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Over-expression in *Escherichia coli* of the ToxA5.1 llama single domain antibody targeting
Clostridium difficile enterotoxin A

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THÈSE DE MScA

Département de Génie Chimique et Biologique

**Surexpression chez *Escherichia coli* de l'anticorps à domaine simple
ToxA5.1 provenant du lama et ayant pour cible l'entérotoxine A produite
par *Clostridium difficile***

**MASc THESIS
Department of Chemical and Biological Engineering**

**Over-expression in *Escherichia coli* of the ToxA5.1 llama single domain
antibody targeting *Clostridium difficile* enterotoxin A**

par/by

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University of Ottawa

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Résumé

Un nouveau type d'anticorps, les anticorps à domaine simple (sdAb), se prête à une multitude d'applications. Le ToxA5.1, capable de lier et de neutraliser l'entérotoxine A produite par *Clostridium difficile* (TcdA), a été développé. Le *C. difficile* résistant aux antibiotiques, causant des diarrhées sévères, est un problème auquel les hôpitaux doivent faire face. Le but de cette étude était de déterminer l'impact, sur l'expression du sdAb ToxA5.1 dans *E. coli* TG1, de divers paramètres. Suite à une optimisation du procédé de production, la température et le contrôle du pH se sont avérés être les paramètres affectant le plus l'expression protéique où 503 mg/L du ToxA5.1 furent produits en bioréacteur. La quantification des protéines fut effectuée à l'aide de mesures densitométriques et l'analyse de la cinétique fut effectuée par analyse Biacore. Les résultats obtenus ont permis une simplification du milieu de culture et de la procédure de purification tout en conservant les propriétés du ToxA5.1.

Abstract

Single domain antibodies (sdAb) have emerged as a family of novel antibodies that have vast applications. Of particular relevance, ToxA5.1, a sdAb capable of binding to, and therefore neutralizing, *Clostridium difficile* toxin A (TcdA) has been developed. The antibiotic resistant version of *C. difficile* bacterium is of major health concern worldwide, particularly in hospital settings, causing severe gastrointestinal disorders. In this study, we investigated various parameters affecting the expression of the ToxA5.1 sdAb in *E. coli* TG1 and optimization of culture conditions revealed that temperature and medium buffering are critical parameters affecting small-scale expression of this recombinant protein. Large-scale expression was achieved in bioreactor at 503 mg/L of recombinant protein. Protein quantification was performed using SDS-PAGE densitometry while binding activity against TcdA was assessed via Biacore. Our studies also led to medium simplification for small-scale expression, shorter expression time and simpler purification process without impairing on the quality of the expressed recombinant protein.

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Collaborator's Contributions

Chapter 3

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Provided help with the experimental procedures by preparing media, conducting the experiments, sampling and monitoring OD for some of the experiments (temperature and buffer effects).

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Constructed the bacterial strain and wrote the relevant Material and Methods section. Also performed the ELISA experiment.

Jamshid Tanha

Revision of the manuscript

Christopher Q. Lan

Correction of the manuscript and corresponding author.

Chapter 4

Greg Hussack

Prepared the purified samples for Biacore testing and provided tables and graphs for the Biacore results. Write up of the relevant Material and Methods sections.

Christian McKinnon

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Noémie Manuelle Dorval Courchesne

Provided assistance with sampling the bioreactors and sample preparation.

Jamshid Tanha

Revision of the manuscript

Christopher Q. Lan

Correction of the manuscript and corresponding author

Chapter 1: Introduction

Progress in recombinant DNA technologies resulted in the production of numerous high-value bioproducts in the form of recombinant proteins and other molecules, which could not have been produced via traditional chemical synthesis and *Escherichia coli* has been one of the most important workhorses for the production of these molecules [1]. To this end, therapeutic molecules are probably the ones having the biggest impact on the humanity. For instance, according to the Center for Disease Control in the United States, over 24 millions people in the USA alone have to fight diabetes and need daily insulin injection. The insulin used by these people is recombinant human insulin, which is produced by recombinant *E. coli* or recombinant yeast.

From the idea of producing a molecule to the actual marketing of the product, molecular biologists must first engineer a bacterial strain that will express the desired molecule. Once strain construction is completed, engineers take over to express the molecules at optimized conditions. In the process of optimisation, several factors can impact on the yield and productivity of the product and it is these factors that must be sought and understood in order to significantly increase the expression level.

The work done for this MASc thesis looked at what impact growth conditions had on the expression in an *E. coli* strain of a particular novel type of antibody, a single domain antibody, that could be used to treat people infected with antibiotic resistant strains of *Clostridium difficile*.

Antibodies are a family of high-value products, which are becoming more and more popular to a great extent because of their versatility. They are used in applications where

high specificity is required ranging from immunostaining to drug delivery directly to a specific target.

Estimated to account for over 30% of the biotechnology market in 2008 [2], antibody production is a fast growing and lucrative market with worldwide sales reaching over \$17 billion for monoclonal antibodies in 2008 [3]. However, monoclonal antibodies have their limitations such as the high production costs associated with mammalian cell culture, immunogenicity that causes efficacy reduction and safety concerns, and large size, which prevents them from accessing immunosilent epitopes or from crossing certain tissue barriers like the blood-brain barrier [4]. In order to circumvent these problems, conventional antibodies have been broken into fragments such as antigen binding (Fab), crystallizing fragments (Fc), variable light chain (V_L) and variable heavy chain (V_H) domains have been linked together (scFv) or rearranged in multimeric units. However, all of the aforementioned fragments are lacking the long serum half-life and binding specificity of conventional immunoglobulins G (IgGs) [3]. These fragments are complicated to engineer since light and heavy domains have to be linked together using flexible peptides. They also have lower binding affinity than the parent molecule [5].

In the quest for even smaller fragments, single domain antibodies were engineered [6]. These small single domain antibodies (sdAb) consisting of only the variable portion of the conventional antibody were demonstrated to be able to bind antigens but with much less strength than their full size counterparts [7, 8]. In 1993, investigating what seemed to be faulty results from an experiment, a group of Belgian researchers discovered that naturally occurring sdAbs were already produced in camelidae [9]. These sdAbs were capable of binding antigen with the same strength as their conventional counterparts,

opening the door to new possibilities using sdAb [9]. The major difference between the synthetically engineered and naturally occurring sdAb reside in the absence of the variable domain of the light chain (V_L) on synthetic sdAb which exposes an hydrophobic surface and therefore impairs on the protein solubility, resulting in aggregation thus a lower yield of soluble proteins [5].

Among many others, one of the applications foreseen for sdAb is toxin binding which can prevent the adverse effect of toxins in the human body. To this end, a recombinant *E. coli* TG1 strain has been engineered by the Antibody Engineering Group from the Institute for Biological Science of the National Research Council Canada in Ottawa to express sdAb ToxA5.1 that is capable of binding and thus neutralizing the enterotoxin A (TcdA) excreted by the bacterium *Clostridium difficile*. Since *C. difficile* causes toxin-mediated diseases, it is believed that the binding of the sdAb to TcdA will reduce the severity of the symptoms *C. difficile* associated diseases (CDAD) as it has been the case with the use of mAb [10-17].

The work done for this thesis aimed at ascertaining whether or not the sdAb ToxA5.1 could be expressed in bioreactor at levels sufficient to support up-coming animal studies investigating the potential of ToxA5.1 as a toxin neutralizing agent. This was achieved by first looking at how parameters such as inducer concentration, supplemental carbon source, medium buffering and temperature affected sdAb ToxA5.1 expression in flasks culture. Then, once parameters affecting the expression were identified, bioreactors were used to express, in large quantity, the desired ToxA5.1 sdAb under different concentrations of glucose and glycerol.

1.1 References

1. Chemier, J.A., et al., *Trends in microbial synthesis of natural products and biofuels*. Advances in enzymology and related areas of molecular biology, 2009. **76**: p. 151-217.
2. Holliger, P. and P.J. Hudson, *Engineered antibody fragments and the rise of single domains*. Nature Biotechnology, 2005. **23**(9): p. 1126-1136.
3. Gibbs, W.W., *Nanobodies*. Scientific American, 2005. **293**(2): p. 78-83.
4. Muruganandam, A., et al., *Selection of phage-displayed llama single-domain antibodies that transmigrate across human blood-brain barrier endothelium*. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2002. **16**(2): p. 240-242.
5. Muyldermans, S., *Single domain camel antibodies: Current status*. Reviews in Molecular Biotechnology, 2001. **74**(4): p. 277-302.
6. McFarland, L.V., et al., *Nosocomial acquisition of Clostridium difficile infection*. New England Journal of Medicine, 1989. **320**(4): p. 204-210.
7. Dick, H.M., *Single domain antibodies*. BMJ (Clinical research ed.), 1990. **300**(6730): p. 659-660.
8. Gussow, D., et al., *Single domain antibodies (dAb's): Binding activities from E. coli by expression of a repertoire of immunoglobulin variable domains*. Fresenius' Journal of Analytical Chemistry, 1990. **337**(1): p. 47.
9. Hamers-Casterman, C., et al., *Naturally occurring antibodies devoid of light chains*. Nature, 1993. **363**(6428): p. 446-448.
10. Babcock, G.J., et al., *Human monoclonal antibodies directed against toxins A and B prevent Clostridium difficile-induced mortality in hamsters*. Infection and Immunity, 2006. **74**(11): p. 6339-6347.
11. Casadevall, A., E. Dadachova, and L.A. Pirofski, *Passive antibody therapy for infectious diseases*. Nature Reviews Microbiology, 2004. **2**(9): p. 695-703.
12. Corthier, G., et al., *Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against Clostridium difficile toxin A*. Infection and Immunity, 1991. **59**(3): p. 1192-1195.
13. Lyerly, D.M., et al., *Passive immunization of hamsters against disease caused by Clostridium difficile by use of bovine immunoglobulin G concentrate*. Infection and Immunity, 1991. **59**(6): p. 2215-2218.
14. Taylor, C.P., et al., *Open-label, dose escalation phase I study in healthy volunteers to evaluate the safety and pharmacokinetics of a human monoclonal antibody to Clostridium difficile toxin A*. Vaccine, 2008. **26**(27-28): p. 3404-3409.
15. Salcedo, J., et al., *Intravenous immunoglobulin therapy for severe Clostridium difficile colitis*. Gut, 1997. **41**(3): p. 366-370.
16. Sougioultzis, S., et al., *Clostridium difficile toxoid vaccine in recurrent C. difficile-associated diarrhea*. Gastroenterology, 2005. **128**(3): p. 764-770.
17. Kuijper, E.J., et al., *Emergence of Clostridium difficile-associated disease in North America and Europe*. Clinical Microbiology and Infection, 2006. **12**(12 SUPPL. 6): p. 2-18.

Chapter 2: Literature review

2.1 Engineering background

2.1.1 Effects of parameters on growth and recombinant protein expression

At the molecular biology level, several strategies can be used in order to increase the expression of recombinant proteins. For instance, in certain expression systems such as *E. coli*, codon usage or codon bias used in the recombinant protein sequence can enhance the expression as *E. coli* favours certain codons more than others [1, 2]. Another option is to select a strong promoter or use high copy number vectors, both of which would lead to a higher protein expression. A stronger promoter will yield large number of mRNA thus more proteins and a larger number of vector copies will yield more protein factories per bacterium thus more expressed proteins. In addition, the choice of expression vector (bacterial RNA polymerase or T7 bacteriophage RNA polymerase), the choice of *E. coli* strain and the protein sequence can also greatly affect the expression levels of a protein [2].

Once the recombinant strain is constructed, engineers would be able to manipulate the growth and expression conditions to optimize recombinant protein expression. Factors having the most effects on recombinant proteins expression are inducer selection and concentration, temperature at which growth and expression take place, fermentation medium pH and composition, cell growth rate and production of metabolic by-products.

2.1.2 Inducer selection and concentration

Organisms used for recombinant protein expression often possess a mechanism, which can be turned on at a desired time, to initiate expression. This mechanism is usually

under the control of an inducer, a chemical substance, that is added to the fermentation medium when induction conditions are favourable. A commonly used inducer for recombinant protein expression is Isopropyl- β -D-1-thiogalactopyranoside (IPTG), which is functional for expression systems employing the lac operon. This compound can enable the transcription of the lac promoter since it is a lactose analog, which stops the repression of the gene located downstream of the operon. When lactose analogs are present, they bind to the repressor, which is attached to the DNA, causing its release thus enabling RNA polymerases to access the DNA in order to initiate the transcription process. Once added to the fermentation broth, IPTG concentration remains constant due to the presence of a sulphur bond, which is not hydrolysable thus preventing IPTG biodegradation.

It is also possible to use lactose as an inducer. Several strategies have been tested [3-5] and the choice of strategy depends on the protein to be expressed. Using lactose as an inducer can greatly reduce the cost of fermentation since IPTG is an expensive chemical that is suitable for small-scale expression but in large-scale fermentation, cost associated with its use can become prohibitive. In addition to the price, large amount of IPTG needed for larger bioreactor would also be of concern because of the potential toxicity of IPTG when used for therapeutic proteins production [6, 7].

The concentration of inducer can also play a crucial role in the expression yield. It has been reported that *E. coli* can be induced with as low as 0.1 mM IPTG while some systems may require up to 2 mM [8]. This all depends on the induction timing (when the inducer is added to the fermentation broth), the duration of the induction period and the

temperature at which induction takes place [9]. As for many systems in biological science, parameters must be tailored for a specific system and inducer is no exception [10].

2.1.3 Temperature

Temperature influences both cell growth and recombinant protein expression in all bacterial strains. Lower temperatures have an effect on the expression of certain genes. It was demonstrated that expression of OmpA gene, responsible for the synthesis of transmembrane proteins, is less efficient at temperature lower than 28°C when compared to temperature of 37°C [11]. As another example, Bortolussi and Ferrieri reported the absence of their target protein, an antigen, when cells were grown at temperature lower than 30°C for a short period of time [12].

The effects of temperature on cell growth can be largely described by the Arrhenius equation. Lower biomass concentration at higher temperature can be explained by the fact that *E. coli* growth follows an Arrhenius type equation with an optimal temperature range between 23°C and 37°C outside which growth rate diminishes drastically [13]. On the other hand, lower temperature, despite reduced growth rates, might be desirable to enhance protein solubility [14]. When expression temperatures are lowered, fewer proteins are expressed, as discussed previously. This reduction in protein expression causes a reduction of the intracellular protein concentration which in turns reduces protein aggregation. When proteins aggregate, they tend to form inclusion bodies which are insoluble. Following cell lysis, these inclusion bodies remain enclosed in the bacterial debris and further denaturing steps are required for the extraction of the recombinant proteins in a soluble form.

2.1.4 pH

Fermentation medium pH is of great importance for proper cell growth and protein expression as environmental pH greatly affects the behaviour of the outer membrane [15, 16]. When cells are in the presence of low or high pH, gene transcription of stress response proteins is induced by the pH variation and the metabolism resources are utilized in the stress response caused by harsh environmental conditions. Consequently, less resources are allocated to cell growth or to the expression of other types of proteins than those implicated in the stress response [17]. Furthermore, as will be discussed later, organic acids can penetrate cell membrane and interfere with proton driven pumps. Lower environmental pH causes lower intracellular pH due to organic acid penetration and intracellular protein conformations are affected by the increase of protons, preventing them from performing their intended tasks.

2.1.5 Medium composition and carbon source

In *E. coli* culture, medium composition can vary depending on the particular strain used or the particular target protein to express. From minimal medium to complex medium containing component such as amino acids and other compounds whose identification and quantification cannot be determined, each of them have its advantages and disadvantages. The minimal mineral medium is usually used in laboratory settings to select certain strains or to grow wild type bacteria but is not able to provide other factors or supplements found in complex medium that could yield the highest biomass. Defined medium enables a control of every component in the media and can be used to investigate the effect of a particular component on cell growth or protein expression. On the other hand, complex media cannot be used to ascertain the effect of some components, as the exact composition

of this media is unknown. One of the advantages of these complex media is the growth factors and other trace elements provided by the yeast extract usually found in the ingredients, which help bacteria thrive. A supplemental carbon source can be added to any of the above medium providing an energy source supporting cell growth. The most common sources of carbon are glucose and glycerol but other sources such as lactose, sucrose, mannose, succinate, fructose, or xylose can also be used [18-20] each of them having an effect on specific growth rate with glucose yielding the highest growth rate of all the carbon sources [21].

The carbon source used by bacteria can serve as an energy source in the form of ATP (adenosine triphosphate) and NADH₂ (nicotinamide adenine dinucleotide), which are the end products of total oxidation of the carbon molecule in the presence of oxygen. When *E. coli* is grown aerobically, part of its energy comes from the Krebs cycle also known as TCA (tricarboxylic acid) cycle, which can be seen in Figure 2-1. For a glucose molecule, the cycle is completed twice since glucose is broken into two pyruvate molecules each going once in the cycle for a total yield of six ATPs. If no favourable carbon sources such as sugars or sugar alcohols are present in the medium, bacteria can also utilize other carbon sources such as amino acids to support cell growth. This is one of the possible reasons why, when *E. coli* is grown in complex medium without sugar supplementation, the pH rises as when amino acid utilization releases ammonia.

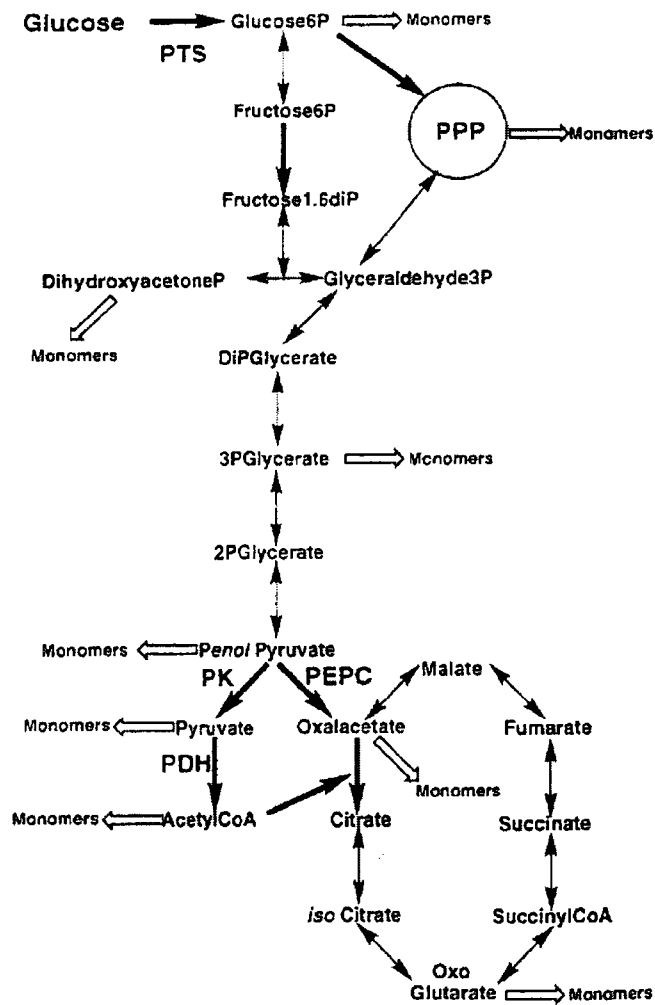


Figure 2-1: Metabolism of glucose entering the Krebs cycle [21].

2.1.5.1 Glucose catabolism

Aerobic catabolism of glucose can be separated into three phases: 1) Embden-Meyerhof-Parnas pathway (EMP), 2) Krebs cycle, and 3) Electron transport chain. In the EMP pathway, a glucose molecule is broken into two pyruvate molecules which enter the Krebs cycle and are converted into CO_2 and NADH. NADH is used, in several metabolic reactions, as an electron carrier. The Krebs cycle serves three purposes which are: 1) to provide electrons via NADH for respiratory pathways and biosynthesis, 2) to provide carbon backbone for anabolism and finally, 3) to generate energy in the form of ATP and

this may be accomplished using the pool of NADH produced [22]. Growth on glucose is the fastest with a specific growth rate of 0.94 h^{-1} but it is also the one producing the most by-product due to glucose overflow in the TCA [21] as can be seen in Figure 2-2.

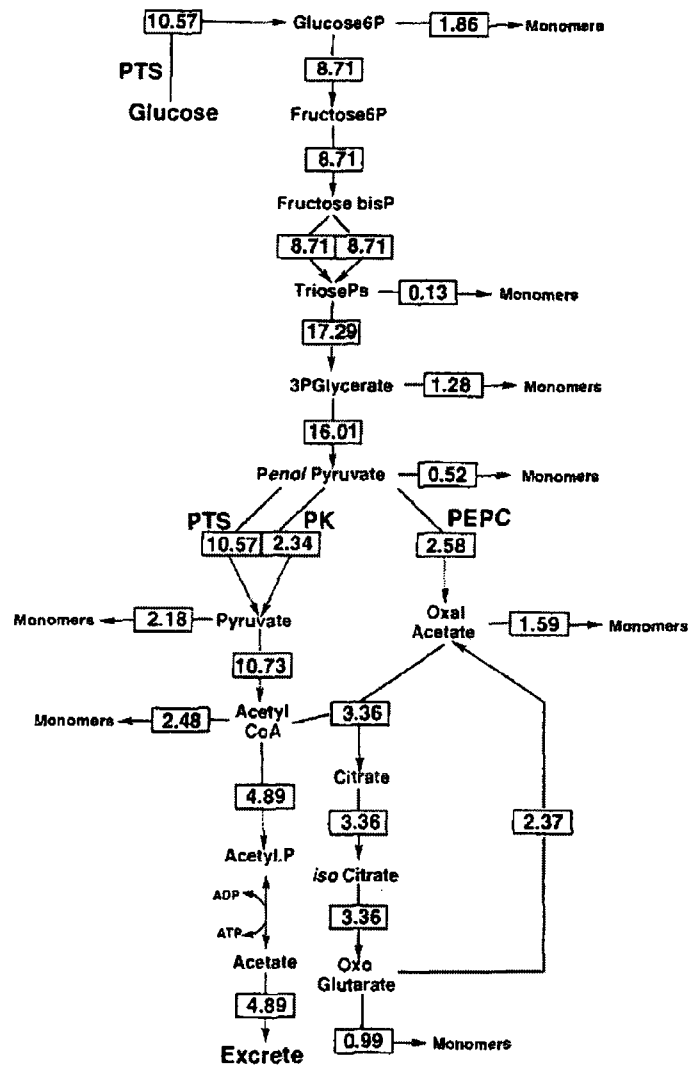


Figure 2-2: Flux analysis of *E. coli* grown on glucose [21].

2.1.5.2 Glycerol catabolism

E. coli can also use glycerol for metabolic reactions. Growth on this carbon source is slower than growth on glucose (0.70 h^{-1} compared to 0.94 h^{-1}), probably because only 66% of the carbon atoms are delivered to the Krebs cycle. It is interesting to notice that, as shown in Figure 2-3, glycerol catabolism does not produce acetate and the only major products associated with glycerol utilization are biomass, CO_2 and water [21].

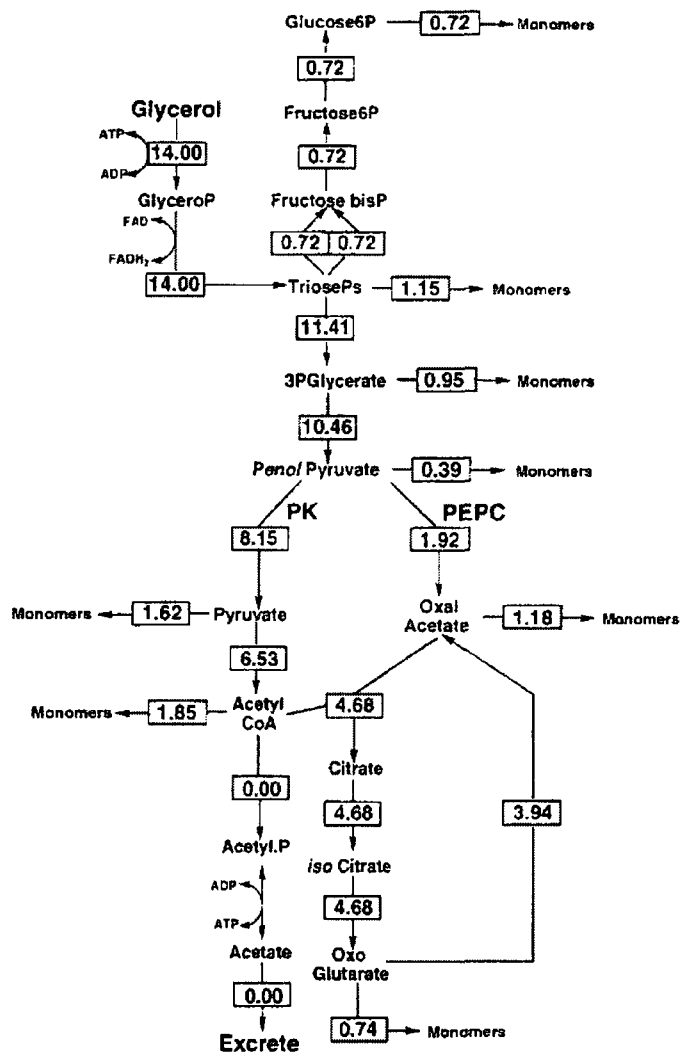


Figure 2-3: Flux analysis of *E. coli* grown on glycerol [21].

2.1.5.3 Acetate catabolism

Acetate can also be used for cell growth but it is much less effective as the specific growth rate on acetate is only 0.43 h^{-1} . Since acetate is an anabolite of the acetyl CoA, catabolism of acetate implies that the reverse reactions must be carried out, consuming ATP in the process, as can be seen on Figure 2-4.

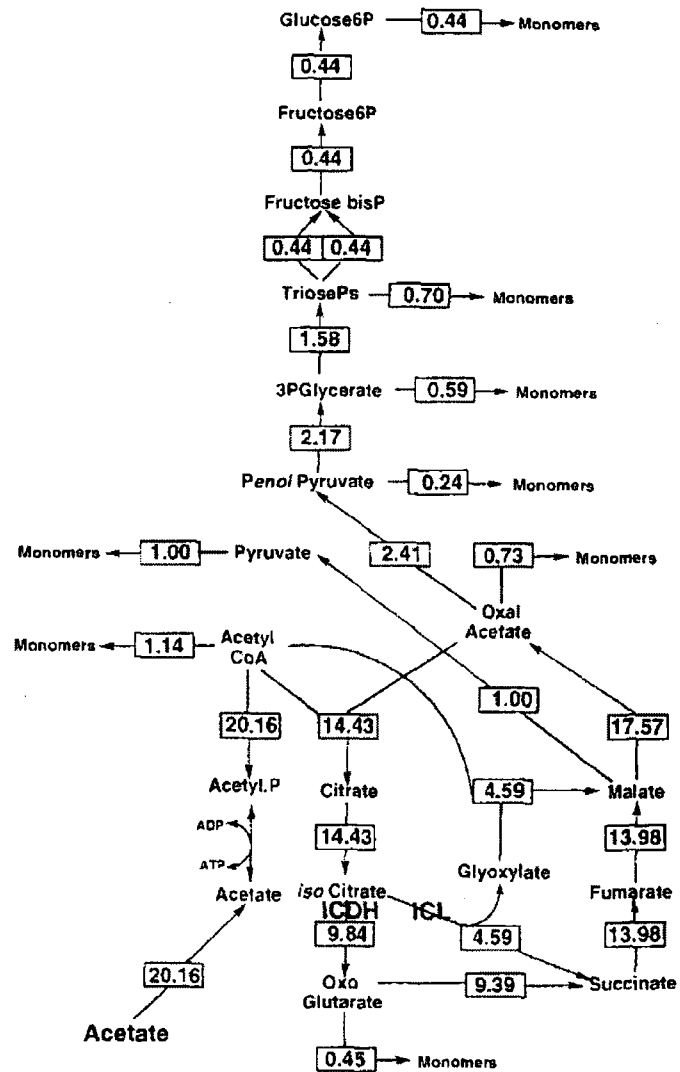


Figure 2-4: Flux analysis of *E. coli* grown on acetate [21].

2.1.6 Critical growth rate

The growth rate of the bacterial culture depends on several factors. First, a source of carbon must be present to provide the carbon backbone of cell materials and the bio-energy for cell metabolism. For aerobic growth, oxygen must also be present as the final electron acceptor. When other factors are not limiting, it was reported that increasing initial glucose concentration corresponded to increasing specific growth rate [23]. Maximal growth rate does not necessarily lead to maximization of the biomass or desirable products. For instance, when *E. coli* cells are grown in high concentration of glucose, an overflow of the acetyl CoA pool to the Krebs cycle yields higher concentration of acetate [21, 24-36]. It has been reported that *E. coli* metabolism favours the maximization of ATP production [37] and this is why when excess glucose is present in the medium, acetate formation is favoured as a secondary pathway for ATP production since it produces the second largest pool of ATP and NADH₂ [29]. It has also been reported that the use of complex medium (e.g. yeast extract or tryptone) results in acetate formation at lower growth rate (dilution rate of 0.2 h⁻¹) than the use of a defined medium (dilution rate of 0.35 h⁻¹) [38]. As it will be seen in the next section, acetate is a metabolic by-product that may have an inhibitory effect on both cell growth and recombinant protein expression. Since acetate formation is related to cell growth rate, several strategies have been attempted to reduce its formation by controlling the specific growth rate under a critical value [27, 31, 39, 40]. The critical growth rate, defined as the rate at which *E. coli* produces minimal amount of acetate, can be achieved by controlling glucose uptake and dissolved oxygen. These strategies include controlling the rate at which bacteria are growing by controlling the rate of glucose consumption or by addition of chemicals that can alter the consumption of glucose [24, 25].

2.1.7 Acetate and other by-products formation

As discussed above, acetate formation is closely related to the specific growth rate which is related to the glucose concentration in the medium [28]. Acetate has been shown to cause cessation of cell growth when its concentration is higher than 10 g/L [41, 42] but can affect growth at concentration as low as 0.5 g/L [32]. Furthermore, acetate also has inhibitory effects on protein expression as it specifically inhibits oxygen and glucose uptake, resulting in the reduction of recombinant proteins expression [39, 43]. It is also suspected of reducing the proton motive pump by disturbing transmembrane pH gradient [31, 44]. Cherrington *et al.* reported that short-chain acids could impair on RNA, DNA and other macromolecules synthesis [45]. They hypothesised that not only the protons would be responsible for this inhibition but also, upon diffusion in the cell, organic acids would dissociate and resulting anions could impair the synthesis of these macromolecules. Other metabolic by-products such as lactate, the second preferred by-product excreted from an overflowing glucose metabolism which once protonated yields lactic acid [21], pyruvate and ethanol can also be detrimental to cell growth and protein expression [27].

2.2 Molecular Biology background

2.2.1 Recombinant protein expression

Recombinant DNA techniques are common practices since the late 60's and in order to facilitate the manipulation of recombinant DNA, vectors have been developed enabling bacterial strains to express proteins that are not intrinsic. An expression vector or plasmid usually consists in circular double stranded DNA, which must contain an origin of replication, a promoter sequence, a selection marker and a multiple cloning site. The origin of replication must be present for the bacteria to replicate the plasmid DNA once it is inside

the cytoplasm thus increasing the number of copies of the plasmid present in each bacterium. The promoter sequence is a sequence on which the RNA polymerase must bind to initiate DNA transcription leading to mRNA that will subsequently be translated into proteins. The selection marker is used to ensure that only the bacteria bearing the vector will grow. The multiple cloning site enables directional cloning of the desired gene in the bacteria. For a gene to be transcribed into mRNA, it must be in the proper orientation (5'-3') and this is achieved by inserting specific restriction enzyme sites to the gene product when it is amplified via the polymerase chain reaction [46].

Once inside the bacteria, the bacterial machinery replicates the vector and when proper conditions exist, protein expression will occur. As discussed previously, an inducer is required to promote protein expression when an inducible promoter is used. The inducer system is a set of genes, an operon, to which the RNA polymerase must bind in order to initiate transcription of the DNA into mRNA, which will be translated into protein. The widely used lac operon is a set of genes involved in the lactose catabolism. When no lactose is available, catabolite repression of the operon takes place. This is achieved by the binding of a repressor molecule to the operon, which prevents the RNA polymerase from binding the operon. If lactose is available, metabolite of lactose, allolactose, binds to the repressor protein, modifying its conformation and preventing its binding to the operon thus enabling the RNA polymerase to initiate DNA transcription. Since the recombinant protein gene is located downstream of the lac operon, the recombinant DNA gets translated with the other lac operon genes [46].

2.3 *Clostridium difficile* associated diseases (CDAD) and sdAb ToxA5.1

2.3.1 *Clostridium difficile*

Clostridium difficile is a sporulating anaerobic Gram-positive bacterium, which has been the leading and deadliest cause of nosocomial diarrhea colitis around the world [47, 48]. In the USA alone, it is estimated that 3 million cases per year associated to *C. difficile* diseases (CDAD) are costing over a 1 billion dollars in health care services [49]. The emergence of these outbreaks is tightly associated with the overconsumption of broad-spectrum antimicrobial drugs [50] as elimination of the normal gut flora by these antimicrobial drugs is essential for *C. difficile* colonization [51].

The pathogenicity of *C. difficile* is associated with the production of enterotoxin A (TcdA) and cytotoxin B (TcdB) [47, 48, 52-55], which belong to the family of large clostridial toxins/glycosyltransferase. TcdA (308 kDa) and TcdB (270 kDa) are amongst the largest reported toxin to date and both target intracellular components Rho, Rac, and Cdc42 [47], which are proteins responsible for the regulation of the formation of actin filament-based structures and also for the assembly of adhesion sites needed for extra-cellular matrices [56].

2.3.2 Toxin A

Once the toxins are excreted in the gut by *C. difficile*, they are up taken into the target cells, intestinal epithelial cells, via surface receptor-mediated endocytosis and gain access to the cytosol [55, 57-59]. Inside the cytosol, TcdA and TcdB start to modulate host cell physiology by interfering with protein responsible for the production of the actin skeleton, cell junctions and by interfering with cell signalization [55].

2.3.3 Antibody therapy

It has been demonstrated that the severity and the duration of the CDAD is linked to the amount of toxin present [60] and that large quantities of anti-TcdA IgG are sufficient to prevent CDAD resurgence in patients [61, 62]. In order to prevent CDAD, immunoglobulin-directed therapy was investigated and several reported protection effects in the murine model [61, 63-69]. By binding to the toxin, the antibody can prevent the toxin from binding to its intended receptor on the epithelial cell. Since it has been reported that TcdA can be neutralized by monoclonal antibodies, the use of a novel type of antibodies, single domain antibodies that are antibodies found in camelidae and cartilaginous fish [70], could also be used as toxin inactivator as reported in some papers [39, 71].

2.4 Single domain antibodies

2.4.1 Discovery, Structure and Properties

Discovered in 1993 [70], camelidae sdAb, termed VHH, have similar structure to human variable domain (VH). These molecules are comprised of three complementary determining regions (CDR) providing antigen binding and four framework region constituting the core structure of the molecule [72]. SdAb have several unique properties, which make them very promising therapeutic agents. The major difference between synthetically engineered and naturally occurring sdAb is that the absence of the variable domain of the light chain (V_L) on synthetic sdAb exposes hydrophobic surface impairing on the protein solubility, resulting in aggregation thus low yield of soluble proteins [73].

The major advantage of sdAb is their small size. With a molecular weight of around 15 kDa, sdAb are much smaller than conventional antibodies (~160 kDa) and engineered fragments (~55 kDa for heavy chain and ~25 kDa for light chain). This property provides easy molecular manipulations in terms of cloning and expression since the information for the antibody is located on a single gene, which requires only a small set of defined primers for PCR reactions [74, 75]. Ease of manipulations leads to a faster overall production of the antibody with a period of about four months from immunization of the llama to the production of the antibody in *E. coli* [76, 77]. Shorter times in the development of sdAb make them cheaper to produce and for a wider range of targets [74]. Furthermore, their small size enables them to reach surface epitopes that are either hidden in crevasses or inaccessible due to conformation (immunosilent) to conventional antibodies [74, 76]. Being able to cross tissue barrier such as the brain barrier [78] is a great advantage to deliver payload of therapeutics agent to areas that are not accessible to their conventional counterparts. Another advantage related to small size is the density of biosensors that can be achieved on a detection surface.

As mentioned previously, these antibodies are highly soluble, which makes production of high concentrations advantageous since no precipitation occurs and therefore no reduction in the activity of the molecules. SdAb are also very stable with a shelf life of several months at room temperature. They can also withstand temperature as high as 90°C, extreme pH as well as digestion from proteases [72-76, 79, 80]. These properties enable oral administration of sdAb, which could minimize immunogenic reaction seen when mAb are injected in the blood stream [81]. Furthermore, it has been demonstrated that repeated injections of sdAb did not yield any immunogenic reaction in mice and this might be due to

a structural similarity between VHH and VH (which is the human form of the variable heavy chain domain) [72]. Unfortunately, not all sdAb will yield successful *in vivo* response as reported in Conrath *et al.* [82]. But improving proteolytic stability through molecular manipulations yielded satisfactory results in the survival rate of piglets exposed to a bacterial toxin [83]. Furthermore, if longer serum life is required, sdAb can be fused with albumin [84, 85] or immunoglobulin molecules [83], which provides significantly extended serum life.

Finally, producing sdAb is relatively less expensive than mAb. Since they do not require extensive folding and therefore can be produced in prokaryotic cells (e.g. *E. coli*) or in eukaryotic microbial cells (e.g. yeasts) of which fermentation is less expensive in both capital and operation cost, and easier to scale-up. Downstream processing of microbial fermentation broth is also less extensive [74, 76, 77]. Harmsen and De Haard have summarized some of the advantages of sdAb, which are shown in Table 2-1.

Table 2-1: Advantages of camelidae single domain antibodies [72].

Advantage	Molecular basis
Facile genetic manipulation	Single-domain nature
Increased functional size of immune libraries	No decrease in library size because of reshuffling of VL and VH domains
Facile production of multivalent formats	More flexible linker design and no mispairing of VL and VH domains
Facile production of oligoclonal preparations from single cells	No mispairing of VL and VH domains
High physicochemical stability	Efficient refolding due to increased hydrophilicity and single-domain nature
High solubility	Increased hydrophilicity
Recognition of hidden antigenic sites	Small size and extended flexible CDR3
Rapid tissue penetration, fast clearance	Small size
Well expressed	Efficient folding due to increased hydrophilicity and single-domain nature

It is generally agreed among researchers that the most significant advantages of sdAb over conventional antibodies are their small size and high solubility. However, no sdAb are currently in clinical trials, as further studies on animal models are required.

2.4.2 Potential Applications of sdAb

The potential applications for sdAb are the same as for conventional antibodies except with a larger scope due to the size difference as discussed above.

2.4.2.1 Detection and Biosensors

As with conventional antibodies, sdAb can be used as detection agents in several instances [86-88]. When coupled to radioactive isotopes or fluorescent markers, sdAb enables real time detection of cancer cells (lung and prostate), pathogens or toxins [79]. Due to their small size, sdAb serving as biosensors enable a quicker and more sensitive response (sub-nanogram/mL levels) since high concentration on surface material can be achieved [79]. It was also shown that sdAb have the potential to bind low molecular weight haptens with very high affinity [89].

2.4.2.2 Fusion Partners

Another application of sdAb is their use with a conjugated partner [90]. Of these tandems, the complex sdAb-enzyme is often used. The antibodies bind the target and the enzyme converts a prodrug (inactive form of a drug that requires a modification for toxic activation) into a lethal chemical in the vicinities of the target cancer cells [74, 76]. SdAb can also be fused with peptides that have a toxic activity against target cells or with liposomes carrying drugs.

2.4.2.3 Toxin Inactivation

The ability of sdAb to bind with high specificity is also used against toxin to prevent them from binding to their intended receptors to neutralize toxins. This strategy has been used successfully in murine models to prevent tooth caries [91] or to inactivate the

toxin of venom bites [92]. This strategy was also used against the tetanus toxin with good results [93]. Several other sdAb have been reported to bind to toxins, Goldman *et al.* reported ricin toxin binding [80], Liu *et al.* reported sdAb binding to three toxins: staphylococcal enterotoxin B (SEB), ricin, and botulinum toxin A (BoNT/A) complex toxoid [71] while Shuntao *et al.* reported a sdAb binding and inactivating the ricin toxin [39].

2.4.2.4 Cancer and Other Diseases Diagnostic and Treatment

One of the great hopes for sdAb is the possibility for cancer diagnostic and treatment. As discussed previously, sdAb can be used as imaging system being able to locate and bind to specific target like cancer cells. Several papers related the use of sdAb against cancers cells [81, 94, 95]. The target of choice is the epithelial growth factor receptor found in large quantity on cancer cells. Several other papers related the use of sdAb against these receptors in order to inhibit growth factors from reaching the cells or as part of detection procedures [96-100]. Furthermore, sdAb may be used in the treatment of inflammatory and infectious diseases. They may also be used for the treatment of Alzheimer's [76], Huntington and Parkinson diseases [101].

2.4.3 Production of sdAb

In order to satisfy the demand for antibodies, large-scale production must be carried out in a cost effective manner. Production of large quantities of mAb still is hindered because scale-up of process is complicated and facilities are costly to build and to operate [74-76]. As mentioned previously, sdAb do not require extensive folding or glycosylation in order to be functional, making the production of this kind of antibodies using

recombinant bacteria and yeast possible, which can have a significant impact on the costs associated with their production.

2.4.3.1 Comparison between Mammalian, yeast and bacterial systems

Mammalian or insect cells are used for the production of large, complete antibodies molecules such as IgG requiring proper folding and linking for functionality while bacteria and yeast are preferred for the production of antibody fragments such as Fab (requiring only minor folding and linking), multimeric fragments and sdAb [75, 102]. The reason for this is that prokaryotic cells do not have the complex machinery needed for proper protein folding nor the proper cellular environment for disulfide bonding that higher eukaryotic cell possesses. It is to be noted that *E. coli* could become a major expression system for sdAb since there is no need to export the protein, via exporting signal peptides, in the reducing periplasmic space, as it is the practice for Fab production, since they do not require disulfide bond formation [103]. This practice was used when fragments requiring assembly of different parts were trying to be produce in *E. coli* [72, 75]. Nevertheless, information is scarce on *E. coli*, especially at relatively large-scales [99]. In the end, the choice of an expression system is based on knowledge and on the individual protein that has to be expressed since different proteins will expressed at different levels depending on the system used [72, 77] and there is no universal system for the production of sdAb as production levels are dependent on the protein sequence.

2.4.3.2 Prokaryotic cells

Prokaryotic cells, mainly *E. coli*, have several advantages when it comes to protein expression [104]. The first one is the short period of time between the moment a sequence

of a desired protein becomes known and the final expression optimization of this protein in *E. coli* [75, 77, 105]. This is greatly due to the extensive molecular biology information available on *E. coli*, which makes cloning, transformation and selection a very efficient process. Furthermore, fermentation can be done in fairly cheap and readily available installations, which reduces capital costs. Operation costs are also significantly lower than mammalian cell culture since the fermentation techniques are fairly simple and the medium is inexpensive. Another advantage of *E. coli* system is that there is no possibility for virus contamination. However, a major drawback is the possibility of endotoxin contamination [106] and therefore purification of the protein must be well performed, and even in that case, extra care must be taken as some immunogenic response triggering agent such as lipopolysaccharide can be tightly bound to the purified protein. In order to circumvent the endotoxin problem, sdAb could be produced in bacteria that are GRAS (generally regarded as safe) such as lactic acid producing bacteria [107]. This could even serve as a delivery system, with the production of sdAb directly in the gut where antibodies would be needed to fight parasitic or bacterial infections.

Early clinical tests using sdAb have, however, shown very promising results. It is apparently of significant relevance to develop optimized protocols for the cost-effective, large-scale production of this new therapeutic agent [108].

2.4.3.3 Yields and experimental conditions in *E. coli* system

As mentioned previously, very few papers were specifically aiming at studying sdAb production in *E. coli* but it was possible to find information on fragment production (Fab and dimeric miniantibodies). In 1996, Horn *et al.* reported an optimized production of scFv of about 4 g/L in a fed-batch culture grown for 33 h at a cell density of 145 g/L on

glucose mineral salt medium. Of these, 80% were functionally assembled [109]. This number is surprisingly high when compared to the other result obtained by Corsideo and Wang (2004) where optimized expression conditions for the production of a Fab yielded a low 16 mg/L (shake flasks in LB media) [110]. The source of the variation between the two experiments is hard to pinpoint. First, the cells have not been grown on the same medium. The first group used a mineral salt medium while the second group used a standard media for *E. coli* culture and also batch culture cannot be compared with flask culture. It is clear however that the production can greatly vary (from 16 mg/L to 4000 mg/L) and that fed-batch is definitively the route to take for expression of any protein as multiple papers have proven this point. However, in this thesis, batch cultivation was performed in order to gather critical information on kinetic of bacterial growth as well as on ToxA5.1 sdAb expression.

Concerning sdAb production, Rahbarizadeh *et al.* produced up to 42 mg/L in batch culture using LB media (8 h induction time at 37°C) under optimized conditions [99]. This study shows that the production of sdAb can vary greatly. Other papers reported higher sdAb production even though they were not aimed at optimizing the production but merely producing sdAb for further biochemistry experiments. Tanha *et al.* reported a sdAb production of 80 mg/L [111] and in other papers, the amount of sdAb produced varies from 10 to 100 mg/L [80, 89, 92, 99, 100] in unoptimized similar flask conditions. As mentioned in Harmsen and De Haard, sdAb varying only by a few amino acids can have highly variable expression levels [72].

2.4.3.4 Eukaryotic cells

Most of the studies available on the production of sdAb are mainly done in eukaryotic cells. A few studies have been done in plants [112] or in fungi [113] but most of the studies have been performed in yeasts. This might be due to the fact that yeast systems were used with greater success in earlier stages of antibody production and expertise was transferred to sdAb production. Similar to prokaryotic system, yeast systems have been in place for a long time and are popular for fermentation where secretion of the desired protein in the culture medium is needed. Yeast systems require more elaborated procedures than prokaryotic systems regarding molecular manipulation. The cloning, transformation and selection processes are done in an *E. coli* strain bearing a shuttle vector. This vector is designed to be functional both in *E. coli* and in the yeast. Once the vector is constructed and the potential clones have been selected in *E. coli* cells, the vector is isolated and transformed into the yeast for the final step, the production step. Yeasts are grown in fed-batch fermentation, using relatively inexpensive media. Similar to the fermentation using *E. coli*, yeast fermentation is much less expensive for sdAb production than mammalian cell culture. However, yeast tend to grow a lot slower than *E. coli* but the fact that cells do not need to be lysed to access the protein due to protein excretion in the fermentation broth, compensate for this slower growth.

One of the drawbacks of protein production using recombinant yeast, is the possible increase in immunogenic reaction caused by the addition of high mannose sugars yeasts add to protein [114].

2.4.3.5 Yields and experimental conditions in yeast systems

Production of antibody fragments has been well studied in yeast and fungi. Gasser and Mattanovich have compiled an extensive list of fragments produced in different types of yeast and fungi reporting yield, culture conditions and media [77]. This list can be seen in Table 2-2.

From this list, it is clear that fermentation in bioreactors using fed-batch system produces significantly larger amount of fragments, at magnitudes of g/L compared to shake flask cultures where only mg/L were obtained. This could be transposed to sdAb and bioreactor fermentation should definitively be used for the production of the antibodies.

As mentioned previously, most of the studies done on sdAb production were conducted in yeast systems. As with *E. coli*, fragments have also been produced in yeast. In *Saccharomyces cerevisiae* shake flask culture, production of 100 mg/L was achieved [115]. Although bioreactor fermentations seemed to produce higher yield of fragments, it is to be noted that this is not the case for every fragments. It was reported by Thomassen *et al.* that 1.3 kg of VHH were produced in a 15 m³ fermentor [116] corresponding to a concentration of 42 mg/L. In another experiment, the yeast *Pichia pastoris* was used to produce sdAb in fermentation setting and it too yielded a low protein concentration of only 10-15 mg/L of culture [117].

Host	Antibody	Antigen	Promoter ^a	Titer (mg l ⁻¹)	Cultivation	Culture medium	Comments	Reference
<i>A. awamori</i>	mAb	Hu1D10	<i>glcA</i>	200	Shake flask	Complex	Glucoamylase fusion (LC + HC)	Ward et al. (2004)
<i>A. awamori</i>	mAb	Trastuzumab	<i>glcA</i>	900	Shake flask	Complex	Glucoamylase fusion (LC + HC)	Ward et al. (2004)
<i>A. awamori</i>	Kappa LC	Trastuzumab	<i>glcA</i>	1,500	Shake flask	Complex	Glucoamylase fusion (LC)	Ward et al. (2004)
<i>A. awamori</i>	Gamma HC	Trastuzumab	<i>glcA</i>	300	Shake flask	Complex	Glucoamylase fusion (HC)	Ward et al. (2004)
<i>A. awamori</i>	Fab	Trastuzumab	<i>glcA</i>	1,200	Shake flask	Complex	Glucoamylase fusion (LC + HC)	Ward et al. (2004)
<i>A. awamori</i>	scFv	Lysozyme	<i>exlA</i>	10-30	Shake flask	Complex	2-6 fold; glucoamylase fusion	Frenken et al. (1998)
<i>A. awamori</i>	scFv	Lysozyme	<i>exlA</i>	50-80	Shake flask	Complex	2-6 fold; glucoamylase fusion	Frenken et al. (1998)
<i>A. awamori</i>	scFv	4715	<i>exlA</i>	200	Fermentation	Complex	2-6 fold; glucoamylase fusion	Frenken et al. (1998)
<i>A. awamori</i>	scFv	Lysozyme	Xylanase	14.5	Fermentation	Complex	15-fold; induction time, double C- and N-source concentration, less inducer	Sotiriadis et al. (2001)
<i>A. awamori</i>	scFv	Lysozyme	Xylanase	108	Fermentation	Complex	15-fold; induction time, double C- and N-source concentration, less inducer	Sotiriadis et al. (2001)
<i>T. reesei</i>	Fab	2-phenyloxazolone	<i>CBHI</i>	0.3-1	Shake flask	?		Nyyssonen et al. 1993
<i>T. reesei</i>	Fab	2-phenyloxazolone	<i>CBHI</i>	5-40	Shake flask	?		Nyyssonen et al. 1993
<i>T. reesei</i>	Fab	2-phenyloxazolone	<i>CBHI</i>	150	Fermentation	?	50-fold; CBHI fusion	Nyyssonen et al. 1993
<i>P. pastoris</i>	scFv	Leukaemia inhibitory factor	<i>AOX1</i>	>100	Shake flask	Minimal		Ridder et al. 1995
<i>P. pastoris</i>	scFv	Desipramine	<i>AOX1</i>	250	Shake flask	Minimal		Eldin et al. 1997
<i>P. pastoris</i>	scFv	CD7	<i>AOX1</i>	60	Shake flask	Minimal		Eldin et al. 1997
<i>P. pastoris</i>	scFv	A33	<i>AOX1</i>	4,880	Fermentation	Defined	0.5% Methanol induction; pH 3.0	Damasoano et al. 2004
<i>P. pastoris</i>	scFv	<i>E. coli</i> /BA11	<i>AOX1</i>	3,500	Fermentation	Defined	Oxygen-limited control of methanol uptake	Khatri and Herfmann 2006
<i>P. pastoris</i>	scFv	Colorectal cancer/1c9-cca.1	<i>AOX1</i>	1,200	Fermentation	Minimal		Freyre et al. 2000
<i>P. pastoris</i>	Bivalent scFv	CA125-ovarian carcinoma	<i>AOX1</i>	100	Shake flask	Minimal		Luo et al. 1998
<i>P. pastoris</i>	Fab	Atrazine/K411B	<i>AOX1</i>	40	Fermentation	Complex		Lange et al. 2001
<i>P. pastoris</i>	Fab	IgE-receptor	<i>AOX1</i>	40	Shake flask	Minimal		Takahashi et al. (2000)
<i>P. pastoris</i>	Fab	HIV1/2FS	<i>GAP</i>	18	Shake flask	Complex	1.9-fold; HAcCl, PDI over-expression	Gasser et al. 2006

Table 2-2: Summary of antibody fragments produced in yeasts and fungi [77].

2.5 Conclusion

It has been shown that the production of the sdAb is of commercial value and that the sdAb we are trying to produce has an important application, which is to fight drug resistant *C. difficile* diseases. Furthermore, it has also been shown that bioreactor fermentation should be used for this production if large amount of sdAb are needed.

Experimental results in the form of research papers concerning sdAb expression will be presented in the next two chapters. The first paper focuses on ascertaining which parameters have the greatest effects on sdAb expression while the second paper investigates the effect of carbon source and concentration on the expression of the sdAb ToxA5.1 in bioreactors.

2.6 References

1. Gouy, M. and C. Gautier, *Codon usage in bacteria: correlation with gene expressivity*. Nucleic Acids Research, 1982. **10**(22): p. 7055-7074.
2. Schumann, W. and L.C.S. Ferreira, *Production of recombinant proteins in Escherichia coli*. Genetics and Molecular Biology, 2004. **27**(3): p. 442-453.
3. Kotik, M., et al., *High-level expression of a fungal pyranose oxidase in high cell-density fed-batch cultivations of Escherichia coli using lactose as inducer*. Protein Expression and Purification, 2004. **36**(1): p. 61-69.
4. Gombert, A.K. and B.V. Kilikian, *Recombinant gene expression in Escherichia coli cultivation using lactose as inducer*. Journal of Biotechnology, 1998. **60**(1-2).
5. Neubauer, P., et al., *Maximizing the expression of a recombinant gene in Escherichia coli by manipulation of induction time using lactose as inducer*. Applied Microbiology and Biotechnology, 1992. **36**(6): p. 739-744.
6. Figge, J., et al., *Stringent regulation of stably integrated chloramphenicol acetyl transferase genes by E. coli lac repressor in monkey cells*. Cell, 1988. **52**(5): p. 713-722.
7. Makrides, S.C., *Strategies for achieving high-level expression of genes in Escherichia coli*. Microbiological Reviews, 1996. **60**(3): p. 512-538.
8. Cho, S., et al., *Effect of iso-propyl-thio- β -D-galactoside concentration on the level of lac-operon induction in steady state Escherichia coli*. Biochemical and Biophysical Research Communications, 1985. **128**(3): p. 1268-1273.
9. Yildir, C., Z.I. Önsan, and B. Kirdar, *Optimization of starting time and period of induction and inducer concentration in the production of the restriction enzyme EcoRI from recombinant Escherichia coli 294*. Turkish Journal of Chemistry, 1998. **22**(3): p. 221-226.
10. Turner, P., O. Holst, and E.N. Karlsson, *Optimized expression of soluble cyclomaltodextrinase of thermophilic origin in Escherichia coli by using a soluble fusion-tag and by tuning of inducer concentration*. Protein Expression and Purification, 2005. **39**(1): p. 54-60.
11. Afonyushkin, T., et al., *Temperature-dependent stability and translation of Escherichia coli ompA mRNA*. Biochemical and Biophysical Research Communications, 2003. **311**(3): p. 604-609.
12. Bortolussi, R., P. Ferrieri, and P.G. Quie, *Influence of growth temperature of Escherichia coli on K1 capsular antigen production and resistance to opsonization*. Infection and Immunity, 1983. **39**(3): p. 1136-1141.
13. Herendeen, S.L., R.A. VanBogelen, and F.C. Neidhardt, *Levels of major proteins of Escherichia coli during growth at different temperatures*. Journal of Bacteriology, 1979. **139**(1): p. 185-194.
14. Chalmers, J.J., et al., *Effects of temperature on Escherichia coli overproducing β -lactamase or human epidermal growth factor*. Applied and Environmental Microbiology, 1990. **56**(1): p. 104-111.
15. Zilberstein, D., et al., *Escherichia coli intracellular pH, membrane potential, and cell growth*. Journal of Bacteriology, 1984. **158**(1): p. 246-252.

16. Felle, H., et al., *Quantitative measurements of membrane potential in Escherichia coli*. Biochemistry, 1980. **19**(15): p. 3585-3590.
17. Tucker, D.L., N. Tucker, and T. Conway, *Gene expression profiling of the pH response in Escherichia coli*. Journal of Bacteriology, 2002. **184**(23): p. 6551-6558.
18. Andersson, C., et al., *Effect of different carbon sources on the production of succinic acid using metabolically engineered Escherichia coli*. Biotechnology Progress, 2007. **23**(2): p. 381-388.
19. Sung, K.L. and J.D. Keasling, *Effect of glucose or glycerol as the sole carbon source on gene expression from the Salmonella prpBCDE promoter in Escherichia coli*. Biotechnology Progress, 2006. **22**(6): p. 1547-1551.
20. Hempfling, W.P. and S.E. Mainzer, *Effects of varying the carbon source limiting growth on yield and maintenance characteristics of Escherichia coli in continuous culture*. Journal of Bacteriology, 1975. **123**(3): p. 1076-1087.
21. Holms, H., *Flux analysis and control of the central metabolic pathways in Escherichia coli*. FEMS Microbiology Reviews, 1996. **19**(2): p. 85-116.
22. Shuler, M.L. and F. Kargi, *Bioprocess Engineering, Basic Concepts*. 2nd ed. 2002, Upper Saddle River, NJ: Prentice Hall PTR. 553.
23. Marison, I.W. and U. Von Stockar, *The application of a novel heat flux calorimeter for studying growth of Escherichia coli W in aerobic batch culture*. Biotechnology and Bioengineering, 1986. **28**(12): p. 1780-1793.
24. Chou, C.H., G.N. Bennett, and K.Y. San, *Effect of modified glucose uptake using genetic engineering techniques on high-level recombinant protein production in Escherichia coli dense cultures*. Biotechnology and Bioengineering, 1994. **44**(8): p. 952-960.
25. Chou, C.H., G.N. Bennett, and K.Y. San, *Effect of modulated glucose uptake on high-level recombinant protein production in a dense Escherichia coli culture*. Biotechnology Progress, 1994. **10**(6): p. 644-647.
26. Christensen, M.L. and N.T. Eriksen, *Growth and proton exchange in recombinant Escherichia coli BL21*. Enzyme and Microbial Technology, 2002. **31**(4): p. 566-574.
27. De Mey, M., et al., *Minimizing acetate formation in E. coli fermentations*. Journal of Industrial Microbiology and Biotechnology, 2007. **34**(11): p. 689-700.
28. Eiteman, M.A. and E. Altman, *Overcoming acetate in Escherichia coli recombinant protein fermentations*. Trends in Biotechnology, 2006. **24**(11): p. 530-536.
29. Han, K., H.C. Lim, and J. Hong, *Acetic acid formation in Escherichia coli fermentation*. Biotechnology and Bioengineering, 1992. **39**(6): p. 663-671.
30. Hansen, R. and N.T. Eriksen, *Activity of recombinant GST in Escherichia coli grown on glucose and glycerol* Process Biochemistry, 2007. **42**(8): p. 1259-1263.
31. Luli, G.W. and W.R. Strohl, *Comparison of growth, acetate production, and acetate inhibition of Escherichia coli strains in batch and fed-batch fermentations*. Applied and Environmental Microbiology, 1990. **56**(4): p. 1004-1011.
32. Nakano, K., et al., *Influence of acetic acid on the growth of Escherichia coli K12 during high-cell-density cultivation in a dialysis reactor*. Applied Microbiology and Biotechnology, 1997. **48**(5): p. 597-601.

33. San, K.Y., et al., *Strategies in high-level expression of recombinant protein in Escherichia coli*. Annals of the New York Academy of Sciences, 1994. **721**: p. 257-267.
34. Sandén, A.M., et al., *Limiting factors in Escherichia coli fed-batch production of recombinant proteins*. Biotechnology and Bioengineering, 2003. **81**(2): p. 158-166.
35. Xu, B., et al., *Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with Escherichia coli*. Applied Microbiology and Biotechnology, 1999. **51**(5): p. 564-571.
36. Xu, B., M. Jahic, and S.O. Enfors, *Modeling of overflow metabolism in batch and fed-batch cultures of Escherichia coli*. Biotechnology Progress, 1999. **15**(1): p. 81-90.
37. Majewski, R.A. and M.M. Domach, *Simple constrained-optimization view of acetate overflow in E. coli*. Biotechnology and Bioengineering, 1990. **35**(7): p. 732-738.
38. Meyer, H.P., C. Leist, and A. Fiechter, *Acetate formation in continuous culture of Escherichia coli K12 D1 on defined and complex media*. Journal of Biotechnology, 1984. **1**(5-6): p. 355-358.
39. Shuntao, W., et al., *A novel designed single domain antibody on 3-D structure of ricin A chain remarkably blocked ricin-induced cytotoxicity*. Molecular Immunology, 2006. **43**(11): p. 1912-1919.
40. Korz, D.J., et al., *Simple fed-batch technique for high cell density cultivation of Escherichia coli*. Journal of Biotechnology, 1995. **39**(1): p. 59-65.
41. Jae Gu, P., R. Joon Shick, and J.M. Lebeault, *Physiological constraints in increasing biomass concentration of Escherichia coli B in fed-batch culture*. Biotechnology Letters, 1987. **9**(2): p. 89-94.
42. Fass, R., T.R. Clem, and J. Shiloach, *Use of a novel air separation system in a fed-batch fermentative culture of Escherichia coli*. Applied and Environmental Microbiology, 1989. **55**(5): p. 1305-1307.
43. Kleman, G.L. and W.R. Strohl, *Acetate metabolism by Escherichia coli in high-cell-density fermentation*. Applied and Environmental Microbiology, 1994. **60**(11): p. 3952-3958.
44. El-Mansi, E.M.T. and W.H. Holms, *Control of carbon flux to acetate excretion during growth of Escherichia coli in batch and continuous cultures*. Journal of General Microbiology, 1989. **135**(11): p. 2875-2883.
45. Cherrington, C.A., M. Hinton, and I. Chopra, *Effect of short-chain organic acids on macromolecular synthesis in Escherichia coli*. Journal of Applied Bacteriology, 1990. **68**(1): p. 69-74.
46. Ausubel, F.M., R. Brent, and R.E. Kingston, *Current Protocols in Molecular Biology*. 1998, Hoboken, NJ: John Wiley and Sons, Inc. 5300.
47. Bobak, D.A., *The molecular pathogenesis of Clostridium difficile-associated disease*. Current Infectious Disease Reports, 2008. **10**(2): p. 111-115.
48. Kelly, C.P., C. Pothoulakis, and J.T. Lamont, *Clostridium difficile colitis*. New England Journal of Medicine, 1994. **330**(4): p. 257-262.
49. Oldfield Iii, E.C., *Clostridium difficile-associated diarrhea: Risk factors, diagnostic methods, and treatment*. Reviews in Gastroenterological Disorders, 2004. **4**(4): p. 186-195.

50. Gorbach, S.L., *Antibiotics and Clostridium difficile*. New England Journal of Medicine, 1999. **341**(22): p. 1690-1691.
51. Tonna, I. and P.D. Welsby, *Pathogenesis and treatment of Clostridium difficile infection*. Postgraduate Medical Journal, 2005. **81**(956): p. 367-369.
52. Blondeau, J.M., *What have we learned about antimicrobial use and the risks for Clostridium difficile-associated diarrhoea?* Journal of Antimicrobial Chemotherapy, 2009. **63**(2): p. 238-242.
53. Burakoff, R., et al., *Effects of purified Clostridium difficile toxin A on rabbit distal colon*. Gastroenterology, 1995. **109**(2): p. 348-354.
54. Lee, J.Y., et al., *Clostridium difficile toxin A promotes dendritic cell maturation and chemokine CXCL2 expression through p38, IKK, and the NF- κ B signaling pathway*. Journal of Molecular Medicine, 2009. **87**(2): p. 169-180.
55. Voth, D.E. and J.D. Ballard, *Clostridium difficile toxins: Mechanism of action and role in disease*. Clinical Microbiology Reviews, 2005. **18**(2): p. 247-263.
56. Allen, W.E., et al., *Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages*. Journal of Cell Science, 1997. **110**(6): p. 707-720.
57. Florin, I. and M. Thelestam, *Internalization of Clostridium difficile cytotoxin into cultured human lung fibroblasts*. BBA - Molecular Cell Research, 1983. **763**(4): p. 383-392.
58. Henriques, B., I. Florin, and M. Thelestam, *Cellular internalisation of Clostridium difficile toxin A*. Microbial Pathogenesis, 1987. **2**(6): p. 455-463.
59. Mitchell, M.J., B.E. Laughon, and S. Lin, *Biochemical studies on the effect of Clostridium difficile toxin B on actin in vivo and in vitro*. Infection and Immunity, 1987. **55**(7): p. 1610-1615.
60. Burdon, D.W., R.H. George, and G.A.G. Mogg, *Faecal toxin and severity of antibiotic-associated pseudomembranous colitis*. Journal of Clinical Pathology, 1981. **34**(5): p. 548-551.
61. Corthier, G., et al., *Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against Clostridium difficile toxin A*. Infection and Immunity, 1991. **59**(3): p. 1192-1195.
62. Kyne, L., et al., *Association between antibody response to toxin A and protection against recurrent Clostridium difficile diarrhoea*. Lancet, 2001. **357**(9251): p. 189-193.
63. Babcock, G.J., et al., *Human monoclonal antibodies directed against toxins A and B prevent Clostridium difficile-induced mortality in hamsters*. Infection and Immunity, 2006. **74**(11): p. 6339-6347.
64. Casadevall, A., E. Dadachova, and L.A. Pirofski, *Passive antibody therapy for infectious diseases*. Nature Reviews Microbiology, 2004. **2**(9): p. 695-703.
65. Lyerly, D.M., et al., *Passive immunization of hamsters against disease caused by Clostridium difficile by use of bovine immunoglobulin G concentrate*. Infection and Immunity, 1991. **59**(6): p. 2215-2218.
66. Taylor, C.P., et al., *Open-label, dose escalation phase I study in healthy volunteers to evaluate the safety and pharmacokinetics of a human monoclonal antibody to Clostridium difficile toxin A*. Vaccine, 2008. **26**(27-28): p. 3404-3409.
67. Salcedo, J., et al., *Intravenous immunoglobulin therapy for severe Clostridium difficile colitis*. Gut, 1997. **41**(3): p. 366-370.

68. Sougioultzis, S., et al., *Clostridium difficile* toxoid vaccine in recurrent *C. difficile*-associated diarrhea. *Gastroenterology*, 2005. **128**(3): p. 764-770.
69. Wilcox, M.H., *Descriptive study of intravenous immunoglobulin for the treatment of recurrent Clostridium difficile diarrhoea*. *Journal of Antimicrobial Chemotherapy*, 2004. **53**(5): p. 882-884.
70. Hamers-Casterman, C., et al., *Naturally occurring antibodies devoid of light chains*. *Nature*, 1993. **363**(6428): p. 446-448.
71. Liu, J.L., G.P. Anderson, and E.R. Goldman, *Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library*. *BMC Biotechnology*, 2007. **7**.
72. Harmsen, M.M. and H.J. De Haard, *Properties, production, and applications of camelid single-domain antibody fragments*. *Applied Microbiology and Biotechnology*, 2007. **77**(1): p. 13-22.
73. Muyldermans, S., *Single domain camel antibodies: Current status*. *Reviews in Molecular Biotechnology*, 2001. **74**(4): p. 277-302.
74. Holliger, P. and P.J. Hudson, *Engineered antibody fragments and the rise of single domains*. *Nature Biotechnology*, 2005. **23**(9): p. 1126-1136.
75. Arbabi-Ghahroudi, M., J. Tanha, and R. MacKenzie, *Prokaryotic expression of antibodies*. *Cancer and Metastasis Reviews*, 2005. **24**(4): p. 501-519.
76. Gibbs, W.W., *Nanobodies*. *Scientific American*, 2005. **293**(2): p. 78-83.
77. Gasser, B. and D. Mattanovich, *Antibody production with yeasts and filamentous fungi: On the road to large scale?* *Biotechnology Letters*, 2007. **29**(2): p. 201-212.
78. Muruganandam, A., et al., *Selection of phage-displayed llama single-domain antibodies that transmigrate across human blood-brain barrier endothelium*. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 2002. **16**(2): p. 240-242.
79. Saerens, D., et al., *Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing*. *Analytical Chemistry*, 2005. **77**(23): p. 7547-7555.
80. Goldman, E.R., et al., *Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library*. *Analytical Chemistry*, 2006. **78**(24): p. 8245-8255.
81. Cortez-Retamozo, V., et al., *Efficient Cancer Therapy with a Nanobody-Based Conjugate*. *Cancer Research*, 2004. **64**(8): p. 2853-2857.
82. Conrath, K.E., et al., *Emergence and evolution of functional heavy-chain antibodies in Camelidae*. *Developmental and Comparative Immunology*, 2003. **27**(2): p. 87-103.
83. Harmsen, M.M., et al., *Escherichia coli F4 fimbriae specific llama single-domain antibody fragments effectively inhibit bacterial adhesion in vitro but poorly protect against diarrhoea*. *Veterinary Microbiology*, 2005. **111**(1-2): p. 89-98.
84. Coppieters, K., et al., *Formatted anti-tumor necrosis factor α VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis*. *Arthritis and Rheumatism*, 2006. **54**(6): p. 1856-1866.

85. Roovers, R.C., et al., *Efficient inhibition of EGFR signalling and of tumour growth by antagonistic anti-EGFR Nanobodies*. *Cancer Immunology, Immunotherapy*, 2007. **56**(3): p. 303-317.
86. Rothbauer, U., et al., *Targeting and tracing antigens in live cells with fluorescent nanobodies*. *Nature Methods*, 2006. **3**(11): p. 887-889.
87. Presta, L.G., *Engineering antibodies for therapy*. *Current Pharmaceutical Biotechnology*, 2002. **3**(3): p. 237-256.
88. Olichon, A. and T. Surrey, *Selection of genetically encoded fluorescent single domain antibodies engineered for efficient expression in Escherichia coli*. *Journal of Biological Chemistry*, 2007. **282**(50): p. 36314-36320.
89. Alvarez-Rueda, N., et al., *Generation of llama single-domain antibodies against methotrexate, a prototypical hapten*. *Molecular Immunology*, 2007. **44**(7): p. 1691-1701.
90. Stockwin, L.H. and S. Holmes, *Antibodies as therapeutic agents: Vive la renaissance!* *Expert Opinion on Biological Therapy*, 2003. **3**(7): p. 1133-1152.
91. Krüger, C., et al., *Therapeutic effect of llama derived VHH fragments against Streptococcus mutans on the development of dental caries*. *Applied Microbiology and Biotechnology*, 2006. **72**(4): p. 732-737.
92. Stewart, C.S., C.R. MacKenzie, and J. Christopher Hall, *Isolation, characterization and pentamerization of α -cobrotoxin specific single-domain antibodies from a naïve phage display library: Preliminary findings for antivenom development*. *Toxicon*, 2007. **49**(5): p. 699-709.
93. Arbabi Ghahroudi, M., et al., *Selection and identification of single domain antibody fragments from camel heavy-chain antibodies*. *FEBS Letters*, 1997. **414**(3): p. 521-526.
94. Cortez-Retamozo, V., et al., *Efficient tumor targeting by single-domain antibody fragments of camels*. *International Journal of Cancer*, 2002. **98**(3): p. 456-462.
95. Groner, B., C. Hartmann, and W. Wels, *Therapeutic antibodies*. *Current Molecular Medicine*, 2004. **4**(5): p. 539-547.
96. Vokes, E.E. and E. Chu, *Anti-EGFR therapies: clinical experience in colorectal, lung, and head and neck cancers*. *Oncology (Williston Park, N.Y.)*, 2006. **20**(5 Suppl 2): p. 15-25.
97. Roovers, R.C., G.A.M.S. Van Dongen, and P.M.P. Van Bergen En Henegouwen, *Nanobodies in therapeutic applications*. *Current Opinion in Molecular Therapeutics*, 2007. **9**(4): p. 327-335.
98. Revets, H., P. De Baetselier, and S. Muyldermans, *Nanobodies as novel agents for cancer therapy*. *Expert Opinion on Biological Therapy*, 2005. **5**(1): p. 111-124.
99. Rahbarizadeh, F., et al., *High expression and purification of the recombinant camelid anti-MUC1 single domain antibodies in Escherichia coli*. *Protein Expression and Purification*, 2005. **44**(1): p. 32-38.
100. Omidfar, K., et al., *Production of a novel camel single-domain antibody specific for the type III mutant* *Tumor Biology*, 2004. **25**(5-6): p. 296-305.
101. Messer, A. and J. McLear, *The therapeutic potential of intrabodies in neurologic disorders: Focus on Huntington and Parkinson diseases*. *BioDrugs*, 2006. **20**(6): p. 327-333.

102. Filpula, D., *Antibody engineering and modification technologies*. Biomolecular Engineering, 2007. **24**(2): p. 201-215.
103. Power, B.E., et al., *High-level temperature-induced synthesis of an antibody VH-domain in Escherichia coli using the PelB secretion signal*. Gene, 1992. **113**(1): p. 95-99.
104. Fernandez, L.A., *Prokaryotic expression of antibodies and affibodies*. Current Opinion in Biotechnology, 2004. **15**(4): p. 364-373.
105. Plückthun, A., *Antibody engineering: Advances from the use of Escherichia coli expression systems*. Nature Biotechnology, 1991. **9**(6): p. 545-551.
106. Wakelin, S.J., et al., *"Dirty little secrets"-Endotoxin contamination of recombinant proteins*. Immunology Letters, 2006. **106**(1): p. 1-7.
107. Yeh, C.M., et al., *Extracellular expression of a functional recombinant Ganoderma lucidum immunomodulatory protein by Bacillus subtilis and Lactococcus lactis*. Applied and Environmental Microbiology, 2008. **74**(4): p. 1039-1049.
108. Gill, D.S. and N.K. Damle, *Biopharmaceutical drug discovery using novel protein scaffolds*. Current Opinion in Biotechnology, 2006. **17**(6): p. 653-658.
109. Horn, U., et al., *High volumetric yields of functional dimeric miniantibodies in Escherichia coli, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions*. Applied Microbiology and Biotechnology, 1996. **46**(5-6): p. 524-532.
110. Corisdeo, S. and B. Wang, *Functional expression and display of an antibody Fab fragment in Escherichia coli: Study of vector designs and culture conditions*. Protein Expression and Purification, 2004. **34**(2): p. 270-279.
111. Tanha, J., et al., *Selection by phage display of llama conventional VH fragments with heavy chain antibody VHH properties*. Journal of Immunological Methods, 2002. **263**(1-2): p. 97-109.
112. Ismaili, A., et al., *Production and characterization of anti-(mucin MUC1) single-domain antibody in tobacco (Nicotiana tabacum cultivar Xanthi)*. Biotechnology and Applied Biochemistry, 2007. **47**(1): p. 11-19.
113. Joosten, V., et al., *Production of bifunctional proteins by Aspergillus awamori: Llama variable heavy chain antibody fragment (VHH) R9 coupled to Arthromyces ramosus peroxidase (ARP)*. Journal of Biotechnology, 2005. **120**(4): p. 347-359.
114. Gerngross, T.U., *Advances in the production of human therapeutic proteins in yeasts and filamentous fungi*. Nature Biotechnology, 2004. **22**(11): p. 1409-1414.
115. Frenken, L.G.J., et al., *Isolation of antigen specific Llama V(HH) antibody fragments and their high level secretion by Saccharomyces cerevisiae*. Journal of Biotechnology, 2000. **78**(1): p. 11-21.
116. Thomassen, Y.E., et al., *Large-scale production of VHH antibody fragments by Saccharomyces cerevisiae*. Enzyme and Microbial Technology, 2002. **30**(3): p. 273-278.
117. Rahbarizadeh, F., et al., *Over expression of anti-MUC1 single-domain antibody fragments in the yeast Pichia pastoris*. Molecular Immunology, 2006. **43**(5): p. 426-435.

Chapter 3: Over-expression in *Escherichia coli* of single domain antibody against *Clostridium difficile* toxin A (TcdA)

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3.1. Abstract

The need for large-scale and cost effective antibody production is driving research towards smaller recombinant antigen binding fragments. To this end, single domain antibodies (sdAb), isolated originally from natural sources such as llama, have emerged as the most promising candidates. Their small size and simple structure facilitate genetic manipulations and, most importantly, enable their production in prokaryotic systems. In this study, we investigated the expression of an sdAb against *C. difficile* toxin A (TcdA), sdAb ToxA5.1, in *E. coli* TG1. Protein quantification was performed using SDS-PAGE densitometry while binding activity against toxin A was confirmed using ELISA. We found that system TG1- pSJF2H was not tightly regulated as 306 mg/L of protein, corresponding to a protein cell content of 155 mg protein/g DCW, were expressed in the absence of an inducer. However, the addition of an inducer did enhance protein expression and 377 mg/L sdAb ToxA5.1, corresponding to a cell content of 241 mg protein/g DCW, was achieved with the induction of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 24 h at 37°C. Temperature was demonstrated to have a significant effect on protein

expression. At an IPTG concentration of 0.01 mM, induction at 25°C did not yield detectable amount of protein while induction at 40°C yielded only 295 mg/L (197 mg protein/g DCW). The best protein expression was obtained at 37°C, which was 355 mg/L, corresponding to a protein cell content of 197 mg protein/g DCW. Glycerol was shown to be more favourable as a carbon source than glucose in terms of protein expression in flasks, with 30% higher protein produced compared to glucose. Medium buffering was found critical as no protein was detected when no buffering agent was included in the medium, which resulted in a final pH of lower than 5.5. A one-step purification of protein from crude lysate using magnetic beads was proven to be sufficient for protein recovery. The proteins thus purified had similar binding activity to those purified using immobilized metal affinity chromatography (IMAC) from on periplasmic extract followed by overnight dialysis.

3.2. Introduction

Estimated to account for over 30% of the biotechnology market in 2008 [1], antibody production is a fast growing and lucrative market with worldwide sales reaching over \$17 billion for monoclonal antibodies (mAb) alone in 2008 [2]. However, mAb have their limitations such as high production costs, immunogenicity, and relatively large size. The high production costs of mAbs can be mainly attributed to the fact that mammalian cell lines are required for their production and mammalian cell culture and protein recovery from mammalian cell culture are complex and costly. Furthermore, the relatively large size of mAb represents potentially strong immunogenicity and may also prevent them from accessing immunosilent epitopes. In order to circumvent these problems, conventional antibodies have been broken into a variety of different fragments such as antigen binding

fragment (Fab), crystallizing fragments (Fc), variable light chain (V_L), and variable heavy chain (V_H) domains linked together (scFv) or rearranged in multimeric units. However, all the of the aforementioned fragments lack the long serum half-life of conventional immunoglobulins G (IgGs) [2]. In the quest for even smaller and more active fragments, single domain antibodies have been engineered [3]. These small single domain antibodies (sdAb) consist of only the variable portion of the conventional antibody but are still able to bind antigens, although with much less avidity and strength than their counterparts [4, 5]. In 1993, a group of Belgian researchers discovered that sdAb were naturally produced in camelidae with antigen binding capabilities similar to their full-size conventional counterparts [6]. These naturally occurring sdAbs are comprised of three complementary determining regions (CDR) that provide antigen binding capacity and four framework regions that constitute the core structure of the molecule [7].

SdAb have several unique advantages, which make them very promising therapeutic agents. The primary advantage of sdAb is their small size. With a molecular weight of around 15 kDa, sdAb are much smaller than conventional antibodies (~160 kDa) and engineered fragments (~55 kDa for heavy chain and ~25 kDa for light chain). This property provides easy molecular manipulations in terms of cloning and expression since the information for the antibody is located on a single gene, which requires only a small set of defined primers for PCR reactions [1, 8].

The ease of DNA manipulation leads to a shorter development period of the antibody [2, 9]. Shorter development time reduces production costs and enables screening for a wider range of targets [1]. Since they do not require post-translation processing (e.g. folding and glycosylation), sdAb can be produced in prokaryotic cells (*E. coli*) [8] or in

eukaryotic microbes (yeasts). Microbial fermentation is less expensive, in terms of both capital and operation costs, and easier to scale-up than mammalian cell culture. The downstream processing for recombinant protein recovery from fermentation broth is also remarkably less costly than that from mammalian cell cultures [1, 2, 9].

Small sdAb are highly soluble and also very stable with a shelf life of several months at room temperature. They can also withstand extreme temperature (as high as 90°C), extreme pH and digestion from proteases[1, 2, 7, 8, 10-12]. These properties enable oral administration of sdAb, which could minimize immunogenic reaction [13] and is also more cost-effective and more acceptable to patients, especially children.

SdAb potentially have the same applications as that of conventional antibodies but with a larger scope due to the size difference. Some of these applications include: 1) detection and biosensors [10, 14-17]; 2) use as a conjugated partner for targeted drug delivery [1, 2, 18]; 3) cancer diagnosis and treatment [13, 19-25] ; and 4) toxin binding and inactivation [26-28]. Of particular relevance, several sdAb have been reported to be capable of binding to different toxins. Some of these toxin-binding adAb include ricin toxin binding sdAb reported by Glodman *et al.* [12], sdAb binding to three toxins: staphylococcal enterotoxin B (SEB), ricin, and botulinum toxin A (BoNT/A) complex toxoid, which was reported by Liu *et al.* [29], and another sdAb binding and inactivating the ricin toxin, which was reported by Shuntao *et al.* [30]. The present study aims at investigating the parameters affecting the expression of a new toxin-binding sdAb, sdAb ToxA5.1 targeting *Clostridium difficile* enterotoxin A [31].

3.3. Material and Methods

3.3.1. Expression vector and bacterial strain

DNA encoding ToxA5.1, a llama single-domain antibody with specificity for *C. difficile* toxin A [31], was cloned into the expression vector pSJF2H [8] via BbsI and BamHI (New England Biolabs, Mississauga, ON) restriction sites. Protein expression was performed in TG1 *E. coli* cells purchased from Invitrogen (Carlsbad, CA).

3.3.2. Media

Inoculum medium: Modified 2x YT medium which contained 16 g/L enzymatic Tryptone (Fluka, Buchs, Switzerland), 10 g/L yeast extract (Oxoid, Basingstoke, England) and 5 g/L NaCl (Sigma-Aldrich Corporation St. Louis, MO) plus 0.4% glycerol (Fisher Scientific, Pittsburgh, PA) or 2% glucose (Sigma-Aldrich Corp.), 12.32 g/L K₂HPO₄ (Sigma-Aldrich Corp.), 2.22 g/L KH₂PO₄ (Fisher Scientific) and 100 µg/mL ampicillin (Fisher Scientific), were used throughout the studies as inoculum media. Glycerol or glucose was used to match the carbon source of fermentation medium.

Fermentation Medium: Medium used for fermentation was in general the same as the inoculum medium. However, individual components may be changed as indicated in the text to investigate their effects on cell growth and protein expression. When parameters other than carbon source were investigated, 0.4% glycerol was used as carbon source.

3.3.3. Inoculum preparation

To prepare inoculum, glycerol stock cultures, which were stored at -80°C in an ultralow temperature freezer, were thawed at room temperature and transferred to LB agar plates containing 100 µg/mL ampicillin. Single colonies were used to inoculate 125 mL

flasks containing 10 mL of inoculum medium and grown at 37°C overnight at 200 rpm before being used as inoculum.

3.3.4. Bacterial cultivation and induction

Flasks (250 ml) containing 50 mL of fresh medium were inoculated with 1% (v/v) of the inoculum culture, which had been centrifuged (12 000 rpm for 2 min) and resuspended in 1 mL fresh broth; then incubated at 37°C and 200 rpm in shaker. Induction using IPTG (Isopropyl β -D-1-thiogalactopyranoside, Fisher Scientific) was conducted when cultures reached 0.3 - 0.5 OD₆₀₀, which is the predetermined mid-exponential phase. The concentration of IPTG was 0.01 mM, except when the effects of inducer concentration were investigated. In those cases, IPTG concentrations ranged from 0.0001 mM to 1 mM.

3.3.5. Cell lysis

Cells were harvested at different time intervals (2, 4, 6, 8, and 24 h) as follows: 1 mL of broth was centrifuged at 12 000 rpm for 2 min in a microcentrifuge at room temperature. Supernatant was discarded and pellet was frozen at -20°C for at least 15 min. Lysis buffer (300 μ L/pellet) was prepared by mixing 1X Fast Break lysis reagent (Promega Corporation, Madison, WI), 1 mM phenylmethylsulphonyl fluoride (PMSF) (Bio Basic Inc., Markam, ON) and 3 U DNase 1 (Promega Corp.). Cell lysis was performed on a rocking platform at room temperature for 30 min.

3.3.6. Protein quantification

Crude cell lysates were loaded on 4-15% discontinuous SDS-PAGE gels [48]. Standards of previously purified and quantified sdAb (275, 225, 151, 112, and 75 ng) were loaded on every gel to provide quantitative estimation. Proteins were fixed using 10%

acetic acid and 25% isopropanol for 15 min and stained for 60 min using Fermentas PageBlue Protein Staining Solution (Glen Burnie, MD). Quantification of scanned gels was achieved via densitometry using ImageJ software [49].

3.3.7. Western Blotting and ELISA

Purification of ToxA5.1 was achieved using MagneHIS Protein Purification System beads (Promega Corp.) on crude cell lysates, according to the manufacturer's instructions. Western blot analysis was performed using 15% discontinuous SDS-PAGE gels. Proteins were transferred to PVDF membranes (Pall Corporation, East Hills, NY), blocked with powdered skim milk, followed by incubation with mouse anti-His₆ IgG (GE Life Sciences Piscataway, NJ) primary antibody (1:3000 in PBS+0.05% Tween, 60 min). After washing with PBS+T (3 x 5 min), goat anti-mouse IgG conjugated with alkaline phosphatase (Cedarlane Laboratories, Burlington, NC) was applied (1:3000 in PBS+T, 60 min). Proteins were detected after washing with PBS+T (3 x 5 min) using AP Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA).

To assess the activity of purified ToxA5.1, an ELISA was performed. First, a 96-well plate was coated overnight with *C. difficile* toxin A (2.5 µg/ml diluted in PBS, 100 µl/well, 4°C). The plate was then blocked with 1% casein (diluted in PBS, 200 µL/well) for 2 hours at 37°C. Primary antibodies (100 µl/well) were serially diluted in PBS from a starting concentration of 0.5 µg/mL (31 nM), incubated at room temperature for 1 hour, and was followed by 5 washes with PBS-Tween 20 (0.05%). Secondary antibody (rabbit anti-His₆-HRP IgG, Cedarlane Laboratories) diluted 1:2500 in PBS was added at 100 µL per well, incubated for 1 hour at room temperature, and was followed by 5 PBS-T washes. HRP substrate (KPL substrate kit Mandel Scientific, Guelph, ON) was added at 100 µL per

well for 3 min. The reaction was stopped by the addition of 100 μ l of 1M phosphoric acid per well and plates were read at 450 nm wavelength.

3.4. Results and Discussion

3.4.1. Confirmation of ToxA5.1 expression

As shown in Figure 3-1, western blot results confirmed that a hexahistidine tag bearing protein of approximately 17 kDa, which is the anticipated size for the sdAb, was expressed. The identity and binding activity of the purified protein was then confirmed using ELISA (Figure 3-2) for which *C. difficile* toxin A was used as the target. Binding affinity of sdAb purified from crude lysate using MagneHis purification system was tested against purified sdAb (periplasmic extraction, IMAC purification and overnight dialysis) of known binding affinity. Results showed comparable binding activity between the two samples, indicating that neither the high-level expression of the sdAb nor the single-step purification from crude lysate using magnetic beads impaired the binding capability of the sdAb.

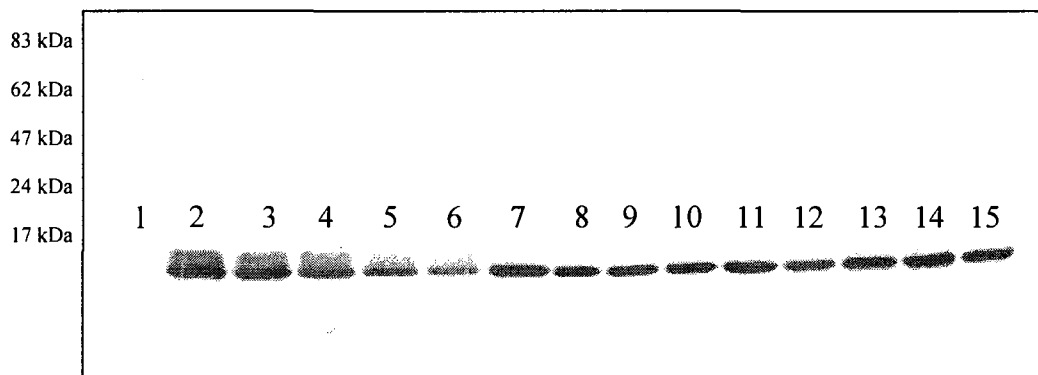


Figure 3-1: Western blot of hexahistidine tag on sdAbToxA5.1 using anti-his mouse IgG as primary antibody and goat anti-mouse IgG conjugated to alkaline phosphatase as secondary antibody. Lanes: 1 marker, 2-6 sdAb ToxA5.1 standards (275, 225, 151, 112, and 75 ng), 7-9 0.0001 mM IPTG, 10-12 0.01 mM IPTG, and 13-15 1 mM IPTG.

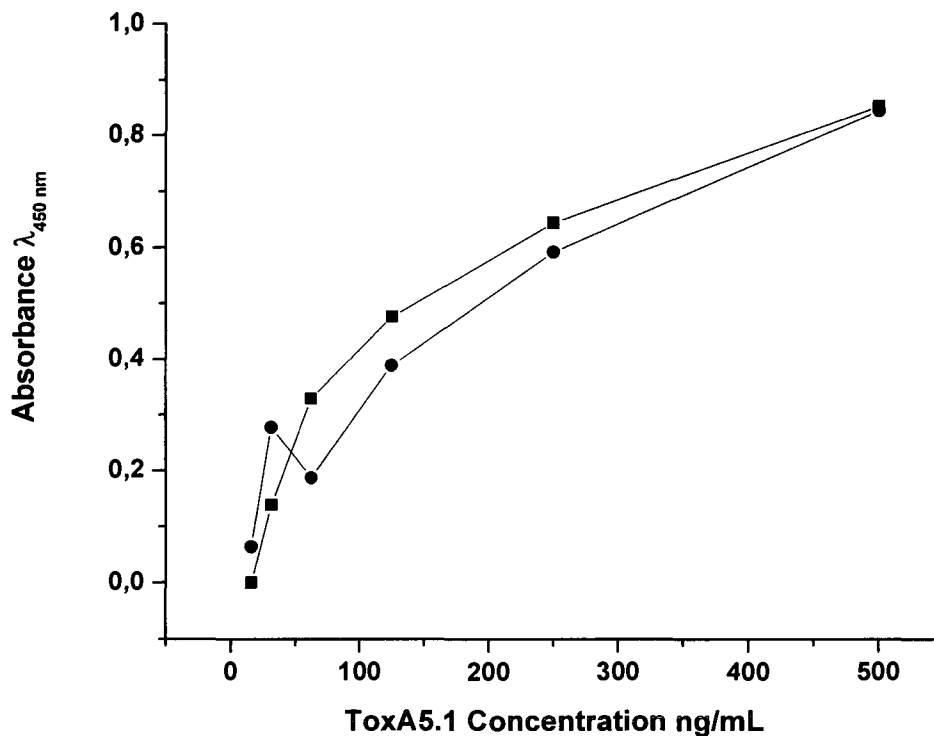


Figure 3-2: Binding of ToxA5.1 to *C. difficile* toxin A revealed by ELISA assay on hexahistidine tag. ToxA5.1 from IMAC purification following periplasmic extract and overnight dialysis in PBS (-■-) and ToxA5.1 from magnetic beads purification applied to crude lysate (-●-).

3.4.2. Effect of inducer concentration on cell growth and ToxA5.1 expression

As shown in Figure 3-3 and Table 3-1, cell growth of *E. coli* TG1 was adversely affected at high concentration (1 mM) of the inducer as shown by the maximum specific growth rate (μ_{\max}). However, inducer concentrations lower than 0.01 mM seemed to have no significant effects on cell growth. As shown in Figure 3-3, the 1 mM culture started to deviate from the other cultures hours after induction. This reduction in biomass production might be due to metabolic load from sdAb production. When inducer is added to the media, the cells shifted from cell growth to protein expression, causing a drop in the growth rate.

This phenomenon was reported to be more significant with complex media (e.g. LB) than with minimal medium (e.g. M9) [50]. As a result, final biomass concentration varied about 20% between no inducer (1.97 g/L DCW) and 1 mM inducer concentration (1.57 g/L DCW) while protein expression varied about 23%, which were 306 mg/L and 377 mg/L respectively.

Table 3-1: Effects of IPTG concentrations on cell growth kinetics and ToxA5.1 expression.

[IPTG]	μ_{\max}	[Final ToxA5.1]	Final ToxA5.1 cell content
mM	h^{-1}	mg/L	mg Protein /g DCW
0	0.808	306 ± 2	155 ± 5
0.0001	0.815	314 ± 6	165 ± 5
0.01	0.806	355 ± 18	197 ± 10
1	0.794	377 ± 11	241 ± 7

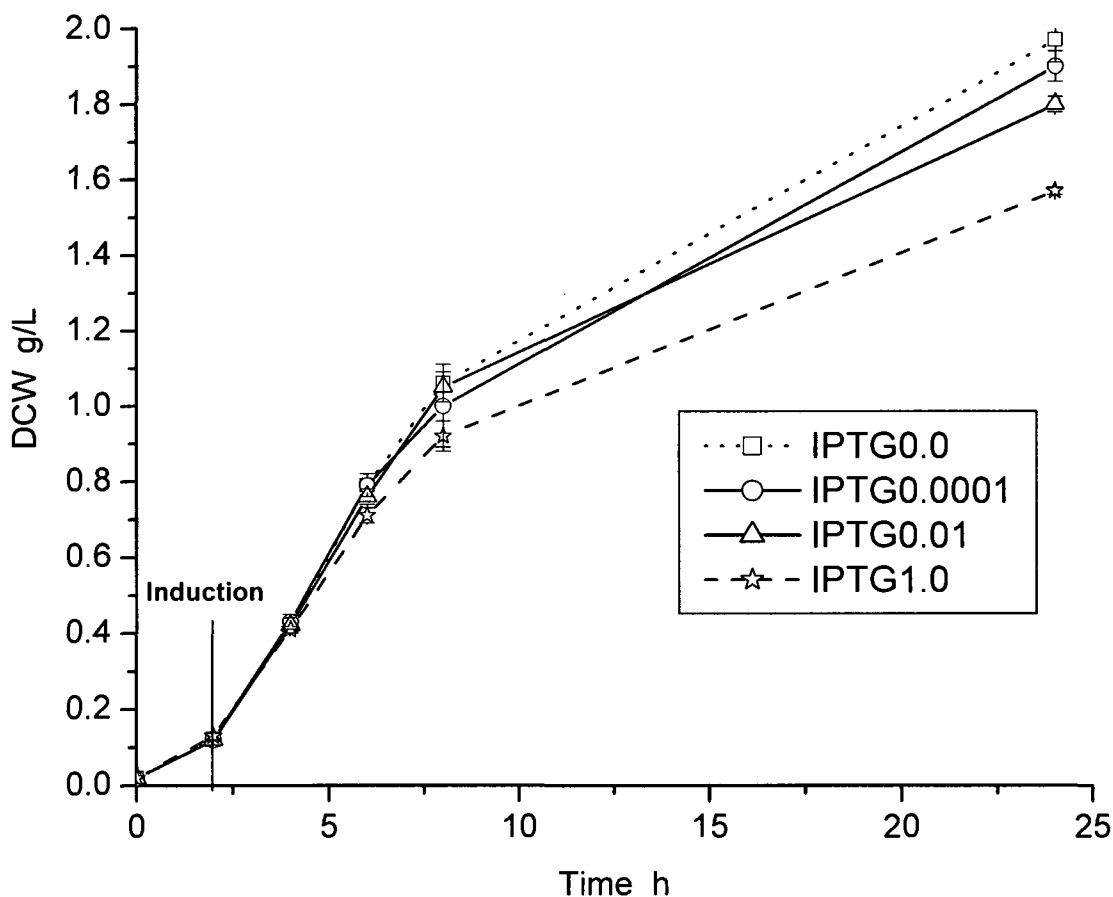


Figure 3-3 Effects of IPTG concentrations on growth of *E. coli* TG1+ToxA5.1 in buffered 2x YT + 0.4% glycerol.

The effects of IPTG concentration on protein expression are shown in Table 3-1. As shown in the table, 306 mg/L of sdAb was produced when no inducer was added, indicating plasmid pSJF2H, which was derived from a high copy number vector, pUC8 [51-53], did not have a tight expression regulation. In other words, the repressor may not fully bind to the operator site on the lac operon, allowing a certain level of transcription of the genes downstream of the promoter [52, 54]. The other factors that might also have contributed to the high-level basal expression are 1) the pSJF2H is a high-copy number plasmid [54]; 2) the use of a complex medium (i.e., the modified 2x YT medium in our

case) may facilitate recombinant protein expression [55], and 3) cells entered stationary phase soon after the induction, where leaky expression was enhanced by nutrient limitation that causes increase of the cAMP pool [55]. This phenomenon might be lessened by using glucose in the media as glucose metabolites are preventing the formation of the CAP-cAMP complex needed for DNA transcription of the lac operon promoter [56, 57] while glycerol has no effect on the promoter repression [58]. When 0.0001 mM IPTG was added in the mid-exponential phase to induce recombinant protein expression, the sdAb production increased slightly to 314 mg/L, corresponding to an increase of 3% in comparison to the non-induced culture. By further increasing IPTG concentration 100-fold to 0.01 mM, the sdAb production increase to 355 mg/L, corresponding to an increase of 13% in comparison to that obtained with 0.0001 mM IPTG. Another 100-fold increase of IPTG to 1 mM yielded a protein concentration of 377 mg/L, corresponding to an increase of 6%. Similar trends of the effects of IPTG concentration on Fab (antigen binding fragment) expression in *E. coli* was reported for IPTG concentrations ranging from 0.02 to 2 mM [59].

It is quite obvious that in the tested range, as shown in Table 3-1, 1 mM IPTG resulted in the highest protein productivity and protein cell content (241 mg/g DCW). However, since IPTG is quite costly and there was only a mild increase (6%) of protein production when 1 M instead of 0.01 mM IPTG was used, the latter was used for all subsequent experiments.

3.4.3. Effect of buffering and carbon source on cell growth and ToxA5.1 expression

The effects of the phosphate buffer and that of two different carbon sources, glucose and glycerol, on cell growth and protein expression are shown in Table 3-2.

Clearly, buffering showed significant effect on the specific growth rates. When glycerol was used as the carbon source, the maximal specific growth rates (in the exponential phase) were 0.806 and 0.857 h⁻¹ with and without buffering, respectively. Similar results were observed with glucose. The specific growth rates were 0.831 h⁻¹ and 0.859 h⁻¹ with and without buffering, respectively. Apparently, the phosphate buffer was at a level inhibitive to cell growth because it resulted in noticeable decrease of the maximum specific growth rate (μ_{\max}) with both glucose and glycerol as the carbon source. The inhibitive effects of high concentration of phosphate buffer to *E. coli* and other bacteria are well documented. One possible reasons of this inhibitive effect is that increased salt concentration or potassium ions might interfere with sugar uptake, which is regulated by membrane proteins [60, 61]. Nevertheless, the maximum biomass concentration obtained with buffered media were 1.22 and 1.88 g/L with glucose and glycerol, respectively, significantly higher than that obtained with unbuffered media, which were 1.11 and 1.42 g/L with glucose and glycerol, respectively. These results show that in the presence of a buffer in the medium, the culture was maintained in the favourable pH range (Table 3-2), allowing longer cell growth period. On the other hand, when the media were not buffered, production of organic acids, primarily acetic acid, quickly brought the culture pH down to the unfavourable range, resulting in shortened cell growth period and less final biomass concentration despite of the relatively high specific growth rate in the exponential phase.

As shown in Table 3-2, glycerol was able to support high cell densities of 1.80 and 1.42 g/L in buffered and unbuffered media, respectively, significantly higher than the corresponding final biomass concentrations obtained with glucose, which were 1.11 and 1.22 g/L, respectively. The lower biomass concentrations obtained with the glucose media

can be attributed to acetate production inhibiting cell growth. It is a well established phenomenon that high concentration of glucose in the media leads to higher cell growth rates under which acetate production is enhanced due to the overflow of glucose in the TCA cycle producing an excess of acetyl CoA resulting in acetate [62-75]. On the other hand, it was reported that glycerol catabolism does not lead to the accumulation of acetic acid in a defined medium [69].

However, it is interesting to observe that the pH value dropped below 5.5 for both glucose and glycerol media in the absence of buffer, indicating that organic acids were indeed produced in the media even when glycerol was used as the carbon source. The pH drop of the glycerol medium (Table 3-2) can be attributed to the use of a complex medium in this study, which may lead to the formation of acetic acid or other organic acids due to the consumption of amino acids and other organic compounds rich in yeast extract and peptone [76].

Table 3-2: Effects of carbon source and pH buffering on cell growth kinetics and ToxA5.1 expression.

	pH	μ_{\max} h ⁻¹	[Final ToxA5.1] mg/L	[Final biomass] g/L	Final ToxA5.1 cell content mg Protein/g DCW
Unbuffered Glucose	4.97	0.859	< 10	1.11 ± 0.03	< 9
Buffered Glucose	7.39	0.831	264 ± 9	1.22 ± 0.01	185 ± 10
Unbuffered Glycerol	5.46	0.857	< 10	1.42 ± 0.07	< 7
Buffered Glycerol	7.26	0.806	355 ± 18	1.80 ± 0.02	196 ± 10

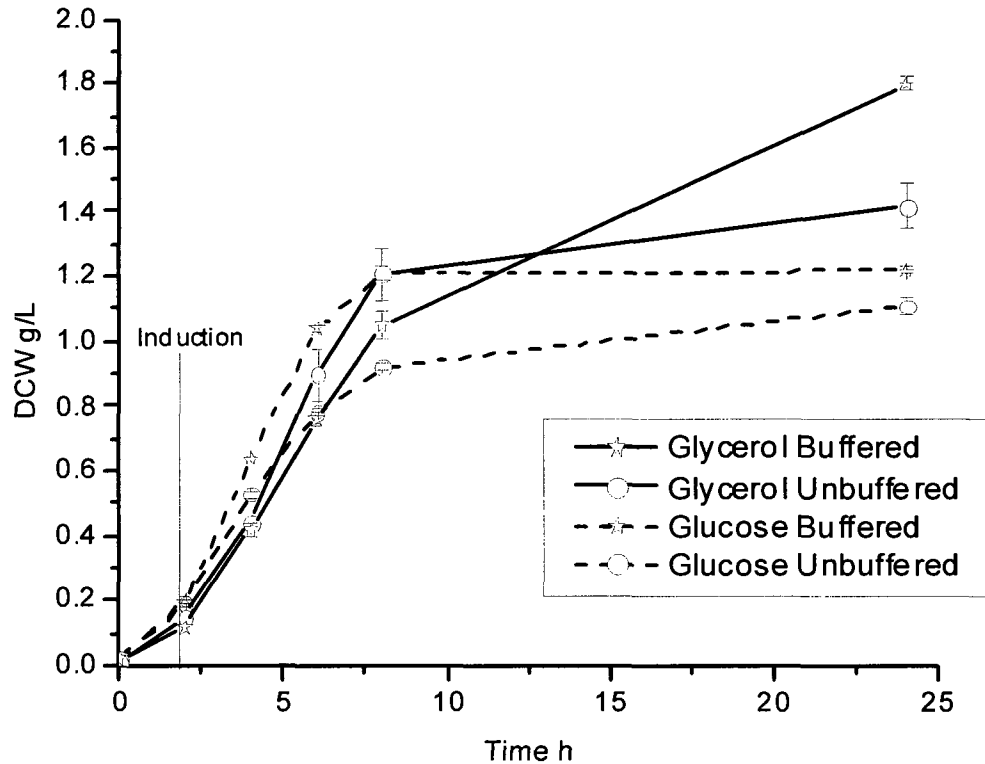


Figure 3-4: Effects of carbon source and medium buffering on cell growth of *E. coli* TG1+ToxA5.1 sdAb 2x YT + carbon source.

As shown in Table 3-2, buffering had a major impact on protein expression, which was lower than the detectable level for both glycerol and glucose when the media were not buffered. As aforementioned, the pH values of both unbuffered glucose and glycerol media were below 5.5. It is hypothesized that the acidification of the medium was the primary reason for the low protein expression level. For the buffered media, protein expression was 264 mg/L and 355 mg/L for glucose and glycerol respectively, indicating that glycerol is a more favourable carbon source for protein expression.

3.4.4. Effect of temperature on cell growth and ToxA5.1 expression

Temperature was also found to be another parameter that had critical effects on the cell growth and protein expression. As shown in Table 3-3, the specific growth rate was

0.52, 0.81, and 0.77 h⁻¹, when the cultivation temperature was 25, 37, and 40°C, respectively. Indicating that 37°C was the optimal temperature among the ones tested for the cell growth of *E. coli* TG1+ToxA5.1 plasmid. It is interesting to notice that the final biomass concentrations were the highest at 25°C (2.08 g/L) and lowest at 40°C (1.3 g/L).

It has been well established that increase of temperature would lead to the increase of rates of two opposite reactions: the reactions that lead to cell growth and the endogenous metabolic reactions that consume cell materials to sustain the viability of cells. When the environmental temperature is lower than the optimal temperature, increase of temperature will result in increased cell growth reaction as opposed to endogenous metabolic reaction, leading to the increase of the overall or net cell growth rate. On the other hand, when the temperature is higher than the optimal temperature, temperature increase will result in increased endogenous metabolism, leading to a decrease in the new specific cell growth rate [77]. Apparently, 40°C was above the optimal temperature for the cell growth of *E. coli*. The unfavourably high temperature resulted in lower biomass yield and therefore lower final biomass concentration than when the temperature was lowered at 37°C or 25°C.

As also shown in Table 3-3, the cultivation temperature also had a significant effect on protein. In fact, although the highest final biomass concentration (2.08 g/L) was obtained at 25°C, no detectable protein was expressed at such a temperature. It was reported that lower temperature might have an effect on the expression of certain genes [78, 79]. For instance, it was demonstrated that the expression of OmpA gene, which is present in the plasmid used in this study, is less efficient at temperature lower than 28°C when compared to a temperature of 37°C [80]. Similar absence of the recombinant protein

K1 capsular antigen expression was reported by Bortolussi and Ferrieri [81] when *E. coli* cells were grown at a temperature lower than 30°C for a short period of time.

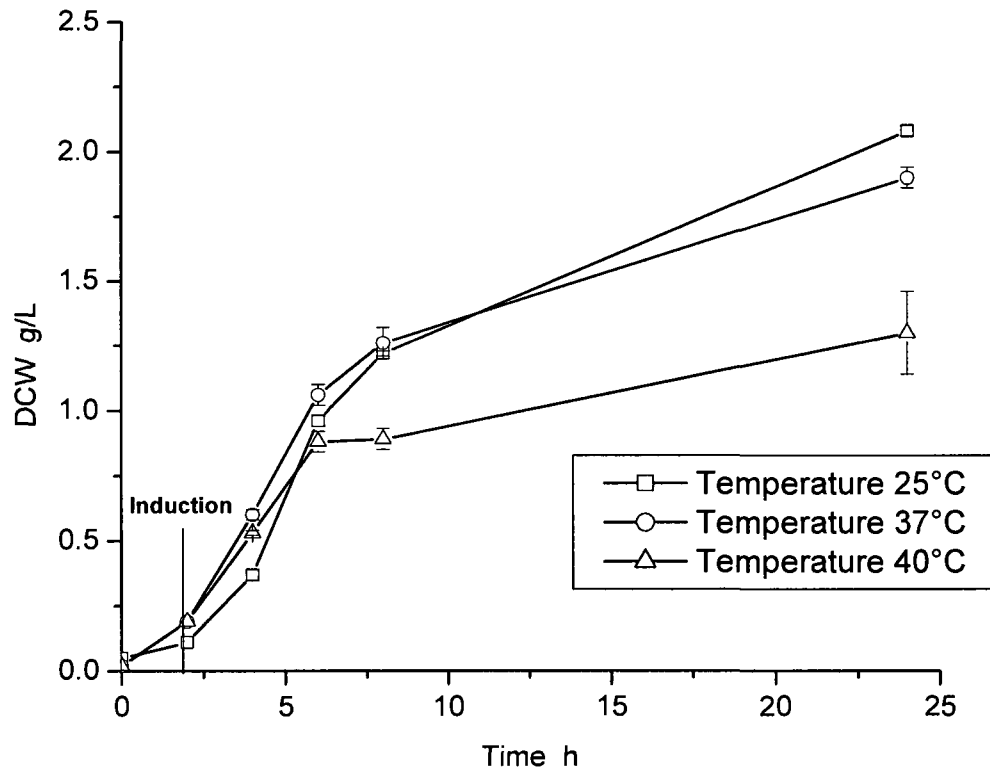


Figure 3-5: Effect of fermentation temperature on cell growth of *E. coli* TG1+ToxA5.1 sdAb in buffered 2X YT+ 0.4% glycerol.

The protein concentrations were 355 mg/L and 295 mg/L at 37°C or 40°C, respectively. It is interesting to note that although the overall protein yield was significantly higher at 37°C than at 40°C, the total protein cell content was lower at 37°C (197 mg sdAb/g DCW) than that at 40°C (227 mg sdAb/g DCW). It is also worth noting that, as shown in Figure 3-6, there exists a band at 19 kDa when the operating temperature was 40°C. It is hypothesized that this band is the ToxA5.1 with an intact pelB leader sequence, which has a molecular weight of approximately 2 kDa. This indicates that the leader

sequence was not properly cleaved, possibly because that the high temperature of 40°C reduced the efficiency of the cleaving enzyme.

Table 3-3: Effects of fermentation temperature on cell growth kinetics and ToxA5.1 expression.

Temperature °C	μ_{\max} h ⁻¹	[Final biomass] g/L	[Final protein] mg/L	Final protein cell content mg Protein/g DCW
25	0.52	2.08 ± 0.02	< 10	< 4.8
37	0.81	1.90 ± 0.04	355 ± 18	197 ± 10
40	0.77	1.30 ± 0.16	295 ± 1	227 ± 27

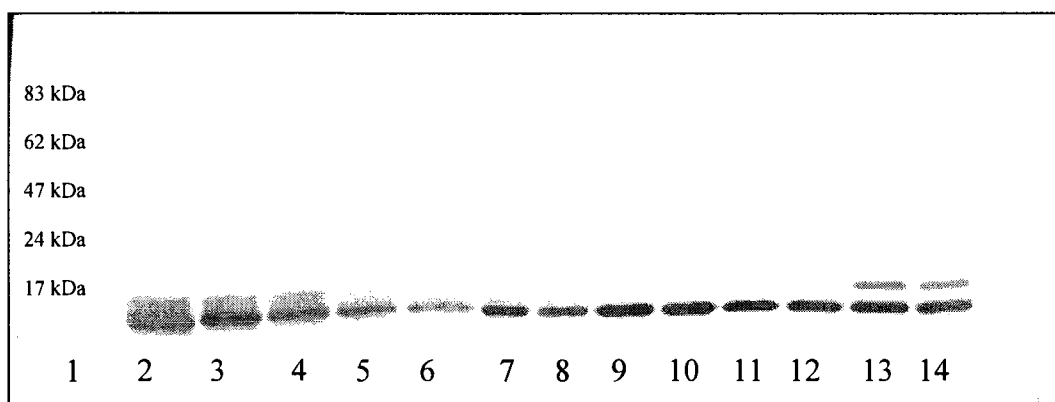


Figure 3-6: Western blot of hexahistidine tag on sdAb ToxA5.1 under different induction temperatures using anti-his mouse IgG as primary antibody and goat anti-mouse IgG conjugated to alkaline phosphatase as secondary antibody. Lanes: 1 marker, 2-6 sdAb ToxA5.1 standards (275, 225, 151, 112, and 75 ng), 7-8 37°C 0.01 mM IPTG glucose, 9-12 37°C 0.01 mM IPTG glycerol, and 13-14 40°C 0.01 mM IPTG glycerol.

3.5 Conclusion

In conclusion, approximately 377 mg/L sdAb ToxA5.1 was expressed using recombinant *E. coli* TG1 hosting pSJF2H plasmid, i.e., using the modified 2x YT medium containing 0.4% glycerol and 100 mM phosphate buffer at 37°C induced with 1 mM of IPTG. It was shown that temperature and pH buffering had significant effects on cell

growth and protein expression. No protein expression was observed at 25°C. On the other hand, the highest protein cell content was observed at 40°C, a temperature substantially higher than the optimal temperature for cell growth and protein expression, 37°C. A second band carrying the hexahistidine tag was observed in the Western blot lane of samples purified from the 40°C culture, indicating the enzymes responsible for the cleavage of the leader sequence might become less efficient at such as the temperature. It was also concluded that the system, i.e., *E. coli* TG1- pSJF2H was not tightly regulated under the tested conditions as a significant amount of basal expression was observed at the absence of induction. Inducer concentration, especially in the high concentration range, was demonstrated to have significant effects on cell growth and protein expression. The use of immobilized metal ions magnetic beads for one-step purification of protein from crude cell lysate was sufficient to recover sdAb ToxA5.1 protein.

3.6 References

1. Holliger, P. and P.J. Hudson, *Engineered antibody fragments and the rise of single domains*. Nature Biotechnology, 2005. **23**(9): p. 1126-1136.
2. Gibbs, W.W., *Nanobodies*. Scientific American, 2005. **293**(2): p. 78-83.
3. McFarland, L.V., et al., *Nosocomial acquisition of Clostridium difficile infection*. New England Journal of Medicine, 1989. **320**(4): p. 204-210.
4. Dick, H.M., *Single domain antibodies*. BMJ (Clinical research ed.), 1990. **300**(6730): p. 659-660.
5. Gussow, D., et al., *Single domain antibodies (dAb's): Binding activities from E. coli by expression of a repertoire of immunoglobulin variable domains*. Fresenius' Journal of Analytical Chemistry, 1990. **337**(1): p. 47.
6. Hamers-Casterman, C., et al., *Naturally occurring antibodies devoid of light chains*. Nature, 1993. **363**(6428): p. 446-448.
7. Harmsen, M.M., et al., *Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins*. Vaccine, 2005. **23**(41): p. 4926-4934.
8. Arbabi-Ghahroudi, M., R. Mackenzie, and J. Tanha, *Selection of Non-aggregating V(H) Binders from Synthetic V (H) Phage-Display Libraries*. Methods Mol Biol, 2009. **525**: p. 1-30.
9. Gasser, B. and D. Mattanovich, *Antibody production with yeasts and filamentous fungi: On the road to large scale?* Biotechnology Letters, 2007. **29**(2): p. 201-212.
10. Saerens, D., et al., *Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing*. Analytical Chemistry, 2005. **77**(23): p. 7547-7555.
11. Muyldermans, S., *Single domain camel antibodies: Current status*. Reviews in Molecular Biotechnology, 2001. **74**(4): p. 277-302.
12. Goldman, E.R., et al., *Facile generation of heat-stable antiviral and antitoxin single domain antibodies from a semisynthetic llama library*. Analytical Chemistry, 2006. **78**(24): p. 8245-8255.
13. Cortez-Retamozo, V., et al., *Efficient Cancer Therapy with a Nanobody-Based Conjugate*. Cancer Research, 2004. **64**(8): p. 2853-2857.
14. Rothbauer, U., et al., *Targeting and tracing antigens in live cells with fluorescent nanobodies*. Nature Methods, 2006. **3**(11): p. 887-889.
15. Presta, L.G., *Engineering antibodies for therapy*. Current Pharmaceutical Biotechnology, 2002. **3**(3): p. 237-256.
16. Olichon, A. and T. Surrey, *Selection of genetically encoded fluorescent single domain antibodies engineered for efficient expression in Escherichia coli*. Journal of Biological Chemistry, 2007. **282**(50): p. 36314-36320.
17. Alvarez-Rueda, N., et al., *Generation of llama single-domain antibodies against methotrexate, a prototypical hapten*. Molecular Immunology, 2007. **44**(7): p. 1691-1701.
18. Stockwin, L.H. and S. Holmes, *Antibodies as therapeutic agents: Vive la renaissance!* Expert Opinion on Biological Therapy, 2003. **3**(7): p. 1133-1152.
19. Cortez-Retamozo, V., et al., *Efficient tumor targeting by single-domain antibody fragments of camels*. International Journal of Cancer, 2002. **98**(3): p. 456-462.

20. Groner, B., C. Hartmann, and W. Wels, *Therapeutic antibodies*. Current Molecular Medicine, 2004. **4**(5): p. 539-547.
21. Vokes, E.E. and E. Chu, *Anti-EGFR therapies: clinical experience in colorectal, lung, and head and neck cancers*. Oncology (Williston Park, N.Y.), 2006. **20**(5 Suppl 2): p. 15-25.
22. Roovers, R.C., et al., *Efficient inhibition of EGFR signalling and of tumour growth by antagonistic anti-EGFR Nanobodies*. Cancer Immunology, Immunotherapy, 2007. **56**(3): p. 303-317.
23. Revets, H., P. De Baetselier, and S. Muyldermans, *Nanobodies as novel agents for cancer therapy*. Expert Opinion on Biological Therapy, 2005. **5**(1): p. 111-124.
24. Rahbarizadeh, F., et al., *High expression and purification of the recombinant camelid anti-MUC1 single domain antibodies in Escherichia coli*. Protein Expression and Purification, 2005. **44**(1): p. 32-38.
25. Omidfar, K., et al., *Production of a novel camel single-domain antibody specific for the type III mutant EGFR* Tumor Biology, 2004. **25**(5-6): p. 296-305.
26. Krüger, C., et al., *Therapeutic effect of llama derived VHH fragments against Streptococcus mutans on the development of dental caries*. Applied Microbiology and Biotechnology, 2006. **72**(4): p. 732-737.
27. Stewart, C.S., C.R. MacKenzie, and J. Christopher Hall, *Isolation, characterization and pentamerization of α -cobrotoxin specific single-domain antibodies from a naïve phage display library: Preliminary findings for antivenom development*. Toxicon, 2007. **49**(5): p. 699-709.
28. Arbabi Ghahroudi, M., et al., *Selection and identification of single domain antibody fragments from camel heavy-chain antibodies*. FEBS Letters, 1997. **414**(3): p. 521-526.
29. Liu, J.L., G.P. Anderson, and E.R. Goldman, *Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library*. BMC Biotechnology, 2007. **7**.
30. Shuntao, W., et al., *A novel designed single domain antibody on 3-D structure of ricin A chain remarkably blocked ricin-induced cytotoxicity*. Molecular Immunology, 2006. **43**(11): p. 1912-1919.
31. Hussack, G., J. Tanha, and C.R. MacKenzie, *Llama single domain antibody binding Clostridium difficile toxin A*. to be submitted, 2009.
32. Bobak, D.A., *The molecular pathogenesis of Clostridium difficile-associated disease*. Current Infectious Disease Reports, 2008. **10**(2): p. 111-115.
33. Kelly, C.P., C. Pothoulakis, and J.T. Lamont, *Clostridium difficile colitis*. New England Journal of Medicine, 1994. **330**(4): p. 257-262.
34. Oldfield Iii, E.C., *Clostridium difficile-associated diarrhea: Risk factors, diagnostic methods, and treatment*. Reviews in Gastroenterological Disorders, 2004. **4**(4): p. 186-195.
35. Gorbach, S.L., *Antibiotics and Clostridium difficile*. New England Journal of Medicine, 1999. **341**(22): p. 1690-1691.
36. Tonna, I. and P.D. Welsby, *Pathogenesis and treatment of Clostridium difficile infection*. Postgraduate Medical Journal, 2005. **81**(956): p. 367-369.

37. Blondeau, J.M., *What have we learned about antimicrobial use and the risks for Clostridium difficile-associated diarrhoea?* Journal of Antimicrobial Chemotherapy, 2009. **63**(2): p. 238-242.
38. Burakoff, R., et al., *Effects of purified Clostridium difficile toxin A on rabbit distal colon.* Gastroenterology, 1995. **109**(2): p. 348-354.
39. Lee, J.Y., et al., *Clostridium difficile toxin A promotes dendritic cell maturation and chemokine CXCL2 expression through p38, IKK, and the NF- κ B signaling pathway.* Journal of Molecular Medicine, 2009. **87**(2): p. 169-180.
40. Voth, D.E. and J.D. Ballard, *Clostridium difficile toxins: Mechanism of action and role in disease.* Clinical Microbiology Reviews, 2005. **18**(2): p. 247-263.
41. Allen, W.E., et al., *Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages.* Journal of Cell Science, 1997. **110**(6): p. 707-720.
42. Florin, I. and M. Thelestam, *Internalization of Clostridium difficile cytotoxin into cultured human lung fibroblasts.* BBA - Molecular Cell Research, 1983. **763**(4): p. 383-392.
43. Henriques, B., I. Florin, and M. Thelestam, *Cellular internalisation of Clostridium difficile toxin A.* Microbial Pathogenesis, 1987. **2**(6): p. 455-463.
44. Mitchell, M.J., B.E. Laughon, and S. Lin, *Biochemical studies on the effect of Clostridium difficile toxin B on actin in vivo and in vitro.* Infection and Immunity, 1987. **55**(7): p. 1610-1615.
45. Burdon, D.W., R.H. George, and G.A.G. Mogg, *Faecal toxin and severity of antibiotic-associated pseudomembranous colitis.* Journal of Clinical Pathology, 1981. **34**(5): p. 548-551.
46. Corthier, G., et al., *Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against Clostridium difficile toxin A.* Infection and Immunity, 1991. **59**(3): p. 1192-1195.
47. Kyne, L., et al., *Association between antibody response to toxin A and protection against recurrent Clostridium difficile diarrhoea.* Lancet, 2001. **357**(9251): p. 189-193.
48. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-685.
49. Abramoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with imageJ.* Biophotonics International, 2004. **11**(7): p. 36-41.
50. Bentley, W.E., et al., *Plasmid-encoded protein: The principal factor in the 'metabolic burden' associated with recombinant bacteria.* Biotechnology and Bioengineering, 1990. **35**(7): p. 668-681.
51. Chambers, S.P., et al., *Plasmid pMTL153: A high copy number version of pAT153 and its use to obtain high expression of the Pseudomonas carboxypeptidase G2 gene.* Applied Microbiology and Biotechnology, 1988. **29**(6): p. 572-578.
52. Nielsen, B.L., V.C. Willis, and C.Y. Lin, *Western blot analysis to illustrate relative control levels of the lac and ara promoters in Escherichia coli.* Biochemistry and Molecular Biology Education, 2007. **35**(2): p. 133-137.
53. Hoedemaeker, F.J., et al., *A single chain Fv fragment of P-glycoprotein-specific monoclonal antibody C219. Design, expression, and crystal structure at 2.4 Å resolution.* Journal of Biological Chemistry, 1997. **272**(47): p. 29784-29789.

54. Anthony, L.C., H. Suzuki, and M. Filutowicz, *Tightly regulated vectors for the cloning and expression of toxic genes*. Journal of Microbiological Methods, 2004. **58**(2): p. 243-250.
55. Grossman, T.H., et al., *Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability*. Gene, 1998. **209**(1-2): p. 95-103.
56. Griffiths, A.J.F., et al., *An Introduction to genetic analysis*. 2000, New York: W.H. Freeman.
57. Reznikoff, W.S., *The lactose operon-controlling elements: A complex paradigm*. Molecular Microbiology, 1992. **6**(17): p. 2419-2422.
58. Lin, E.C., *Glycerol dissimilation and its regulation in bacteria*. Annual Review of Microbiology, 1976. **30**: p. 535-578.
59. Corisdeo, S. and B. Wang, *Functional expression and display of an antibody Fab fragment in Escherichia coli: Study of vector designs and culture conditions*. Protein Expression and Purification, 2004. **34**(2): p. 270-279.
60. Hénin, J., et al., *Diffusion of glycerol through Escherichia coli aquaglyceroporin GlpF*. Biophysical Journal, 2008. **94**(3): p. 832-839.
61. Wang, Y., K. Schulten, and E. Tajkhorshid, *What makes an aquaporin a glycerol channel? A comparative study of AqpZ and GlpF*. Structure, 2005. **13**(8): p. 1107-1118.
62. Chou, C.H., G.N. Bennett, and K.Y. San, *Effect of modified glucose uptake using genetic engineering techniques on high-level recombinant protein production in Escherichia coli dense cultures*. Biotechnology and Bioengineering, 1994. **44**(8): p. 952-960.
63. Chou, C.H., G.N. Bennett, and K.Y. San, *Effect of modulated glucose uptake on high-level recombinant protein production in a dense Escherichia coli culture*. Biotechnology Progress, 1994. **10**(6): p. 644-647.
64. Christensen, M.L. and N.T. Eriksen, *Growth and proton exchange in recombinant Escherichia coli BL21*. Enzyme and Microbial Technology, 2002. **31**(4): p. 566-574.
65. De Mey, M., et al., *Minimizing acetate formation in E. coli fermentations*. Journal of Industrial Microbiology and Biotechnology, 2007. **34**(11): p. 689-700.
66. Eiteman, M.A. and E. Altman, *Overcoming acetate in Escherichia coli recombinant protein fermentations*. Trends in Biotechnology, 2006. **24**(11): p. 530-536.
67. Han, K., H.C. Lim, and J. Hong, *Acetic acid formation in Escherichia coli fermentation*. Biotechnology and Bioengineering, 1992. **39**(6): p. 663-671.
68. Hansen, R. and N.T. Eriksen, *Activity of recombinant GST in Escherichia coli grown on glucose and glycerol* Process Biochemistry, 2007. **42**(8): p. 1259-1263.
69. Holms, H., *Flux analysis and control of the central metabolic pathways in Escherichia coli*. FEMS Microbiology Reviews, 1996. **19**(2): p. 85-116.
70. Luli, G.W. and W.R. Strohl, *Comparison of growth, acetate production, and acetate inhibition of Escherichia coli strains in batch and fed-batch fermentations*. Applied and Environmental Microbiology, 1990. **56**(4): p. 1004-1011.
71. Nakano, K., et al., *Influence of acetic acid on the growth of Escherichia coli K12 during high-cell-density cultivation in a dialysis reactor*. Applied Microbiology and Biotechnology, 1997. **48**(5): p. 597-601.

72. Yildir, C., Z.I. Önsan, and B. Kirdar, *Optimization of starting time and period of induction and inducer concentration in the production of the restriction enzyme EcoRI from recombinant Escherichia coli 294*. Turkish Journal of Chemistry, 1998. **22**(3): p. 221-226.
73. Sandén, A.M., et al., *Limiting factors in Escherichia coli fed-batch production of recombinant proteins*. Biotechnology and Bioengineering, 2003. **81**(2): p. 158-166.
74. Xu, B., et al., *Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with Escherichia coli*. Applied Microbiology and Biotechnology, 1999. **51**(5): p. 564-571.
75. Xu, B., M. Jahic, and S.O. Enfors, *Modeling of overflow metabolism in batch and fed-batch cultures of Escherichia coli*. Biotechnology Progress, 1999. **15**(1): p. 81-90.
76. Teich, A., et al., *Amplification of ColE1 related plasmids in recombinant cultures of Escherichia coli after IPTG induction*. Journal of Biotechnology, 1998. **64**(2-3): p. 197-210.
77. Herendeen, S.L., R.A. VanBogelen, and F.C. Neidhardt, *Levels of major proteins of Escherichia coli during growth at different temperatures*. Journal of Bacteriology, 1979. **139**(1): p. 185-194.
78. Robbe-Saule, V., et al., *Effect of growth temperature on Crl-dependent regulation of σ activity in Salmonella enterica serovar Typhimurium*. Journal of Bacteriology, 2008. **190**(13): p. 4453-4459.
79. Kovářová, K., A.J.B. Zehnder, and T. Egli, *Temperature-dependent growth kinetics of Escherichia coli ML 30 in glucose-limited continuous culture*. Journal of Bacteriology, 1996. **178**(15): p. 4530-4539.
80. Afonyushkin, T., et al., *Temperature-dependent stability and translation of Escherichia coli ompA mRNA*. Biochemical and Biophysical Research Communications, 2003. **311**(3): p. 604-609.
81. Bortolussi, R., P. Ferrieri, and P.G. Quie, *Influence of growth temperature of Escherichia coli on K1 capsular antigen production and resistance to opsonization*. Infection and Immunity, 1983. **39**(3): p. 1136-1141.

Chapter 4: Large-scale expression of llama single domain antibody targeting *Clostridium difficile* toxin A in *Escherichia coli*

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4.1 Abstract

The drug resistant strains of *Clostridium difficile* are a major health concern in hospital settings. The diseases associated with these bacteria are toxin-mediated which offers an option for treating the disease. Toxin inactivation via antibodies therapy can drastically reduce the morbidity associated with the disease and in this study, we have investigated the expression of a llama single domain antibody targeting *C. difficile* toxin A. The ToxA5.1 sdAb was expressed in 3L bioreactors under different carbon sources and concentrations. Lower initial glucose concentrations yielded a higher expression rate with a total of 503 mg/L of sdAb expressed using 2 g/L of glucose. Expression of sdAb under glycerol was not concentration dependent as higher (30 g/L) and lower concentrations (5 g/L) yielded similar expression. The higher concentration of glycerol had an impact on periplasmic leakage as 41% of the total sdAb were found in the broth. Using magnetic beads for the purification from crude cell lysate did not impair the binding ability of the sdAb ToxA5.1 to toxin A as shown by Biacore results.

4.2 Introduction

Nosocomial infections are a great source of health concerns especially the drug resistant strains of *Clostridium difficile* that are responsible for causing severe diarrhea in patients undergoing antibiotic therapy. Toxins excreted by *C. difficile*, enterotoxin A and cytotoxin B, cause pseudomembranous colitis by disrupting actin skeleton and cell junctions as a result of binding to colon epithelial cell receptors [1, 2]. As *C. difficile*-associated diseases (CDAD) are toxin-mediated, inactivating the toxins would result in a reduction of severity and duration of the symptoms as well as the mortality rate as shown in murin model [3] and hamster model [4]. Several non-antibody toxin-binding molecules were investigated (rifaximin, nitazoxanide, difimicin and ramoplanin) in the hope of inactivating the toxin and three were tested clinically (Cholestyramine, Synsorb 90, and Tolevamer). Unfortunately, none of them can perform the task without side effects [5].

Immunoglobulin-directed therapy was also investigated and several reported the protection effects [3, 4, 6-11]. By binding to the toxin, the antibody can prevent the toxin from binding to its intended receptor on the epithelial cell. Since it has been reported that TcdA can be neutralized by monoclonal antibodies, the use of a novel type of antibodies, single domain antibodies that are antibodies naturally found in camelidae and cartilaginous fish [12], could also be used as toxin inactivator as reported in [13, 14]. Their simple structure enables the production in prokaryotic systems since no extensive folding or glycosylation is required to ensure functionality. Prokaryotic cells, mainly *E. coli*, have several advantages when it comes to protein expression [15]. The first one is the short period of time between the moment a sequence of a desired protein becomes known and the final expression optimization of this protein in *E. coli* [16-18]. Furthermore,

fermentation can be accomplished at low cost in readily available installations. As a result, capital costs are significantly reduced. Operation costs are also significantly lower since the fermentation techniques are fairly simple and the media is inexpensive. In this study, we have investigated the effects of different carbon sources at different concentration on the expression of the ToxA5.1 sdAb against TcdA in batch fermentation cultures.

4.3 Material and Methods

4.3.1 Bacterial strain and plasmid

DNA encoding ToxA5.1, a llama single-domain antibody with specificity for *C. difficile* toxin A [19], was cloned into the expression vector pSJF2H [20] via BbsI and BamHI (New England Biolabs, Mississauga, ON) restriction sites. Protein expression was performed in TG1 *E. coli* cells purchased from Invitrogen (Carlsbad, CA). Recombinant strain were grown and screened for the best sdAb producer. The best expressing recombinant strain was stored as stock in 15% glycerol at -80°C . Patches were grown on LB plate containing 100 $\mu\text{g}/\text{mL}$ of ampicillin at 37°C for 6 h and inoculum was prepared in inoculum media containing 16 g/L Enzymatic tryptone, 20 g/L Yeast extract, 5 g/L NaCl, and carbon source and grown overnight at 37°C and 200 rpm in shaker.

4.3.2 Fermentation in bioreactors

New-Brunswick Scientific (Edison, NJ) BioFlo 110 3 L bioreactors with a working volume of 1.5 L were used. Temperature (37°C) and pH (7.0) measured with a Mettler Toledo probe (Columbus, OH) were kept constant, dissolved oxygen (DO) measured with an Mettler Toledo (Columbus, OH) polarographic DO probe was set at 20% saturation.

Airflow was kept constant at 1 LPM and agitation was varied by the controller from 300 to 900 rpm in order to keep DO values above 20%.

The bioreactors were inoculated using an overnight culture grown in the same medium as used for bioreactor fermentations with a volume sufficient to achieve an initial OD₆₀₀ of approximately 0.1. OD₆₀₀ was monitored and induction with 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was performed at OD₆₀₀ of approximately 0.6 which was confirmed to correspond to mid-exponential phase.

4.3.3 Sampling

1 mL samples were taken every 2 h over a 24 h induction period and were centrifuged on benchtop microcentrifuge for 2 min at 12 000 rpm. Supernatant (clarified broth) was separated from the pellet and kept for analysis. All samples were stored at -80°C prior to analysis.

4.3.4 Protein quantification and purification

Cell lysis buffer (300 μL/pellet) was prepared with 1X Promega Corporation (Madison, WI) Fast Break lysis reagent, 1 mM phenylmethylsulphonyl fluoride (PMSF) (Bio Basic Inc., Markam, ON) and 3 U DNase 1 (Promega Corp.) and cell lysis was carried out on a rocking platform at room temperature for 30 min.

Soluble fraction of crude cell lysates were loaded on 4-15% discontinuous SDS-PAGE gels [21]. Standards of previously purified and quantified sdAb (225, 151, 112, and 75 ng) were loaded on every gel to provide quantitative estimation. Proteins were fixed using 10% acetic acid and 25% isopropanol for 15 min and stained for 60 min using Fermentas PageBlue Protein Staining Solution (Glen Burnie, MD) followed by overnight

destaining in distilled water. Quantification of scanned gels was achieved via densitometry using ImageJ software [22]. The amount of protein reported is the sum of the proteins in the fermentation broth (culture supernatant after initial centrifugation step) and proteins in the cell pellets. Purification of the sdAb was carried out using Promega MagneHIS Protein Purification System (Promega Corp.) according to manufacturer's instructions.

4.3.5 Binding kinetics

ToxA5.1 produced in bioreactors supplemented with either glucose or glycerol was analyzed by surface plasmon resonance to determine if the sdAbs retained their binding affinity for *C. difficile* toxin A. Briefly, ToxA5.1 from both bioreactor cultures was purified using MagneHIS Protein Purification System magnetic beads from crude cell lysate according to the manufacturer's instruction and subjected to size exclusion chromatography on a Superdex 75 gel filtration column as described in [23]. Eluted fractions were collected and used for surface plasmon resonance analysis on a Biacore 3000 instrument (GE Healthcare, Montreal, QC). *C. difficile* toxin A was immobilized on CM5 dextran chips and binding data was collected and analyzed as previously reported by Hussack *et al.* [19].

4.3.6 Glucose, glycerol and acetate

The concentrations of glucose, glycerol and acetate were determined using an Agilent 1200 unit (Agilent Technologies, Foster City, CA) with a Shodex SH-1011 column (Showa Denko K.K., Kawasaki, Japan) and a Shodex SG-1011 guard column. Samples of 50 μ L were loaded and the mobile phase (H_2SO_4 5 mM) was run at 0.6 mL/min. Glucose and glycerol were quantified using RI detector while acetate was quantified using UV detector ($\lambda=210$ nm).

4.4 Results and Discussion

4.4.1 Effect of glucose concentrations on cell growth

The effect of glucose concentration on cell growth can be seen in Figure 4-1A.

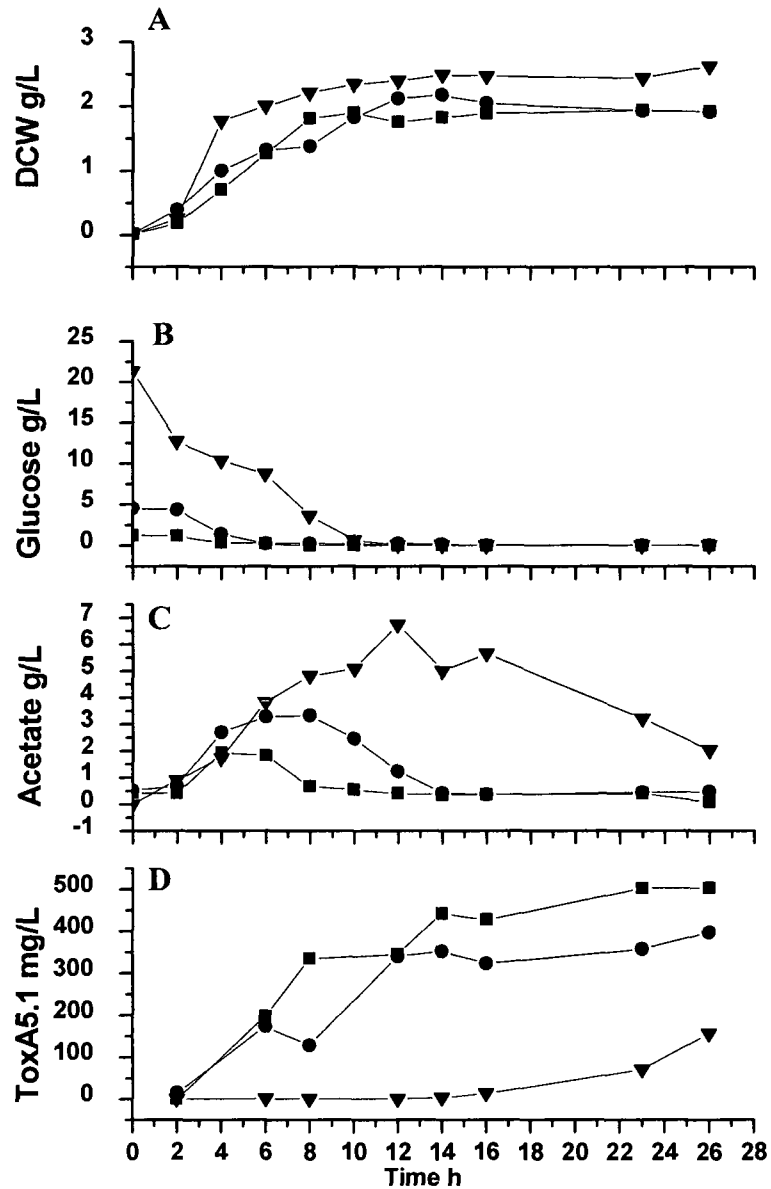


Figure 4-1: Effects of 2 g/L (—■—), 5 g/L (—●—), and 20 g/L (—▼—) initial glucose concentrations on Biomass production on a dry weight basis, Glucose consumption, Acetate production and sdAb ToxA5.1 expression.

After induction which took place 2h into the fermentation, the maximum specific growth rates (μ_{\max}) for the cultures are correlated to the glucose concentration in the media. Increasing initial glucose concentration corresponding to increasing specific growth rate as reported in [24]. As can be seen in Table 4-1 specific growth rates were 0.875, 1.035 and 1.165 h⁻¹ when the initial glucose concentrations were of 2, 5 and 20 g/L, respectively.

Table 4-1: Kinetic parameters calculated from the average of the 8 to 16 h points for glucose as carbon source.

		Glucose g/L		
		2	5	20
S ₀	(g/L)	1.22	4.55	21.31
P	(mg/L)	375	327	3
X _M	(g/L)	1.83	2.04	2.38
Y _{X/S}		1.50	0.45	0.11
Y _{P/S}	(mg/g)	307	72	0
Y _{P/X}	(mg/g)	204	160	1
Acetate	(g/L)	1.93	3.33	6.75
μ_{\max}	(h ⁻¹)	0.875	1.035	1.165

It appears that when no or small quantities of proteins are expressed, the growth rates are higher which supports the metabolic load theory stating that when recombinant proteins are expressed, less resources are allocated to cell growth [25]. Furthermore, the final biomass concentration also increases as 1.83, 2.04 and 2.38 g/L of biomass (dry cell weight) were obtained for the 2, 5 and 20 g/L glucose cultures, respectively. The biomass yield coefficients (Y_{X/S}) were 1.50, 0.45, and 0.11 g DCW/g of glucose present initially,

respectively (Table 4-1). Carbon source consumption can be seen in Figure 4-1B. It is to be noted that glucose gets depleted from the media after 6 h for initial concentrations of 2 and 5 g/L. This implies that the rate at which glucose is consumed is higher with higher concentration. In the 20 g/L culture, glucose was exhausted after 12 h of fermentation. Since the glucose consumed does not yield higher concentration of ToxA5.1 sdAb nor significantly higher biomass, the excess glucose consumed is either utilized for acetate production or wasted [26]. It has been reported that *E. coli* metabolism favours the maximization of ATP production [27] and this is why when excess glucose is present in the medium, acetate formation is favoured as a secondary source of energy as it produces the second largest pool of ATP and NADH₂ [26]. Acetate formation is directly related to growth rate, which is in turn related to substrate consumption [28]. This would explain why more acetate is produced as the initial glucose concentration increased. It has been reported that the use of complex media (e.g. yeast extract or tryptone) results in acetate formation at lower growth rate (dilution rate of 0.2 h⁻¹) than a defined media (dilution rate of 0.35 h⁻¹) [29]. The lowest glucose concentration we have tested was higher than the 0.75 g/L value reported by Marisson and von Stockar [24] and complex medium was used such that acetate production was expected. It was reported that acetate showed inhibitive effects on cell growth and protein expression to concentration as low as 0.5 g/L [30] and starts to be detrimental at concentration above 10 g/L [31, 32].

Furthermore, acetate production diverts energy which could have been used for biomass or protein production [33]. Once glucose has been depleted from the medium, acetate that was formed due to glucose utilization gets consumed as a secondary carbon source as shown in Figure 4-1C and this phenomenon was previously widely observed [34,

35]. The acetate concentration does not seem to have a strong inhibition effect on cell growth as the 20 g/L culture yielded the maximum biomass of all the concentrations tested. Acetate concentration reached a maximum value of 6.75 g/L while the maximum acetate concentration for the 2 and 5 g/L glucose cultures were 1.83 and 3.33 g/L respectively. Those values being below the threshold of 10 g/L, strong growth inhibition is not apparent.

4.4.2 Effect of glucose concentration on ToxA5.1 expression

It was clear from Figure 4-1D that a higher concentration of initial glucose in the medium did not yield higher concentration of expressed ToxA5.1 sdAb. The highest expression was achieved on the lowest initial glucose concentration of 2 g/L for a final ToxA5.1 concentration (extracellular and intracellular) of 503 mg/L. As can be seen on Figure 4-1D, 20 g/L initial glucose concentration seems to inhibit sdAb expression as no detectable amount of sdAb can be seen until the fourteenth hour when glucose was depleted from the medium and acetate concentration started decreasing. The 5 g/L culture follows the expression rate of the 2 g/L culture up until the twelfth hour after which the 5 g/L culture remained at an average value of 327 mg/L compared to 375 mg/L for the 2 g/L culture (Table 4-1). From this table it can be seen that the yield coefficient $Y_{P/X}$ are of 204, 160 and 1 mg sdAb/g DCW for the 2, 5 and 20 g/L cultures, respectively.

In Figure 4-1D, the final concentrations of expressed ToxA5.1 sdAb for the 5 and 20 g/L initial glucose cultures were of 397 and 156 mg/L respectively. The fraction of intra and extracellular sdAb can be seen in Table 4-2. The absence of protein in the 20 g/L culture could be the result of one or more of the following reasons: 1) catabolite repression of the lac operon [36] 2) specific growth rate [37-40], and 3) acetate inhibition [26, 28, 38, 40-48]. Catabolite repression of the lac operon occurs when high concentration of glucose

catabolites prevent the formation of the CAP-cAMP (catabolite activation protein and cyclic adenosine monophosphate) [49, 50] which the RNA polymerase requires for binding to the lac operon site. Without this binding, no mRNAs are produced thus no protein synthesis under the lac promoter is possible. If glucose concentration was the sole responsible for inhibition, there would be protein expression after depletion of glucose to levels similar to the 5 g/L culture.

Table 4-2: Intracellular and extracellular ToxA5.1 fractions and intracellular concentration expressed as mg ToxA5.1/L of fermentation broth.

	Intracellular Fraction %	Extracellular	Intracellular ToxA5.1 mg/g DCW
Glycerol			
5 g/L	89	11	75
30 g/L	59	41	48
Glucose			
2 g/L	89	11	234
5 g/L	93	7	193
20 g/L	87	13	51

Another reason for the low expression of sdAb could be the higher growth rate sustained under the different initial glucose concentrations as seen in Table 4-1. This relates to the concept of critical growth rate, which is the growth rate minimizing acetate formation, which might explain the protein inhibition when specific growth rates were higher as reported by several [37-40]. The critical growth rate theory has been investigated and growth rate below 0.45 h^{-1} proved to increase the expression of a human interferon alpha 1 in *E. coli* [51]. Growth rates below 0.7 h^{-1} proved to increase the expression of a

human growth hormone while growth rate above 0.8 h^{-1} reduced the specific expression of the same recombinant protein by a factor of 4 [52]. Furthermore, acetate has an inhibitory effect on protein expression as it specifically inhibits oxygen and glucose uptake, reducing expression of recombinant proteins [35, 53]. It is also suspected of reducing the proton motive pump by decreasing the pH gradient across the cytoplasmic membrane in the cells [54, 55]. Cherrington *et al.* reported that short-chain acids could impair RNA, DNA and other macromolecules synthesis [56]. They hypothesized that not only the protons would be responsible for this inhibition but, upon diffusion in the cell, organic acids would dissociate and the anions could also impair on the synthesis of these macromolecules. This argument supports the results observed in this investigation since the 2 g/L initial glucose concentration culture has the highest yield coefficient of protein expressed per gram of carbon used ($Y_{P/S}$) and the highest yield of protein per gram of biomass produced ($Y_{P/X}$) with 307 mg sdAb/g glucose and 204 mg sdAb/g biomass while the lowest yield coefficients, both $Y_{P/S}$ and $Y_{P/X}$ were observed in the 20 g/L culture.

4.4.3 Effect of inoculum age

As shown in Figure 4-2A, the 2-days old inoculum culture at 2 g/L glucose yielded less total sdAb than the 1-day old inoculum culture (average $272 \pm 18 \text{ mg/L}$ vs 503 mg/L respectively). It is a known fact that inoculum age plays a major role in fermentation and it is reported that even as short difference of 12 h in the inoculum age can have a serious impact on the protein expression [57, 58]. The sdAb expression was similar for the 2-days old and 1-day old inoculum until 4 h after induction at which time the 1-day old inoculum yielded more sdAb (Figure 4-2A). For the 2-days old inoculum, sdAb expression diminished around the 8th hour. The lower protein expression of the 2-days old inoculum

culture correlates with a biomass production that was higher than for the 1-day old inoculum. It is also to be noted that the consumption of glucose (Figure 4-2B) was faster as, after 4 h of fermentation, glucose was depleted from the medium while for the 1-day old inoculum depletion occurred at 8 h into the fermentation.

Despite the fact that glucose consumption was faster in the 2-days old inoculum, the rate of acetate formation (Figure 4-2C) seems to have remained similar with maximum of 1.70 ± 0.05 g/L for the 2-days old inoculum and 1.93 g/L for the 1-day old inoculum but the rate of acetate consumption was faster again for the 2-days old inoculum. The fact that less sdAb were produced while the biomass concentration was higher (Figure 4-2D) tends to suggest that less bacteria were harbouring the plasmid [25]. This would also explain the rapid increase in biomass and higher final biomass concentration for the 2-days old inoculum (2.11 ± 0.01 g/L) compared to the 1-day old inoculum (1.91 g/L). If bacteria are not bearing the plasmid, the growth rate is not impaired by the metabolic load resulting from induction as discussed previously.

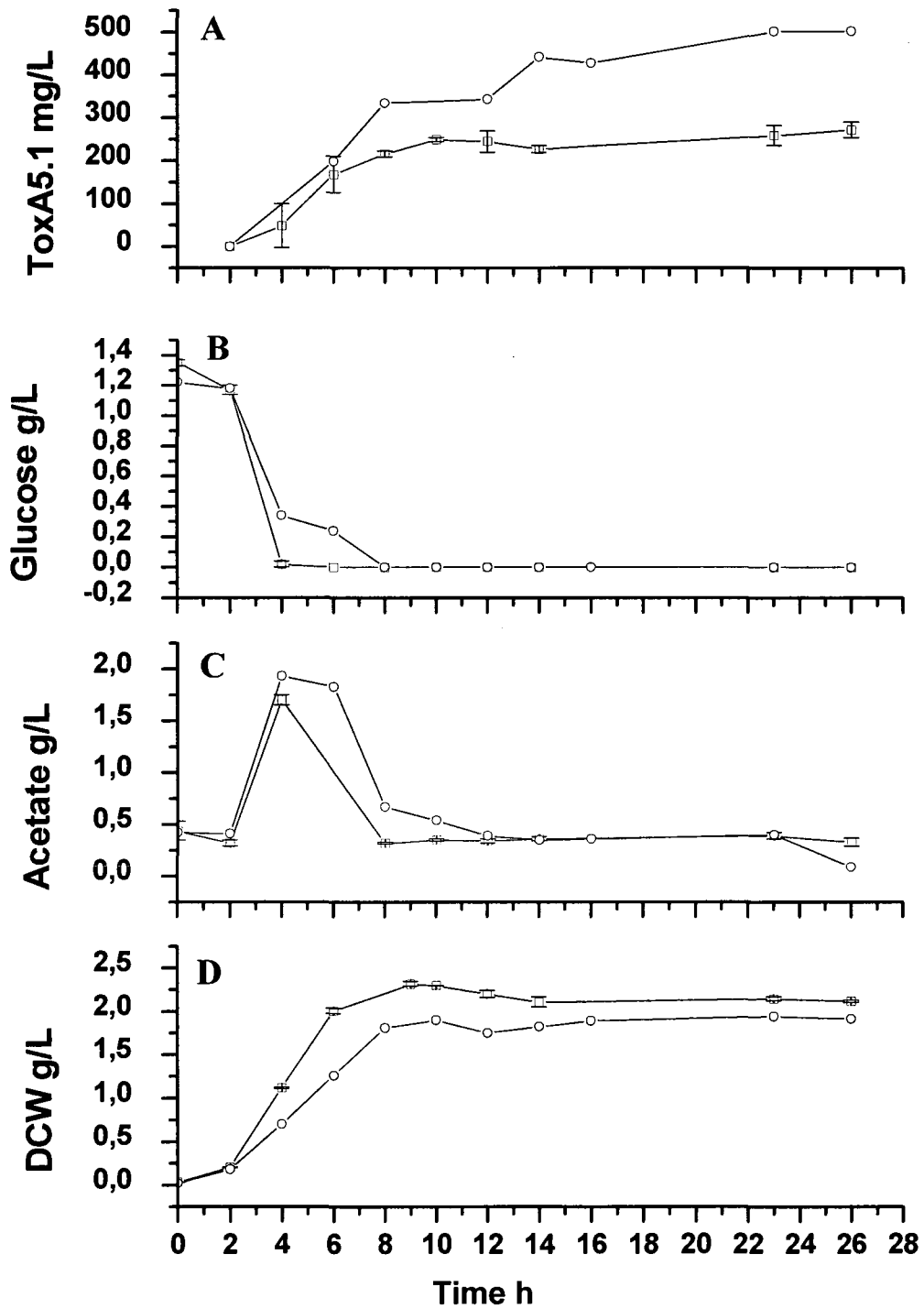


Figure 4-2: Comparing effects of 1-day old inoculum (—○—) and 2-days old inoculum (—□—) on Biomass production on a dry weight basis, Glucose consumption, Acetate production and sdAb ToxA5.1 expression.

4.4.4 Growth and ToxA5.1 expression on glycerol

Growth and sdAb expression using different concentrations of glycerol was also investigated. The highest and lowest concentrations of glycerol tested yielded similar results in terms of sdAb expression with a final total expression of 184 mg/L and 186 mg/L for initial substrate concentration of 5 and 30 g/L glycerol, respectively. This suggests that glycerol did not have a significant impact on ToxA5.1 expression and that even if residual concentrations of glycerol are present, the final concentration of ToxA5.1 is the same. This also suggests that glycerol is not a limiting factor in the expression of the ToxA5.1 sdAb. Expression patterns for both concentrations are similar as can be seen in Figure 4-3A where at 8 h into the fermentation, a peak of sdAb expression was achieved (243 and 219 mg/L for 5 and 30 g/L glycerol respectively). It is not understood why the concentration of ToxA5.1 tends to decrease with time after the peak concentration. It is hypothesized that cellular lysis (data not shown) could be responsible for the degradation of the sdAb. Cell lysis would cause the release of proteases from the cytoplasm which are absent from the periplasmic space. Since the concentration of proteases increases in the fermentation broth, degradation of the ToxA5.1 is thought to occur. The final DCW concentration was 2.17 and 2.19 g/L for the 5 and 30 g/L cultures respectively (Figure 4-3B). Glycerol depletion occurred after 8 h of fermentation for the 5 g/L culture while the final glycerol concentration for the 30 g/L culture was 14 g/L (Figure 4-3C). Again, glycerol does not seem to be a limiting factor for the growth of the cell as residual glycerol did not yield larger biomass concentrations. Acetate formation reached a maximum concentration of 2.7 g/L and 3.78 g/L respectively for the 5 and 30 g/L cultures (Figure 4-3D). Acetate

formation is not said to be dependant on glycerol metabolism [59] but is rather a byproduct from the degradation of another carbon source found in the complex medium used [29].

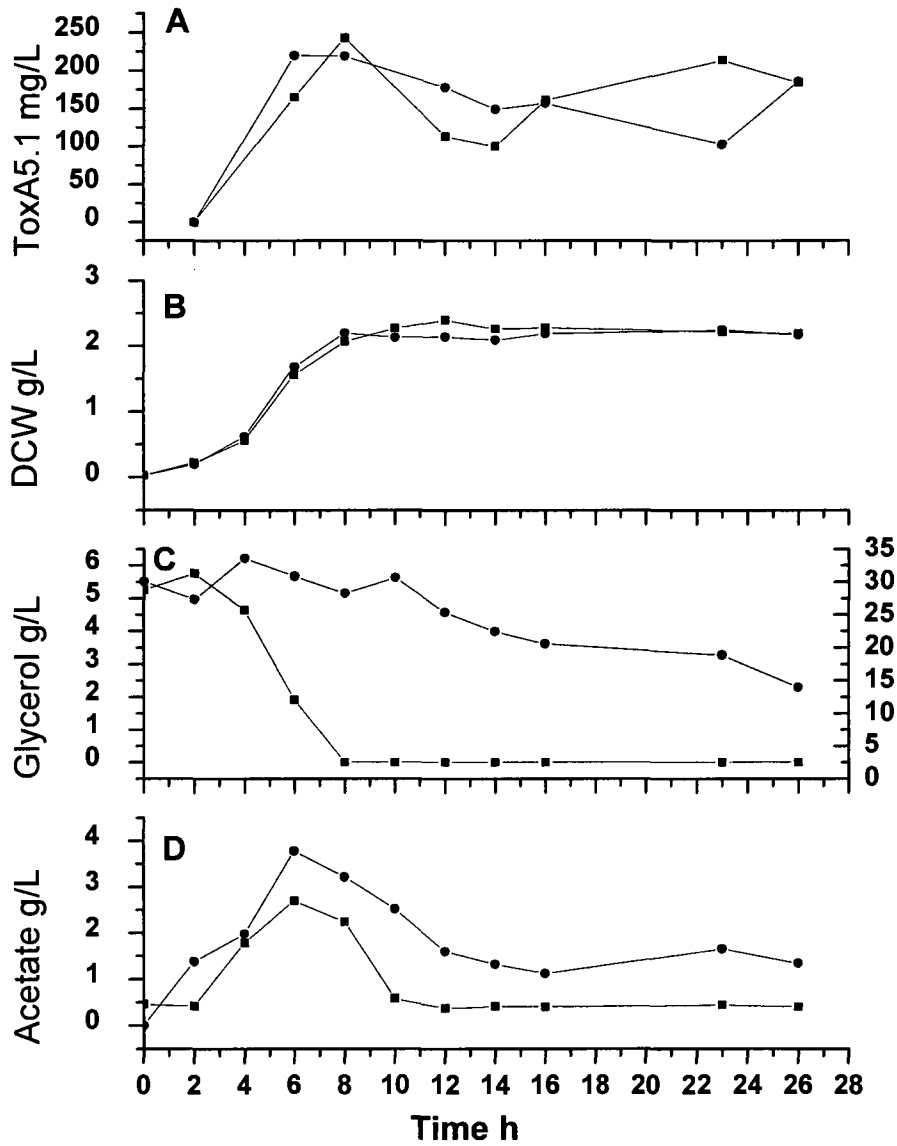


Figure 4-3: Effects of 5 g/L (—■—) and 30 g/L (—●—) initial glycerol concentrations on Biomass production on a dry weight basis, Glycerol consumption, Acetate production and sdAb ToxA5.1 expression.

4.4.5 Periplasmic leakage

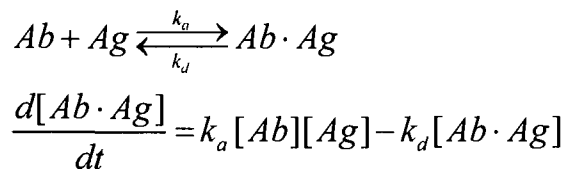
The expressed sdAb ToxA5.1 is exported to the periplasmic space of the bacteria where the reducing environment and the presence of disulfide-binding enzymes enables proper protein folding [60] which might not otherwise occur in the cytoplasm. In order to migrate from the cytoplasm to the periplasm, an export peptide, *pelB* sequence, is fused to the protein via the expression vector. During the transport to the periplasm, this sequence gets cleaved by a signal peptidase yielding a mature protein in the periplasm space [61].

Extracellular sdAb were observed when both sources of carbon were used (Table 4-2) and accounted for approximately 10% of the total sdAb expressed with the exception of the 30 g/L glycerol culture when it accounted for 41% of the total sdAb expressed. Pressure on the outer membrane caused by the accumulation of sdAb cannot explain this phenomenon as for the 2 g/L glucose culture, 89% of the sdAb expressed (more than 440 mg/L) was recovered from the periplasmic space without causing membrane rupture while a concentration of 100 mg/L was recovered from periplasmic space of the 30 g/L glycerol culture. It has been reported that the addition of glycine or a surfactant like Triton-X resulted in a higher periplasmic leakage from *E. coli* cells producing F_v fragments against TNF-alpha [62]. It was hypothesized that this leakage was a result of membrane destabilization [63, 64]. It is also hypothesised that the high concentration of glycerol in the fermentation medium might have affected the structural integrity of the outer membrane causing cell lysis as there were less total proteins in the cultures pellets (data not shown). Effect of the fermentation period might also lead to increased membrane permeability as stated by Shibui and Nagahari who observed an increase of Fab fragment in the medium after 10 h of fermentation, which they attributed to random diffusion through the outer

membrane or to another mechanism [65]. Another explanation for this high concentration of sdAb in the broth might be that when high concentration of glycerol are present in the environment, risk of metabolic flooding can occur, resulting in the production of an antibiotic component, methylglyoxal, from a derivative of glycerol (dihydroxyacetone phosphate) [66, 67] which would explain the cell lysis observed in the 30 g/L glycerol culture (data not shown).

4.4.6 Binding kinetics

Samples of purified sdAb expressed under both carbon sources were subjected to Biacore analysis in order to confirm the binding ability of the ToxA5.1 sdAb to TcdA was conserved. This analysis had for objective to verify that the carbon source did not impair the binding ability of the sdAb since it was reported that the use of glycerol during the expression of a GST yielded higher activities compared to glucose (76% and 32% respectively) [68]. Our results confirmed that ability of the sdAb to bind TcdA was unaffected as can be seen in Table 4-3. Kinetic constants are obtained by using a simple binding model equation where:



Ab:	Antibody
Ag:	Antigen
Ab·Ag:	Antibody·Antigen complex
k _a :	Association rate constant (M ⁻¹ s ⁻¹)
k _d :	Dissociation rate constant (s ⁻¹)

$$K_D = \frac{k_d}{k_a} = \frac{[Ab \cdot Ag]}{[Ab][Ag]}$$

K_D : Affinity constant (M)

Table 4-3 Binding kinetics for ToxA5.1 sdAb obtained from Biacore data.

Kinetic constants	Flask Periplasmic extraction	Bioreactor Glucose Magnetic beads	Bioreactor Glycerol Magnetic beads
k_a (1/M s)	1.60×10^6	1.29×10^6	1.20×10^6
k_d (1/s)	5.00×10^{-3}	5.05×10^{-3}	5.05×10^{-3}
K_D (M)	3.00×10^{-9}	3.91×10^{-9}	4.23×10^{-9}

Association constants are similar with values of 1.29×10^6 and 1.20×10^6 1/M s respectively for glucose and glycerol. Both dissociation constants for glucose and glycerol are 5.05×10^{-3} 1/s and affinity constants are 3.91×10^{-9} and 4.23×10^{-9} M respectively. These values are similar to the value of the ToxA5.1sdAb reported by Hussack *et al.* [19]. The carbon source and large-scale expression did not seem to interfere with binding affinity of the sdAb. Furthermore, the purification method does not seem to pose a problem. Samples from the bioreactor cultures were purified from total cell lysate using magnetic beads and compared with samples from flask experiment purified using periplasmic extraction followed by overnight dialysis and IMAC purification. Samples purified from the two different methods showed similar binding behaviour as can be seen in Figure 4-4.

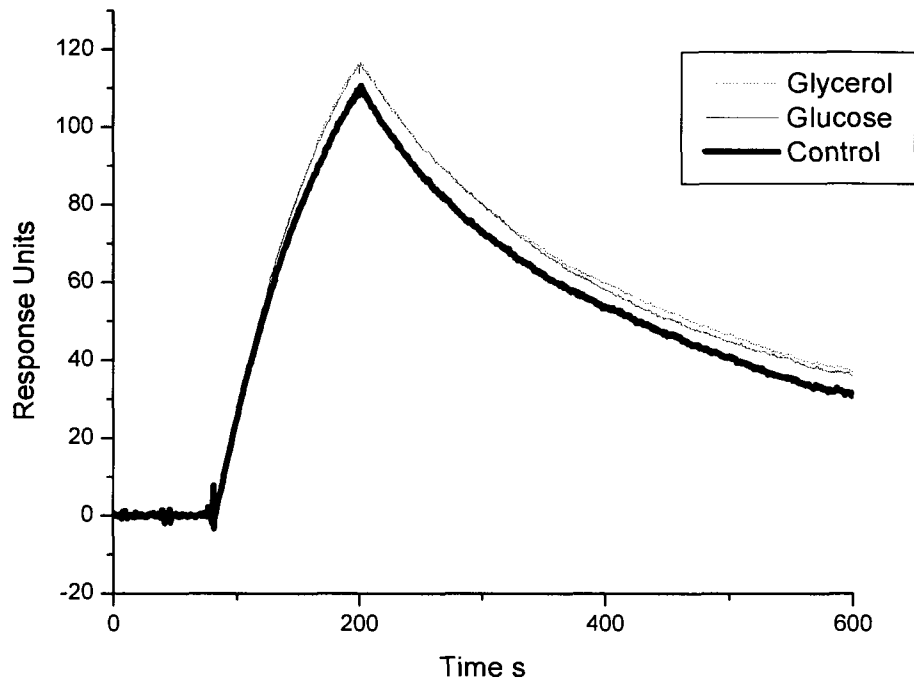


Figure 4-4: Biacore binding results of sdAB ToxA5.1 samples purified from periplasmic extract (control) and magnetic beads (glycerol and glucose samples).

4.5 Conclusion

Over-expression of the ToxA5.1 sdAb against *C. difficile* toxin A was achieved at 503 mg/L in a stirred-tank bioreactor with 1.5 L working volume using complex medium (2x YT) and glucose as carbon source. Lower initial glucose concentrations yielded higher ToxA5.1 expression and it is hypothesized that acetate inhibition caused by the higher glucose uptake rate might be responsible for this phenomenon. Low and high glycerol concentrations, of 5 and 30 g/L, respectively, yielded similar expression patterns as well as final ToxA5.1 concentration at the end of the 24 h fermentation culture. When high concentration of glycerol is used as the carbon source, periplasmic leakage occurs and the

concentration of ToxA5.1 recovered from the broth accounted for 41% of the total ToxA5.1 recovered. It was also demonstrated, using Biacore analysis, that neither the carbon source (glucose or glycerol) nor the purification procedures using magnetic beads on crude lysate as opposed to dialyzed periplasmic extract affected the binding activity of the ToxA5.1 sdAb to the TcdA.

4.6 References

1. Bobak, D.A., *The molecular pathogenesis of Clostridium difficile-associated disease*. Current Infectious Disease Reports, 2008. **10**(2): p. 111-115.
2. Voth, D.E. and J.D. Ballard, *Clostridium difficile toxins: Mechanism of action and role in disease*. Clinical Microbiology Reviews, 2005. **18**(2): p. 247-263.
3. Corthier, G., et al., *Protection against experimental pseudomembranous colitis in gnotobiotic mice by use of monoclonal antibodies against Clostridium difficile toxin A*. Infection and Immunity, 1991. **59**(3): p. 1192-1195.
4. Babcock, G.J., et al., *Human monoclonal antibodies directed against toxins A and B prevent Clostridium difficile-induced mortality in hamsters*. Infection and Immunity, 2006. **74**(11): p. 6339-6347.
5. Weiss, K., *Toxin-binding treatment for Clostridium difficile: a review including reports of studies with tolevamer*. International Journal of Antimicrobial Agents, 2009. **33**(1): p. 4-7.
6. Casadevall, A., E. Dadachova, and L.A. Pirofski, *Passive antibody therapy for infectious diseases*. Nature Reviews Microbiology, 2004. **2**(9): p. 695-703.
7. Lyerly, D.M., et al., *Passive immunization of hamsters against disease caused by Clostridium difficile by use of bovine immunoglobulin G concentrate*. Infection and Immunity, 1991. **59**(6): p. 2215-2218.
8. Taylor, C.P., et al., *Open-label, dose escalation phase I study in healthy volunteers to evaluate the safety and pharmacokinetics of a human monoclonal antibody to Clostridium difficile toxin A*. Vaccine, 2008. **26**(27-28): p. 3404-3409.
9. Salcedo, J., et al., *Intravenous immunoglobulin therapy for severe Clostridium difficile colitis*. Gut, 1997. **41**(3): p. 366-370.
10. Sougioultzis, S., et al., *Clostridium difficile toxoid vaccine in recurrent C. difficile-associated diarrhea*. Gastroenterology, 2005. **128**(3): p. 764-770.
11. Wilcox, M.H., *Descriptive study of intravenous immunoglobulin for the treatment of recurrent Clostridium difficile diarrhoea*. Journal of Antimicrobial Chemotherapy, 2004. **53**(5): p. 882-884.
12. Hamers-Casterman, C., et al., *Naturally occurring antibodies devoid of light chains*. Nature, 1993. **363**(6428): p. 446-448.
13. Liu, J.L., G.P. Anderson, and E.R. Goldman, *Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library*. BMC Biotechnology, 2007. **7**.
14. Shuntao, W., et al., *A novel designed single domain antibody on 3-D structure of ricin A chain remarkably blocked ricin-induced cytotoxicity*. Molecular Immunology, 2006. **43**(11): p. 1912-1919.
15. Fernandez, L.A., *Prokaryotic expression of antibodies and affibodies*. Current Opinion in Biotechnology, 2004. **15**(4): p. 364-373.
16. Arbabi-Ghahroudi, M., J. Tanha, and R. MacKenzie, *Prokaryotic expression of antibodies*. Cancer and Metastasis Reviews, 2005. **24**(4): p. 501-519.
17. Gasser, B. and D. Mattanovich, *Antibody production with yeasts and filamentous fungi: On the road to large scale?* Biotechnology Letters, 2007. **29**(2): p. 201-212.
18. Plückthun, A., *Antibody engineering: Advances from the use of Escherichia coli expression systems*. Nature Biotechnology, 1991. **9**(6): p. 545-551.

19. Hussack, G., J. Tanha, and C.R. MacKenzie, *Llama single domain antibody binding Clostridium difficile toxin A*. to be submitted, 2009.
20. Arbabi-Ghahroudi, M., R. Mackenzie, and J. Tanha, *Selection of Non-aggregating V(H) Binders from Synthetic V (H) Phage-Display Libraries*. *Methods Mol Biol*, 2009. **525**: p. 1-30.
21. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. *Nature*, 1970. **227**(5259): p. 680-685.
22. Abràmoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with imageJ*. *Biophotonics International*, 2004. **11**(7): p. 36-41.
23. To, R., et al., *Isolation of monomeric human VHs by a phage selection*. *Journal of Biological Chemistry*, 2005. **280**(50): p. 41395-41403.
24. Marison, I.W. and U. Von Stockar, *The application of a novel heat flux calorimeter for studying growth of Escherichia coli W in aerobic batch culture*. *Biotechnology and Bioengineering*, 1986. **28**(12): p. 1780-1793.
25. Bentley, W.E., et al., *Plasmid-encoded protein: The principal factor in the 'metabolic burden' associated with recombinant bacteria*. *Biotechnology and Bioengineering*, 1990. **35**(7): p. 668-681.
26. Han, K., H.C. Lim, and J. Hong, *Acetic acid formation in Escherichia coli fermentation*. *Biotechnology and Bioengineering*, 1992. **39**(6): p. 663-671.
27. Majewski, R.A. and M.M. Domach, *Simple constrained-optimization view of acetate overflow in E. coli*. *Biotechnology and Bioengineering*, 1990. **35**(7): p. 732-738.
28. Eiteman, M.A. and E. Altman, *Overcoming acetate in Escherichia coli recombinant protein fermentations*. *Trends in Biotechnology*, 2006. **24**(11): p. 530-536.
29. Meyer, H.P., C. Leist, and A. Fiechter, *Acetate formation in continuous culture of Escherichia coli K12 D1 on defined and complex media*. *Journal of Biotechnology*, 1984. **1**(5-6): p. 355-358.
30. Nakano, K., et al., *Influence of acetic acid on the growth of Escherichia coli K12 during high-cell-density cultivation in a dialysis reactor*. *Applied Microbiology and Biotechnology*, 1997. **48**(5): p. 597-601.
31. Jae Gu, P., R. Joon Shick, and J.M. Lebeault, *Physiological constraints in increasing biomass concentration of Escherichia coli B in fed-batch culture*. *Biotechnology Letters*, 1987. **9**(2): p. 89-94.
32. Fass, R., T.R. Clem, and J. Shiloach, *Use of a novel air separation system in a fed-batch fermentative culture of Escherichia coli*. *Applied and Environmental Microbiology*, 1989. **55**(5): p. 1305-1307.
33. March, J.C., M.A. Eiteman, and E. Altman, *Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in Escherichia coli*. *Applied and Environmental Microbiology*, 2002. **68**(11): p. 5620-5624.
34. Xu, B., et al., *Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with Escherichia coli*. *Applied Microbiology and Biotechnology*, 1999. **51**(5): p. 564-571.
35. Xu, B., M. Jahic, and S.O. Enfors, *Modeling of overflow metabolism in batch and fed-batch cultures of Escherichia coli*. *Biotechnology Progress*, 1999. **15**(1): p. 81-90.

36. Čerić, S. and Z. Kurtanjek, *Model identification, parameter estimation, and dynamic flux analysis of coli central metabolism*. Chemical and Biochemical Engineering Quarterly, 2006. **20**(3): p. 243-253.
37. Kahru, A. and R. Vilu, *On characterization of the growth of Escherichia coli in batch culture*. Archives of Microbiology, 1983. **135**(1): p. 12-15.
38. Korz, D.J., et al., *Simple fed-batch technique for high cell density cultivation of Escherichia coli*. Journal of Biotechnology, 1995. **39**(1): p. 59-65.
39. Warny, M., et al., *Human antibody response to Clostridium difficile toxin A in relation to clinical course of infection*. Infection and Immunity, 1994. **62**(2): p. 384-389.
40. Sandén, A.M., et al., *Limiting factors in Escherichia coli fed-batch production of recombinant proteins*. Biotechnology and Bioengineering, 2003. **81**(2): p. 158-166.
41. Chou, C.H., G.N. Bennett, and K.Y. San, *Effect of modified glucose uptake using genetic engineering techniques on high-level recombinant protein production in Escherichia coli dense cultures*. Biotechnology and Bioengineering, 1994. **44**(8): p. 952-960.
42. Chou, C.H., G.N. Bennett, and K.Y. San, *Effect of modulated glucose uptake on high-level recombinant protein production in a dense Escherichia coli culture*. Biotechnology Progress, 1994. **10**(6): p. 644-647.
43. De Mey, M., et al., *Minimizing acetate formation in E. coli fermentations*. Journal of Industrial Microbiology and Biotechnology, 2007. **34**(11): p. 689-700.
44. Lara, A.R., et al., *Engineering Escherichia coli to improve culture performance and reduce formation of by-products during recombinant protein production under transient intermittent anaerobic conditions*. Biotechnology and Bioengineering, 2006. **94**(6): p. 1164-1175.
45. San, K.Y., et al., *Strategies in high-level expression of recombinant protein in Escherichia coli*. Annals of the New York Academy of Sciences, 1994. **721**: p. 257-267.
46. Sun, W.J., et al., *Acetate inhibition on growth of recombinant E. coli and expression of fusion protein TGF α -PE40*. Biotechnology Letters, 1993. **15**(8): p. 809-814.
47. Tebtoub, S.S. and A. Khellaf, *Detecting the production of acetate in fed-batch fermented process: Application to Escherichia coli cultivations*. WSEAS Transactions on Systems, 2006. **5**(8): p. 1907-1911.
48. Tsuji, K., et al., *Over-production of Thermus protease in dense culture of Escherichia coli using two carbon sources*. Biotechnology Techniques, 1997. **11**(6): p. 395-398.
49. Griffiths, A.J.F., et al., *An Introduction to genetic analysis*. 2000, New York: W.H. Freeman.
50. Reznikoff, W.S., *The lactose operon-controlling elements: A complex paradigm*. Molecular Microbiology, 1992. **6**(17): p. 2419-2422.
51. Riesenber, D., et al., *High cell density fermentation of recombinant Escherichia coli expressing human interferon alpha 1*. Applied Microbiology and Biotechnology, 1990. **34**(1): p. 77-82.
52. Jensen, E.B. and S. Carlsen, *Production of recombinant human growth hormone in Escherichia coli: Expression of different precursors and physiological effects of*

- glucose, acetate, and salts*. Biotechnology and Bioengineering, 1990. **36**(1): p. 1-11.
53. Kleman, G.L. and W.R. Strohl, *Acetate metabolism by Escherichia coli in high-cell-density fermentation*. Applied and Environmental Microbiology, 1994. **60**(11): p. 3952-3958.
 54. El-Mansi, E.M.T. and W.H. Holms, *Control of carbon flux to acetate excretion during growth of Escherichia coli in batch and continuous cultures*. Journal of General Microbiology, 1989. **135**(11): p. 2875-2883.
 55. Luli, G.W. and W.R. Strohl, *Comparison of growth, acetate production, and acetate inhibition of Escherichia coli strains in batch and fed-batch fermentations*. Applied and Environmental Microbiology, 1990. **56**(4): p. 1004-1011.
 56. Cherrington, C.A., M. Hinton, and I. Chopra, *Effect of short-chain organic acids on macromolecular synthesis in Escherichia coli*. Journal of Applied Bacteriology, 1990. **68**(1): p. 69-74.
 57. Pan, H., et al., *Optimization of culture conditions to enhance cis-epoxysuccinate hydrolase production in Escherichia coli by response surface methodology*. Biochemical Engineering Journal, 2008. **42**(2): p. 133-138.
 58. Sunitha, K., et al., *Statistical optimization of seed and induction conditions to enhance phytase production by recombinant Escherichia coli*. Biochemical Engineering Journal, 2000. **5**(1): p. 51-56.
 59. Holms, H., *Flux analysis and control of the central metabolic pathways in Escherichia coli*. FEMS Microbiology Reviews, 1996. **19**(2): p. 85-116.
 60. Shokri, A., A.M. Sandén, and G. Larsson, *Cell and process design for targeting of recombinant protein into the culture medium of Escherichia coli*. Applied Microbiology and Biotechnology, 2003. **60**(6): p. 654-664.
 61. Choi, J.H. and S.Y. Lee, *Secretory and extracellular production of recombinant proteins using Escherichia coli*. Applied Microbiology and Biotechnology, 2004. **64**(5): p. 625-635.
 62. Yang, J., et al., *One hundred seventy-fold increase in excretion of an FV fragment-tumor necrosis factor alpha fusion protein (sFV/TNF- α) from Escherichia coli caused by the synergistic effects of glycine and Triton X-100*. Applied and Environmental Microbiology, 1998. **64**(8): p. 2869-2874.
 63. Hammes, W., K.H. Schleifer, and O. Kandler, *Mode of action of glycine on the biosynthesis of peptidoglycan*. Journal of Bacteriology, 1973. **116**(2): p. 1029-1053.
 64. Kragh-Hansen, U., *Transitional steps in the solubilization of protein-containing membranes and liposomes by nonionic detergent*. Biochemistry, 1993. **32**(6): p. 1648-1656.
 65. Shibui, T. and K. Nagahari, *Secretion of a functional Fab fragment in Escherichia coli and the influence of culture conditions*. Applied Microbiology and Biotechnology, 1992. **37**(3): p. 352-357.
 66. Morgan, F.J. and F.G. Priest, *Lethal catabolism of glycerol by Bacillus amyloliquefaciens*. FEMS Microbiology Letters, 1981. **10**(3): p. 257-260.
 67. Lin, E.C., *Glycerol dissimilation and its regulation in bacteria*. Annual Review of Microbiology, 1976. **30**: p. 535-578.
 68. Hansen, R. and N.T. Eriksen, *Activity of recombinant GST in Escherichia coli grown on glucose and glycerol* Process Biochemistry, 2007. **42**(8): p. 1259-1263.

Chapter 5: Conclusion and recommendations

5.1 Conclusion

Of the factors affecting the expression of sdAb ToxA5.1 in flask, pH and temperature have the most impact on sdAb ToxA5.1 yields. In bioreactor batch fermentations, the inoculum plays a major role in the final yield of expressed sdAb. It was found that using low initial glucose concentration in the medium yielded more ToxA5.1 sdAb. Overall the initial goal of this thesis, which was to ascertain the possibility of producing sdAb ToxA5.1 in large quantity, was achieved; it was possible to produce up to 500 mg/L in a stirred-tank bioreactor using a 1.5 L working volume. Furthermore, the medium used for expression was simple and the expression period was shortened by using an expression temperature of 37°C. The purification steps were shortened and simplified, compared to periplasmic extraction and overnight dialysis, by using magnetic beads applied directly to crude cell lysate.

5.2 Recommendations

In the light of the results obtained, fed-batch operation seems to be the next step for the large-scale production of the ToxA5.1 sdAb. Fed-batch strategy will allow control of the cell growth rate and thus a control of acetate formation, which is known to cause protein expression inhibition. Recovery of the ToxA5.1 from large-scale production could also be investigated. The use of different techniques for cell lysis, such as sonication, chemical lysis or high pressures, as well as different purification schemes, IMAC or other system able to purify antibodies, such as the c-Myc, the Protein A or the Protein D systems, could be investigated for maximizing the recovery yield of this valuable product.

The fact that *E. coli* enterotoxin could be co-purified with the sdAb using traditional methods could be addressed by the use of a novel purification system involving heat treatment as sdAbs are resistant to high temperature. It would also be possible to adapt the expression and purification protocol of sdAb derived from the first paper to high-throughput screening of potential high expressing antigen binder clones during the earlier stages and by doing so, increase the amount of sdAb produced in bioreactor. Finally, modeling of the periplasmic expression of the sdAb could be investigated.