figure 4-1 and **Figure 4-2** show fermentations with *E. coli* Strain B and Strain A expressing B1 scFv at 35°C. The figures are exemplary for fermentations with H6- and A4 scFv fragments. Batch phase of Strain B was always finished faster than batch phase of Strain A indicating faster growth on and adaption to glucose. As shown in **Figure 4-1** batch phase of B1 scFv fragment was 9.6 h for Strain B compared to 12.1 h for Strain A.

A sudden increase of the DO level indicated the end of the batch phase caused by the depletion of glucose. Exponential feeding was subsequently started with a growth rate of μ = 0.15 h^{-1} which was maintained for 16.5 h. Afterwards, exponential feed was changed to constant feed to guarantee sufficient oxygen supply during induction period. Full induction with 1 mM IPTG (calculated in relation to the final fermentation volume) was applied for 5 h. According to a growth limited fed batch, the glucose level was perpetually monitored and kept at a minimum in exponential- and constant feed phase.

During exponential- and constant feed phase both strains showed similar growth behaviour as shown in **Figure 4-2**. However, biomass formation in Strain B proceeded after induction with IPTG but diminished in Strain A after the second hour of induction. Consequently, DCW-values were lower for Strain A than for Strain B at the fermentation-end (52 g/L compared to 60 g/L).

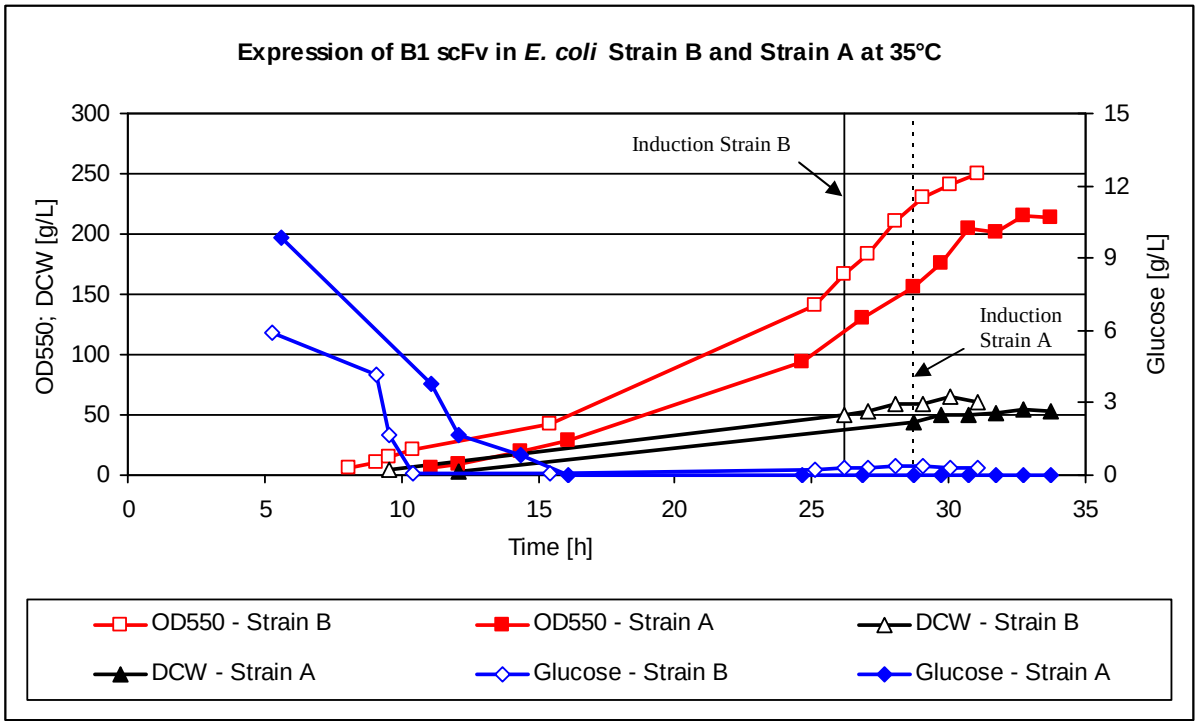

Figure 4-1: Comparison of B1 scFv expression in *E. coli* Strain B and Strain A; total fermentation

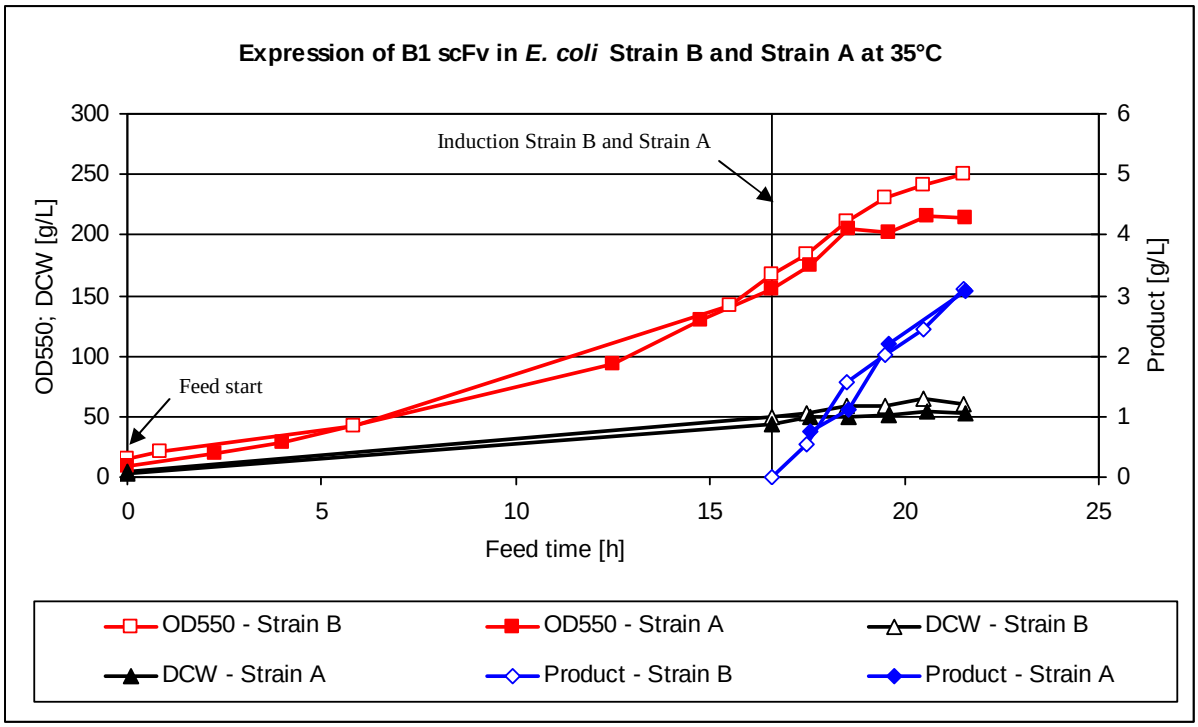

Figure 4-2: Comparison of B1 scFv expression in *E. coli* Strain B and Strain A; feed phase

For protein analysis from cell lysates, cell disruption by ultrasonication was applied. The scFv fragments were identified by their molecular weights using SDS-PAGE and quantified by densitometry. **Figure 4-3** shows a representative Coomassie stained gel of A4 scFv expressed in *E. coli* Strain B:

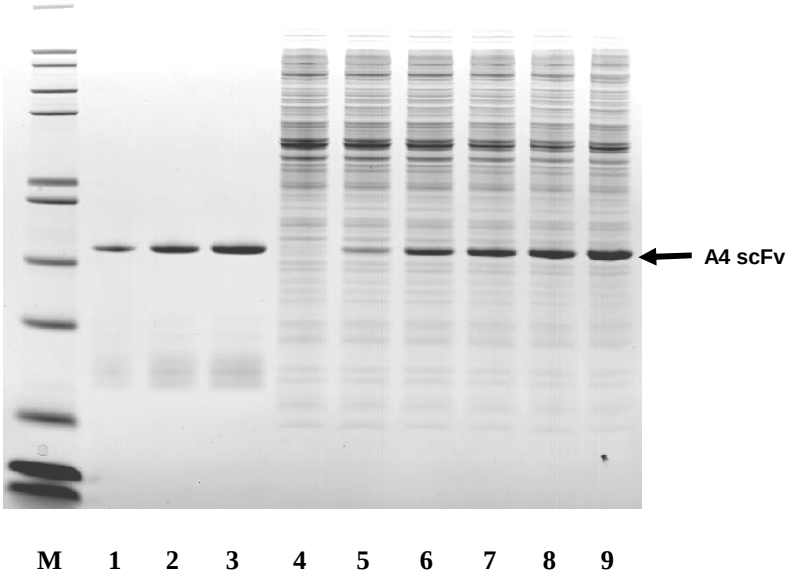

Figure 4-3: SDS-PAGE (12%) of total protein fraction of *E. coli* Strain B expressing A4 scFv at 35°C in 20 L scale: (M) Mark 12 molecular weight marker [200.0; 116.3; 97.4; 66.3; 55.4; 36.5; 31.0; 21.5; 14.4; 6.0; 3.5; 2.5 kDa]; (1-3) A4 scFv standard [0.4; 0.8; 1.2 µg]; (4) uninduced; (5) induced for 1 h; (6) induced for 2 h; (7) induced for 3 h; (8) induced for 4 h; (9) induced for 5 h;

The intensity of the Coomassie stained A4 scFv bands increased from lane 4 to lane 9 indicating continuous product formation until fermentation-end. Specific product concentration (**Table 4-6**) as well as volumetric product concentration (**Table 4-7**) increased throughout the induction period for all scFv fragments. Except for H6 scFv, the maxima of specific product concentration and volumetric product concentration were obtained after 5 hours of induction for all fermentations. H6 scFv expressed in *E. coli* Strain A reached its maximum after 4 hours of induction. The overall maximum of specific product concentration (132 mg/g DCW) and volumetric product concentration (8.4 g/L) was obtained for A4 scFv expressed in *E. coli* Strain B.

Table 4-5: Comparison of maximum cytosolic H6 scFv concentrations: results from Wagner, 2008 and the present work

Maximum cytosolic product concentration of H6 scFv [g/L]			
E. coli Strain B		*E. coli* Strain A	
(Wagner, 2008)	Present work	(Wagner, 2008)	Present work
0.2	6.7	0.1	5.9

In **Table 4-5** maximum intracellular H6 scFv concentrations obtained by Wagner, 2008 and in the present work are compared. A potential explanation for the considerable differences of volumetric product concentrations could be the codon optimized gene sequences of H6 scFv used in the present work. For more details on effects of non-host-codon-usage on recombinant protein expression see **Chapter 2.2.3**. Previous works reported volumetric product concentrations in the gram per liter range for intracellular scFv fragment expression as IBs in *E. coli* (Martineau *et al.*, 1998). The high volumetric product concentrations obtained in the present work (**Table 4-5**) are in accordance with these results.

Considering the variation of SDS-PAGE results and DCW values, the specific product concentrations of both strains expressing H6- and A4 scFv were comparable (**Table 4-6**). The specific product concentrations of both strains expressing B1 scFv only reached about 50% the amount of H6- and A4 scFv expression. For fermentations with B1 scFv (**Figure 4-1**), DCW-values were lower for Strain A than for Strain B at the fermentation-end (52 g/L compared to 60 g/L). The same observations were made for H6- and A4 scFv. **Figure 4-1** shows fermentations of B1 scFv and is exemplary for fermentations of H6- and A4 scFv as well.

These findings explain the comparable maximum volumetric product concentrations of H6- and A4 scFv for each individual expression host (6.7 g/L and 8.4 g/L for Strain B; 5.9 g/L and 6.3 g/L for Strain A). However, Strain B yielded significantly higher volumetric product concentrations for H6- and A4 scFv than Strain A (**Table 4-7**). A possible explanation are higher maximum DCW values for fermentations with Strain B than for Strain A (**Figure 4-1**). Volumetric B1 scFv concentrations only reached 3.1 g/L in both expression hosts. This is only about 50% of H6- and A4 scFv and can be explained by 50% lower specific product concentrations for B1 scFv in both hosts. DCW values were higher for Strain B than for Strain

A (60g/L and 52 g/L; **Figure 4-1**). Nevertheless, both strains yielded 3.1 g/L of B1 scFv at fermentation-end. This is caused by higher specific B1 scFv concentrations in Strain A compared to Strain B (59 mg/g DCW and 52 mg/g DCW).

Table 4-6: Specific product concentrations of scFv fragments throughout induction period

	Specific product concentration [mg/g DCW]					
	H6 scFv		**A4 scFv**		**B1 scFv**	
Induction time	B[i]	A[ii]	B[i]	A[ii]	B[i]	A[ii]
0 h	8	–	2	–	0	–
1 h	40	34	28	31	10	15
2 h	72	74	67	61	27	22
3 h	84	88	86	77	34	43
4 h	98	**109**	100	111	38	–
5 h	**111**	87	**132**	**122**	**52**	**59**

Table 4-7: Volumetric product concentrations of scFv fragments throughout induction period

	Volumetric product concentration [g/L]					
	H6 scFv		**A4 scFv**		**B1 scFv**	
Induction time	B[i]	A[ii]	B[i]	A[ii]	B[i]	A[ii]
0 h	0.4	–	0.1	–	–	–
1 h	2.1	1.6	1.5	1.6	0.5	0.8
2 h	4.1	3.9	3.9	3.2	1.6	1.1
3 h	4.9	4.7	5.6	4.2	2.0	2.2
4 h	6.3	**5.9**	6.8	5.5	2.4	–
5 h	**6.7**	4.8	**8.4**	**6.3**	**3.1**	**3.1**

Remarkably, values for specific product concentration (**Table 4-6**) and volumetric product concentration (**Table 4-7**) of B1 scFv only reached about one half the amount of A4- and H6 scFv. **Figure 4-4** shows the Coomassie stained gel of B1 scFv expressed in *E. coli* Strain B:

[i] *E. coli* Strain B

[ii] *E. coli* Strain A

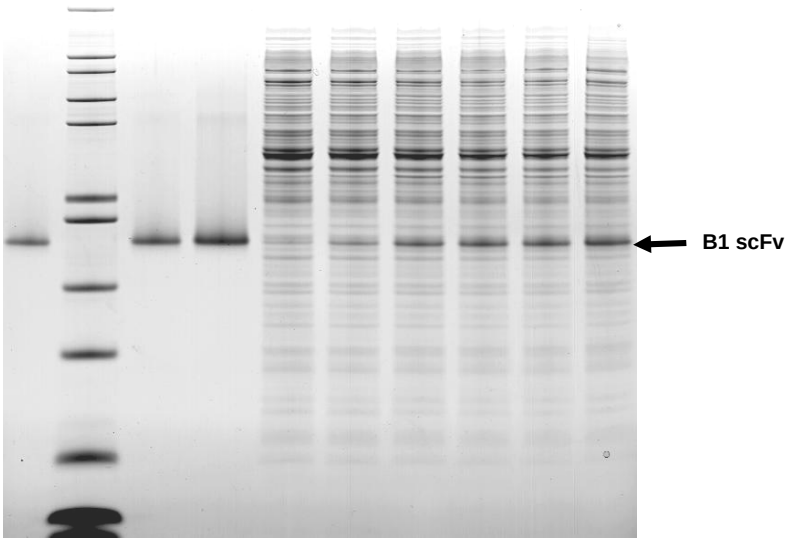

Figure 4-4: SDS-PAGE (12%) of total protein fraction of *E. coli* Strain B expressing B1 scFv at 35°C in 20 L scale: (M) Mark 12 molecular weight marker [200.0; 116.3; 97.4; 66.3; 55.4; 36.5; 31.0; 21.5; 14.4; 6.0; 3.5; 2.5 kDa]; (1-3) B1 scFv standard [0.4; 0.8; 1.2 µg]; (4) uninduced; (5) induced for 1 h; (6) induced for 2 h; (7) induced for 3 h; (8) induced for 4 h; (9) induced for 5 h;

In this work, the reason for poor B1 scFv expression levels as IBs could not be determined. It might be caused by its length of 253 aa compared to 243 aa for H6 scFv and 236 aa for A4 scFv. Differences in theoretical pI of 9.06 compared to 4.99 for H6 scFv and 8.35 for A4 scFv (**Table 3-3**) might also have an influence on its expression.

It was assumed, that a portion of B1 scFv was expressed not only as IBs but also soluble in the cytosol. Subsequently, the influence of different cultivation temperatures on the *in vivo* aggregation, expression level, and purity of B1 scFv was investigated (**Chapter 4.3.2**).

4.3.1.1 Product purity

Product purity was determined according to **Chapter 3.8.2.4**. Values for product purities are listed in **Table 4-8**:

Table 4-8: Product purity of different scFv fragments at fermentation-end

scFv fragment	Host	Lane purity [%]	IB purity [mg/g IB]
H6	B[i]	refolding not possible	
	A[ii]		
A4	B[i]	63	381
	A[ii]	69	290
B1	B[i]	47	218
	A[ii]	52	281

For H6 scFv, refolding was not possible. A potential explanation is an unpaired cysteine residue in the H6 scFv polypeptide chain interacting with unpaired cysteine residues of other H6 scFv fragments.

According to **Table 4-8**, lane purities and IB purities greatly depend on the nature of the expressed scFv fragment and not on the expression host. In addition, IB purities depend on the applied expression host as well. As discussed in **Chapter 3.8.2.4**, IB purity has more validity than lane purity. Therefore, A4 scFv expressed in *E. coli* Strain B is favourable in terms of purity (381 mg/g IB). IB purities of B1- and A4 scFv expressed in *E. coli* Strain A were about 100 mg/g IB lower. The least IB purity was obtained by B1 scFv expressed in Strain B yielding only 218 mg/g IB.

4.3.2 Expression of B1 scFv at different temperatures

As stated in **Chapter 4.3.1**, values for specific product concentrations (**Table 4-6**) as well as volumetric product concentrations (**Table 4-7**) of B1 scFv only reached about 50% compared to A4- and H6 scFv. It was assumed, that a portion of B1 scFv was expressed not only as IBs but also in a soluble form in the cytosol. It is well known (also see **Chapter 2.2.4**) that higher cultivation temperatures increase growth rates, protein synthesis rates (Sorensen and Mortensen, 2005), and *in vivo* aggregation of recombinant proteins (Kiefhaber *et al.*, 1991) in

[i] *E. coli* Strain B

[ii] *E. coli* Strain A

E. coli (Schein, 1989; Vasina and Baneyx, 1997). Consequently, fermentations of *E. coli* Strain B and Strain A expressing B1 scFv at higher (42°C) and lower (28°C) cultivation temperature were performed. The aim was to investigate the influence of different cultivation temperatures on the *in vivo* aggregation, expression level, and purity of B1 scFv. As an outcome, the insoluble (as IBs) or soluble *in vivo* expression should be evaluated as an expression strategy for B1 scFv.

Shortly before induction, temperature shifts from 35°C to 28°C and 42°C were applied. **Table 4-9** gives an overview of expression features:

Table 4-9: Expression of B1 scFv at different temperatures

scFv fragment	Expression temperature			Linker peptide	Expression host
	28°C	35°C	42°C		
B1	P	P	P	$(Gly_4Ser)_3$	*E. coli* Strain B and Strain A

For fermentations at 35°C the same data as described in **Chapter 4.3.1** was used. Apart from temperature, the fermentations at 28°C and 42°C expression temperature were carried out according to standard operating parameters (**Table 3-16**) in 20 L scale. A standard fed batch protocol (**Table 4-10**) was applied:

Table 4-10: Standard fed batch protocol for the expression of B1 scFv with temperature shifts from 35°C to 28°C and 42°C in *E. coli* Strain B and Strain A

PRE CULTURE	Medium	**Table 3-12**
	Inoculum	*E. coli* Strain B or Strain A + pET30a comprising B1scFv
	Inoculation ratio	1:1000 (inoculum from MCB)
BATCH	Medium	**Table 3-13**
	Inoculation ratio	150 mL pre culture (OD_{550} between 1.0 and 3.0) were inoculated to 13 L batch medium
FED BATCH	Feed Medium	**Table 3-14**
	Exponential feed	$\mu = 0.15\ h^{-1}$
	pO_2 controlled feed Constant feed	$pO_2 \geq 20\%$ (expression temperature 28°C) 47.5 mL/h*L batch (617.8 mL/h; ρ=1.22) (expression temperature 42°C)

Cultivation temperature during batch phase was 35°C for all fermentations. Therefore, batch phase was comparable with fermentations of B1 scFv at 35°C (**Figure 4-1**). Batch phase of Strain B was always shorter compared to batch phase of Strain A as already observed and discussed in **Chapter 4.3.1**. A sudden increase of the DO level indicated the end of the batch phase caused by the depletion of glucose. Exponential feed was subsequently started with a growth rate of $\mu = 0.15\ h^{-1}$.

For fermentations with temperature shifts from 35°C to 28°C, exponential feeding was maintained for 16.0 h. It is well known (Sorensen and Mortensen, 2005) that reducing the cultivation temperature results in reduced growth rates and therefore lower glucose consumption rates in *E. coli*. To accomplish the adaption to a lower growth rate at 28°C, a pO_2-controlled feed was applied. The pO_2-level was set to $\geq$ 20% by fixing the rotational speed of the stirrer, the aeration, and the pressure. pO_2-levels below this value indicated increased *E. coli* growth resulting from an overabundance of glucose in the medium and *vice versa*. Therefore, the feed rate could quickly be adapted. Measurement of glucose level was not applicable for fast adaption of feed rates to prevent glucose accumulation in the medium because it could only be monitored offline. The cultivation temperature was 35°C during the first hour of pO_2-controlled feed letting the controller and the culture adapt to the new conditions. After the first hour of pO_2-controlled feed, the cultivation temperature was decreased to 28°C which was maintained for an additional hour for adaption reasons. After the second hour of pO_2-controlled feed, full induction with 1 mM IPTG (calculated in relation to the final fermentation volume) was applied for 6 h. For fermentation diagrams of *E. coli* Strain B and Strain A see **Figure 4-5** and **Figure 4-6**.

For fermentations with temperature shifts from 35°C to 42°C, the exponential feed was maintained for 16.5 h. 15 h after exponential feed start, the cultivation temperature was raised from 35°C to 42°C to let the culture adapt to the new conditions. After exponential feed a constant feed was applied. The constant feed guaranteed sufficient oxygen supply during the induction period. Simultaneously with feed change, full induction with 1 mM IPTG (calculated in relation to the final fermentation volume) was applied for 6 h. The glucose level was at a minimum during exponential- and constant feed phase and perpetually monitored according to a growth limited fed batch. For fermentation diagrams see **Figure 4-5** and **Figure 4-6**.

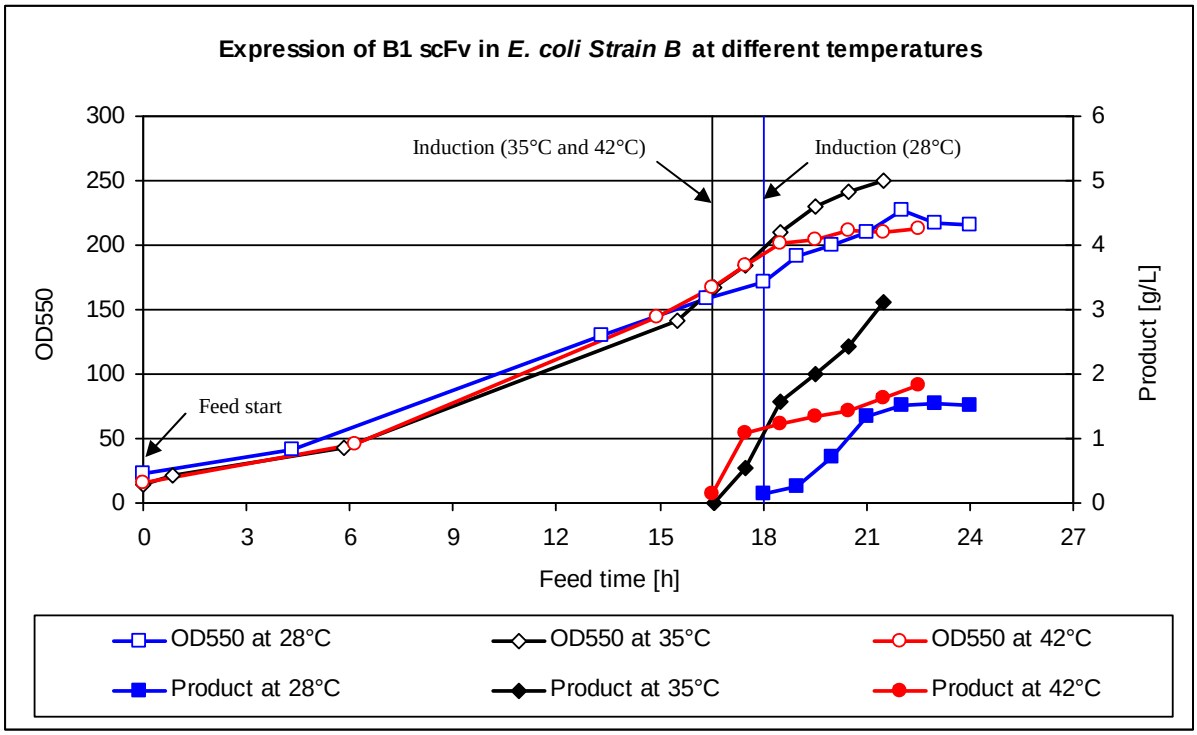

Figure 4-5: Comparison of B1 scFv expression in *E. coli* Strain B at different temperatures; feed phase

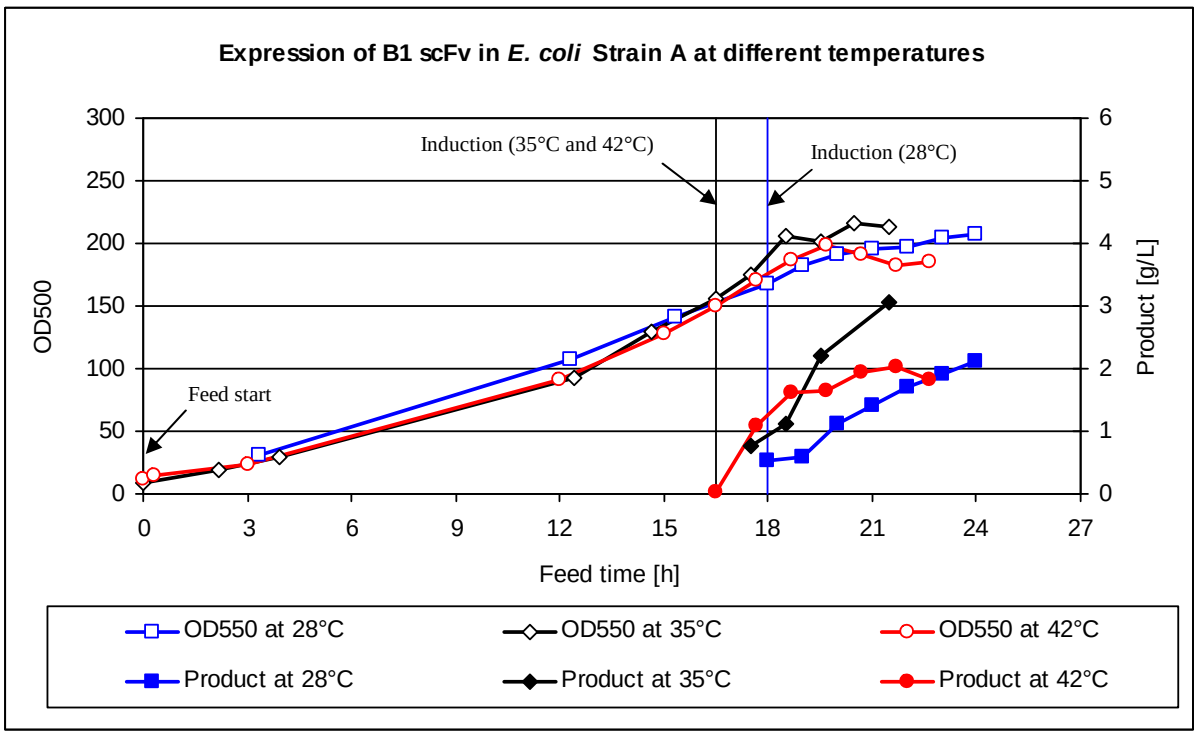

Figure 4-6: Comparison of B1 scFv expression in *E. coli* Strain A at different temperatures; feed phase

For protein analysis from cell lysates, cell disruption by ultrasonication was applied. The scFv fragments were identified by their molecular weights using SDS-PAGE and quantified by densitometry. Specific product concentration (**Table 4-11**) as well as volumetric product concentration (**Table 4-12**) increased throughout the induction period for all fermentations. The maxima of specific product concentration and volumetric product concentration were reached 5 h or 6 h after induction. Considering the variation of SDS-PAGE, the volumetric product concentrations for both strains at 35°C and 42°C expression temperature were comparable (**Table 4-12**). At 28°C expression temperature however, the volumetric B1 scFv concentration was significantly higher in Strain A compared to Strain B at the fermentation-end (2.1 g/L and 1.5 g/L). After induction Strain A showed diminished biomass formation compared to Strain B as indicated by OD_{550} trends (**Figure 4-5** and **Figure 4-6**). As a consequence, throughout induction period the specific product concentration was usually significantly higher for Strain A compared to Strain B at the same induction temperature (**Table 4-11**).

At 42°C induction temperature, Strain B and Strain A showed highest product concentrations of all fermentations after the first hour of induction (1.0 g/L and 1.1 g/L). The increase of product concentrations greatly attenuated afterwards, however (**Table 4-12**, **Figure 4-5**, and **Figure 4-6**). At the fermentation-end the values were 1.8 g/L for both, Strain B and Strain A and therefore much lower compared to 35°C expression temperature. Previous works described higher recombinant protein expression rates after a sudden increase of the cultivation temperature (Shaw and Ingraham, 1967). In the present work, these observations could be confirmed for the first hour of induction. Afterwards, the expression hosts could not maintain the high protein expression rates.

At 28°C expression temperature the volumetric product concentration in Strain B and Strain A was low (0.2 g/L and 0.6 g/L) after 1 induction-hour. This complies with data from other authors who reported decreased expression efficiencies at lower cultivation temperatures (Shaw and Ingraham, 1967; Vasina and Baneyx, 1996) (also see **Chapter 2.2.4**). Owing to the low temperature of 28°C, steady product formation was observed after induction (**Table 4-12**). Similar volumetric product concentrations were observed at the fermentation-end compared to fermentations at 42°C expression temperature. After 6 hours of induction, Strain B and Strain A reached 1.5 g/L and 2.1 g/L at 28°C induction temperature.

Both strains reached maximum OD_{550} values as well as maximum volumetric- and specific B1 scFv concentrations at 35°C expression temperature (**Table 4-12**, **Figure 4-5**, and **Figure 4-6**). After 5 hours of induction, OD_{550} values were 250 for Strain B and 214 for Strain A. Volumetric product concentrations were 3.1 g/L for both (**Table 4-12**). Specific product concentration was 52 mg/g DCW compared to 59 mg/g DCW (**Table 4-11**). Therefore, maximum product formation is obtained at 35°C.

Table 4-11: Specific B1 scFv concentrations throughout induction period

	Specific B1 scFv concentration [mg/g DCW]					
	28°C		**35°C**		**42°C**	
Induction time	B[i]	A[ii]	B[i]	A[ii]	B[i]	A[ii]
0 h	3	12	–	–	3	0
1 h	4	11	10	15	21	26
2 h	11	–	27	22	24	33
3 h	22	27	34	43	26	36
4 h	26	32	38	–	26	43
5 h	**28**	35	**52**	**59**	30	**45**
6 h	26	**40**	–	–	**34**	42

Table 4-12: Volumetric B1 scFv concentrations throughout induction period

	Volumetric B1 scFv concentration [g/L]					
	28°C		**35°C**		**42°C**	
Induction time	B[i]	A[ii]	B[i]	A[ii]	B[i]	A[ii]
0 h	0.1	0.5	–	–	0.1	0.0
1 h	0.2	0.6	0.5	0.8	1.1	1.1
2 h	0.7	1.1	1.6	1.1	1.2	1.6
3 h	1.3	1.4	2.0	2.2	1.4	1.6
4 h	1.5	1.7	2.4	–	1.4	1.9
5 h	**1.5**	1.9	**3.1**	**3.1**	1.6	**2.0**
6 h	1.5	**2.1**	–	–	**1.8**	1.8

Previous work described increased cytosolic aggregation of recombinant protein by increasing the expression temperature (Kiefhaber *et al.*, 1991). Other authors reported increased *in vivo*

[i] *E. coli* Strain B

[ii] *E. coli* Strain A

solubility of recombinant proteins by decreasing the expression temperature (Schein, 1989; Vasina and Baneyx, 1997). The data in the present work clearly show that the overall *in vivo* production of recombinant B1 scFv is not enhanced by increasing or lowering the expression temperature of *E. coli* Strain B and Strain A. To investigate whether altering the expression temperature affects the *in vivo* solubility of recombinant B1 scFv, additional experiments were performed (**Chapter 4.3.1**).

4.3.2.1 IB formation and soluble expression in the cytosol

The *in vivo* solubility of B1 scFv in *E. coli* Strain B and Strain A was determined by Western blot analysis from end-of-fermentation samples. For Strain A expressing B1 scFv at 35°C, an additional sample 1 h before fermentation-end was analyzed. Cell walls were enzymatically degraded applying BugBuster® reagent (**Chapter 3.8.2.2**). The soluble B1 scFv fraction was separated from the insoluble fraction (IBs) by centrifugation. Afterwards, samples were treated with LDS_{red} and separared by SDS-PAGE. Western blot was accomplished as described in **Chapter 3.8.3**:

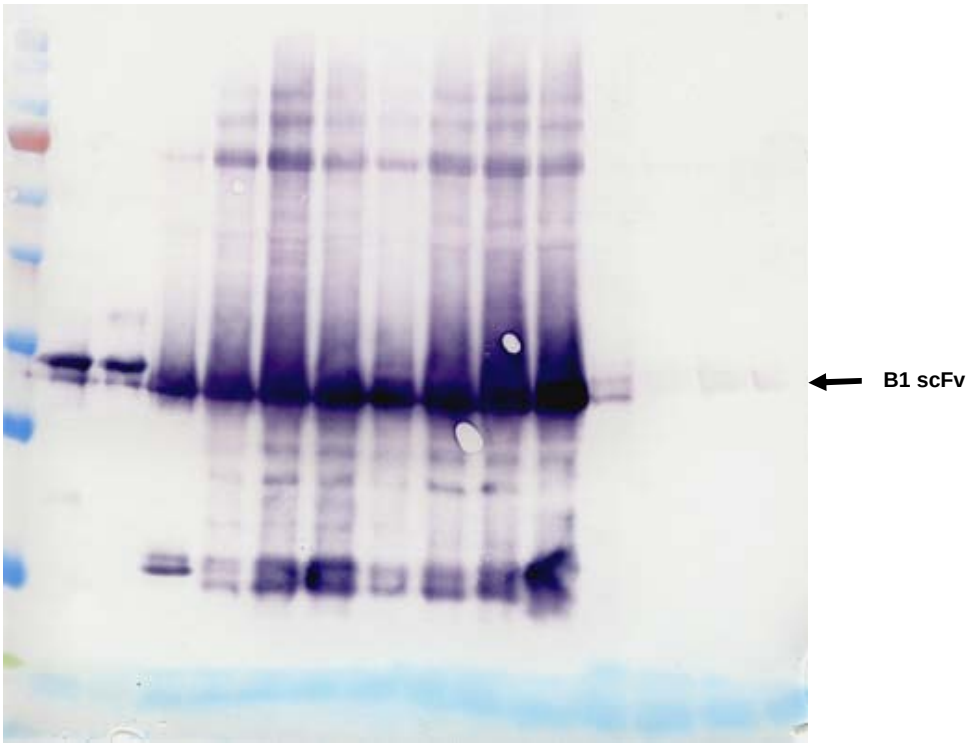

Figure 4-7: Western blot of insoluble and soluble protein fractions of *E. coli* Strain B and Strain A expressing B1 scFv in 20 L scale at different temperatures: (M) PageRuler™ Prestained Protein Ladder [170; 130; 100; 70; 55; 40; 35; 25; 15; 10 kDa]; (P) positive control of H6 scFv; (S) B1 scFv standard [1.2 µg]; (1) Strain B at 28°C – insoluble (2) Strain B at 35°C – insoluble (3) Strain B at 42°C – insoluble (4) Strain A at 28°C – insoluble (5) Strain A at 35°C – insoluble (6) Strain A at 35°C – insoluble (7) Strain A at 42°C – insoluble (8) Strain B at 28°C – soluble (9) Strain B at 35°C – soluble (10) Strain B at 42°C – soluble (11) Strain A at 28°C – soluble; the sample in lane 5 was derived one hour before fermentation-end. All other samples were derived from fermentation-end:

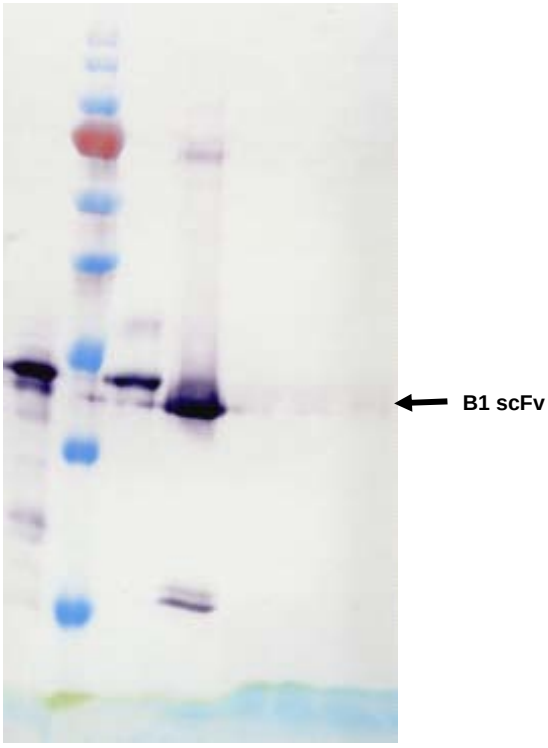

Figure 4-8: Western blot of soluble protein fractions of *E. coli* Strain A expressing B1 scFv in 20 L scale at different temperatures: (M) PageRuler™ Prestained Protein Ladder [170; 130; 100; 70; 55; 40; 35; 25; 15; 10 kDa]; (P) positive control of H6 scFv; (S) B1 scFv standard [1.2 µg]; (1) Strain A at 35°C – soluble (2) Strain A at 35°C – soluble (3) Strain A at 42°C – soluble; the sample in lane 1 was derived one hour before fermentation-end. The other samples were derived from fermentation-end

B1 scFv was only detected in the insoluble fractions (**Figure 4-7**: lanes 1 **–** 7). The bands in lane 8 (**Figure 4-7**) and lane 1 (**Figure 4-8**) are most likely a result of B1 scFv diffusion from adjacent lanes. The presented data clearly indicate that expression temperature does not affect intracellular solubility of B1 scFv. It also shows that the *in vivo* solubility of B1 scFv is not

affected by the expression host (*E. coli* Strain B and Strain A). Shifting the expression temperature from 35°C to 42°C or 28°C decreases the volumetric- and specific product concentration up to 50% as determined by SDS-PAGE analysis (compare **Table 4-13**).

Table 4-13: Concentration of B1 scFv expressed at different temperatures; fermentation-end

B1 scFv			
Temperature shift to [°C]	**Host**	**Volumetric B1 scFv concentration [g/L]**	**Specific B1 scFv concentration [mg/g DCW]**
28°C	B[i]	1.5	26
	A[ii]	2.1	40
35°C	B[i]	3.1	52
	A[ii]	3.1	59
42°C	B[i]	1.8	34
	A[ii]	1.8	42

These data are not in accordance with Schein and Vasina and Baneyx who reported increased *in vivo* solubility of recombinant protein at lower cultivation temperatures (Schein, 1989; Vasina and Baneyx, 1997). They are also not in accordance with Kiefhaber *et al.* who reported increased cytosolic aggregation of recombinant protein at higher cultivation temperatures (Kiefhaber *et al.*, 1991). Irrespective of cultivation temperature of *E. coli* strains Strain B and Strain A, B1 scFv was only expressed as IBs in the cytoplasm. Therefore, only the insoluble (as IBs) expression strategy in the cytoplasm is feasible for B1 scFv. Moreover, altering the expression temperature does not increase the overall volumetric- or specific B1 scFv concentrations as proposed in the beginning of **Chapter 4.3.2**.

4.3.2.2 Product purity

Product purity was determined as described in **Chapter 3.8.2.4**.

[i] *E. coli* Strain B

[ii] *E. coli* Strain A

Table 4-14: Product purity of B1 scFv expressed at different temperatures; fermentation-end

B1 scFv			
Temperature shift to [°C]	**Host**	**Lane purity [%]**	**IB purity [mg/g IB]**
28°C	B[i]	28	100
	A[ii]	36	157
35°C	B[i]	47	218
	A[ii]	52	281
42°C	B[i]	31	133
	A[ii]	35	200

According to **Table 4-14**, lane purity is comparable between *E. coli* Strain B and Strain A at same expression temperatures. However, IB purity is higher by 57-67 mg/g IB for Strain A compared to Strain B at the same expression temperature. Therefore, Strain A is the favourable expression host in terms of purity.

Lane purity is comparable between fermentations performed at 28°C and 42°C. Fermentations performed at 35°C have about 20% higher lane purity than fermentations performed at 28°C and 42°C. IB purities at 35°C are higher between 18-181 mg/g IB than for the other temperatures. Therefore, 35°C is the favourable expression temperature in terms of purity.

As stated in **Chapter 4.3.1**, values for specific product concentrations (**Table 4-6**) as well as volumetric product concentrations (**Table 4-7**) of B1 scFv only reached about 50% compared to A4- and H6 scFv. These poor expression levels could not be countervailed by altering the expression temperature (**Chapter 4.3.2**). In another approach, four different linker peptides connecting the variable domains were introduced in B1 scFv replacing the $(Gly_4Ser)_3$ linker. These B1 scFvs comprising the newly introduced linkers were investigated in terms of intracellular solubility, expression level, and purity in *E. coli* Strain A (**Chapter 4.3.3**).

[i] *E. coli* Strain B

[ii] *E. coli* Strain A

4.3.3 Expression of B1 scFv comprising different linker peptides

As discussed in **Chapter 2.2.5**, the linker peptide connecting the two scFv domains affects several properties of the corresponding scFv fragment. Three of them are especially important when investigating the influence of different linker peptides on the yield, intracellular solubility, and purity of B1 scFv: **(i)** The linker peptide influences aggregation (Whitlow *et al.*, 1993; Desplancq *et al.*, 1994; Raag and Whitlow, 1995) and thermodynamic stability (Robinson and Sauer, 1998). **(ii)** An overabundance of polar residues within the linker sequence favours the solubility of scFv fragments and avoids intercalation within or between variable domains during scFv fragment folding (Huston *et al.*, 1991). **(iii)** On the other hand, studies with scFv fragments comprising different linkers show that expression levels (Tang *et al.*, 1996) as well as folding properties are mainly influenced by the domains and not by the linker (Hennecke *et al.*, 1998). Therefore, the linker peptide is considered as a 'passive connector' (Hennecke *et al.*, 1998).

Four different linker peptides (**Chapter 2.2.5**) were introduced into the existing B1 scFv, replacing the commonly used $(Gly_4Ser)_3$-linker (Huston *et al.*, 1991). **Table 4-15** gives an overview of utilized linker peptides and expression features:

Table 4-15: Expression of B1 scFv comprising newly introduced linker peptides

scFv fragment	Expression temperature	Linker peptide	Expression host
B1	37°C	L205	*E. coli* Strain A
		L218	
		LLB18	
		E-Linker	

At first, fermentations at 35°C in 20 L scale were performed with B1 scFv comprising the linkers L205 and LLB18. These fermentations were carried out according to standard operating parameters (**Table 3-16**). A standard fed batch protocol (**Table 4-16**) was applied:

Table 4-16: Standard fed batch protocol for B1 scFv expression comprising different linker peptides; 20 L scale

PRE CULTURE	Medium	**Table 3-12**	
	Inoculum	*E. coli* Strain A + pET30a comprising B1scFv (L205/L218/LLB18/E-Linker)	
	Inoculation ratio	1:1000 (inoculum from MCB)	
BATCH	Medium	**Table 3-13**	
	Inoculation ratio	150 mL pre culture (OD_{550} between 1.0 and 3.0) were inoculated to 13 L batch medium	
FED BATCH	Feed Medium	**Table 3-14**	
	Exponential feed	$\mu = 0.15\ h^{-1}$	for 16.5 h
	Constant feed, induction	47.5 mL/h*L batch (617.8 mL/h; ρ=1.22)	for 6 h

During fermentations a rapid increase of media viscosity resulted in insufficient oxygen supply. As a consequence the fermentations of B1 scFv comprising L205 and LLB18 had to be aborted after 15.2 h and 14.8 h total fermentation time, respectively. Reruns with the same fermentation conditions were performed with B1 scFv comprising the linkers L205, L218, and E-Linker. A rapid increase of media viscosity for fermentations with B1 scFv comprising L205 and L218 resulted in insufficient oxygen supply again. The fermentation with L218 had to be aborted 23.7 h after total fermentation time. The fermentation of L205 had to be aborted after the third hour of induction. The fermentation applying B1 scFv comprising the E-Linker did not show an increase of medium viscosity and could be carried out until the scheduled fermentation-end. A correlation between culture broth viscosity and oxygen transfer was already observed by other authors (Krishna Rao *et al.*, 2008). The exact cause for the rapid increase of media viscosity could not be identified. As described by Balasundaram *et al.*, a reason for increased viscosity might be the release of chromosomal DNA into the culture broth caused by cell lysis (Balasundaram *et al.*, 2009).

Modifications to the standard operating parameters (**Table 3-16**) and the standard fed batch protocol (**Table 4-16**) were made to circumvent the problem of increased media viscosity. One approach was to raise the fermentation temperature from 35°C to 37°C. Another

approach was to raise the fermentation temperature from 35°C to 37°C and to add complex media components to batch- and feed-media (10 g/L bacto yeast extract and 20 g/L soy peptone). Subsequent fermentations in 20 L scale were performed employing B1 scFv comprising the linker L205. Both fermentations could be carried out until the scheduled fermentation-end.

Consequently, fermentations of B1 scFv comprising the linkers L205, L218, LLB18, and E-Linker were performed at 37°C. The fermentations were accomplished in 5 L scale applying media without complex additions. An adapted standard fed batch protocol as listed in **Table 4-17** was applied:

Table 4-17: Standard fed batch protocol for B1 scFv expression comprising different linkers; 5 L scale

PRE CULTURE	Medium	**Table 3-12**	
	Inoculum	*E. coli* Strain A + pET30a comprising B1scFv (L205/L218/LLB18/E-Linker)	
	Inoculation ratio	1:1000 (inoculum from MCB)	
BATCH	Medium	**Table 3-13**	
	Inoculation ratio	35 mL pre culture (OD_{550} between 1.0 and 3.0) were inoculated to 3.5 L batch medium	
FED BATCH	Feed Medium	**Table 3-14**	
	Exponential feed	$\mu = 0.15\ h^{-1}$	for 16.5 h
	Constant feed, induction	47.5 mL/h*L batch (166.3 mL/h; ρ=1.22)	for 6 h

The fermentations of B1 scFv comprising L205, LLB18, and E-Linker showed similar courses of fermentation. The trend of B1 scFv comprising LLB18 in **Figure 4-9** is exemplary for fermentations with B1 scFv comprising L205 and E-Linker:

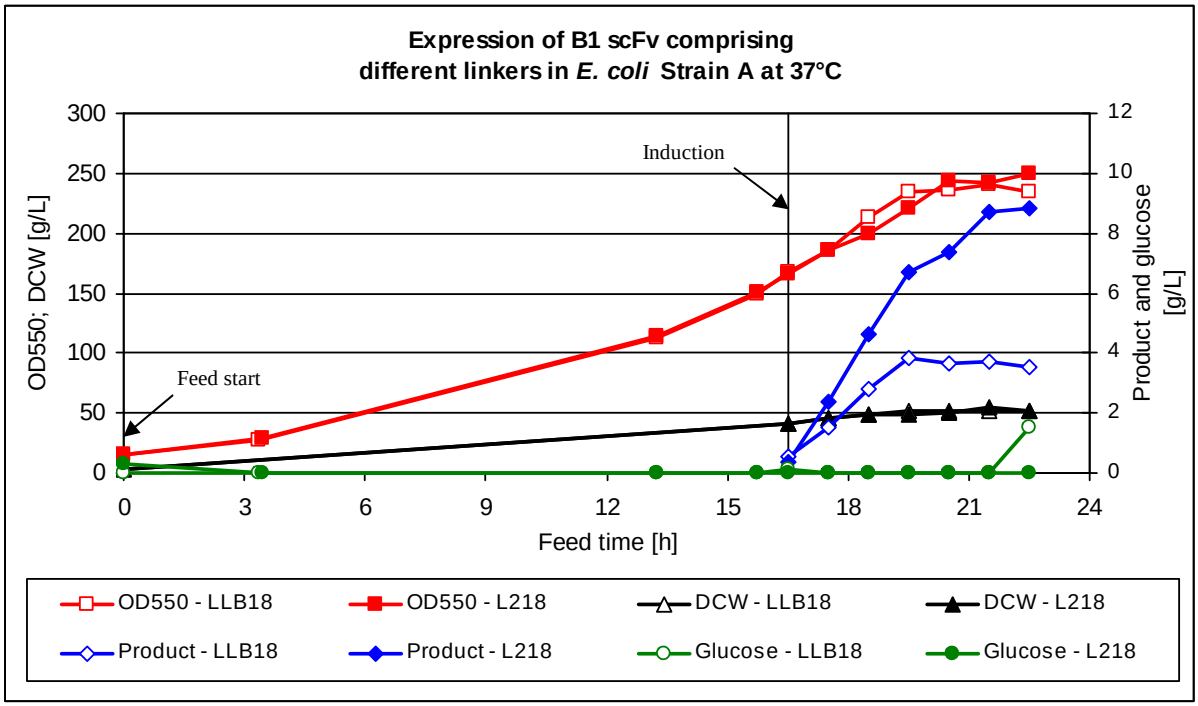

Figure 4-9: Comparison of B1 scFv expression comprising Linkers LLB18 and L218; feed phase

After exponential feed, cultures were fully induced with 1 mM IPTG (calculated in relation to the final fermentation volume) and constant feed was applied for 6 h. Until the third hour of induction, biomass- and product formation continued without attenuation (**Table 4-18** and **Table 4-19**). Afterwards, cultures stopped biomass- and product formation resulting in constant OD_{550}-, DCW-, and product concentration values until fermentation-end. Glucose accumulated in the media only at the fermentation-end.

As shown in **Figure 4-9**, the fermentation of B1 scFv comprising L218 continued biomass formation until the fourth hour of induction. Volumetric product concentration increased until the fifth hour of induction. Afterwards, the culture stopped biomass- and product formation resulting in constant OD_{550}-, DCW-, and product concentration values until fermentation-end. Throughout the fermentation, glucose did not accumulate in the medium. Volumetric product concentrations of B1 scFv comprising L218 were 4.9-5.7 g/L, specific product concentrations were 92-110 mg/g DCW higher compared to B1 scFv comprising L205, LLB18, or E-Linker (**Table 4-18** and **Table 4-19**).

Maximum specific product concentrations of B1 scFv comprising L205, LLB18, and E-Linker were reached after 3 hours of induction compared to 6 hours of induction for L218. Similarly, the maximum volumetric product concentrations of B1 scFv comprising LLB18 were reached after 3 hours of induction, for L205 and E-Linker after 4 hours of induction compared to 6 hours of induction for L218.

Table 4-18: Specific B1 scFv concentrations throughout induction period; host: *E. coli* Strain A

	Specific B1 scFv concentration [mg/g DCW]			
Induction time	L205	L218	LLB18	E-Linker
0 h	8	9	13	7
1 h	30	51	33	26
2 h	56	96	57	44
3 h	**78**	136	**75**	**60**
4 h	76	145	69	–
5 h	76	161	72	58
6 h	73	**170**	69	59

Table 4-19: Volumetric B1 scFv concentrations throughout induction period; host: *E. coli* Strain A

	Volumetric B1 scFv concentration [g/L]			
Induction time	L205	L218	LLB18	E-Linker
0 h	0.3	0.4	0.6	0.3
1 h	1.4	2.4	1.5	1.2
2 h	2.8	4.6	2.8	2.1
3 h	**4.0**	6.7	**3.8**	3.0
4 h	**4.0**	7.4	3.6	**3.2**
5 h	**4.0**	8.7	3.7	2.8
6 h	3.7	**8.9**	3.5	2.8

Figure 4-10 shows the Coomassie stained gel of B1 scFv comprising LLB18. It is exemplary for B1 scFv comprising L205 and E-Linker. **Figure 4-11** shows the Coomassie stained gel of B1 scFv comprising L218.

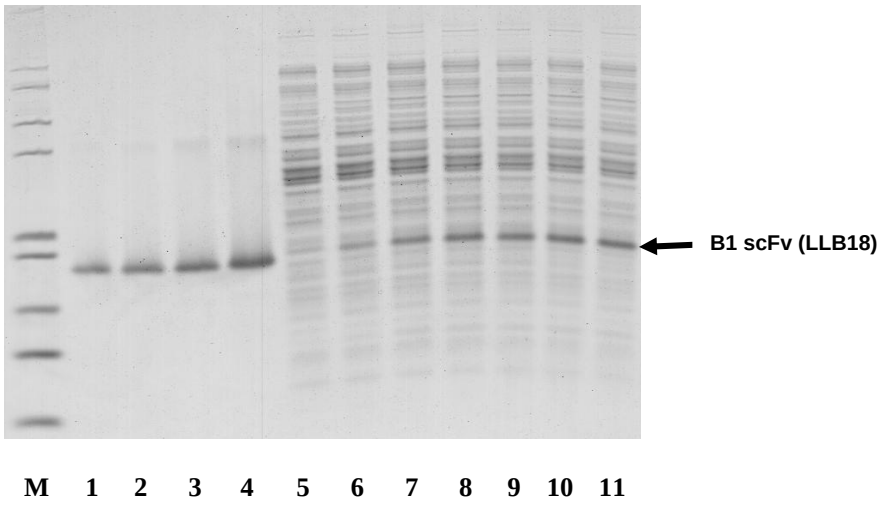

Figure 4-10: SDS-PAGE (4-12%) of total protein fraction of *E. coli* Strain A expressing B1 scFv comprising LLB18 in 5 L scale at 37°C: (M) Mark 12 molecular weight marker [200.0; 116.3; 97.4; 66.3; 55.4; 36.5; 31.0; 21.5; 14.4; 6.0; 3.5; 2.5 kDa]; (1-4) B1 scFv E-Linker standard [0.4; 0.6; 0.8; 1.2 µg]; (5) uninduced; (6) induced for 1 h; (7) induced for 2 h; (8) induced for 3 h; (9) induced for 4 h; (10) induced for 5 h; (11) induced for 6 h;

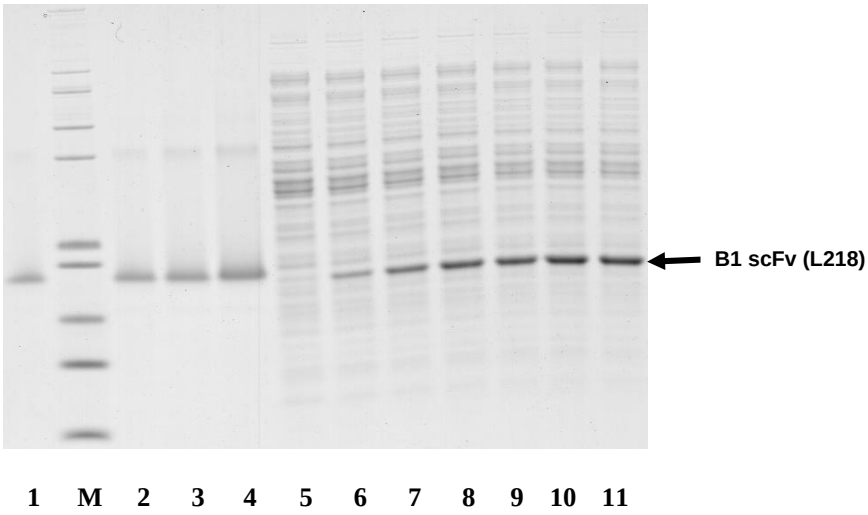

Figure 4-11: SDS-PAGE (4-12%) of total protein fraction of *E. coli* Strain A expressing B1 scFv comprising L218 in 5 L scale at 37°C: (M) Mark 12 molecular weight marker [200.0; 116.3; 97.4; 66.3; 55.4; 36.5; 31.0; 21.5; 14.4; 6.0; 3.5; 2.5 kDa]; (1-4) B1 scFv E-Linker standard [0.4; 0.6; 0.8; 1.2 µg]; (5) uninduced; (6) induced for 1 h; (7) induced for 2 h; (8) induced for 3 h; (9) induced for 4 h; (10) induced for 5 h; (11) induced for 6 h;

As a result the volumetric concentration of B1 scFv was significantly enhanced by altering the sequence and length of the linker peptide. The linker L218 lead to increased volumetric B1 scFv concentrations of 4.9-5.7 g/L and increased specific concentrations of 92-110 mg/g

DCW compared to the other investigated linkers. The increased productivity can be attributed to many of L218's amino acids such as glutamic acid or lysine which have charged residues (Huston *et al.*, 1988; Bird *et al.*, 1988; Whitlow *et al.*, 1993). Additionally, this linker exhibits increased proteolytic stability resulting from a proline interspersed in the polypeptide chain (Whitlow *et al.*, 1993).

4.3.3.1 IB formation and soluble expression in the cytosol

The fraction of *in vivo* soluble B1 scFv comprising the four different linker peptides expressed in *E. coli* Strain A was determined. For protein detection, Western blots could not be applied because no primary antibodies directed against the different linker peptides were available. Instead, SDS-PAGE with subsequent Coomassie- and silver staining were utilized for protein detection (**Chapter 3.8.2.3**). Samples were derived from fermentation-ends. **Figure 4-12** and **Figure 4-13** show Coomassie- and silver stained gels to detect the soluble fraction of B1 scFv comprising the LLB18 linker. The gels are exemplary for fermentations with B1 scFv comprising L205, L218, and E-Linker as well:

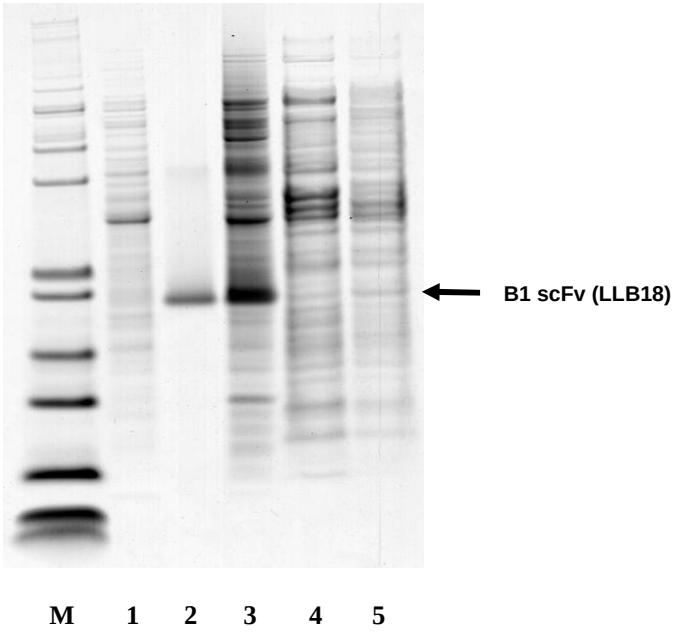

Figure 4-12: SDS-PAGE (4-12%) with subsequent Coomassie staining of cytosolic insoluble and soluble protein fractions. The samples were not diluted. The host was *E. coli* Strain A expressing B1 scFv comprising LLB18 in 5 L scale at 37°C: (M) Mark 12 molecular weight marker [200.0; 116.3; 97.4; 66.3; 55.4; 36.5; 31.0; 21.5; 14.4;

6.0; 3.5; 2.5 kDa]; (1) uninduced Strain A - insoluble; (2) B1 scFv LLB18 standard [1 µg]; (3) induced Strain A - insoluble; (4) uninduced Strain A - soluble; (5) induced Strain A - soluble;

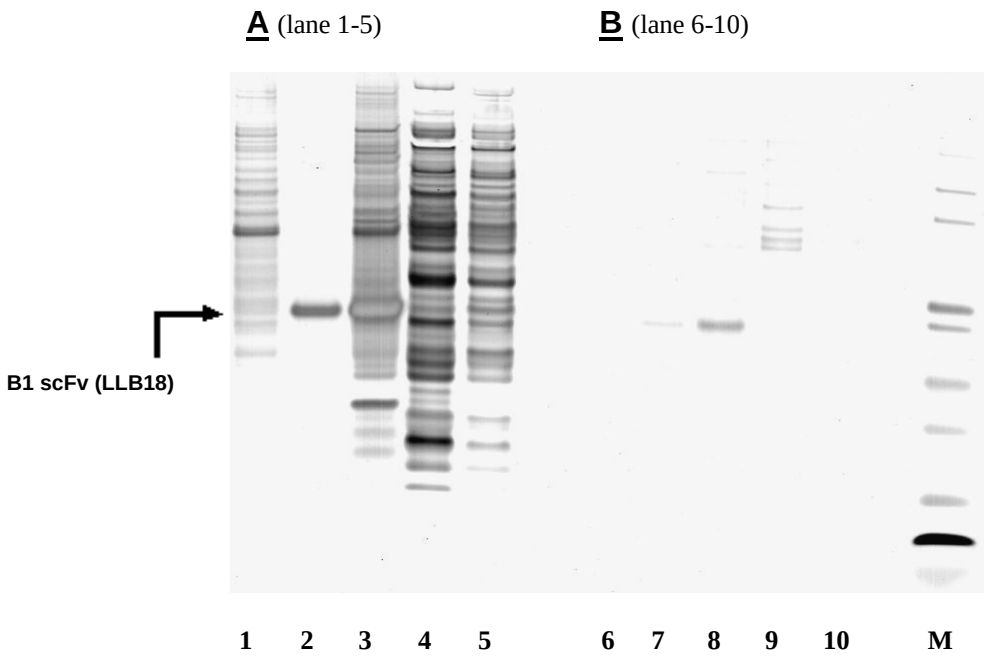

Figure 4-13: SDS-PAGE (4-12%) with subsequent silver staining of cytosolic insoluble and soluble protein fractions. Samples of (**A**) were diluted 1:10; Samples of (**B**) were diluted 1:100. The host was *E. coli* Strain A expressing B1 scFv comprising LLB18 in 5 L scale at 37°C: (M) Mark 12 molecular weight marker [200.0; 116.3; 97.4; 66.3; 55.4; 36.5; 31.0; 21.5; 14.4; 6.0; 3.5; 2.5 kDa]; (1 and 6) uninduced Strain A - insoluble; (2 and 7) B1 scFv LLB18 standard [0.1 and 0.01 µg]; (3 and 8) induced Strain A - insoluble; (4 and 9) uninduced Strain A - soluble; (5 and 10) induced Strain A - soluble;

By applying Coomassie- and silver staining it was observed that none of the four linkers led to intracellular solubility of the B1 scFv. B1 scFv comprising the linkers L205, L218, LLB18, and E-Linker was only detected in the intracellular insoluble fraction. The presented data clearly indicate that altering the linker peptide does not affect the intracellular solubility of B1 scFv.

4.3.3.2 Product purity

Product purity was determined as described in **Chapter 3.8.2.4**.

Table 4-20: Product purity of B1 scFv comprising different linker peptides; expression host: *E. coli* Strain A; fermentation-end

Linker	Lane purity [%]	IB purity [mg/g IB]
L205	61	329
L218	80	558
LLB18	60	515
E-Linker	50	261

According to **Table 4-20**, purity within a SDS-PAGE lane is comparable for B1 scFv comprising L205, LLB18, and E-Linker (50% – 61%). B1 scFv comprising L218 has significantly higher purity within a SDS-PAGE lane (80%).

The dependency of specific B1 scFv concentration [mg/g IB] on the applied linker peptide is remarkable (**Table 4-20**). Values range from 261 mg/g IB for E-linker up to 558 mg/g IB for L218.

Therefore, B1 scFv comprising L218 not only features the highest volumetric- and specific [mg/g DCW] concentrations amongst all applied linker peptides, but also exhibits the highest purity within a SDS-PAGE lane (80%) and the highest specific concentration [mg/g IB]. These results make the linker L218 an interesting option for further investigations with other scFv fragments.

4.4 Expression of Fab fragments in *E. coli*

Another aim of this study was to evaluate the feasibility of intracellular Fab-fragment-expression in *E. coli*. Antigen-binding fragments (Fabs) are heterodimeric proteins which require at least one interchain-disulfide bond formation. This disulfide bond has a major contribution to interchain-binding energy and therefore to Fab stability as described in **Chapter 2.1.2**. As mentioned in **Chapter 2.2.2**, some *E. coli* strains such as the Origami

strains (Novagen, Germany) provide an oxidizing milieu in the cytoplasm which enables intracellular disulfide bond formation (Sorensen and Mortensen, 2005). Routinely applied *E. coli* strains such as Strain B and Strain A possess a reducing milieu within the cytoplasm which does not allow intracellular disulfide bond formation, however.

Another difficulty in cytosolic Fab expression in *E. coli* is the molar ratio of the light- and heavy chain. It has to be in a certain range for efficient folding and assembly of Fab fragments (Schlatter *et al.*, 2005). The heavy chain tends to aggregate in the cytoplasm if its folding is not assisted by sufficient amounts of light chain.

4.4.1 Separate expression of light- and heavy chain of A4 Fab

In this work the separate expression of A4 Fab light- and heavy chain was investigated. *E. coli* Strain A was used as host. The chains were expressed as IBs in the reducing cytosol. Fermentations according to standard operating parameters (**Table 3-16**) and a standard fed batch protocol (**Table 4-21**) were performed:

Table 4-21: Standard fed batch protocol for differential expression of light- and heavy chain of A4 Fab 5 L scale

PRE CULTURE	Medium	**Table 3-12**	
	Inoculum	*E. coli* Strain A + pET30a comprising A4_Fab_LC_bacMiG / A4_Fab_HC_bacMiG	
	Inoculation ratio	1:1000 (inoculum from MCB)	
BATCH	Medium	**Table 3-13**	
	Inoculation ratio	35 mL pre culture (OD_{550} between 1.0 and 3.0) were inoculated to 3.5 L batch medium	
FED BATCH	Feed Medium	**Table 3-14**	
	Exponential feed	$\mu = 0.15\ h^{-1}$	for 16.5 h
	Constant feed, induction	47.5 mL/h*L batch (166.3 mL/h; ρ=1.22)	for 6 h

As shown in **Figure 4-14** the fermentations of *E. coli* Strain A expressing the individual chains of A4 Fab showed similar courses. Both cultures generated 49 g/L DCW until cultures were induced. After induction, Strain A expressing A4 Fab light chain generated more biomass. At the fermentation-end DCW and OD_{550} values were 66 g/L and 316 for the light chain compared to 59 g/L and 274 for the heavy chain (**Figure 4-14**). Glucose did not accumulate in the media throughout fermentations of both chains.

After induction the volumetric product concentration of both A4 Fab chains steadily increased until the fourth hour of induction (**Figure 4-14** and **Table 4-22**). This is also shown by the Coomassie stained gels of A4 Fab light- and heavy chain (**Figure 4-15** and **Figure 4-16**). After the fourth hour of induction the increase of volumetric product concentration attenuated resulting in 14.2 g/L for the light chain and 11.7 g/L for the heavy chain at fermentation-end. Specific concentrations as well as volumetric concentrations of both A4 Fab chains showed similar courses. Maximum specific concentrations were reached at the fermentation-end for both Fab chains yielding 217 mg/g DCW for the light chain and 198 mg/g DCW for the heavy chain.

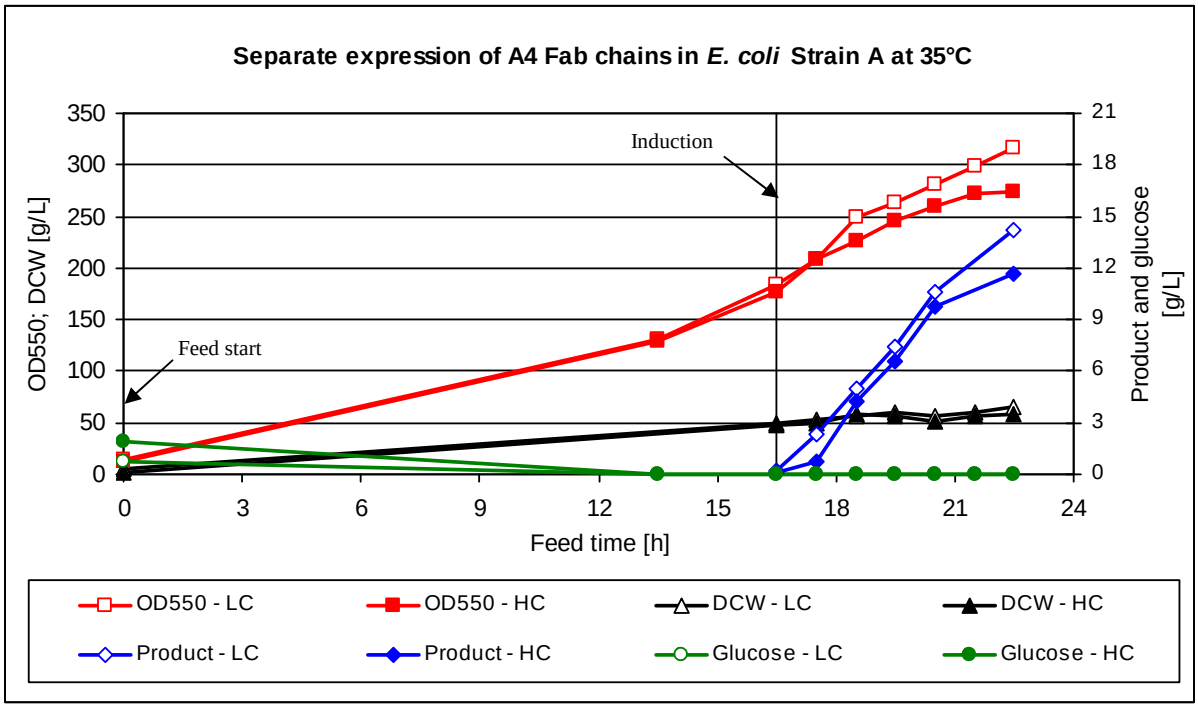

Figure 4-14: Comparison of differential expression of A4 Fab chains in *E. coli* Strain A at 35°C; feed phase

Table 4-22: Volumetric- and specific concentrations of A4 Fab (LC and HC) throughout induction period; host: *E. coli* Strain A

	Volumetric concentration [g/L]		**Specific concentration [mg/g DCW]**	
Induction time	LC	HC	LC	HC
0 h	0.2	0.1	5	2
1 h	2.3	0.7	44	15
2 h	5.0	4.3	87	73
3 h	7.4	6.6	123	114
4 h	10.6	9.8	186	189
5 h	–	–	–	–
6 h	**14.2**	**11.7**	**217**	**198**

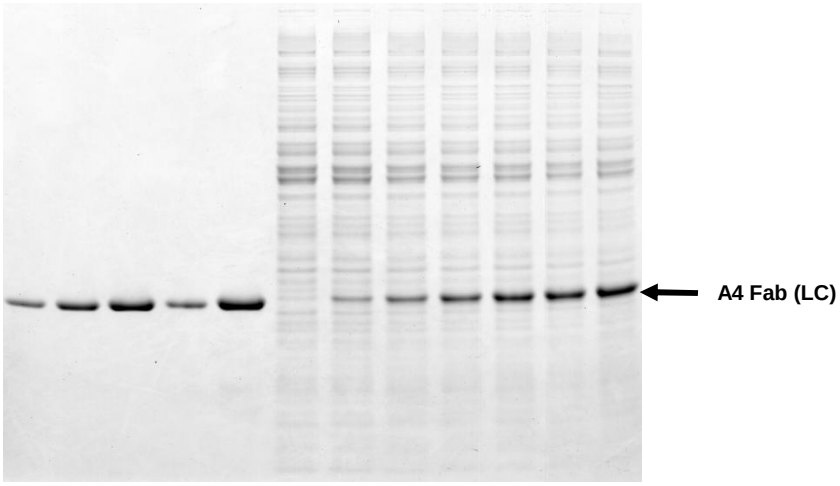

Figure 4-15: SDS-PAGE of total protein fraction of *E. coli* Strain A expressing the light chain of A4 Fab in 5 L scale at 35°C: (1-3) A4 scFv standard in LCHC orientation [0.5; 1.1; 1.6 µg]; (4-5) A4 scFv standard in HCLC orientation [0.5; 1.6 µg]; (6) uninduced (7) induced for 1 h; (8) induced for 2 h; (9) induced for 3 h; (10) induced for 4 h; (11) induced for 5 h; (12) induced for 6 h;

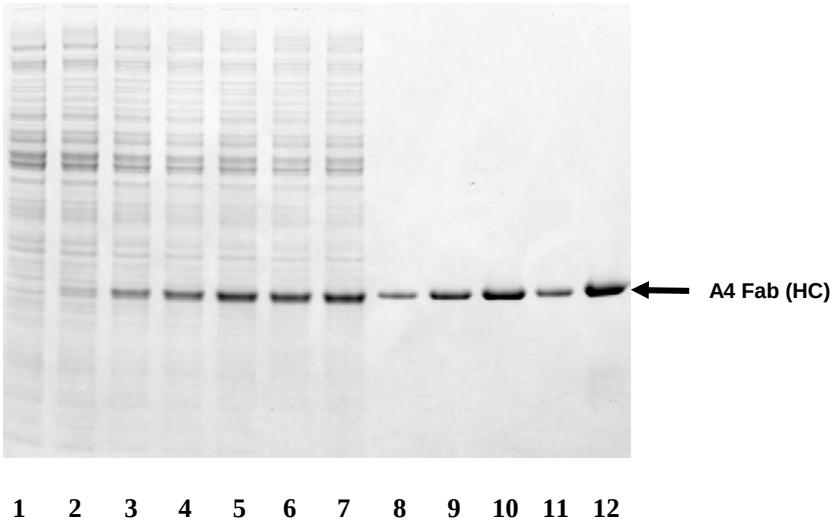

Figure 4-16: SDS-PAGE of total protein fraction of *E. coli* Strain A expressing the heavy chain of A4 Fab in 5 L scale at 35°C: (1) uninduced; (2) induced for 1 h; (3) induced for 2 h; (4) induced for 3 h; (5) induced for 4 h; (6) induced for 5 h; (7) induced for 6 h; (8-10) A4 scFv standard in LCHC orientation [0.5; 1.1; 1.6 µg]; (11-12) A4 scFv standard in HCLC orientation [0.5; 1.6 µg];

Previous work reported maximum volumetric concentrations of 3.1 g/L Fab fragment which was intracellular expressed as IBs in *E. coli* (Arbabi-Ghahroudi *et al.*, 2005). In the present work, significantly higher maximum expression levels were obtained for the individual Fab chains: 14.2 g/L for A4 Fab light chain and 11.7 g/L for the heavy chain. However, the obtained volumetric concentrations for the A4 Fab chains may not be applicable to Fab chains of other mAbs because expression levels can greatly depend upon the utilized protein. Additionally, the purity of the individual A4 Fab chains was investigated (**Chapter 4.4.1.1**).

4.4.1.1 Product purity

Product purity was determined as described in **Chapter 3.8.2.4**.

Table 4-23: Product purity of A4 Fab (LC and HC);
expression host: *E. coli* Strain A; fermentation-end

Molecule	Lane purity [%]	IB purity [mg/g IB]
LC	87	479
HC	80	442

Considering the variation of SDS-PAGE, the A4 Fab chains feature similar purities within SDS-PAGE lanes (**Table 4-23**). Specific concentrations (mg/g IB) are comparable as well. Overall, slightly higher purities were obtained for A4 light chain.

The presented data suggest that intracellular and separate expression of the light- and heavy chain of A4 Fab is a valuable expression strategy. To evaluate its full potential, further studies on refolding and reassembling of the two chains have to be performed. Results from these studies will finally reveal the value of the presented expression strategy. In subsequent investigations, chains of other Fab fragments could be separately expressed in *E. coli* to further extend this platform technology.

5. CONCLUSIONS

Recent advances in antibody-engineering have led to a range of novel and innovative antibody therapeutics that are now entering the biopharmaceutical market (Pavlou and Belsey, 2005). However, most antibody based therapies require high dosage which results in a great demand and remarkable costs (Farid, 2007). A promising alternative to full-length antibodies are their fragments. These smaller domain variants can be produced in microbial host systems in large amounts and therefore less costly (Joosten *et al.*, 2003). Generic manufacturing platforms are valuable tools in the pharmaceutical industry. They facilitate choosing the most suitable expression system for a specific antibody product. Therefore, faster process development can be achieved which is crucial to meet short time-lines to clinical trials and market release as well as to stay competitive on the market. Despite their similar structure, antibody fragments turned out to be variable in their production. Therefore, particular attention must be paid to each individual format. This corresponds to findings from previous research: On the one hand, different antibody fragments were expressed at different levels in the same host (Miller *et al.*, 2005); On the other hand, the choice of the expression system had an impact on yields of one specific antibody fragment (Robin *et al.*, 2003).

In this study, the *E. coli* strains Strain B and Strain A under the control of the T7 promoter were used as expression systems. They are well established and enable short and inexpensive processes (Arbabi-Ghahroudi *et al.*, 2005). The feasibility of expression of two antibody fragments, namely the single-chain fragment (scFv) and the antigen-binding fragment (Fab), was evaluated. The scFv fragments were derived from the therapeutic monoclonal antibodies H6 (Kunert *et al.*, 2002), B1, and A4 (both from Boehringer Ingelheim Pharma, Germany). The Fab fragment was derived from the therapeutic monoclonal antibody A4 (Boehringer Ingelheim Pharma, Germany).

High yields for the cytoplasmic scFv-fragment-expression as IBs were previously reported (Martineau *et al.*, 1998). Contrary, intracellular expression of Fab fragments is not state-of-the-art in *E. coli* (Plückthun and Skerra, 1989). This is caused by its inability to form disulfide bonds within the reducing environment of the cytosol. These interchain-disulfide-bonds are necessary to link the V_H-C_H1 and the V_L-C_L of the heterodimeric Fab fragment, however. To

countervail this limitation, the light- and heavy chain of the A4 Fab were separately expressed as IBs in the cytosol of *E. coli* in this study.

In the present work, all investigated antibody fragments were expressed at high levels in the gram per liter range and high purities. Wagner, 2008 reported maximum cytosolic H6 scFv concentrations of 240 mg/L in *E. coli* Strain B and 50 mg/L in Strain A. These low volumetric concentrations were possibly caused by non-optimized *E. coli* codon usage. In the present work, only *E. coli* codon optimized gene sequences were applied resulting in 6.7 g/L H6 scFv in Strain B and 5.9 g/L in Strain A. Therefore, the expression level of H6 scFv in Strain B was increased 28-fold and in Strain A 118-fold by applying codon optimization. This complies with former observations from Wagner, 2008 for Fab fragments where a 100-fold increase of the expression level was observed when using codon optimized genes. As a result, codon optimized gene sequences proved to be crucial for heterologous protein expression in *E. coli*.

Maximum volumetric concentrations of B1 scFv were 3.1 g/L in *E. coli* Strain B and Strain A. This was only half the amount of A4- and H6 scFv expression levels (**Chapter 4.3.1**). As a consequence, the influence of different expression temperatures on B1 scFv yield, intracellular solubility, and purity was subsequently investigated. Both, raising and lowering the expression temperature to 42°C and 28°C, resulted in lower yields compared to initial expression temperature of 35°C. Maximum B1 scFv concentrations at 42°C and 28°C ranged between 1.5 g/L and 2.1 g/L in both *E. coli* strains. In addition, highest purities of B1 scFv were obtained at 35°C. None of the investigated expression temperatures caused intracellular solubility of B1 scFv. For these reasons, 35°C proved to be the ideal expression temperature in terms of B1 scFv yield and purity.

As described in **Chapter 2.2.5** the linker peptide connecting the two variable domains within the scFv fragment is considered to influence aggregation (Whitlow *et al.*, 1993), thermodynamic stability (Robinson and Sauer, 1998), and solubility (Tang *et al.*, 1996). However, expression levels (Tang *et al.*, 1996) and folding properties (Hennecke *et al.*, 1998) of a scFv fragment are rather influenced by the domains than by the linker. For these cases the linker is thought to be a ‘passive connector’ (Hennecke *et al.*, 1998). To reveal the influence of different linker peptides on B1 scFv expression yield, intracellular solubility, and purity further studies were made. Four different linker sequences were introduced into the existing B1 scFv replacing the original $(Gly_4Ser)_3$-linker (**Chapter 2.2.5**). E-linker, L205, and LLB18 yielded comparable volumetric B1 scFv concentrations between 3.2 g/L and 4.0 g/L. L218 yielded significantly higher volumetric B1 scFv concentration of 8.9 g/L. Especially the

linkers L205, LLB18, and L218 contained several charged, polar, and therefore hydrophilic residues favouring linkers' solubility (Huston *et al.*, 1988; Bird *et al.*, 1988; Pantoliano *et al.*, 1991; Whitlow *et al.*, 1993; Hennecke *et al.*, 1998). Contrary to findings from Dolezal *et al.* the intracellular solubility of B1 scFv was not enhanced by additional polar residues within the linker sequence (Dolezal *et al.*, 2003). As a result, none of the four linker peptides caused intracellular solubility of B1 scFv. At the fermentation-end, the IB-purities of antibody-fragments were determined. After IB purification from cell lysates, the specific product concentration referred to the whole IB [mg/g IB] was measured. IB-purity of B1 scFv was widely scattered ranging from 261 mg/g IB (E-Linker) to 558 mg/g IB (L218). To conclude, B1 scFv comprising L218 yielded highest volumetric concentrations and highest purities of all investigated linkers. These findings make the linker L218 an interesting option for further studies within other scFv fragments.

Wagner, 2008 investigated the periplasmic expression of H6 Fab in *E. coli* Strain B. Expression levels of H6 Fab were low and insufficient for quantification with densitometry. Moreover, SDS-PAGE revealed that most recombinant protein resulted from unassembled H6 Fab light- and heavy chain. In the present study, the chains of A4 Fab were separately expressed as IBs in *E. coli* Strain A. Expression yields of 14.2 g/L and 11.7 g/L for the light- and heavy chain were obtained. The achieved IB-purities were 479 mg/g IB for the light chain and 442 mg/g IB for the heavy chain. Therefore, differential expression of A4 Fab chains proved to be a valuable expression strategy. To evaluate its full potential, further studies on refolding and reassembling of the two chains have to be made. Results from these studies will finally reveal the value of the presented expression strategy. In subsequent investigations, individual chains of other Fab fragments could be expressed in *E. coli* to further extend this platform technology.

In terms of the manufacturing platform, two antibody fragments (scFv and Fab) derived from three therapeutic monoclonal antibodies (H6, B1, and A4) were evaluated in this study. Concerning feasibility, expression of every single investigated format is possible in *E. coli* Strain B and Strain A. In every case, yields in the gram per liter range with varying purities were obtained. For future investigations it will be interesting how far the experiences made in this study can be used for the expression of other antibody fragments. Further model antibodies will have to be introduced to the platform technology applying the same generic modules to make more general statements.

6. Appendix

6.1 Abbreviations

aa	amino acid
Ab	antibody
AEPDT	antibody-enzyme prodrug therapy
AGE	agarose gel electrophoresis
BOKU	University of Natural Resources and Applied Life Sciences, Vienna
bp	base pair
BSA	Bovine serum albumin
C domain	constant domain
CDR	complementarity determining region
CHO	Chinese hamster ovary
C_H	constant domain of the heavy chain
C_L	constant domain of the light chain
DCW	dry cell weight
DEPC	Diethylpyrocarbonate
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EDTA	ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
e.g.	exempli gratia
E-linker	extended linker peptide
Fab	antigen-binding fragment
FAPα	fibroblast activation protein alpha
Fc	fragment crystallizable
FFG	Austrian Forschungsförderungsgesellschaft
FR	framework region
g	gram
h	hour

HC	heavy chain
HIV	human immunodeficiency virus
HQ	high quality
IAM	Institute of Applied Microbiology
IB	inclusion body
Ig	immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kbp	kilo base pairs
kDa	kilo Dalton
kV	kilovolt
L	liter
L205	linker peptide L205
L218	linker peptide L218
LB	lysogeny broth
LC	light chain
LDS	SDS-PAGE smple buffer
LLB18	linker peptide LLB18
M	molar concentration
mAb	monoclonal antibody
MCB	master cell bank
MES	2-(N-morpholino)ethanesulfonic acid
mg	miligrm
min	minute
mL	milliliter
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
Mr	relative molar mass
μ	growth rate
μg	microgram
μL	microliter
μm	micrometer
ng	nanogram
nm	nanometer

OD_{550}	optical density measured at 550 nm
PBST	Phosphate Buffered Saline Tween-20
PCR	polymerase chain reaction
pDNA	plasmid DNA
pH	potentia hydrogenii
pI	isoelectric point
pO_2	partial pressure of oxygen
P. pastoris	*Pichia pastoris*
ρ	density
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
scFv	single-chain fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	super optimal broth with additional glucose
TBE	Tris/Borate/EDTA
tRNA	transfer RNA
UV	ultraviolet
V	volt
vvm	volume per volume per minute
V domain	variable domain
V_H	N-terminal variable domain of the heavy chain
V_{HH}	camelide heavy-chain antibody
V_L	N-terminal variable domain of the light chain
v/v	volume per volume
WCB	working cell bank
WCW	wet cell weight
w/v	weight per volume
$Y_{P/X}$	yield coefficient: product/biomass [$g*g^{-1}$]

7. References

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