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Lysis of Escherichia coli for the Recovery of Pentamerised Single-Domain Antibody Used for the Gender Specific Separation of Bovine Sperm

THÈSE DE M.Sc.A.

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ABSTRACT

Gender of animal offspring is of great interest to farmers where gender selection is achieved via the separation of male-bearing from female-bearing sperms prior to performing artificial insemination. A start-up company (Ab Biotech Inc.) has developed a technique for gender selection based on the production of an intracellular single-domain antibody (sdAb) using the bacterium *Escherichia coli* capable of sexing bovine sperm. The purpose of this research was to provide a recommendation to Ab Biotech Inc. for the lysis of *E. coli*. An efficient lysis technique was required in order to release the intracellular sdAb. In the dairy industry, sexing for female calves is preferred since male calves are not useful for the purpose of milk production. Multiple lysis techniques were tested in order to provide a feasible recommendation for Ab Biotech Inc. These techniques included high pressure homogenization, sonication, bead milling and enzymatic/chemical lysis using lysozymes and Triton X-100. Required lysis time, extent of lysis and potential operating costs were contributing factors for determining an optimal technique. The extent of lysis was determined by quantifying the total amount of released protein using SDS-PAGE densitometry. It was recommended to choose bead milling for potential process upscaling since a large amount of fractional lysis (0.70) was obtained over a short amount of lysis time (3 min) with an inexpensive ($9.50/kg) 0.3 mm mixture of glass beads.

RÉSUMÉ

Le sexe de la progéniture des animaux est d'un grand intérêt pour les agriculteurs, où la sélection du sexe est obtenue par la séparation des spermes menant à un mâle ou une femelle avant que l'insémination artificielle ne soit effectuée. Une entreprise en démarrage (Ab Biotech Inc.) a mis au point une technique de sélection du sexe de la race bovine basée sur la production d'un anticorps à domaine unique (ATDU) intracellulaire par la bactérie *Escherichia coli* (*E. coli*). L'objectif principal de cette thèse était de fournir une recommandation à Ab Biotech Inc. pour la sélection d'une méthode pour la lyse des cellules *E. coli*. Une technique de lyse efficace est requise afin de libérer un anticorps à domaine unique (ATDU) intracellulaire utilisé pour le sexage des spermes bovins. Dans l'industrie laitière, le sexage des veaux femelles est préféré car les veaux mâles ne sont pas utiles aux fins de la production de lait. De multiples techniques de lyse ont été étudiées afin de fournir une recommandation la plus appropriée pour Ab Biotech Inc. Les techniques de lyse qui furent considérées sont l'homogénéisation à haute pression, la sonication, le broyage par billes et la lyse enzymatique/chimique avec les lysozymes et le Triton X-100. La durée de la lyse, le pourcentage de lyse obtenue et les coûts potentiels d'exploitation ont été les principaux facteurs considérés pour déterminer la technique de lyse la plus appropriée pour cette application. Le pourcentage de lyse est déterminé par la quantité totale de protéines libérées qui est obtenue par densitométrie des gels de SDS-PAGE. Il a été recommandé de choisir le broyage par billes pour la mise à l'échelle du procédé de lyse puisque cette technique de lyse a donné le plus grand pourcentage de lyse (70 %) obtenu pour un temps de lyse relativement court (3 min). De plus le mélange de billes ayant un diamètre moyen de 0.3 mm est relativement peu dispendieux (9.50 $/kg).

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TABLE OF CONTENTS

Abstract ii
Résumé iii
Acknowledgements iv
Table of Contents v
List of Figures viii
List of Tables ix
Nomenclature x
Chapter 1: Introduction 1
Chapter 2: Literature Review 5
2.1 Flow cytometry 5
2.1.1 Methodology 5
2.1.2 Drawbacks 7
2.2 Pentameric single-domain antibodies for sperm sexing 9
2.3 Production strain: E. coli 10
2.3.1 Importance of *E. coli* lysis 11
2.3.2 The *E. coli* cell wall 11
2.4 Methods of lysis 12
2.4.1 Chemical lysis 13
2.4.2 Enzymatic lysis (lysozymes) 14
2.4.3 High pressure homogenization 16
2.4.4 Sonication 18
2.4.5 Bead milling 21
2.5 Quantification of the extent of lysis 24
2.5.1 Direct cellular analysis 24
2.5.2 Indirect analysis (quantification of cellular products) 26
2.6 Conclusion 31
Chapter 3: Methods and Materials 33
3.1 Introduction 33

3.2 Protein (antibody) description 33
3.3 Host organism (E. coli) 35
3.4 E. coli production 36
3.4.1 Fermentation performed in flasks 36
3.4.2 Fermentation performed in bioreactors 38
3.5 Lysis Procedures 40
3.5.1 Methods and materials for Section 4.1 40
3.5.2 Methods and materials for Section 4.2.2 40
3.5.3 Methods and materials for Section 4.2.3 41
3.5.4 Methods and materials for Section 4.3 41
3.5.5 Methods and materials for Section 4.4 43
3.6 SDS-PAGE 44
3.6.1 SDS-PAGE procedure 44
3.6.2 SDS-PAGE analysis 46
3.7 Western blot analysis 47
Chapter 4: Evaluation of lysis techniques 51
4.1 High pressure homogenization 51
4.1.1 Results and discussion 52
4.1.2 Summary 54
4.2 Bead milling 54
4.2.1 Bead milling: effect of bead to lysate ratio 55
4.2.2 Bead milling: cell growth cycle point and time 55
4.2.3 Bead milling: effect of bead size 63
4.3 Sonication 69
4.3.1 Results and discussion 70
4.3.2 Model development 73
4.3.3 Summary 77
4.4 Synergistic lysis using lysozyme and Triton X-100 78
4.4.1 Results and discussion 79
4.4.2 Summary 81
4.5 Overall Comparison 81
Chapter 5: Conclusion and Recommendations 84

5.1 Conclusion 84
5.2 Recommendations 84
Chapter 6: References 86

LIST OF FIGURES

Figure 1-1: Flow sheet depicting the forms of lysis presented throughout the document 4
Figure 2-1: Mechanism of sperm sexing through flow cytometry 6
Figure 2-2: A visual comparison between a monomeric sdAb and a pentameric antibody 10
Figure 2-3: An illustration of the cellular wall of *E. coli* bacteria 12
Figure 2-4: Lysis mechanism of peptidoglycan through the use of lysozymes 15
Figure 2-5: High pressure homogenization mechanism 16
Figure 2-6: Typical sonication set-up 19
Figure 2-7: Continuous bead milling schematic diagram 21
Figure 3-1: A typical SDS-PAGE of *E. coli* proteins 47
Figure 3-2: An example of results obtained for SDS-PAGE and western blot 49
Figure 4-1: SDS-PAGE of *E. coli* proteins. 52
Figure 4-2: Fractional release of proteins durinf HPH experimentation 53
Figure 4-3: *E. coli* TG1 growth curve for Fermentation 1 57
Figure 4-4: *E. coli* TG1 growth curve for Fermentation 2 57
Figure 4-5: *E. coli* TG1 growth curve for Fermentation 3 58
Figure 4-6: A-C: SDS-PAGE of *E. coli* proteins for Fermentation 3 59
Figure 4-7: A comparison of the amount of fractional lysis of *E. coli* cells over 9 min of bead milling with cells that were harvested from the exponential or stationary phase. 60
Figure 4-8: Effect of bead milling time on cell lysate temperature 62
Figure 4-9: Microscopic images of glass beads used for the operation of milling *E. coli* 64
Figure 4-10: Particle size distribution of the “0.1 mm” diameter beads 64
Figure 4-11: Particle size distribution of the “0.3 mm” diameter bead mixture 65
Figure 4-12: Particle size distribution of the “0.5 mm” diameter beads 65
Figure 4-13: SDS-PAGE of *E. coli* proteins from bead milling experiment comparing lysis as a function of glass diameter 66
Figure 4-14: Comparison of the amount of fractional lysis of *E. coli* cells over 6 min of bead milling from the bead milling experiment comparing lysis as a function of glass bead diameter 67
Figure 4-15: The effects output control, duty cycle, optical density and time (D) on the fractional amount of lysis during sonication 72
Figure 4-16: A plot of the predicted amount (using Equation (4.1)) of fractional lysis versus the amount of experimental lysis during sonication 74
Figure 4-17: Pareto plot for initial model of sonication experimentation 75
Figure 4-18: A plot of the predicted amount (using Equation (4.2)) 76
Figure 4-19: SDS-PAGE of *E. coli* proteins from experimentation with lysozymes and Triton X-100 79
Figure 4-20: A comparison of the amount of fractional lysis of *E. coli* cells during experimentation with lysozymes and Triton X-100 80

LIST OF TABLES

Table 2-1: Approximate extinction coefficients for 1 mg/mL protein solutions 27
Table 3-1: A breakdown of the values of each parameter during the sonication experimentation 43
Table 4-1: Cellular growth phase classification for cell fermentations 1, 2 and 3. 58
Table 4-2: A breakdown of the values of each parameter during the sonication experimentation, as well as experimental and predictive results (using Equations (4.1) and (4.2)) 71

NOMENCLATURE

A	Absorbance
B	Beta
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CFU	Colony Forming Unit
DNA	Deoxyribonucleic acid
E	Extinction coefficient
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence in situ hybridization
HRP	Horseradish Peroxidase
GHCl	Guanidine hydrochloride
IM	Inner membrane
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
LPS	Lipopolysaccharides
mAB	Monoclonal antibody
NAG	N-acetylglucosamine
NAM	N-acetlymuramic acid
NTA	Nitrilotriacetic acid
OD	Optical Density
OM	Outer membrane
P	Pellet
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PL	Phospholipid layer
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene fluoride
rcf	Relative centrifugal force
rpm	Revolutions per minute
S	Soluble
sdAb	Single-domain antibody
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SSP	Sex specific protein
T	Total
TB	Terrific Broth
TBST	Tris-buffered saline and Tween 20
UV	Ultraviolet
VhH	Variable heavy fragment

CHAPTER 1: INTRODUCTION

The agricultural industry has been transformed in the past half century from small family farms producing a wide variety of products to very large and increasingly specialized operations. This is the case for dairy farms where the majority of the farm operation is oriented to milk production. When cows are artificially inseminated, it is highly desirable to resort to gender selection to increase the occurrence of female calves since male calves have no value, and often negative values, for this type of operation. However, for the beef industry the opposite prevails; male calves would be preferentially selected since the males grow faster than the females [1] resulting in higher beef productivity. For milk production farms, resources used to take care of the unwanted male calves during pregnancy, birth, and infancy are deemed to be wasted since these calves cannot potentially contribute to the specified operation. Therefore, gender selection is highly desirable due to cost and resource saving potential.

Sex predetermination in livestock, to provide the producers with faster genetic progress and greater flexibility, has been the subject of research for many decades. The current technology for gender pre-selection, flow cytometry, relies on the difference in the amount of DNA present in X- and Y-sperms (X sperms create females while Y sperms create males). For cattle the DNA content is approximately 4 % greater in X-chromosomal sperms [2]–[5]. Flow cytometry is mostly confined to the cattle breeding industry with relatively good success for pre-sexing except that the sexed sperm doses are too expensive, $80-$100/dose for female straws, for widespread application [2], [5], [6]. Furthermore, sexing sperm with a flow cytometer is considered to be inefficient (42-50 % of each sample is able to be sorted [2]). Because of these reasons, sexed sperm is normally reserved for first pregnancy in heifers where fertility is the highest [2], [7], [8]. Therefore, there is

a clear need for a rapid, economic, efficient and large-scale method for separating X or Y-sperm populations.

One technique, developed by Ab Biotech Inc., is to use specific single-domain pentamerised antibodies that are able to capture and remove male spermatozoa from bull semen enabling the selectivity of female-forming spermatozoa. The antibody is able to attach to antigens specifically found only on the surface of male-forming spermatozoa providing a means of selectively separating males from females through immobilized metal affinity chromatography (IMAC) since the antibody exhibits a histidine-tag (His-tag).

Typically, antibodies are produced in eukaryotic cells since this type of cell contains the cellular machinery needed to provide protein glycosylation and proper protein folding [9]. However, the antibody used in the following research is a single-domain antibody (sdAb) which is much simpler structurally than conventional antibodies hence requiring minimal folding, and does not require glycosylation in order to bind the target epitope [10]. Therefore, this antibody can be produced using a system that is easier and cheaper to maintain such as prokaryotic cells. The host organism that has been chosen for the production of the antibody in this investigation is *Escherichia coli ,* a unicellular gram-negative bacterium which can be grown in large amounts easily and cheaply [11].

The major steps of the production process involve 1) a fermentation to produce high cell concentration, 2) an induction to initiate the production of high amounts of antibodies, 3) cell lysis to expose the antibodies, and 4) the recovery of antibodies from the solution using a targeted separation technique such as IMAC. All steps are paramount for the success of the economic viability of this process. However, this thesis focuses on the cell lysis step which is critical to achieve the highest possible proportion of soluble antibodies available for the purification step. If

a fraction of antibodies is unable to be extracted from cellular debris, the efficiency of the entire process decreases significantly leading to increased operating costs. The lysis step poses significant challenges since there are two cellular walls that need to be breached in order to access the antibodies located within *E. coli* cells. Another challenge consists in ensuring that the antibodies remain stable and active upon release from the cell.

The partner company, Ab Biotech Inc., is working simultaneously on the development of all four production steps including cell lysis at large scale. Large-scale lysis of cells cannot rely on small-scale lysis techniques commonly used in laboratory experiments. To address this problem, various mechanical and chemical/enzymatic lysis techniques were investigated in this thesis. A comparative study was performed to determine which technique would yield a higher relative protein recovery as well as determining which technique could be efficiently scaled-up for potential large-scale operation. This was achieved by identifying which lysis techniques were capable of achieving maximum cell lysis under laboratory-scale conditions. Mechanical lysing techniques that were tested in this investigation include bead milling, high pressure homogenization and sonication. Chemical/enzymatic techniques that were tested include Triton X-100 and lysozymes. The series of experiments performed throughout this investigation and presented in this thesis is summarized in Figure 1-1.

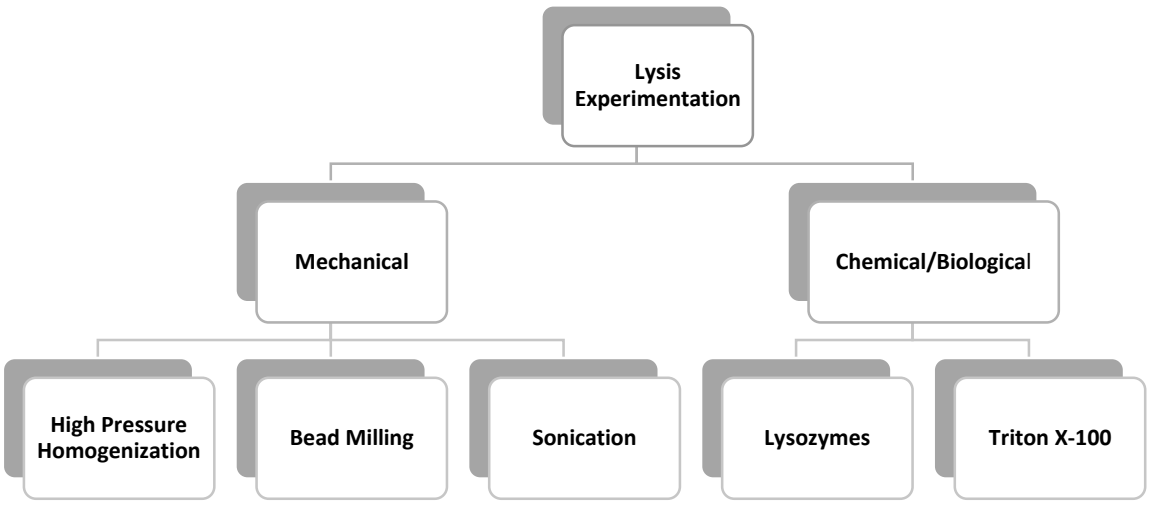

Figure 1-1: Flow sheet depicting the forms of lysis presented throughout the document.

In summary, to meet the company's need, the objective of this research was to develop an efficient lysis method that maximized the amount of soluble pentameric antibodies produced through *E. coli* fermentations for downstream processing. However, throughout the research performed, it was determined that the amount of the target antibody produced was minimal and was therefore difficult to quantify. Since it was difficult to produce high concentrations of the target antibody with the current development of the genetically-modified cellular strain, the focus of this thesis has been expanded to include the quantification of all recovered proteins as a standard for determining the applicability of specific methods of lysing *E. coli* cells.

CHAPTER 2: LITERATURE REVIEW

This chapter briefly presents background information on the methods that are used to separate sperms for gender selection, the production of a specific protein for sexing and the different lysis techniques used to liberate the cell content and make it accessible for specific targeted recovery. This information will help to better understand the challenges that are faced in gender selection and cell lysis.

2.1 Flow cytometry

To this day, flow cytometry is the only viable method for sexing live sperm commercially and is only available through the company Sexing Technologies [7], [12]. The sperm separation is based on the fact that X-sperms contain, in the case of cattle, approximately 4 % [2]–[4] more DNA than the Y-Sperms [2], [13]–[15]. The premise behind flow cytometry is to measure the amount of DNA content of each individual sperm and sex the sperm based upon the resulting measurement. Currently, flow cytometry is able to correctly sex sperm with a 90 % success rate [2], [4], [7], [8], [12], [14]. However, only about 42-50 % of the sperm in each sample are sorted leaving behind more than half of the desired sperm [2].

2.1.1 Methodology

In flow cytometry, the sperm sample is subjected to a fluorescing dye (Hoechst 33342) which is able diffuse through the cell membrane of the sperm and bind with the sperm's DNA [3], [7], [12]–[14]. Since, for cattle, X-sperms have a 4 % higher DNA content than Y-sperms, the X-sperms will fluoresce with 4 % greater intensity than the Y-sperms [7], [12], [14], [16].

The sperm cells are then passed through the flow cytometer exiting the apparatus one at a time. The outlet flowrate of sperm was optimized through the development of a beveled injection needle which ensures the correct orientation of sperm leaving the flow cytometer at high flow rates [3], [7], [14]. Upon exiting the apparatus, the cell sample is broken into droplets by a vibrator [14], [17]. This process typically forms 70 000 to 80 000 drops per second [2]. Each droplet is analyzed by a solid-state laser which detects whether the sperm is an X-sperm or a Y-sperm based upon the level of fluorescence that the sperm emits [12], [14]. If a droplet contains an X-sperm, a positive charge is added to the droplet whereas, if a droplet contains a Y-sperm, a negative charge is added. Each droplet then falls between two electrically-charged plates [2], [14]. One plate emits a positive electric field while the other plate emits a negative electric field. Depending on the charge assigned to the droplet, the droplet will be either drawn toward the positively or the negatively charged plate (the opposite charge to which the droplet has been assigned). The separated sperms are collected at the bottom of each respective plate [2], [14]. Figure 2-1 provides a schematic diagram describing the mechanism for sperm sexing via flow cytometry.

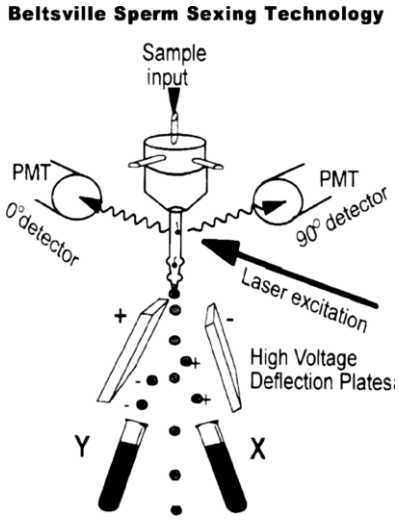

Figure 2-1: Mechanism of sperm sexing through flow cytometry [2].

Along with the Hoechst 3342 fluorescent dye, food colorant is also added to the cell sample prior to being passed through the flow cytometer [3], [7], [12]. Sperm cells with damaged membranes are not able to keep the food colorant from entering the intracellular space. Therefore, the food colourant acts to quench the fluorescent dye lowering the fluorescence [3], [7], [12]. When the damaged sperms are analysed by the laser, they do not meet the threshold of required fluorescence. The damaged sperms are not given a charge and are able to pass freely between the two electrically-charged plates and are discarded. This same principle applies to droplets which 1) do not contain sperms (no fluorescence is observed), 2) contain multiple sperms (fluorescence is too high), or 3) contain sperms which are indistinguishable relative to their DNA content (abnormal amount of fluorescence). In each of these cases, no charge is assigned and the droplets pass freely through the electrically-charged plates and are discarded. After the separation process is complete, the extent of separation can be tested by using Polymerase Chain Reaction (PCR), Fluorescence In Situ Hybridization (FISH) or Sort Reanalysis techniques in order to determine the X-Sperm and Y-Sperm content in the sample [3], [13].

2.1.2 Drawbacks

During the separation process, the sperms can be damaged due to the Hoechst fluorescent dye, the laser or the shear stress due to pressure-driven sperm flow through the apparatus [3], [7], [8], [14], [17], [18]. However, it has been determined that the fluorescent dye has no detrimental effects on the offspring that the sperm fosters [2], [3], [14]. It has been found that lowering the apparatus pressure from 345 to 275 kPa (50 to 40 psi) can significantly lower the amount of damage to sperm cells [3], [7], [8], [14], [17], [18]. The viability of sperm cells is also reduced when they are stored for longer periods prior to separation [3], [8]. Pregnancy success rates dropped from 42 % to 37 % when bovine sperms were stored at 19°C for 20 h prior to sorting [8].

It has also been found that sexed cells degenerate at a faster rate than unsexed cells [3], [19]. Therefore, cryogenic techniques have been developed in order to help preserve sperm cells prior to insemination [3], [14], [20].

Typically, a flow cytometer is able to sort 10 x 10^6 sperms/h [2], [4], [14]. This rate of separation is quite low seeing that a typical dose of frozen bull sperm used for insemination typically contains more than 20 x 10^6 sperms [2], [14], [19]. Even though this is the case, for most bulls, satisfactory fertility is seen with 10 x 10^6 sperms per dose and, for some bulls, high fertility can be seen even at a low dose of 2 x 10^6 sperms. Therefore, the low dose of 2 x 10^6 sperms per insemination is commonly used to reduce the amount of time (and valuable sperms) required to obtain a viable sperm sample for insemination [7], [8], [12], [14]. However, even though some bulls produce semen that exhibits high fertility, heifers are typically chosen over lactating cows for insemination since heifers are considered to be more fertile [2], [7], [8]. Lactating cows can still achieve pregnancy rates comparable to heifers, although somewhat lower, if techniques such as ultrasound, which are cumbersome, are used to determine the lactating cow's fertility window [2], [8], [14]. Proper management of heifers through 1) nutrition 2) disease control, 3) estrus detection, 4) proper semen handling and 5) proper insemination techniques can lead to pregnancy rates of 56 % when using 2 x 10^6 sexed sperms per inseminate compared to 61 % when using 10 x 10^6 unsexed sperms per inseminate [2], [8], [14]. If heifers are mismanaged then the pregnancy rates are very low [2], [7], [8].

Finally, sperm sexing through flow cytometry is considered to be very expensive ($80-$100 per female straw). The apparatus costs over $350 000 and requires skilled operators (which represent training costs) in order to run efficiently [2].

2.2 Pentameric single-domain antibodies for sperm sexing

It has been hypothesized that sperm cells can be sexed based upon sex-specific proteins (SSPs) located on the surface of the cell [20], [21]. Antibodies could then be designed in order to attach specifically to the identified surface protein. Affinity chromatography or magnetic bead separation would then be able to separate the X-sperms from the Y-sperms through the antibody attachment. An approach has been developed to assist in identifying SSPs based upon the hypothesis that sex-specific proteins are evolutionarily more highly conserved than non-SSPs [21].

Rather than developing a monoclonal antibody (mAb) to target a SSP, a single-domain antibody (sdAb also known as a V_HH or nanobody) can be designed for this purpose. Compared to mAb (~160 kDa), sdAbs are significantly smaller (~15 kDa) since they only contain a single variable heavy domain. In comparison, mAbs have 2 light and 2 heavy chains containing both variable and constant regions. The small size of sdAbs allow them to reach antigens on the surface of a cell which would be unreachable by mAbs [22]–[24]. They are 1) very soluble, 2) resistant to high temperatures (up to 90°C), 3) resistant to extreme pH and 4) resistant to protease digestion. Extensive folding and glycosylation are not required to produce sdAbs. Therefore, yeasts or bacteria (such as *Escherichia coli)* can be used for production of sdAbs [22], [23], [25]–[29]. This can greatly reduce production costs compared to eukaryotic production systems.

A major drawback for sdAbs is that they can have a low affinity for surface antigens [30]–[32]. Pentamerization of sdAbs can lead to an increase in affinity to target antigens 1 000-10 000 times greater than monomeric sdAbs [30]–[33]. The sdAbs are fused to a pentamerization protein such as the B subunit of verotoxin (VT1B) (shiga-like toxin) in order to promote pentamerization [24], [30], [32], [33]. The pentamerization process occurs simultaneously as the fused

sdAb/pentamerization protein complex is produced [30], [32], [33]. A comparison between a monomeric sdAb and a pentameric sdAb complex is illustrated below in Figure 2-2.

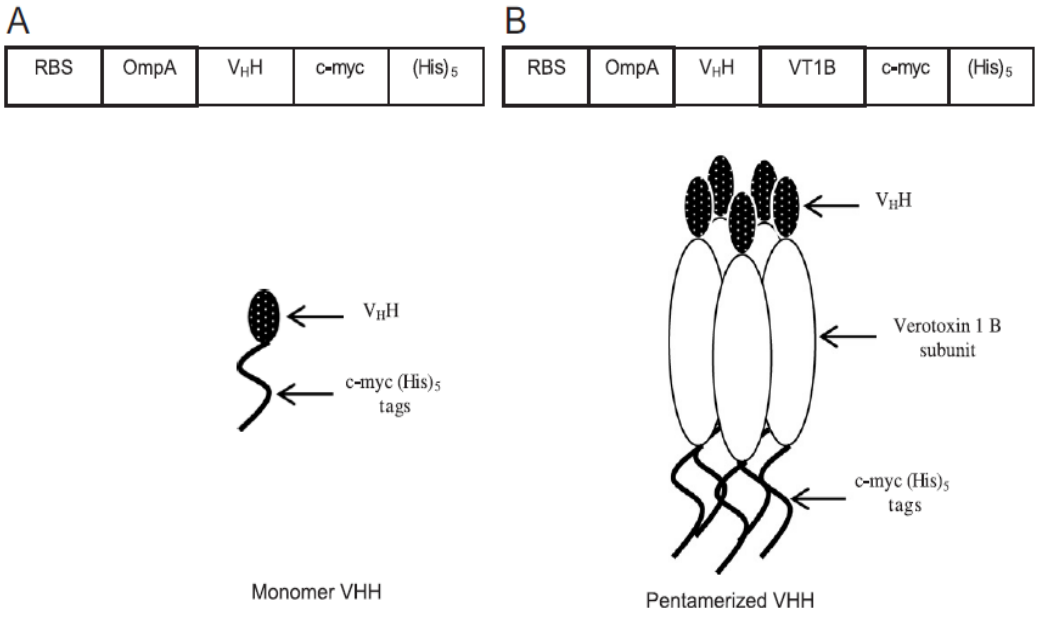

Figure 2-2: A visual comparison between a monomeric sdAb (A) and a pentameric antibody (B). RBS: ribosome binding site. OmpA: OmpA leader sequence. V_HH: single-domain antibody. VT1B: verotoxin 1 subunit B. c-myc: c-myc epitope tag. (His) 5: penta-histidine epitope tag [31].

Even though the pentamerised version is larger than the monomeric version of the sdAb, it has been found that there is no significant change in binding site accessibility to surface antigens [33]. The pentameric version also has 1) excellent thermostability, 2) resistance to proteases (such as trypsin and chymotrypsin), and 3) negligible aggregation (high solubility) [33]. Finally, pentameric sdAb complex can also be produced in bacteria (*E. coli*) or yeasts in very high amounts (up to 200 mg/L) [33].

2.3 Production strain: E. coli

In this investigation, *E. coli* was used to produce the desired protein. In this section, some information on *E. coli* is presented to gain a better understanding on the importance and challenges of producing and recovering the targeted protein.

2.3.1 Importance of *E. coli* lysis

E. coli is a gram-negative bacterium that is an important platform for the production of intracellular products. Many reasons justify the importance of *E. coli*: 1) they are well-known and well-characterized bacteria [11]; 2) they can be grown very quickly to large cell densities [11]; and (3) they can be produced using inexpensive media and fermentation equipment [11]. They have been the subject of extensive studies with their genome being fully decoded and an abundance of genetic engineering tools have been developed for these bacteria, which make them a very well-established platform for both genetic engineering research and expression of recombinant proteins of interest [11]. However, one of the biggest drawbacks to producing desired compounds with *E. coli* is the fact that these cells do not normally secrete proteins to the environment; proteins that are normally produced stay trapped within the confines of the cellular structure. This poses a challenge since the cellular wall of *E. coli* cells now has to be disrupted in order for the intracellular proteins to reach the surrounding environment. The following sections describe possible mechanisms that can be utilized in order to disrupt (or permeabilize) the cellular wall of *E. coli* cells in order to expose a protein of interest.

2.3.2 The *E. coli* cell wall

In order to release intracellular proteins, cells either need to be lysed or become permeable to the proteins located within. As seen in Figure 2-3, the cell wall is composed of three layers: an inner membrane and outer membrane (composed of phospholipids) and a thin peptidoglycan layer (composed of a lattice structure of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) molecules) which is situated in between the two membranes.

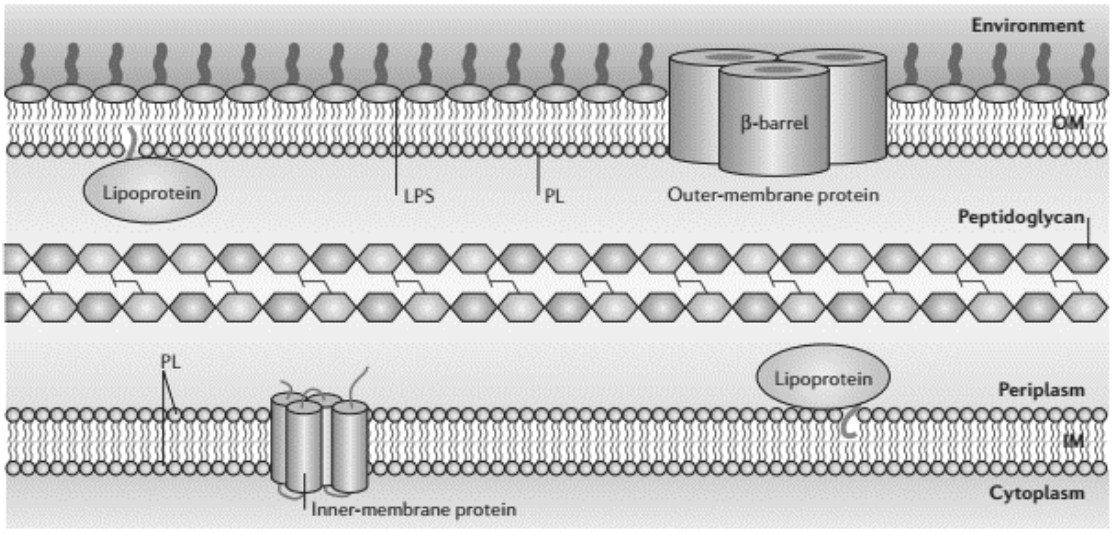

Figure 2-3: An illustration of the cellular wall of *E. coli* bacteria [34].

Proteins of interest can either be found within the inner membrane (cytoplasmic space) of the *E. coli* cell or in the periplasmic space (space between the two membranes) if an export peptide is fused to the recombinant protein. It can be of benefit for the protein of interest to be located within the periplasmic space since only the outer membrane would need to be removed. Since fewer native proteins are found in this space, purification can be considerably simplified [11], [35]. However, if the protein is not engineered to be translocated to the periplasmic space, then both membranes and the peptidoglycan layer of the cell wall need to be lysed. The following methods allow for the recovery of proteins that are located within the *E. coli* cell.

2.4 Methods of lysis

Lysis is the first step in the purification process of intracellular bio-products. There are many variables and challenges to consider when choosing the proper form of lysis since a technique that may be optimal for the recovery of a specific product may be unsuitable for a different product. Therefore, it is suggested to properly analyse and test various forms of lysis to

ensure the highest recovery of the desired product. The following sections illustrate various lysis techniques that can be applied to *E. coli* for cellular product recovery.

2.4.1 Chemical lysis

As the name implies, chemical lysis uses a chemical to lyse the cell by penetrating, destabilizing or disintegrating the cell wall barriers [36].

Triton X-100 is a non-ionic detergent that is able to release intracellular proteins from *E. coli* cells by solubilizing the phospholipids of the inner and outer membranes allowing for the release of intracellular proteins [37]–[39]. Sarkosyl is an anionic detergent that is also able to solubilize the inner membrane of *E. coli* cells [40]. However, Sarkosyl is unable to solubilize the outer membrane of *E. coli.* Therefore, other compounds are also needed such as Triton X-100 to disrupt the outer membrane for the release of intracellular proteins.

Guanidine hydrochloride (GHCl) is a chaotropic agent (disrupts hydrogen binding network between water molecules) which has the ability to solubilize proteins from cytoplasmic membrane fragments [38]. Studies have shown that when both Triton X-100 and GHCl have been utilized together they can have a synergistic effect on the amount of intracellular protein released from *E. coli*. Hettwer and Wang (1989) have shown that for concentrations of 0.1 M GHCl and 2 % (v/v) Triton X-100, 50 % of both intracellular and membrane proteins were released in comparison to almost no protein released when each of these components were used on their own at these concentrations [38].

As stated above, Triton X-100 is able to solubilize the outer and inner membranes of *E. coli* in order to promote protein release [37], [38]. However, this is only the case when there are no divalent cations present in the lysis buffer [41]. When Mg^{2+} ions are present in the lysis buffer,

Triton X-100 is unable to solubilize the outer membrane of the cells since the magnesium ions are able to stabilize the outer membrane from the effects of the detergent. However, EDTA (a chelating agent) is able to remove the divalent magnesium cations enabling Triton X-100 the ability to solubilize the outer and inner membranes of *E. coli* cells [41], [42]. It should be noted that using each of these compounds on their own yields a minimum amount of protein recovery due to poor cell lysis [43]. EDTA has no effect on the peptidoglycan layer found in *E. coli* cells [44]. It has been seen that some strains of *E. coli* like O104:H21 have higher resistance to the effects of EDTA than others like O157:H7 ATCC 43895 [45].

EDTA can also be used with the chaotropic agent urea to initiate *E. coli* cell lysis. As also seen with the chaotropic agent (GHCl), urea is able to solubilize membrane fragments. It has been determined that concentrations of urea ranging from 0.3 mM to 6 M have been effective in releasing intracellular proteins from both logarithmic and stationary phase *E. coli* at levels comparable to mechanical disruption [46]. However, proteins get denatured when high concentrations of urea (2 to 8 M) are used and need to be dialysed in proper buffer to enable refolding in the correct structure [47].

2.4.2 Enzymatic lysis (lysozymes)

Lysozymes are enzymes purified from chicken egg whites that can be used to lyse *E. coli* cells for the recovery of intracellular proteins [48]. Lysozymes can hydrolyse the beta 1-4 linkages between N-acetylmuramic acid and N-acetyl glucosamine units that make up the peptidoglycan layer as illustrated in Figure 2-4 [44], [49]–[52]. The optimal temperature for lysozyme cleavage has been determined to be 35°C [50]. The mechanism of peptidoglycan cleavage can be visualized in Figure 2-4.

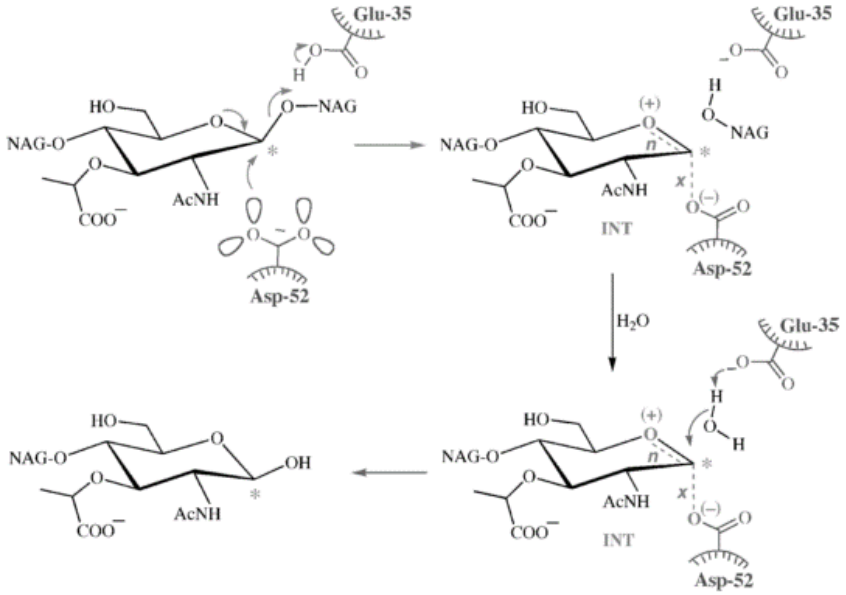

Figure 2-4: Lysis mechanism of peptidoglycan through the use of lysozymes [52].

Gram-positive bacteria are easily lysed using lysozymes since there is no membrane surrounding their large layer of peptidoglycan [50], [53]. In comparison, gram-negative bacteria are more resistant to lysis from lysozyme attack due to a cellular membrane encasing the peptidoglycan layer [50]. Therefore, lysozyme on its own is unable to lyse *E. coli* cells at a very high efficiency since the outer membrane has not been ruptured [50].

When using lysozymes, chemically treating *E. coli* cells with EDTA or Triton X-100 can help disrupt the cellular membrane leading to an enhanced cell lysis. EDTA and Triton X-100 have been demonstrated to have the capability of disrupting the *E. coli* cell's outer membrane [50], [53], [54] enabling lysozymes to gain access to the peptidoglycan layer, and thus causing lysis. It has been determined that optimal conditions for an EDTA/lysozyme synergistic lysis is 100-800 µg/mL of EDTA and 25-50 µg/mL of lysozyme for cells that have been harvested during exponential and late stationary phases [50].

2.4.3 High pressure homogenization

High pressure homogenization is able to lyse *E. coli* cells physically by applying a significantly high shear rate to each of the cells. A cellular suspension under high pressure is forced through a small orifice in the homogenizing unit after which it collides with an impact ring causing cellular lysis. It has been determined that gram-negative bacteria (ex. *E. coli)* are more susceptible to lysis using this technique than gram-positive bacteria [55]. It has also been determined that the effect of high pressure on *E. coli* bacteria is not strain-dependent. Wuytack *et al*. (2009) have determined that the *E. coli* mutant LMM1010, (a strain of *E. coli* resistant to high hydrostatic pressure) has the same resistance to high pressure homogenization as its parental strain MG1655 [55]. A schematic of a typical high pressure homogenizer is depicted in Figure 2-5.

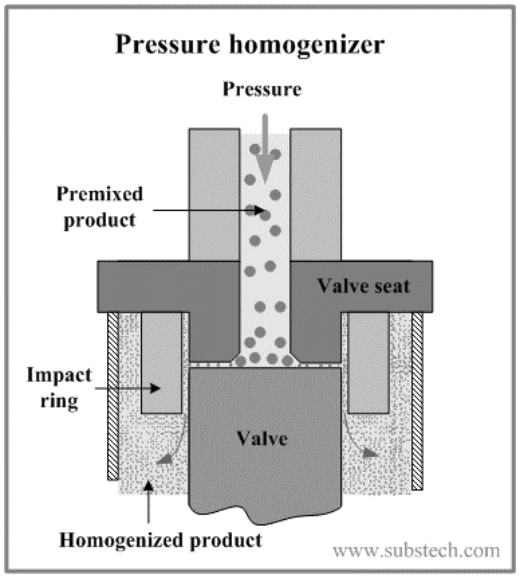

Figure 2-5: High pressure homogenization mechanism [56].

Variables that can be easily manipulated with the high pressure homogenizer apparatus are 1) cell suspension concentration, 2) number of passes through the homogenizer, 3) pressure exerted on the cell suspension and 4) flow rate. It has been demonstrated in literature that lysis efficiency is independent of the cell suspension concentration [57], [58]. Since this is the case, for continuous

processing, the concentration of cells can be increased (while remaining below a concentration that would be too viscous) in order to maintain a highly efficient homogenization that minimizes the power requirement needed.

The main variable that can be adjusted for high pressure homogenization is the pressure exerted on the cells as they flow through the apparatus [57]. Fonseca and Cabral (2002) reported in a study the effect of homogenization pressure on the release of a periplasmic protein. They demonstrated that pressures between 10-25 MPa could not achieve maximum periplasmic protein release even after multiple passes through the system. Protein release throughout the tested range was attributed to point break of the cell envelope [57]. At 50 MPa it was noted that cells were significantly reduced in size and the periplasmic protein release was a function of the number of passes through the system [57]. Finally when a range of 100-150 MPa was tested, it was found that the maximum release of the periplasmic protein was seen after only one pass [57]. This illustrates that an increase in homogenization pressure results in an increase in cellular lysis and periplasmic protein release.

It has been observed that an increase in the initial cellular suspension temperature and a decrease in viscosity leads to an increase in cellular deactivation during high pressure homogenization [59]. When the temperature is increased, the viscosity of the cell suspension subsequently decreases. However, by keeping the viscosity constant with ethylene glycol over a range of temperatures below 45°C, it was observed that the amount of deactivation remained constant [59]. By decreasing suspension viscosity (by varying the concentration of ethylene glycol), higher amounts of deactivation were achieved at constant temperatures that were below 45°C [60]. However, at initial temperatures above 45°C, it was observed that the above trends did

not hold since the heat energy supplied on its own was high enough to contribute to cell deactivation [59].

Pre-treating cell suspensions prior to high pressure homogenization has been shown to increase the amount of total lysis and protein release [61]. The pressure required for maximum protein release decreased from 34.5 MPa to 13.8 MPa when cell suspensions were treated by either GHCl or Triton X-100 [61].

One of the downfalls of high pressure homogenization is the micronization of cellular debris caused by multiple passes through the system [57]. When micronization occurs, cellular debris become so small that downstream purification becomes increasingly difficult since the cellular products can become entrained in the debris [39].

2.4.4 Sonication

Sonication involves the disruption of *E. coli* cells through the use of ultrasonic waves which cause pressure fluctuations around the cell wall leading to a destabilization of the intramolecular cohesive forces that are needed to maintain viable cellular structure [62]. A schematic representation of a sonication device used during laboratory-scale lysis can be seen in Figure 2-6.

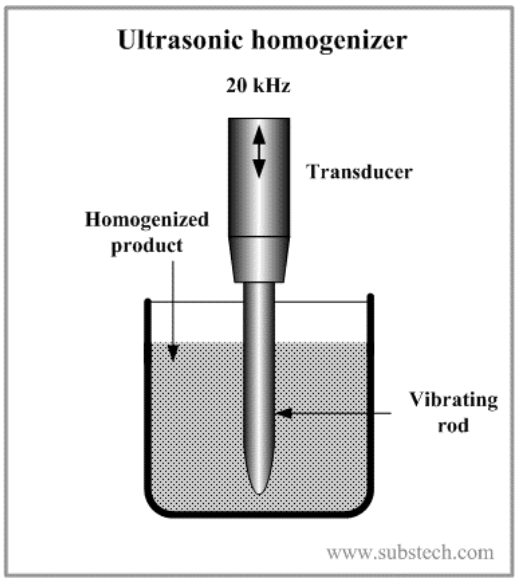

Figure 2-6: Typical sonication set-up [63].

In some cases, lysis and protein recovery efficiencies of sonication are comparable to synergistic lysis methods using lysozymes and EDTA [50] while in other cases, the amount of protein recovered through sonication was determined to be greater than 22 times the amount recovered through using enzymatic lysis [64]. It has also been determined that gram-positive bacteria are more resistant to sonication compared to gram-negative bacteria due to the rigid peptidoglycan structure in their cell wall [65].

The main variables that affect cell lysis and subsequent protein release include power intensity, ultrasonic frequency, volume of the cell suspension and amount of time that the cell suspension is exposed to the ultrasonic waves. It has been shown that providing a higher power intensity, [64], [66]–[69] or using frequencies such as 205 kHz [66] leads to an increase in lysis and protein release. It has also been shown that a lower volume of cell suspension increases the amount of protein released [67], [70]. However, there can potentially be an optimal volume depending on the product being released [64]. For the release of a hepatitis B core antigen through sonication, an optimal cell suspension volume was determined to be 15 mL [64].

Finally, it was shown that protein release was proportional to sonication time [58], [64], [67], [68]. However, based upon a culmination of the other variables, time required to achieve maximum cellular product release can vary substantially from 20 s [65] to 90 min [71]. This is an indication that sonication kinetics depend on the microorganism itself and are influenced by the cell physiology and growth conditions [58]. Therefore, sonication warrants experimentation in order to evaluate its suitability as a lysis technique. Furthermore, it has been demonstrated that prolonged exposure to sonication can lead to temperature increase or to the formation of free radicals both of which could cause protein damage or denaturation [64], [69]–[72]. Therefore, sonication for the release of thermally sensitive products should be performed on ice with resting periods implemented during the process to limit protein denaturation caused by temperature increase [70].

The effect of ionic strength and cell concentration of the cell suspension were also determined to have no effect on the amount of cellular lysis and protein release [67], [69]. This provides a great benefit when lysing large amounts of cells since the concentration of the cells can be maximized reducing the power requirements needed to perform the sonication process.

Various pre-treatments prior to sonication have been shown to promote high degrees of lysis. For example, processing cells through a freeze-thaw cycle prior to sonication has been shown to double the amount of protein release [71]. By enhancing the amount of lysis with a pre-treatment, less time and less acoustic power is needed thus reducing the probability of proteins being damaged [70]–[73].

2.4.5 Bead milling

Another form of mechanical lysis used to lyse *E. coli* cells is bead milling. For industrial purposes, bead milling would be performed on a continuous basis where a cell suspension would be fed into the bead milling chamber containing glass beads. While flowing within the chamber, the suspension is mixed at high speed allowing glass beads to collide with the cells. These collisions break the cellular wall of the *E. coli* cells allowing for the release of intracellular products [69], [74], [75]. After an optimal residence time (to promote maximum lysis), cellular debris along with the released products are passed through a filter that separates the beads from the lysate by retaining the beads within the bead milling chamber. Figure 2-7 provides a visualization of the continuous bead milling design [76].

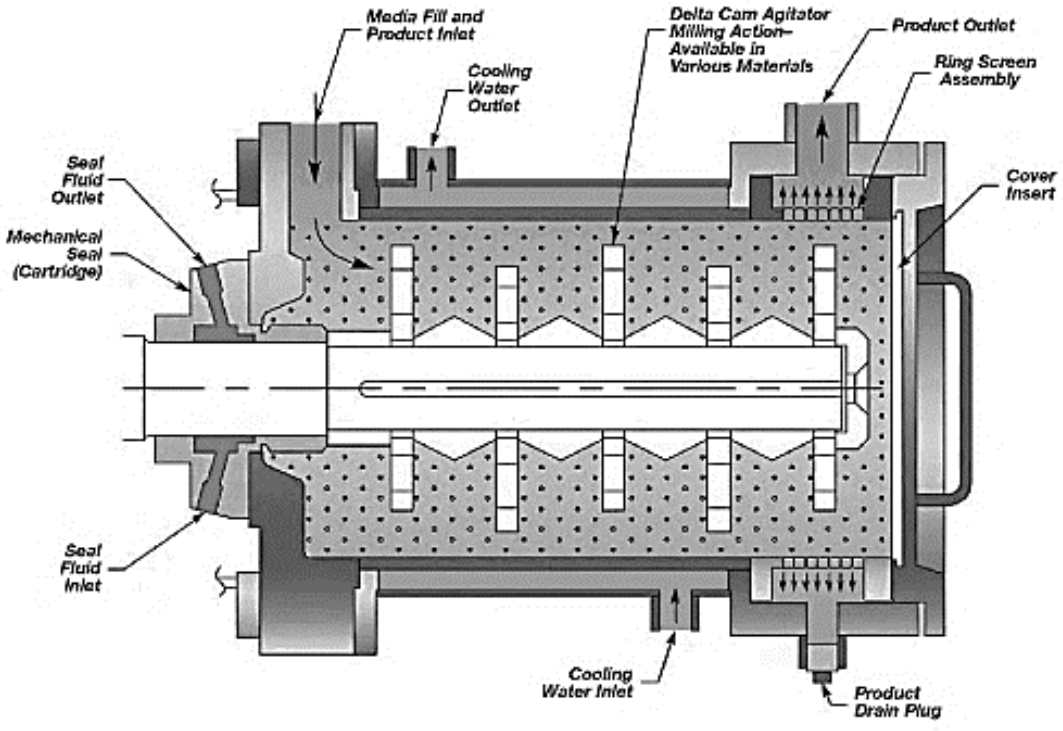

Figure 2-7: Continuous bead milling schematic diagram [77].

There have been very few experiments involving the lysis of *E. coli* cells via bead milling for the purpose of recovering intracellular products. Most bead milling experimentation has been performed on yeast cells [78]. However, based on the limited experiments performed, there are a few key variables that can be adjusted to affect the amount of lysis occurring when using this approach with *E. coli* cells. These variables include the residence time of the cell suspension through the bead milling apparatus, the speed of the impeller, the size of the glass beads and the amount of time lysing the cells.

It has been shown that the amount of cell lysis and protein release was proportional to the residence time in the bead milling apparatus [69], [74] and to the impeller speed [74]. An increase in the impeller speed mixes the cell/bead suspension at higher rates which enables the glass beads to collide with the cells at a higher velocity. Higher velocity collisions lead to higher amounts of cell damage and larger amounts of protein release.

Glass bead sizes were also tested to determine if they would have significant impact on the amount of protein release. Multiple sizes of beads were tested in the range of 0.25 mm to 1.25 mm [69]. It was determined that the optimal bead size needed to release to highest amount of aspartase from *E. coli* K-12 was 0.75 mm. It is hypothesized that smaller beads are too light and fluidize with the slurry causing minimal impact with the cells. In regards to larger bead sizes, it is hypothesized that they are too heavy so they are unable to reach a high enough velocity for cellular lysis [69].

The beads to cell suspension ratio also has a significant impact on the amount of cellular lysis. In a study of the release of aspartase from *E. coli,* it was seen that the amount of beads required to achieve the maximum amount of cellular lysis was 70 % of the total volume of the chamber [69]. For *E. coli* lysing experiments where a bead volume of less than 70 % was used, it

was determined that an insufficient number of collisions between glass beads and cells occurred, preventing maximum lysis. For experimentation above a volume of 70 %, the impeller was unable to move the slurry at high enough speeds to ensure high enough velocities required for maximum lysis [69].

The cellular concentration of cell suspensions has been determined to have no effect on the efficiency of protein release from *E. coli* cells during lysis through bead milling [69]. This was seen during the release of aspartase from *E. coli* where varying cell concentrations from 50 to 250 mg/mL had no impact on cell lysis [69]. This is an important characteristic of the system since there is no change in efficiency as the cell concentration is increased, increasing the cellular concentration will save on power needed to lyse cells and reduce the volume of the outlet stream reducing downstream costs.

It was also determined that under some conditions of bead milling, the protein of interest may end up remaining in the insoluble phase after centrifugation instead of ending up in the soluble phase where it can be easily separated [75]. In the experiment where this was documented, the size of glass beads that were used ranged between 0.3-0.4 mm and the lysis was performed for 20 min [75]. In comparison, high pressure homogenization of the same cells resulted in the protein of interest to be found in the soluble phase instead [75]. There can be two explanations for this. The first explanation would be that the conditions that were used for bead milling were not adequate to properly release the protein of interest from the cells. The second explanation could be that the protein of interest attaches to the membrane of the cell. High pressure homogenization is known to micronize cellular debris, thus being able to remove the protein of interest from its attachment to the cell wall [57]. Therefore, if one is trying to isolate a membrane associated protein the use of bead milling may not be used effectively in this case.

Pre-treatments can also be utilized to increase the amount of lysis seen in bead milling of *E. coli.* Freezing and thawing the *E. coli* cells prior to lysis through bead milling leads to an increase in the amount of cellular lysis and protein release [79]. Two freezing temperatures for *E. coli* pre-treatment have been tested: -4°C and -20°C. It was determined that the damage incurred by cells that were frozen at -4°C was greater than the damage incurred at -20°C [79]. Ice crystals are able to form within the *E. coli* cells under higher freezing temperatures since the cells (and surrounding buffer) freeze at a slower rate. This formation of ice crystals leads to cellular damage enabling a higher amount of cellular lysis after bead milling compared to cells that have not been pre-treated through freezing [79]. Due to increased efficiency through pre-treatment, energy can then be saved during the bead milling process.

2.5 Quantification of the extent of lysis

In this section, various techniques used for the quantification of the extent of cellular lysis are described. The various quantification techniques can be categorized into two distinct forms: direct cellular lysis and indirect cellular lysis. Direct cellular lysis quantification involves observing and quantifying the amount of cellular lysis by performing techniques that are directly related to the cell itself. In contrast, indirect cellular lysis quantification depends upon the quantification of intracellular material such as proteins in order to indirectly determine the amount of cellular lysis that has occurred during the experiment.

2.5.1 Direct cellular analysis

This section briefly describes the lysis quantification techniques that examine the viability of cells following lysis experiments.

2.5.1.1 Microscopic cell count

One method to determine the extent of lysis that has occurred during a lysis experiment is to observe the cell lysate sample under a microscope. A phase-contrast microscope can be used to determine whether or not the cellular structure of individual cells has been compromised. By measuring and comparing the ratio of compromised cells to surviving cells in a lysed sample with the same ratio prevailing in an original sample, the extent of lysis achieved during an experiment can be determined. However, this method can be time-consuming and cumbersome [65].

2.5.1.2 Colony forming units (CFUs)

Another method to directly determine the extent of lysis consists in performing a plate count. Samples are withdrawn from the cellular lysate, serially diluted in buffer then spread on agar plates in order to form colonies. Each colony produced is considered to be a colony forming unit (CFU) which can be directly linked to the amount of lysis that has occurred during the experiment. A lower amount of CFUs indicates a larger extent of lysis and, as a result, a more efficient lysis method. This method is considered time consuming since agar plates must be prepared and inoculated plates must incubate a relatively long time to allow for the growth of the CFUs [65].

2.5.1.3 Optical density at 600 nm

It has been determined that the extent of lysis of *E. coli* cells can be determined by the optical density of the suspension at 600 nm. Therefore, one can determine the extent of lysis by measuring the OD of the cellular suspension at 600 nm prior to the lysis experiment and compare it to the OD (at 600 nm) of the cellular suspension after the lysis experiment. This method can provide a direct quantitative analysis to determine the concentration of viable cells remaining after lysis.[65].

2.5.2 Indirect analysis (quantification of cellular products)

The indirect forms of cellular lysis quantification are based on the determination of the amount of cellular products that are released and separated from *E. coli* cellular constituents. Typically, the quantification is performed on the soluble phase of a supernatant after centrifuging the lysed samples. The amount of released cellular products is then compared to the amount in the soluble phase of the same *E. coli* sample prior to lysis. The difference in protein content between the lysed and unlysed samples provides the extent of the lysis that has occurred. By using these methods of quantification it is assumed that the amount of released cellular products is proportional to the amount of cellular lysis that has occurred.

2.5.2.1 Biuret reaction

One method to measure the amount of proteins that has been released from *E. coli* is the Biuret reaction where a strong-alkaline copper reagent is reacted with the peptide chain of the protein to produce a purple color. The absorbance measurement is performed at 310 nm, i.e. at the near-ultraviolet wavelength. This method is not widely used because of its low sensitivity. To obtain an acceptable quantification, many milligrams of a sample are required [80], [81].

2.5.2.2 UV absorbance of protein at 280 nm

Measuring the ultraviolet absorbance of a protein sample is another way to quantify the amount of proteins in a sample. At 280 nm, proteins absorb in the UV range because of their tyrosine and tryptophan residues. For a sample containing a mixture of proteins, this method can only provide a rough estimate of protein concentration since the amount of tyrosine and tryptophan is variable from one protein to another. This affects the extinction coefficient that is used to calculate the amount of proteins. For tyrosine and tryptophan-enriched proteins, the extinction coefficient can be as high as 2.65 while samples with proteins with no tyrosine or tryptophan would

have extinction coefficients of 0. Therefore, it is best to use this technique to quantify purified proteins of known extinction coefficients. Care must be taken as well to ensure that the solvent does not interfere with the absorbance [81], [82].

2.5.2.3 UV absorbance at 205 nm

In comparison to taking the absorbance of a protein sample at 280 nm, taking the absorption of the sample in the far-UV range (192-220 nm) can be considered to be more sensitive because it is much less affected by the amino acid composition of proteins. Table 2-1 provides an example of the approximate extinction coefficient for 1 mg /mL protein solutions in the range of 192-220 nm.

Table 2-1: Approximate extinction coefficients for 1 mg/mL protein solutions over the 192-220 nm range [81].

Wavelength (nm)	Extinction Coefficient
220	11
215	15
210	20
206	29
205	31
200	45
192	60

It has been determined that the most consistent analysis is obtained for absorption performed at 205 nm wavelength with extinction coefficients in the range of 28.5-33. Equation (2.1) provides a correlation to calculate the extinction coefficient at 205 nm with higher accuracy. The absorbance of the protein solution at 280 nm is also required in order to correct for the tyrosine

and tryptophan content. This equation allows for the extinction coefficient at 205 nm to be predicted with an accuracy of less than 2 % error [83].

$$E_{205}^{1\ mg/mL} = 27.0 + 120 * \frac{A_{280}}{A_{205}} \quad (2.1)$$

The absorbance of the supernatant of a lysate sample can be measured before and after lysis to determine the extent of lysis based on the increase in protein content found in the supernatant (soluble) phase [81].

2.5.2.4 Absorbance of proteins at 595 nm (using Bradford Reagent)

For this method, Coomassie Blue G-250 is dissolved in perchloric acid leading to a red-brown colour (Bradford Reagent). When a protein sample is added to this solution, the colour of the solution turns blue and the absorbance measurement for the quantification of proteins is performed at a wavelength of 595 nm. This method of protein quantification has been found to be sensitive, fast and accurate. The extent of lysis can be determined by quantifying the amount of proteins found in the supernatant of a lysate sample taken before and after lysis [81], [84]–[86].

2.5.2.5 Absorbance at 725 nm using Biuret Reagent and Folin Reagent (Lowry Method)

Another method to quantify proteins is the Lowry method which uses two types of reactions. The first reaction uses the same copper reaction as in the Biuret method, while the second reaction relies on the Folin-Ciocalteau reagent. The Folin-Ciocalteau reagent reacts with the phenols in proteins such as tyrosine. Together, the two reactions create a strong dark blue color when they are contacted with proteins. The sensitivity of this method is quite good providing a measureable colour with only 0.1 mg/mL of proteins. This method allows for the dilution of protein

samples which ensure that some components that are required for protein purification do not interfere with the quantification analysis of the proteins [81], [87]–[89].

2.5.2.6 Bicinchoninic acid (BCA) assay

The bicinchoninic acid (BCA) assay is used to quantify protein samples through the conversion of Cu^{2+} to Cu^{+} [90], [91]. Cu^{2+} can be reduced by either cysteine, cystine, tryptophan, tyrosine or the peptide bond [90]. BCA is then reacted with the resulting Cu^{+} in order to form an intense purple colour who absorbance can be quantified at 562 nm [91]. If the reaction is performed at 37°C, tryptophan, tyrosine and peptide bonds will not be fully oxidized [90]. Raising the temperature of the reaction to 60°C increases the oxidization of these three groups which increases the overall sensitivity of this method [90]. The BCA assay is not affected by detergents and denaturing agents such as urea and guanidinium chloride. However, this assay is sensitive to the presence of reducing sugars [91].

2.5.2.7 Enzymatic assays

A very popular method to determine the efficiency of lysis of *E. coli* cells for the recovery of intracellular products is enzymatic assays [92]. An intracellular enzyme is selected and its activity is quantified prior to and after a lysis experiment. An increase in enzymatic activity is considered to be proportional to the extent of lysis achieved during the lysis experiment. Care must be taken to ensure an excess of substrate in order to avoid the enzymatic activity plateauing at a low activity level. In addition, the solvent must not contain any enzymatic inhibitors that could possibly reduce the activity level of the enzyme.

2.5.2.8 SDS-PAGE quantification

In the SDS-PAGE (Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis) quantification method, protein samples are run on a polyacrylamide gel subjected to an electrical voltage. Proteins are separated vertically along the gel based on their molecular weight [93]. Proteins with lower molecular weights migrate faster through the gel and are found at the lower part of the polyacrylamide gel whereas larger proteins remain near the top of the gel [93]. After migration, the gels are stained with either silver or Coomassie blue type stain in order to visualize the proteins contained within the gel. Once the staining process is completed, the amount of proteins can be quantified based on the intensity of each protein band using computer software such as ImageJ [94].

To determine the amount of total lysis that has occurred throughout an experiment, a sample containing the total amount of proteins in a lysate is run on the gel as well as a sample of the supernatant of the lysate (soluble phase). The extent of lysis is determined by calculating the ratio of the amount of proteins found in the soluble phase to the amount of proteins found in the total lysate sample. The greater the amount of proteins released to the soluble phase, the greater the amount of cellular lysis.

2.5.2.9 Western Blot Analysis

Western blot analysis can also be used to quantify the amount of lysis. Western blots are utilized to determine the presence of a specific protein of interest [95]. The protein samples are run on the same type of gel used for SDS-PAGE. The migrated proteins are then transferred to a membrane where an antibody is added which binds to an epitope on the protein of interest. The antibody is conjugated to an enzyme that is able to be visualized with the addition of a substrate. Through this process, the protein of interest is able to be visualized [95]. A sample containing the

total amount of proteins in a lysate is run on the gel as well as a sample of the supernatant of the lysate (soluble phase). The extent of lysis is determined by calculating the ratio of the amount of proteins found in the soluble phase to the amount of proteins found in the total lysate sample. The greater the amount of proteins released to the soluble phase, the greater the amount of cellular lysis. However, for this method, the protein of interest would need to be an intracellular protein in order to be able to indirectly determine the amount of total lysis. If the protein of interest only localizes in the periplasmic space of the *E. coli* cells, then the amount of cellular lysis that could be comfortably stated could only be the amount of periplasmic lysis.

2.6 Conclusion

Sexing of sperm through the use of antibodies can possibly become a viable alternative to the technique currently used (flow cytometry). Using pentamerised single-domain antibodies (sdAbs) for this purpose provides a protein that has 1) high avidity, 2) high solubility, 3) high thermal stability, 4) resistance to proteases and 4) high production in prokaryotes. If the pentamerised sdAb is not able to be secreted to the extracellular surroundings, then a method of lysis is required for its release and recovery.

Many forms of lysis for the purpose of intracellular protein release have been discussed. These methods have included 1) chemical forms of lysis (Triton X-100, EDTA, GHCl etc.) 2) enzymatic forms of lysis (lysozymes) and finally 3) mechanical forms of lysis (high pressure homogenization, sonication and bead milling). It was seen that for each mechanism of lysis there were multiple variables that could be altered in order to achieve a greater amount of protein release from *E. coli* cells. However, it should be noted that optimal conditions for the release of protein A may not be optimal for the release of protein B. The same can be said when lysing different strains

of *E. coli* cells. Therefore, each system must be tested and optimized on its own in order to determine its ideal set of conditions for each specific protein release.

One must also consider all costs associated with each lysis mechanism in order to determine a suitable method. Costs to release specific proteins will vary depending on the system and how efficient each method is at releasing the protein of interest. Synergistic methods of lysis such as the chemical pre-treatment of cells prior to mechanical disruption should be considered in order to possibly decrease the costs of expensive chemicals and lower the costs of energy required for mechanical disruption.

Many techniques used to quantify the extent of *E. coli* lysis were also presented. A thorough analysis of the system is required as well in order to choose the correct method for quantification.

In conclusion, it can be said that the lysis of *E. coli* cells is a complex process with high variability from one system to the next. Each system must be examined thoroughly in order to choose the correct method of lysis to maximize protein release.

CHAPTER 3: METHODS AND MATERIALS

3.1 Introduction

This chapter outlines methods and materials that were used for all experiments performed during this investigation. The research presented in this thesis was performed in close collaboration with a local start-up company, Ab Biotech Inc., for the production of a protein that is targeted for sex selection of bovine calves, through the use of *E. coli* cells. The specific objective of this research project was to study the lysis of *E. coli* cells in order to enhance targeted protein release, a critical step prior to recovery in the downstream process. The fact that Ab Biotech Inc. was working on improving simultaneously several areas of the protein production resulted in a dynamic research environment to which experiments had to be adapted. The various areas upon which work was performed by the company were: 1) the development of high cell density fermentations to produce high concentrations of cellular antibodies, 2) the induction of cells to express high concentrations of cellular antibodies 3) cell lysis to release the targeted antibodies, and 4) the recovery of the antibodies from the lysate (using immobilized metal affinity chromatography (IMAC)). As a result, experiments needed to accommodate breakthroughs or setbacks occurring in the individual research sections. For example, if some changes were made to improve an upstream process, such as biomass production, the subsequent downstream processes, which included cell lysis for protein recovery were affected. Therefore, the composition of the resulting cellular material being processed in the cell lysis step was highly variable.

3.2 Protein (antibody) description

The proteins produced in this investigation with the *E. coli* cells were single-domain antibodies which have been designed by Ab Biotech Inc. Single-domain antibodies differ from prototypical antibodies where only a single antibody domain is present as opposed to the two heavy

and two light chains found with prototypical antibodies [22]–[24]. The purpose of the produced antibody was to separate male bovine sperms from female bovine sperms for gender selection of offspring. The protein binds specifically to antigens located only on the surface of male-forming sperms. In order to prepare a sexed sperm sample, the antibody is mixed with semen for a period of time that enables the male antigen/antibody binding. Nitrilotriacetic acid (NTA) magnetic nanoparticles are then added allowing for the separation of the male sperms through the coordination bond [96] between a poly-histidine tag present on the antibody and the NTA on the magnetic nanoparticles. The application of a magnetic field, such as a magnet, attracts the magnetic nanoparticles-antibody-male sperm complex. The resulting collected supernatant is now enriched in female sperms.

Two antibodies were used throughout this investigation: 14-2M and 2-3P. Both of these proteins are single-domain antibodies binding male sperms but having slightly different amino acid sequences. Furthermore, these two antibodies differ in their physical form: 14-2M is a monomer while 2-3P is a pentamer. Pentamers are actually five monomer units linked together via a verotoxin sub-unit. A visual comparison between a monomeric and pentameric antibody is schematically presented in Figure 2-2.

It has been shown in previous studies with other sdAbs that pentamerised antibodies have target antigen affinity 1 000-10 000 times greater than monomeric sdAbs [30]–[33]. Therefore, utilization of pentamers over monomers is deemed to be advantageous. However, it should be noted that the monomeric form localizes within the periplasmic space of the *E. coli* cell while the 2-3P form remains in the cytoplasmic space. The localization of the monomeric form in the periplasmic space allows for easier protein release since only one of the two cellular membranes needs to be lysed. There are some lysis techniques, such as osmotic shock lysis, which allow for

selective lysing of only the outer cell membrane in order to selectively release the proteins from the periplasmic space [11], [39].

3.3 Host organism (E. coli)

The system used by Ab Biotech Inc. for the production of their recombinant proteins relies on the gram-negative bacterium *E. coli*. *E. coli* bacteria have been chosen for the production in this case since they have the ability to be grown at high cell densities, enabling high levels of recombinant protein expression using low-cost media [11]. However, the major challenge in using *E. coli* is the fact that they cannot excrete recombinant proteins easily [11].

Excretion of recombinant proteins is highly desirable since the proteins are completely removed from the bacterium and expelled into the growth medium. Translocation of the recombinant protein into the periplasmic space of the cell can also be desirable for protein recovery. The periplasmic space contains relatively few native proteins which facilitates purification of the target protein. Furthermore, the release of the protein of interest from the periplasmic space only requires the outer cell membrane to be disrupted [11]. However, since the protein of interest is neither translocated nor excreted, both cellular membranes need to be disrupted in order to release our protein of interest.

The *E. coli* TG1 (Lucigen, Middleton, WI) expression system was used in this study. *E. coli* stocks (25 % glycerol v/v) (Fisher Scientific, Fairlawn, NJ) were prepared at an optical density of 1.5 (measured using the optical density at $\lambda = 600$ nm (OD_{600})) from a colony of *E. coli* isolated on LB Miller agar (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl, and 15 g/L agar) (Fisher Scientific, Fairlawn, NJ) and frozen at -20°C.

3.4 E. coli production

Fermentations of *E. coli* cells were conducted in flasks, for the ability to conduct numerous experiments simultaneously and in laboratory-scale bioreactors where continuous control of operating variables such as pH, temperature control, and dissolved oxygen was possible. Therefore, a variety of both of the aforementioned methods were used throughout the course of the studies. It is important to note that *E. coli* bacteria prepared for experiments outlined in Sections 3.5.1 and 3.5.3 to 3.5.5 were produced by the staff at Ab Biotech Inc. The bacteria prepared for the experiment outlined in Section 3.5.2 was produced by the author with the aid of Ab Biotech Inc. staff.

3.4.1 Fermentation performed in flasks

Pre-cultures for fermentation conducted in flasks were performed in 250-mL flasks containing 50 mL of Studier ZYM-505 medium [97], with the addition of 50 µL of 100 mg/mL aqueous ampicillin solution (Fisher Scientific, Fairlawn, NJ) , 100 µL of glycerol (Fisher Scientific, Fairlawn, NJ), and 100 µL of the -20 °C *E. coli* stock solution. A 1-L Studier ZYM-505 medium contained: 986 ml H_2O, 10 g tryptone, 5 g yeast extract, 3.55 g Na_2HPO_4 (Sigma-Aldrich, St. Louis, MI), 3.4 g KH_2PO_4 (Fisher Scientific, Fairlawn, NJ), 2.67 g NH_4Cl (Fisher Scientific, Fairlawn, NJ), 0.71 g Na_2SO_4, (Fisher Scientific, Fairlawn, NJ), 2.5 g glycerol, 0.25 g glucose (Fisher Scientific, Fairlawn, NJ) and 200 µL of trace metal solution (50 mM $FeCl_3$ (Fisher Scientific, Fairlawn, NJ), 20 mM $CaCl_2$ (Fisher Scientific, Fairlawn, NJ), 10 mM $MnCl_2$ (Fisher Scientific, Fairlawn, NJ), 10 mM $ZnSO_4$ (Fisher Scientific, Fairlawn, NJ), 2 mM $CoCl_2$ (Sigma-Aldrich, St. Louis, MI), 2 mM $CuCl_2$ (Alfa Aesar, Ward Hill, MA), 2 mM $NiSO_4$ (Fisher Scientific, Fairlawn, NJ), 2 mM Na_2MoO_4 (Sigma-Aldrich, St. Louis, MI), 2 mM H_3BO_3 (Fisher Scientific, Fairlawn, NJ) and 60 mM HCl (EMD, Gibbstown, NJ)). The pre-culture was grown overnight at

a temperature of 30°C on an orbital shaker rotating at 300 rpm (Thermo Scientific MaxQ 5000). After the overnight growth, the optical density was measured at 600 nm (OD_{600}).

Based on the pre-culture optical density (OD_{600}), a specific volume of this pre-culture was added to 1 L of buffered modified TB broth to achieve a final OD_{600} value of 0.1. A 1-L of modified TB broth contained 20 g of LB Miller (Fisher Scientific, Fairlawn, NJ), 2 g of tryptone, 19 g of yeast extract, 2.3 g of KH_2PO_4 $3H_2O$, 16.1 g of K_2HPO_4 (Fisher Scientific, Fairlawn, NJ), 8 mL of 50 % glycerol, 2 mL of $MgSO_4$ (Fisher Scientific, Fairlawn, NJ) solution (30 g/50 mL), 200 µL of trace metal solution (as described above), 1 mL of 100 mg/mL ampicillin sodium salt solution and the remaining volume required was made up of water. All solutions were autoclaved prior to the addition of the pre-culture except for the ampicillin solution which was sterilized using a 0.45 µm filter. The broth was then split into 500-mL flasks, each one containing 250 mL of broth. In order to reduce the amount of foam produced during the fermentation, 100 µL of an aqueous antifoam solution (50 % v/v of antifoam 204 (Sigma-Aldrich, Oakville, ON)) was then added to each flask.

Each flask was monitored for growth and induction was performed at an OD_{600} of 0.6 by adding 250 µL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) aqueous solution (Fisher Scientific, Fairlawn, NJ). The induced cultures were then left for 48 h in an orbital shaker (Thermo Scientific MaxQ 5000) at 30 °C and 300 rpm. The optical density of the cells was monitored as well as the pH by extracting samples from each flask 18 h and 48 h post induction.

After 48 h of growth, the broth from each flask was centrifuged at 2273 rcf (3500 rpm) for 30-45 min at 14°C (Hermle Labortechnik Gmbh Z400K) in order to separate the cells (pellet after centrifugation) from the broth (supernatant after centrifugation). The supernatant was discarded and each pellet was re-suspended in 25 mL of lysis buffer (6.06 g/L Tris Base (Fisher Scientific, Fairlawn, NJ), 1.46 g/L NaCl, pH 7.2). Phenylmethanesulfonylfluoride (PMSF) (BioBasic Inc.,

Markham, ON) solution, a serine protease inhibitor, was added to each re-suspended pellet to a final concentration of 1 mM and, each cell suspension was stored at -20°C until needed for an experiment.

3.4.2 Fermentation performed in bioreactors

3.4.2.1 Batch production

The production of *E. coli* cells in bioreactors was carried out in a 1-L Applikon bioreactor (Foster City, CA) with a working volume of 750 mL. The pre-culture of *E. coli* was prepared by inoculating 25 mL of LB Miller medium with 100 µL of thawed glycerol stock and placed on an orbital shaker at 37°C for 18 h to serve as an inoculum for the bioreactor. Bioreactors containing 750 mL of modified TB medium were inoculated with the pre-culture in order to obtain an optical density of OD_{600} = 0.1. A 1-L solution of modified TB was described in Section 3.4.1 with the following modifications: 1) 12 g of tryptone, 2) 24 g of yeast extract, 3) 10 g of NaCl, 4) 15 g of Bacto-Agar (Fisher Scientific, Fairlawn, NJ), and 5) the exclusion of LB Miller.

The culture conditions were controlled using the Applikon MyControl system. The temperature of the culture medium was kept at a constant temperature of 28°C, or as otherwise mentioned in the text, using a heating blanket and a cooling coil. The pH of the culture medium was monitored with an electrode (Applisense, Applikon) and kept constant at a pH 6.8 through the addition of 2 M sulfuric acid (Fisher Scientific, Fairlawn, NJ) to lower the pH or through the addition of 2 M NaOH (Fisher Scientific, Fairlawn, NJ) to raise the pH. The amount of dissolved oxygen in the medium was monitored using a polarographic electrode (Applisense) and kept constant at 30 % air saturation using filter sterilized compressed air (1 vvm) through a diffuser. When the flow rate of air was not enough to satisfy the oxygen demand of the culture, the controller increased the stirrer speed (2 Rushton-type blades). The stirring speed, initially set to 300 rpm at

the beginning of the fermentation, varied up to 1500 rpm throughout the fermentation process. Foaming was controlled by manual addition of 50 % v/v aqueous antifoam 204 solution.

Induction was performed 17 h into the fermentation with the addition of 10X TB induction medium in order to promote recombinant protein expression. A 1-L of 10X TB induction medium contained 120 g of tryptone, 240 g of yeast extract, 40 g of glycerol and 1 mL of 1 M IPTG solution. The fermentation was then allowed to proceed for an additional 24 h.

The *E. coli* cells were harvested by centrifuging the entire bioreactor contents in a centrifuge (Avanti J-26 XPI, Beckman Coulter) at 1342 rcf and temperature of 4°C for 1 h to pellet the bacteria. The pelleted bacteria were then separated into 5, 10 or 20 g samples in Falcon tubes and frozen at -20°C until needed for experimentation.

3.4.2.2 Fed-batch fermentations

Fed-batch fermentations were performed in the same manner as the batch fermentations outlined in Section 3.4.2.1. However, for fed-batch fermentations, a batch culture containing 400 mL of modified TB medium was grown for 20.5 h prior to induction. At 3.5 h before induction, 300 mL of aqueous feed containing 150 g/L of glucose and 15 g/L of glycerol was added continuously over a span of 3 h to achieve a final working volume of 700 mL. At induction, 10X TB induction media (as described in Section 3.4.2.1) was added until a final concentration of 1X was achieved, in order to promote recombinant protein expression. *E. coli* cells were harvested 20.5 h after induction as described in Section 3.4.2.1.

3.5 Lysis Procedures

3.5.1 Methods and materials for Section 4.1

E. coli cell cultures were grown, induced and stored as specified in Section 3.4.2.1. To initiate a lysis experiment, cells were thawed and suspended in lysis buffer (10 mM HEPES, 29.22 g/L NaCl, pH 7.4) to a final concentration of either 10 % or 20 % (w/v). During lysis experiments, 50 mL cell samples of 10 and 20 % (w/v) *E. coli* cell suspensions were fed through the Avestin Emulsiflex C5 homogenizer at a pressure of 152 MPa (22 000 psi) with a flowrate of 7 mL/min . Each sample was processed through the homogenizer for a total of 3 passes. After each pass, a 500 µL sample of the cell suspension was removed for analysis. To quantify the total protein content in the processed sample, aliquots of each sample were taken and prepared for SDS-PAGE as described in Section 3.6.1.

3.5.2 Methods and materials for Section 4.2.2

Three *E. coli* cell fermentations were performed in 1-L Applikon bioreactors (working volume of 700-750 mL). Fermentation 1 was performed in batch mode as described in Section 3.4.2.1. Fermentation 2 was performed in fed-batch mode as described in Section 3.4.2.2. Finally Fermentation 3 was conducted in fed-batch mode as described in Section 3.4.2.2 with the following modifications: 1) a pre-induction temperature of 37°C and 2) a pre-induction batch phase which lasted 16 h. To shorten the pre-induction step, the temperature was increased thereby reducing the overall fermentation time. After the fermentations were completed, cells were harvested and stored as described in Section 3.4.2.

When a lysis experiment was performed, frozen cell samples were removed, thawed, and suspended in a lysis buffer (10 mM HEPES, 29.22 g/L NaCl, pH 7.4) to achieve 20 % (v/v). EDTA was added to each sample to a final concentration of 3 mM. Each sample was milled using a

BioSpec BeadBeater in the 90-mL chamber with a cell suspension:glass bead (BioSpec 0.1 mm) volume ratio of 1:1. A polycarbonate cooling jacket surrounding the milling chamber was filled with crushed ice in order to prevent overheating of the lysate during experimentation. The BioSpec BeadBeater was operated in a discontinuous cyclic fashion: 1 min on and 1 min off to minimize the increase in temperature. The ice in the cooling jacket was replaced as needed. A lysate sample of 500 µL was taken at 0, 3, 6 and 9 min of total milling time. To analyze the total protein content in the processed sample, aliquots of each sample were taken and prepared for SDS-PAGE (as described in Section 3.6.1).

3.5.3 Methods and materials for Section 4.2.3

E. coli cell fermentations were conducted, induced and stored as specified in Section 3.4.2.2. with the following modifications: 1) cells were grown in a 7.5-L bioreactor (working volume of 5 L), 2) an initial batch volume of 4 L with 1 L of induction medium added at induction time, 3) a pre-induction temperature of 32°C, 4) a pre-induction batch phase which lasted 9.5 h, 5) 3 h prior to induction, feed solution (150 g/L glucose, 15 g/L glycerol) added continuously for 3 h and 6) a post induction phase of 12 h. Cells were lysed as described in Section 3.5.2. A lysate sample of 500 µL was taken at 0, 3, and 6 min of total milling time. To analyze the total protein content in the processed sample, aliquots of each sample were taken and prepared for SDS-PAGE electrophoresis (as described in Section 3.6.1).

3.5.4 Methods and materials for Section 4.3

E. coli cell cultures were grown, induced and stored as specified in Section 3.4.2.1. The day of the sonication experiments, cell pellets were thawed and suspended in lysis buffer (6.06 g/L Tris Base, 1.46 g/L NaCl, pH 7.2) to achieve either a 20, 40 or 60 OD cell suspension corresponding to 10 g/L, 20 g/L and 30 g/L dry weight of *E. coli* cells (where 1 OD unit ≈ 0.5 g/L

[98]). The *E. coli* cells were then lysed using the Branson Sonifier 450 sonicator. During the lysis experimentation with the sonicator, the 50 mL Falcon tube containing 25 mL of the lysate sample was kept in a mixture of ice and water; the ice-water mixture was replaced as needed throughout the experiment. Because of the amount of heat produced by sonication, experiments were performed for the desired time of lysis with a repetitive cycle of 1 minute of sonication followed with 30 s of rest to allow the lysate temperature to decrease. Therefore, a 3 min lysis experiment would consist of 3 1-min lysis cycles with 30 s of rest in between.

Four parameters were tested throughout the experiment: (1) the output power control was used over a range of 2 to 6 (on a scale of 10 where 10 corresponds to the full power of 400 W), (2) the on-off duty cycle (percentage of time the sonicator was on during the experiment) ranged from 20 to 100 %, (3) the optical density (OD) of the cell mixture was varied between 20 and 60, and (5) the total lysis time period was varied between 1 to 5 min. A total of 17 combinations of the four parameters were tested in order to determine the effect of each parameter on the lysis of *E. coli* while minimizing the number of runs. The values of each the four parameters for each run have been tabulated in Table 3-1. Each run was performed three times in order to produce triplicate runs.

Table 3-1: A breakdown of the values of each parameter during the sonication experimentation: A represents Output Control, B represents Duty Cycle, C represents OD and D represents Time (min).

Run	**A**	**B**	**C**	**D**
1	2	20	20	1
2	2	20	20	5
3	2	20	60	1
4	2	20	60	5
5	2	100	20	1
6	2	100	20	5
7	2	100	60	1
8	2	100	60	5
9	6	20	20	1
10	6	20	20	5
11	6	20	60	1
12	6	20	60	5
13	6	100	20	1
14	6	100	20	5
15	6	100	60	1
16	6	100	60	5
17	4	60	40	3

To analyze the total protein content in the processed lysate, 500 µL aliquots of each lysate were taken and prepared for SDS-PAGE electrophoresis (as described in Section 3.6.1).

3.5.5 Methods and materials for Section 4.4

E. coli cell cultures were grown, induced and stored as specified in Section 3.5.3. To perform a lysis experiment, the cells were then thawed and suspended in lysis buffer (10 mM HEPES, 29.22 g/L NaCl, pH 7.4) to a final concentration of 20 % (w/v). Cells were then either lysed solely using lysozymes (1350 U per g of wet cell paste) or lysed using a combination of

lysozymes (1350 U per g of wet cell paste) and Triton X-100 (1 % v/v final concentration). Cell lysates were then incubated for 30-60 min on a rotary shaker at 250 RPM and room temperature (22°C) until the suspension became viscous (an indication of cell lysis due to the release of DNA). To lower the lysate viscosity, 1-2 U of DNase 1 (Thermo Scientific, Rockford, IL) was added to break down nucleic acids. The lysate was further incubated for an additional 30-60 min under the previous conditions. To analyze the total protein content in the processed lysate, 500 µL aliquots of each lysate were taken and prepared for SDS-PAGE (as described in Section 3.6.1).

3.6 SDS-PAGE

3.6.1 SDS-PAGE procedure

For samples loaded on a gel for SDS-PAGE, 50 % (v/v) of the sample is made up of 2X sample buffer prepared as follows: 5.0 mL deionized water, 2.5 mL 0.5 M Tris-HCl (pH 6.8) (Fisher Scientific, Fairlawn, NJ), 2.5 mL of 80 % (v/v) glycerol in water, 7 mg bromophenol blue (Fisher Biotechnology, Fairlawn NJ), 0.4 g sodium dodecyl sulfate (SDS) (Fisher Scientific, Fairlawn, NJ) and 0.31 g dithiothreitol (DTT) (Fisher Scientific, Fairlawn, NJ)). Depending on the lysate sample dilution required, the remainder of the sample was made up of the lysate sample and 1X PBS. A 1-L of 1X PBS contains 8 g NaCl, 0.2 g KCl (Fisher Scientific, Fairlawn, NJ), 1.4 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1 L of dH_2O. For example, a 4X lysate dilution would contain 25 % lysate sample, 25 % PBS and 50 % sample buffer (by volume). The sample now containing the lysate, sample buffer and PBS were placed in a water bath at ~ 90°C for at least 3 min. After incubation in the water bath, the samples were ready to be run on a gel.

For each of the samples, 10 µL were loaded onto the wells on the gel. Depending on the gel, 3 µL of a molecular weight ladder (Precision Plus Protein Dual Colour Standards, Bio-Rad) was added to one lane on the gel while a series of standards containing between 150 ng to 1000 ng

of Bovine Serum Albumin (BSA) (Thermo Scientific, Rockford, IL) were also loaded onto the gel. The molecular weight ladder enables the identification of proteins run on the gel based upon their molecular weight. Since the amount of BSA in each of the standards is known, a standard curve can be obtained for each gel and used to quantify other proteins via densitometry measurement using ImageJ software [94].

After each sample was loaded onto the gels, the gel holder and tank were filled with 1X SDS-PAGE Running Buffer. 1X SDS-PAGE Running Buffer contains 3.03 g of Tris base, 14.4 g of glycine (Fisher Scientific, Fairlawn, NJ), 1 g of SDS, a pH of 8.3 (adjusted using HCl) in a final volume of 1 L (completed to final volume using deionized water). The gels were then run for up to 1 h using a voltage that ranged from 120-180 V (Bio-Rad Mini-Protein Tetra Cell, 4-Gel System).

Following the protein migration, the gels were placed in microwave-safe containers filled with 100 mL of tap water and microwaved for 55 s at 900 W (Salton). The washing step was repeated two more times for a total of three washes in order to remove SDS from the gels. After the third wash, water was discarded and the gel was stained with 10 mL of PAGE-Blue Protein Staining Solution (Thermo Scientific, Rockford, IL) and microwaved for 7 s three times while ensuring that the solution was mixed effectively in between each successive microwave run. The gel was then placed on a rocking platform (Lab Rotator Model: 2304, Thermo Scientific) for 20-30 min. The PAGE-Blue solution was then recovered and the gel was re-suspended in deionized water and placed on a rocking platform for 15 min. After 15 min, the water was discarded and replaced with fresh water. The gel was left overnight and then scanned (Gel Doc Imager system, Bio-Rad) using the Coomassie Blue setting.

3.6.2 SDS-PAGE analysis

In order to determine the extent of lysis that had occurred during an experiment, an assumption was made that the amount of fractional lysis that had occurred is directly proportional to the fractional amount of protein that had been released into the soluble phase from the *E. coli* cells. For this assupmtion to hold true it was assumed that there was no degredation of proteins occuring during the experiment and that lysis of cells results in protein release.

The fractional lysis observed in each experiment was determined by taking the pixel density of all proteins in the soluble phase (e.g. lane S1 of the gel in Figure 3-1) and the total phase (e.g. lane T1 of the gel in Figure 3-1) separately and then dividing the two quantities as shown in Equation (3.1)). ImageJ software enables the user to quantify the amount of protein that has been run on a gel through densitometry. ImageJ can be accessed and downloaded at http://imagej.nih.gov/ij/download.html. Please note that Equation (3.1) is used for all ImageJ analysis throughout this thesis.

$$\text{Fractional lysis} = \frac{\sum \text{Pixel Density of proteins in soluble lane "S"}}{\sum \text{Pixel Density of proteins in total lane "T"}} \qquad (3.1)$$

Figure 3-1 provides an illustrative example of the calculation performed using Equation (3.1). In order to calculate the fractional lysis for sample 1, the sum of the pixel densities of the bands in S1 (soluble protein portion of sample 1 illustrated by the dashed box) is divided by the sum of the pixel densites of the bands in T1 (total protein of sample 1 illustrated by the solid box). In order to determine the fractional lysis for sample 2 the sum of the pixel densities for S2 and T2 would be used in the above calculation instead.

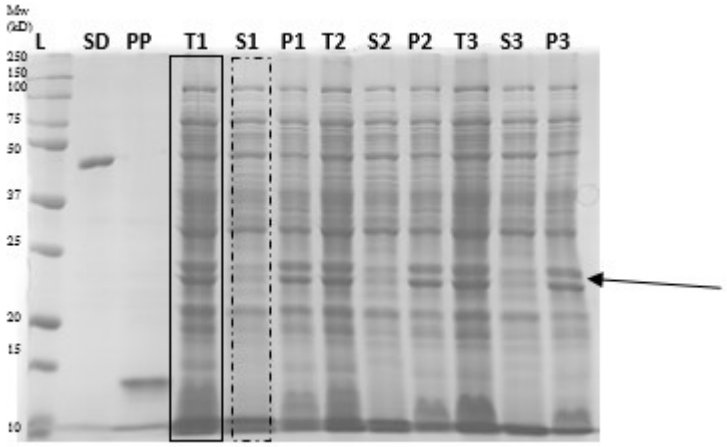

Figure 3-1: A typical SDS-PAGE of *E. coli* proteins. L denotes "protein ladder", SD denotes a BSA standard, PP denotes the purified protein of interest, T denotes total protein fraction, S denotes soluble protein fraction, P denotes insoluble protein fraction. The proteins enclosed within the solid box represent the "total" proteins for sample 1 while the proteins enclosed within the dashed box represent soluble proteins for sample 1. Proteins indicated by the arrow are removed from the quantification analysis. Numerical values denote the cell lysate sample.

It should also be noted that not all of the proteins displayed on each of the gels were included in the analysis for determining the amount of fractional lysis. Figure 3-1 provides an illustrative example of such a case. For all gels that were analysed, it was observed that a set of proteins of c.a. 23-24 kDa (indicated by the arrow in Figure 3-1) did not shift from the total phase to the soluble phase at a rate comparable to that of the other proteins as cells underwent lysis. It is hypothesised that these proteins may be membrane-associated and that they remain attached to the membrane of the *E. coli* cell after lysis and therefore would only be seen in the insoluble phase after centrifugation. Because of this, these proteins cannot accurately represent the extent at which lysis occurs. For this reason, they have been omitted from the calculation of fractional lysis for all gels shown in future sections in order to maintain consistency.

3.7 Western blot analysis

Western blots were performed over the course of experimentation to determine the presence of a specific protein of interest [95]. The protein samples are treated and processed as

they would be for SDS-PAGE. The migrated proteins on the gel are then transferred to a membrane where an antibody is added which binds to an epitope on the protein of interest. The antibody is conjugated to an enzyme that is able to be visualized with the addition of a substrate.

The first step in the process was to prepare and run protein samples on a gel as described in Section 3.6.1. After protein migration, the gel was washed for 15 min in deionized water. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Pall Corporation, East Hills, NY) under 350 mA for 60 min while submerged in a Transfer buffer prepared by mixing 100 mL methanol (VWR BDH Chemicals, Westchester PA), 100 mL of 100 mM CAPS (Sigma Aldrich, Oakville, ON) solution and 800 mL of deionized water per litre of buffer solution.

After transferring the proteins from the gel to the membrane, the membrane was put into a container and washed with TBS-T on a rocking platform for 10 min. TBS-T contains 100 mL of TBS 10X (6.05 g Tris Base, 8.76 g NaCl in 1 L of H_2O, pH adjusted to 7.5 with 1 M HCl), 900 mL of deionized water and 1 mL of 50 % aqueous Tween 20 (Fisher Scientific, Fairlawn, NJ) for each litre of solution. After 10 min, the TBS-T was drained and replaced with blocking solution. The blocking solution contains 100 mL of TBS-T 1X and 1 g of BSA (Fisher Scientific, Fairlawn, NJ) per 100 mL of blocking solution. The membrane was then left to incubate in the blocking solution at room temperature for at least 45 min on a rocking platform.

The blocking solution was removed and the membrane was washed three times for 5 min with TBS-T on a rocking platform. After the third wash, buffer was drained and 2 µL of 1 % Anti-HIS horseradish peroxidase (HRP) antibody (Bethel Rabbit anti-6xHIS-HRP Conjugated, Cedarlane) and 20 mL of blocking buffer were added to the membrane which was then placed on a rocking platform. After 45 min of incubation, the antibody solution was recovered and the membrane was washed three times for 5 min with TBS-T while on a rocking platform.

Next, the membrane was exposed to Western Blotting Substrate which was prepared using a Bio-Rad Conjugate Substrate Kit (#170-6431). For each membrane, the Western Blotting Substrate was composed of 500 µL of HRP CDB (Bio-Rad), 4500 µL of deionized water, 30 µL of HRP color reagent B (Bio-Rad), and 1 mL of HRP color reagent A (Bio-Rad). The substrate was allowed to react with the membrane for a maximum of 45 min until bands formed. The membrane was then washed 1-2 times with deionized water in a container on a rocking platform for 10 min to stop the colorimetric reaction. The membrane was scanned using a Bio-Rad Gel Doc system.

It should be noted that western blots were used in order to detect the presence of the protein of interest in each fermentation. Figure 3-2 provides an example of an SDS-PAGE image and a corresponding western blot (performed for the protein of interest) of a lysis experiment. As can be seen in Figure 3-2, a western blot is required to ensure that there is indeed the protein of interest in this run since the protein of interest cannot be seen with certainty in pane A of Figure 3-2.

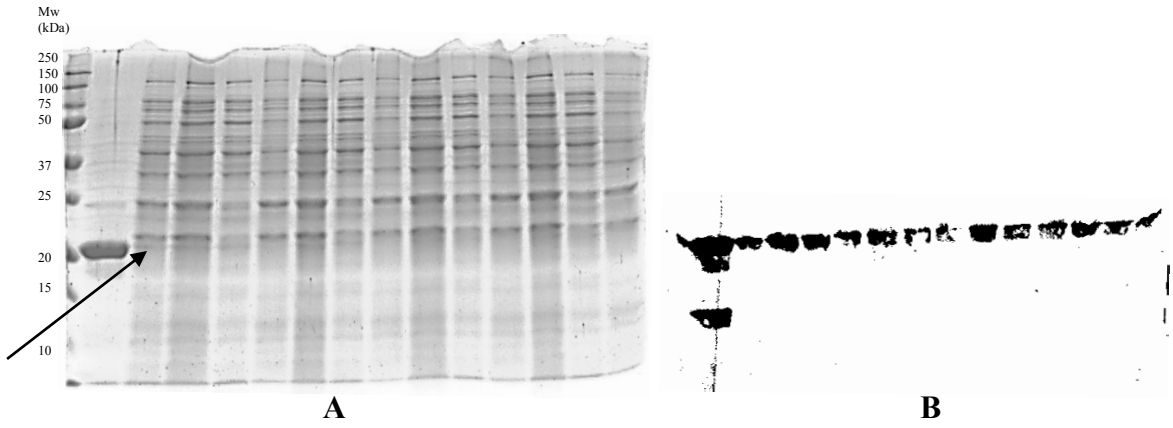

Figure 3-2: An example of results obtained for SDS-PAGE (A) and western blot (B) for a lysis experiment. In pane A, the arrow indicates the location of the protein of interest. Pane B illustrates a western blot performed for the protein of interest.

The amount of protein of interest released is considered to be proportional to the amount of total protein released. This can be verified by comparing the relative intensities of the western blot in pane B of Figure 3-2 to the intensities of proteins found in the SDS-PAGE of pane A of Figure 3-2. It should be emphasised that identical samples were loaded for both figures. It can be seen that the pattern of intensities from lane to lane is identical between the proteins found in pane A and the protein of interest illustrated in pane B. This identical pattern indicates the proportionality of intensity between the protein of interest and the amount of protein within each sample. Therefore, the amount of release of the protein of interest can be inferred based upon the total release of proteins. Western blots were not used to quantify the amount of fractional lysis which occurred during experimentation since they would be too costly and time consuming to produce for each run. Therefore, the quantification of total fractional lysis was performed using the results obtained from SDS-PAGE. Consequently, western blots are not included in future sections.

CHAPTER 4: EVALUATION OF LYSIS TECHNIQUES

The following chapter describes the experiments and analysis performed to determine the optimal lysis technique for the release of proteins from *E. coli* cells. The lysis methods that were examined in this investigation include: 1) high pressure homogenization (Section 4.1), 2) bead milling (Section 4.2), 3) sonication (Section 4.3), and 4) synergistic lysis using lysozymes and Triton X-100 (Section 4.4).

4.1 High pressure homogenization

One method, used industrially, for cell lysis is high pressure homogenization. This lysis method can process up to 50 000 L/h of cell suspension at 7 MPa (21 000 L/h at 25 MPa) (based on the Gaulin 185Q with poppet type valves from APV Homogenizers [99]). In this section, the ability and efficiency of a high pressure homogenizer for *E. coli* cell lysis were studied. High pressure homogenization, also known as French pressure cell press or simply French press, is considered to be an excellent method for cellular disruption [39]. High pressure homogenizers are able to disrupt *E. coli* cells by forcing the cells through a very small orifice via a very high differential pressure. The cell lysates are thereby exposed to an extremely high shear rate and, as a result, cell disruption and lysis occur. Since *E. coli* cells are classified as gram-negative bacteria, they are easier to lyse through high pressure homogenization compared to gram-positive bacteria due to their thicker peptidoglycan layer [55].

In this investigation, experiments were performed with an Emulsiflex C5 (Avestin Inc., Ottawa, Canada) high pressure homogenizer to determine the amount of lysis that could be achieved with equipment that is considered to be the industry benchmark for lysis efficiency. This

allowed for a lysis baseline to be set and used as a comparison between the different lysis techniques for *E. coli* cells. As cell lysis is proportional with the number of passes through the high pressure homogenizer [57], a series of experiments were performed to determine the extent of lysis as well as the potential negative shearing effect on released proteins resulting from several passes through the apparatus.

4.1.1 Results and discussion

Each sample of the lysis experiments was analysed to determine their protein content using SDS-PAGE. A typical gel obtained from SDS-PAGE analysis is shown on Figure 4-1. The "total", "soluble" and "pellet" samples for each successive pass through the homogenizer were run on the gel.

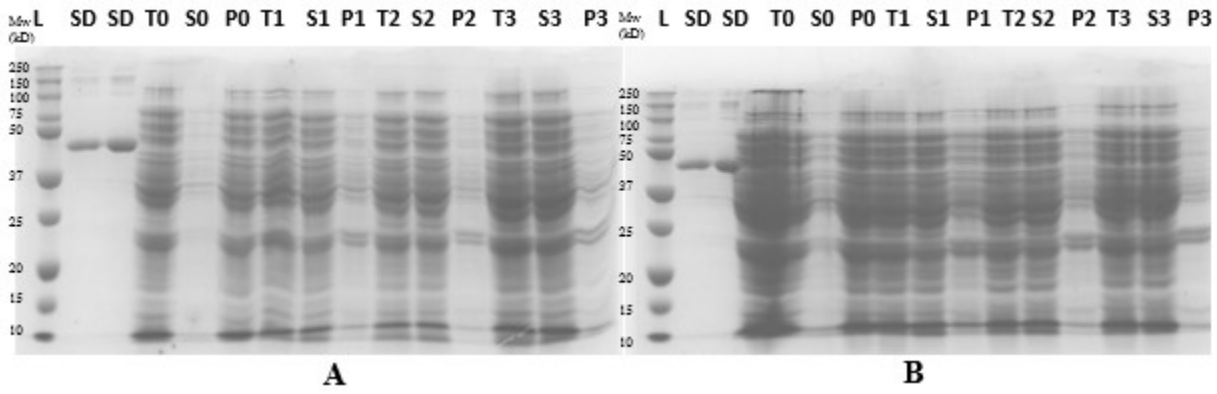

Figure 4-1: SDS-PAGE of *E. coli* proteins. *E. coli* cells were grown in a 1-L bioreactor (750 mL working volume) with a pre-induction and post-induction temperature of 28°C. A 50 mL cell suspension was lysed using an Avestin Emulsiflex C5 high pressure homogenizer for 3 passes at 7 mL/min. L denotes "protein ladder", SD denotes a BSA standard, T denotes total protein, S denotes soluble protein, and P denotes insoluble protein. Numerical values indicate the number of passes through the high pressure homogenizer for that particular sample. Pane A represents a 10 % (w/v) *E. coli* run. Pane B represents a 20 % (w/v) *E. coli* run. Both runs were performed at a 10X dilution on each gel. Please note that the antibody of interest is not displayed in these gels due to the fact that the runs completed above were only performed to screen for a visualization of the total amount of *E. coli* lysis.

Figure 4-1 shows that after a single pass for both the 10 % and 20 % (w/v) samples, almost 100 % of the available proteins have been released from the cells. This is inferred since the lanes containing the soluble proteins, S1, from both samples after the first pass are almost identical in

intensity when compared to the lane that contains the total amount of proteins, T1. It can also be noted that the intensity of the insoluble phase (pellet in lane P1) decreases substantially after one pass indicating that lysis is occurring; the proteins are shifting from the insoluble phase (proteins trapped within the cell) to the soluble phase (proteins released into the extracellular environment). The amount of proteins depicted in Figure 4-1 was quantified and analyzed using ImageJ analysis as described in Section 3.6.1. The results of the analysis can be seen in Figure 4-2. It should be noted that the fractional lysis of the 10 % (w/v) sample after 2 passes was greater than 1 which is not possible. This situation may have occurred due to the inherent error of the quantification method compounded by the fact that the intensities of the two quantified lanes (S2 and T2 in pane A of Figure 4-1) are visually almost identical.

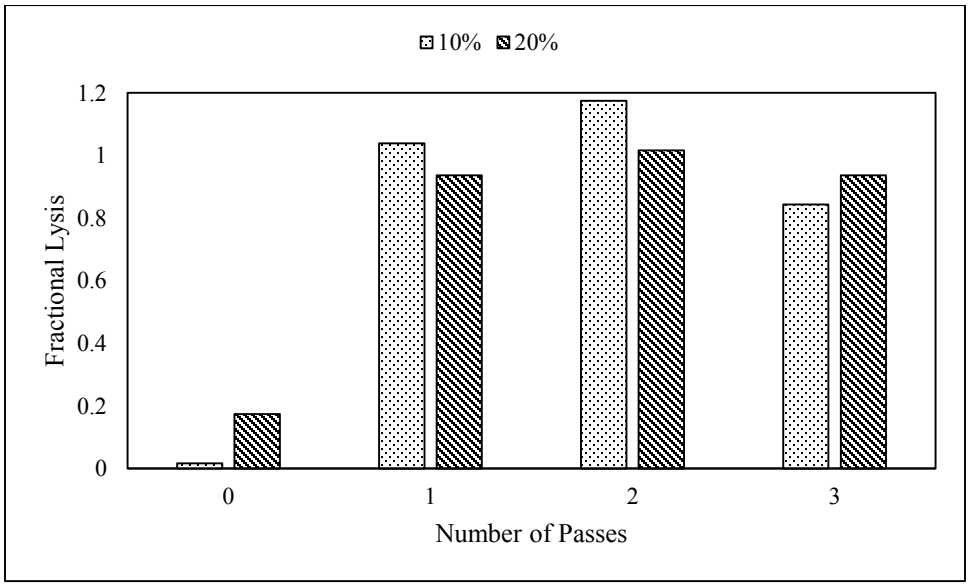

Figure 4-2: Fractional release of proteins from *E. coli* cells based on the number of passes through an Avestin Emulsiflex C5 high pressure homogenizer at 7 mL/min. One run for each cellular concentration (10 % (w/v) and 20 % (w/v)) was performed and compared.

In literature, it has been shown that an increase in the number of passes of an *E. coli* suspension through a high pressure homogenizer leads to an increase in the amount of lysis and therefore an increase in the amount of proteins released [57]. However, it can be seen from Figure

4-2 that total cell lysis seems to occur and that nearly all of the proteins are released from the cells during the first pass through the high pressure homogenizer. Based on this observation, higher density cell suspensions, higher flow rates and lower pressures could be tested to observe if a high amount of lysis would still be maintained. This would allow for the lysis of a greater amount of *E. coli* cells over a shorter period of time at a reduced operating cost.

4.1.2 Summary

It can be concluded that for cell suspensions at 10 % and 20 % (w/v), nearly 100 % of the proteins that are located within the *E. coli* cells are released after one pass through the Avestin Emulsiflex C5 high pressure homogenizer at a flow rate of 7 mL/min and pressure of 152 MPa. Therefore, subsequent passes were not needed to obtain a maximum amount of soluble proteins. Unfortunately, due to the lack of availability of the high pressure homogenizer, only one set of experiments was performed. Other variables that could potentially be tested in the future would be a wider range of cell concentrations of the *E. coli* cell suspension, the flowrate, or homogenizers that contain a differently shaped impact ring. Furthermore, to reduce processing time of larger sample volume, higher capacity units can be used. The yield of fractional lysis could also be improved by pre-treating the *E. coli* cells either chemically or enzymatically in order to weaken or compromise the cellular structure prior to high pressure homogenization [61].

4.2 Bead milling

The following section investigates *E. coli* lysis using bead milling. During bead milling, a cell suspension is mixed at high speeds with glass beads. With the vivid agitation, the glass beads collide with the cells causing lysis and the release of proteins. For the series of bead milling experiments, a 90-mL BioSpec BeadBeater was used to test: 1) the extent of lysis using a laboratory batch-scale bead milling, 2) the effect of lysing cells sampled during the exponential

and stationary growth phases, 3) the required lysis duration, and 4) the effect of the size of the glass beads.

4.2.1 Bead milling: effect of bead to lysate ratio

It has been determined in literature that the bead to lysate ratio of a bead milling experiment has a significant impact on the amount of cellular lysis that can occur. It has been found that in order to achieve a maximum amount of cellular lysis, 70 % of the entire chamber volume must be occupied by glass beads [69]. However, during preliminary testing with a 90-mL BioSpec BeadBeater with 70 % glass bead loading, the apparatus was not able to impart sufficient velocity to the glass beads to cause cell lysis. Therefore, the bead loading percentage was lowered to 50 %. Three reasons motivated lowering the maximum bead loading to 50 %: 1) the apparatus was able to function properly at this ratio, 2) samples were able to be taken directly from the unit during time trials (at 60 % loading, sampling proved to be very difficult), and 3) 50 % bead loading is recommended by the manufacturer to achieve an advertised cell lysis of 90 % [100]. Therefore, results presented in this section were obtained with a glass bead loading percentage of 50 % which corresponds to a cell suspension:glass bead volume ratio of 1:1.

4.2.2 Bead milling: cell growth cycle point and time

The main objective of this section was to investigate if the lysis efficiency was affected by the growth stage of the culture, i.e. the moment at which cells were harvested. Lysis was performed using bead milling for fermentation broth samples collected at different time points in cellular fermentations. The time points chosen were representative of the main two phases in the fermentation: the exponential phase and stationary phase. It should be noted that all samples were taken during stationary and exponential phases that occurred after the induction of the protein of interest. These experiments were performed to see if cells in an exponential growth phase would

be more susceptible to lysis through bead milling compared to cells in a stationary phase. Higher susceptibility in the exponential phase has been demonstrated during experiments with high pressure homogenization [39], [101], osmotic shock [39], [102], enzymatic lysis [39], [103], [104], ultrasound and bead milling [39], [105]. *E. coli* cells in exponential phase are continuously dividing, which make their peptidoglycan structure weaker compared to cells in a stationary phase [39]. Therefore, cells in the exponential phase should lyse more easily [39]. It was then desired to determine if there would be any advantage of lysing the *E. coli* cells at a particular point in the fermentation.

The final objective of this section was to determine the effect that time had on the extent of *E. coli* lysis during bead milling. The purpose of studying this parameter is to determine the optimal amount of time required to efficiently lyse a sample. It has been demonstrated in literature that an increase in lysis time during bead milling results in an increase in the amount of lysis observed [69], [74]. The amount of time required for lysis can have a direct impact on the required volume and the flow rates when designing and operating a continuous bead milling apparatus.

4.2.2.1 Results and discussion

Growth curves from Fermentations 1 to 3 and sample points harvested for lysis are illustrated in Figures 4-3 to 4-5. From these figures, each of the samples taken was either classified as being harvested from the exponential or stationary phase as described in Table 4-1.

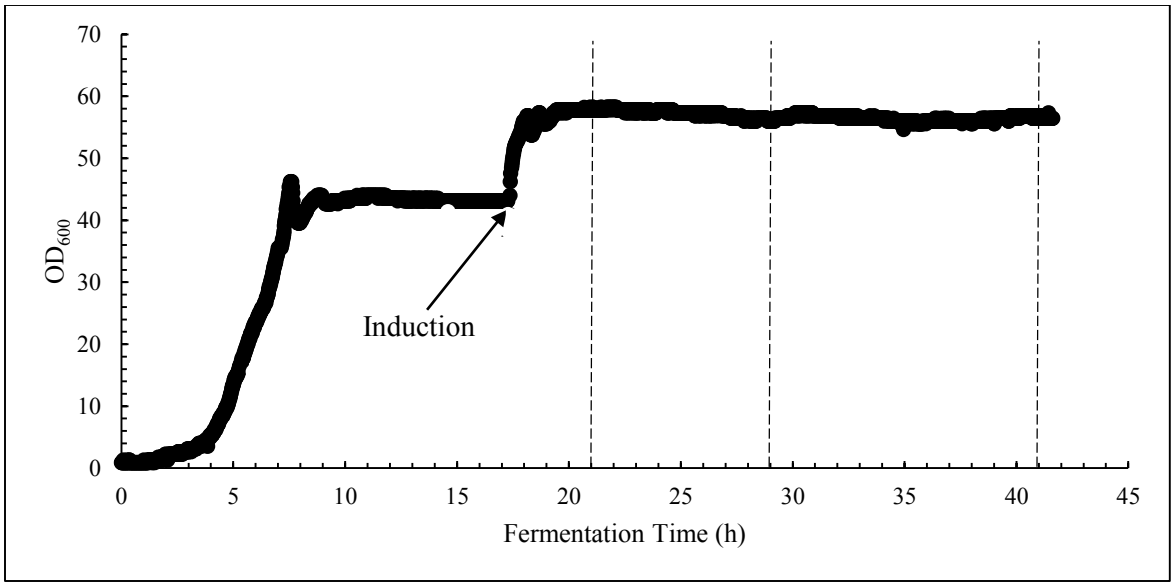

Figure 4-3: *E. coli* TG1 growth curve from batch culture in modified TB medium (4 g/L glycerol at 28°C) with respect to fermentation time. The batch phase, with a volume of 750 mL, was performed for 17 h prior to induction with IPTG. Cell samples for lysis were taken at 4, 12 and 24 h post induction as indicated by dashed lines.

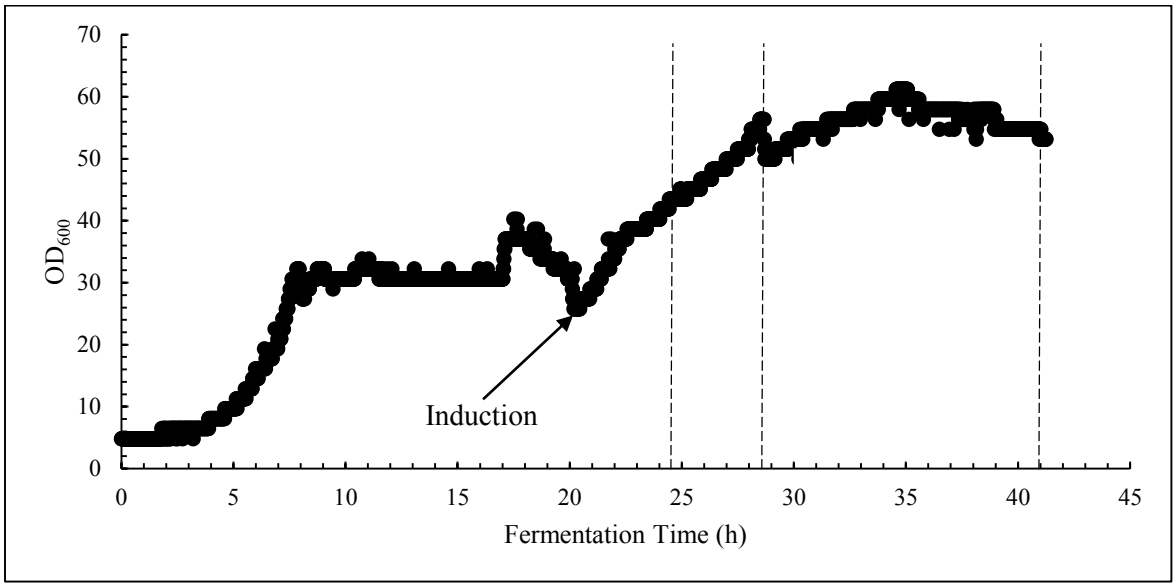

Figure 4-4: *E. coli* TG1 growth curve from fed-batch culture in modified TB medium. The batch phase, with a volume of 400 mL, was performed in TB media, 4 g/L of glycerol at 28°C for 20.5 h prior to induction with IPTG. Fed-batch medium (150 g/L glucose, 15 g/L glycerol) was initiated 3.5 h prior to induction with 300 mL total medium added continuously over 3 h. Cell samples for lysis were taken at 4, 8 and 20.5 h after induction as indicated by dashed lines.

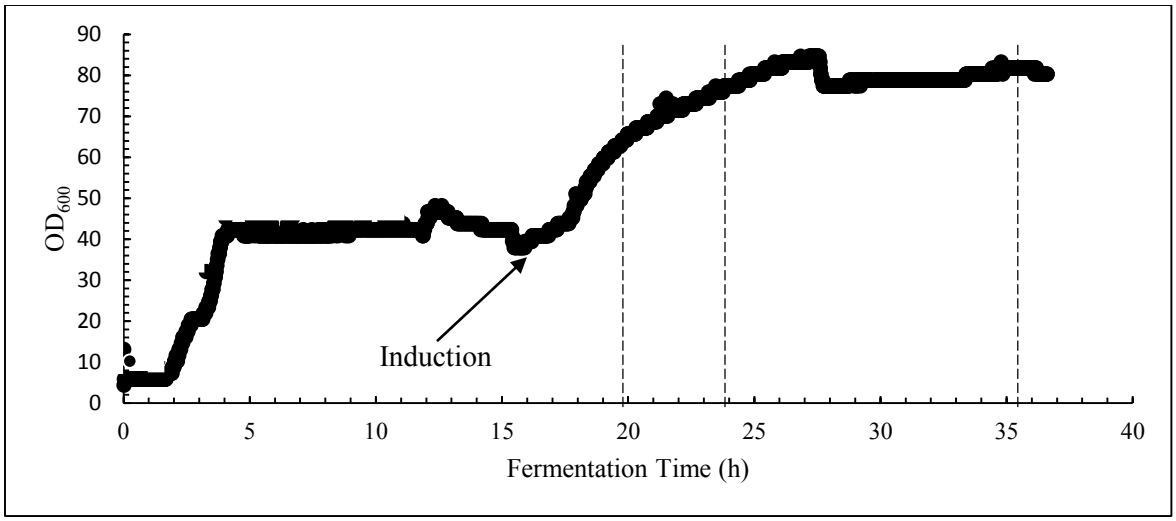

Figure 4-5: *E. coli* TG1 growth curve from fed-batch culture in modified TB medium. The batch phase, with a volume of 400 mL, was performed in TB media, 4 g/L of glycerol at 37°C for 16 h prior to induction with IPTG. Fed-batch medium (150 g/L glucose, 15 g/L glycerol) was initiated 3.5 h prior to induction with 300 mL total medium added continuously over 3 h. After induction, the temperature was decreased to 28°C. Cell samples were taken at 4, 8 and 20.5 h after induction as indicated by dashed lines.

Table 4-1: Cellular growth phase classification for cell fermentations 1, 2 and 3. Classification is based upon the data collected and displayed in Figures 4-3 to 4-5. Samples taken during the increasing linear portion of each figure were classified as being in the exponential phase. Samples taken during the flat portion of each figure were classified as stationary.

Fermentation Number	Sample Time after Induction (h)	Phase Classification
1	4	Stationary
1	12	Stationary
1	24	Stationary
2	4	Exponential
2	8	Exponential
2	20.5	Stationary
3	4	Exponential
3	8	Exponential
3	20.5	Stationary

Figure 4-6 provides a comparison of SDS-PAGE gels for three samples taken at different times after induction. “Total”, “soluble” and “pellet” samples were run on the gel which provide the total, soluble and insoluble fractions of each sample. Comparisons were then made between the “total” and “soluble” samples in order to prepare Figure 4-7.

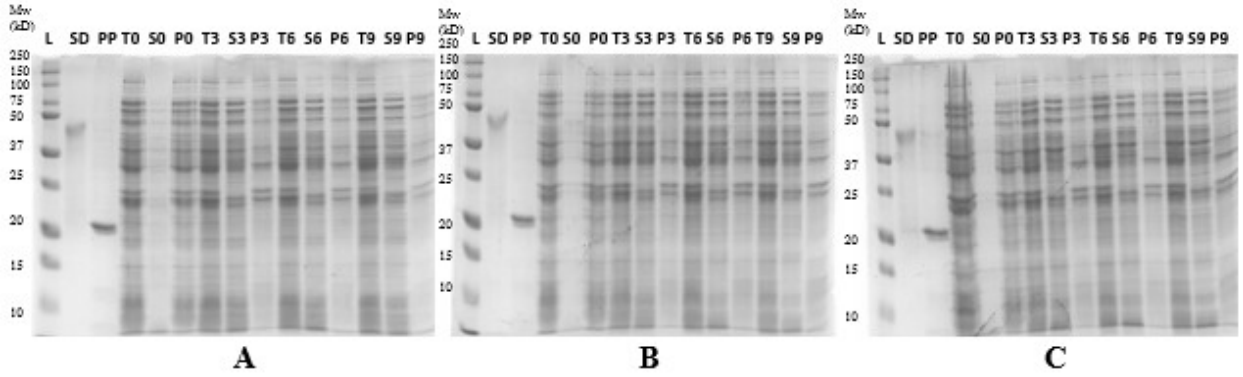

Figure 4-6: A-C: SDS-PAGE of *E. coli* proteins from Fermentation 3. *E. coli* cells were grown in a 1-L bioreactor (750 mL working volume) with a pre-induction and post-induction temperature of 28°C. Cells were lysed using a 90-mL BioSpec BeadBeater chamber, 50 % cell suspension, 50 % 0.1 mm beads, between 0 and 9 min. L denotes “protein ladder”, SD denotes a BSA standard, PP denotes the purified protein of interest, T denotes total protein, S denotes soluble protein, and P denotes insoluble protein. Numerical values indicate at what time of bead milling the sample was taken. A: Cells were harvested 4 h after induction. B: Cells were harvested 8 h after induction C: Cells were harvested 20.5 h after induction.

Figure 4-7 provides a comparison of the fractional cellular lysis achieved with respect to the growth phase and milling time of *E. coli* cells in a 90-mL BioSpec BeadBeater chamber as described in Section 3.5.2. It should be noted that the data displayed in Figure 4-7 has been pooled from bead milling experiments performed on *E. coli* cells from Fermentations 1 to 3. The results of each lysis experiment were tabulated in Figure 4-7 based on the classification of each sample as described in Table 4-1.

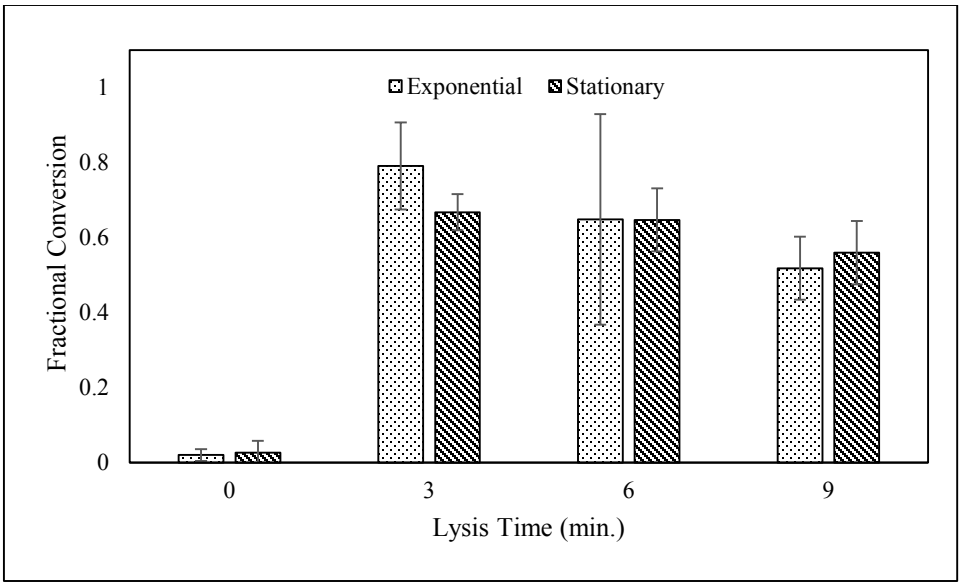

Figure 4-7: A comparison of the amount of fractional lysis of *E. coli* cells over 9 min of bead milling. Cells were harvested during either exponential or stationary phases of the fermentation. *E. coli* cells were lysed using a 90-mL BioSpec BeadBeater chamber and sampled at 3, 6 and 9 min. Cellular lysis was performed using 0.1 mm beads (50 % glass beads: 50 % cellular lysate). Samples were run on SDS-PAGE and evaluated through densitometry using ImageJ software to determine the fractional amount of lysis performed. Fractional conversion of lysis was determined by dividing the amount of protein present in the "soluble" lanes in each gel by the amount of protein present in each corresponding "total" lane. Error bars indicate ±1 standard deviation based on either 4 or 5 trials. Each exponential data set contained 4 data points while each stationary data set contained 5 data points.

It has been seen in literature that *E. coli* cells harvested during the exponential phase are more susceptible to lysis compared to *E. coli* cells harvested during the stationary phase [39], [105]. Figure 4-7 demonstrates the amount of fractional lysis obtained during the above experiment: 0.79 ± 0.12 after 3 min, 0.648 ± 0.28 after 6 min and 0.54 ± 0.08 after 9 min for cells harvested in the exponential phase and 0.67 ± 0.05 after 3 min, 0.65 ± 0.08 after 6 min and 0.56 ± 0.08 after 9 min for cells harvested in the stationary phase. Using Quantum XL (Accessed through www.sigmazone.com/QuantumXL.htm), it has been determined that there is no significant difference between lysing the cells in the exponential phase or in the stationary phase since the p-value for this variable is greater than 0.1 (0.585). Since it has been found that there is no significant difference between lysing cells harvested from either phase it is suggested to harvest *E. coli* cells from the stationary phase for lysis applications. Cells that are harvested during the stationary phase

have completed cellular growth and have therefore produced the maximum concentration of protein possible (in the case of a growth associated proteins) [11]. Furthermore, the fermentation would not have to be as closely monitored as harvesting cells from the exponential phase where a point representing the maximum protein concentration during the exponential phase would have to be identified [11].

In addition, it can be observed from Figure 4-7 that the average amount of lysis found in samples taken after 3 min show an apparent decrease in the overall lysis. Using Quantum XL software, this result has been determined to be statistically significant since the p-value for time for exponential and stationary samples harvested between 3 and 9 min was determined to be less than 0.05 (0.005). This contradicts what is found in literature that an increase in the bead milling time results in an increase of cellular lysis [69], [74]. Since the extent of lysis is calculated based on the quantification of proteins, a decrease in proteins would cause this apparent decrease in lysis. As bead milling time increases, proteins may be destroyed due to the repeated collisions, and shear caused by the glass beads. High temperatures could also contribute to protein degradation. Therefore, the temperature of the bead suspensions for all runs were recorded and illustrated in Figure 4-8.

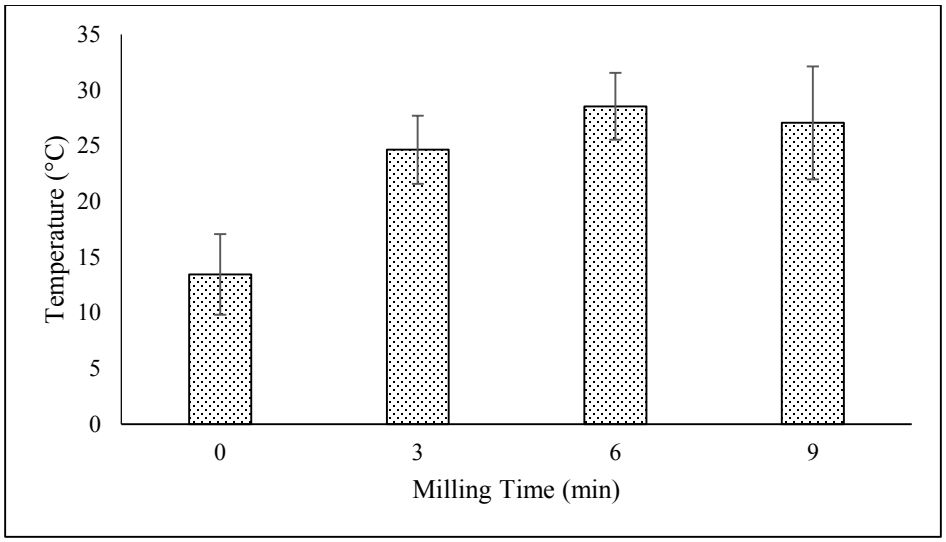

Figure 4-8: Effect of bead milling time on cell lysate temperature. The data was compiled by measuring the temperature of 9 runs of bead milling lysates, using 0.1 mm glass beads (BioSpec) over 0, 3, 6 and 9 min.

From Figure 4-8, it can be seen that the highest average temperature recorded was 29°C which was recorded after a milling time of 6 min. Since this temperature is relatively low, it is highly unlikely that the proteins were degraded due to the temperature. However, it can be noted that a significant amount of lysis does occur after the first 3 min of milling, (0.79 ± 0.12 and 0.67 ± 0.05 for exponential and stationary phases respectively), prior to the observed decrease in lysis. This is an indication that cells can be still be lysed very quickly and efficiently using this technique as long as the lysis is performed for no longer than 3 min.

4.2.2.2 Summary

It was determined that there was no significant difference in lysing cells that have been harvested in either the exponential or stationary phase with the obtained data: 0.79 ± 0.12 after 3 min, 0.648 ± 0.28 after 6 min and 0.54 ± 0.08 after 9 min for cells harvested in the exponential phase and 0.67 ± 0.05 after 3 min 0.65 ± 0.08 after 6 min and 0.56 ± 0.08 after 9 min for cells harvested in the stationary phase. Therefore, it is recommended to lyse cells that have been

harvested in the stationary phase in order to ensure the highest concentration of the protein of interest as possible.

Finally, an apparent decrease was seen in the amount of fractional lysis when the duration of lysis is extended past 3 min. This could be an indication that some of the released proteins are degrading during the bead milling process. It should be noted that the amount of fractional lysis achieved after 3 min of milling is considerable (0.79 ± 0.12 and 0.67 ± 0.05 for the exponential and stationary phases respectively), indicating that bead milling can be a viable lysis technique. Currently it is recommended to mill for no longer than 3 min, however, further testing between 0 and 3 min of bead milling should be performed to determine if the optimal lysis treatment time could be lowered to less than 3 min.

4.2.3 Bead milling: effect of bead size

The objective of this section was to investigate the effect of different glass bead diameters on the lysis of *E. coli* cells using bead milling. It has been recommended by the manufacturer (BioSpec) that the optimal size of glass beads is 0.1 mm diameter to lyse bacterial cells [100]. However, it has been demonstrated in literature that a glass bead size of 0.75 mm instead provides an optimal amount of lysis [69]. Therefore, a comparison should be made between the two bead sizes in order to determine the true optimal size. However, for this experimentation it was not possible to obtain 0.75 mm diameter glass beads due to availability. Instead, three types of beads were used: 0.1 mm and 0.5 mm glass beads from BioSpec which could be purchased for $79.2/kg, and a mixture of lower-cost glass beads with an average size of 0.3 mm from McMaster-Carr (Elmhurst, Illinois) which could be purchased for $9.50/kg. It is to be noted that at the time of the experiment, the company wanted to have tests performed on bacteria producing 14-2M protein. Previous results shown in Sections 4.1.1 and 4.2.1 were based on 2-3P proteins.

4.2.3.1 Results and discussion

Figure 4-9 provides a visual representation of the glass beads utilized during this experimentation. Characteristics and size distribution of the particles are presented in Figures 4-10 to 4-12.

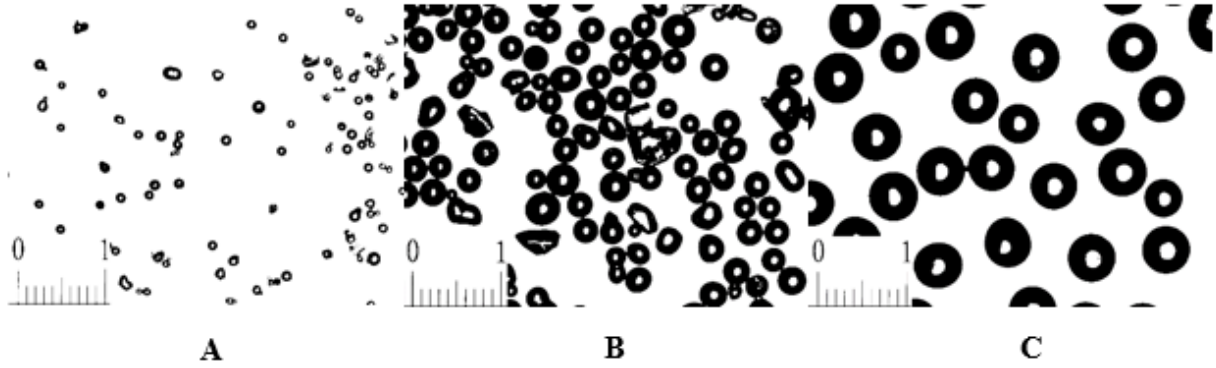

Figure 4-9: A-C Microscopic images of glass beads used for the operation of milling *E. coli*. Images were taken using a light microscope. A: 0.1 mm diameter glass beads (BioSpec) B: 0.3 mm glass beads (average size) (McMaster-Carr) C: 0.5 mm glass beads (BioSpec) Each division on each scale represents 0.1 mm. Please note, there is no void in the centre of each glass bead; the void-like appearance in the centre of each bead is due to the reflection of light. Each image has a magnification of 20X.

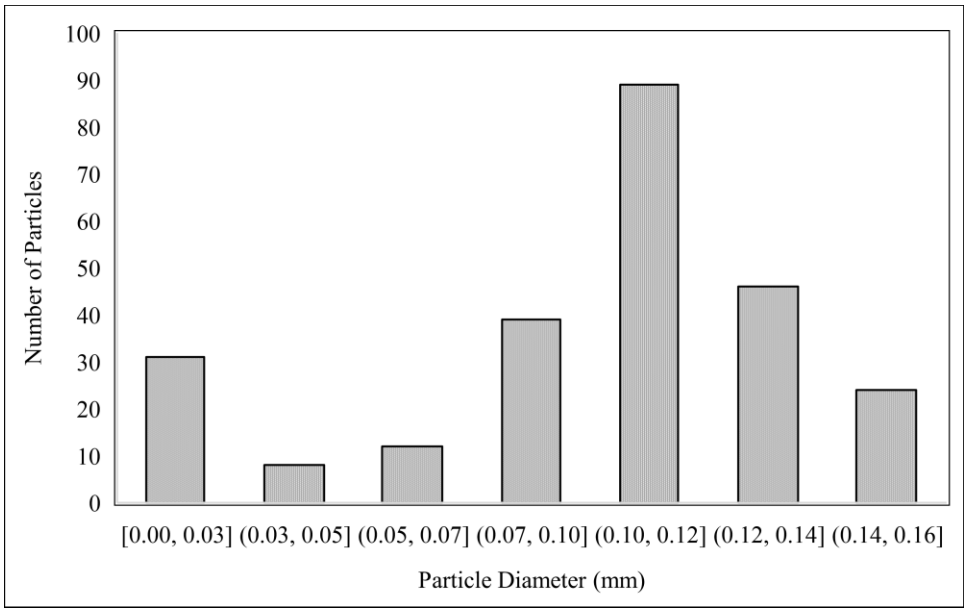

Figure 4-10: Particle size distribution of the "0.1 mm" diameter beads (BioSpec). Beads were characterized by measuring 250 beads and calculating the area of the beads using the software ImageJ. The area of each particle was then converted to a respective diameter. For the "0.1 mm" the average particle size was determined to be 0.097 mm with a standard deviation of 0.041.

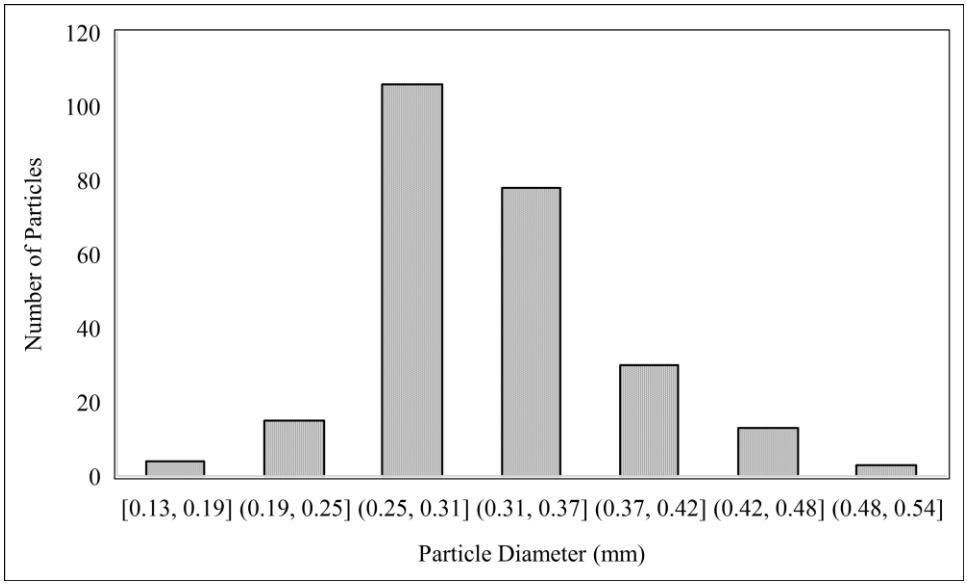

Figure 4-11: Particle size distribution of the "0.3 mm" diameter beads (McMaster-Carr). Beads were characterized by measuring 250 beads and calculating the area of the beads using the software ImageJ. The area of each particle was then converted to a respective diameter. For the "0.3 mm" the average particle size was determined to be 0.318 mm with a standard deviation of 0.061.

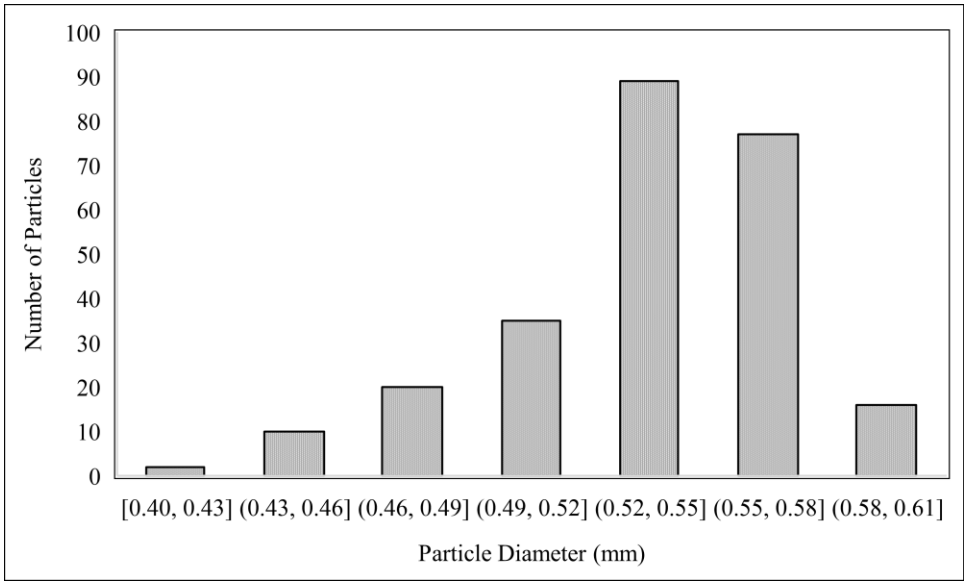

Figure 4-12: Particle size distribution of the "0.5 mm" diameter beads (BioSpec). Beads were characterized by measuring 250 beads and calculating the area of the beads using the software ImageJ. The area of each particle was then converted to a respective diameter. For the "0.5 mm" the average particle size was determined to be 0.539 mm with a standard deviation of 0.037 mm.

It can be observed from Figures 4-10 and 4-12 that the 0.1 mm and 0.5 mm glass beads have a narrow distribution with sizes ranging from 0.07-0.14 mm and 0.49-0.58 mm for the 0.1

mm and 0.5 mm glass beads respectively. The distribution 0.3 mm glass bead mixture in Figure 4-11 has a larger distribution: 0.25-0.42 mm.

E. coli cells were lysed over a span of 6 min using glass beads of 0.1 mm, 0.3 mm or 0.5 mm in diameter. Samples taken at 0, 3 and 6 min were run on SDS-PAGE in order to visualize the amount of overall proteins (total) and the amount of corresponding soluble and insoluble proteins. Typical gels can be seen in Figure 4-13 and were analyzed using ImageJ densitometry (as described in Section 3.6.1) in order to produce the results seen in Figure 4-14.

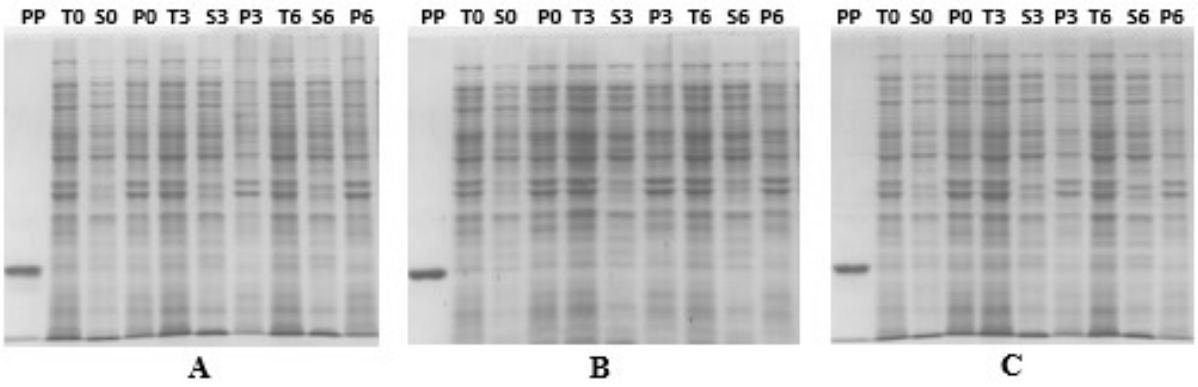

Figure 4-13: A-C: SDS-PAGE of *E. coli* proteins. *E. coli* cells were grown in a 7.5-L bioreactor (working volume 5 L) bioreactor with a pre-induction and post-induction temperatures of 32°C and 28°C respectively. Cells were lysed using a 90-mL BioSpec BeadBeater, 50 % cell suspension, 50 glass beads, between 0 and 6 min. PP denotes the purified protein of interest, T denotes total protein, S denotes soluble protein, and P denotes insoluble protein. Numerical values indicate the time the samples were taken. A: Cells were lysed using 0.1 mm glass beads B: Cells were lysed using 0.3 mm (average size) glass beads C: Cells were lysed using 0.5 mm glass beads.

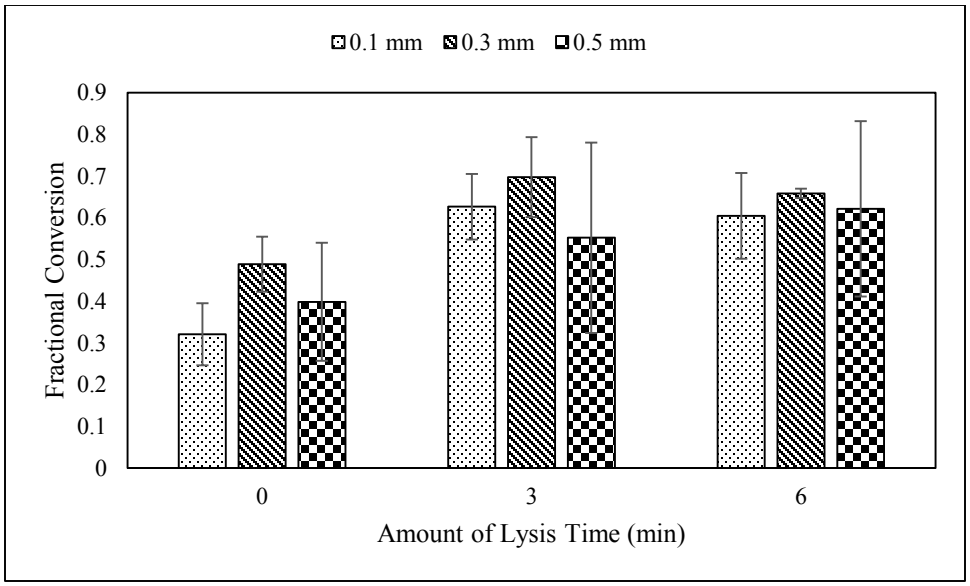

Figure 4-14: Comparison of the amount of fractional lysis of *E. coli* cells over 6 min of bead milling. *E. coli* cells were lysed using a 90-mL BioSpec BeadBeater and sampled at 0, 3 and 6 min. Cellular lysis was performed using 0.1 mm, 0.3 mm (average bead size), or 0.5 mm glass beads (50 % glass beads, 50 % cellular lysate). Samples were run on SDS-PAGE and evaluated through densitometry using ImageJ software to determine the fractional amount of lysis performed. Fractional conversion of lysis was determined by dividing the amount of protein present in the "soluble" lanes in each gel by the amount of protein present in each corresponding "total" lane. Error bars indicate ±1 standard deviation based on 3 trials.

Results of Figure 4-14 show that there is no significant difference between lysing cells using 0.1 mm beads, 0.3 mm (average bead size) beads or the 0.5 mm beads. The amount of fractional lysis obtained after 3 min of lysing was, on average, 0.63 ± 0.08, 0.70 ± 0.10 and 0.55 ± 0.23 for the 0.1 mm, 0.3 mm and 0.5 mm beads, respectively. This implies that beads larger than 0.1 mm and smaller than 0.5 mm can be used in order to lyse *E. coli* cells. Larger beads can be advantageous because they are easier to handle and they have a lower probability of being trapped under seals and damaging equipment. Furthermore, results in Figure 4-14 show that the 0.3 mm beads were able to lyse *E. coli* cells either as well or better than 0.1 mm beads or 0.5 mm beads. Since the 0.3 mm beads are a mixture of multiple bead sizes they can be purchased at a fraction of the cost of beads having a narrow size distribution (ex. 0.1 mm beads or 0.5 mm beads from this experiment). Therefore, it is recommended to use the 0.3 mm bead mixture when scaling up to larger continuous bead milling operations in order to save on production costs.

It can also be noted that the amount of lysis seems to plateau. This is an indication that 3 min of lysis time could be sufficient to achieve the highest quantity of lysis possible through bead milling with these sizes. This phenomenon was also described in Section 4.2.2 with bead milling experiments pertaining to lysis with 0.1 mm glass beads.

It has been reported in literature that using 0.75 mm glass beads leads to a greater amount of lysis (80 %) compared to glass beads ranging from 0.25 mm to 0.5 mm (65 % and 70 % respectively) [69]. It should also be noted that it was found in literature that bead sizes greater or equal to 1 mm in diameter resulted in *E. coli* lysis of less than 50 %. It is difficult to compare these results to the ones stated above since different milling units and different operating conditions were used. However, if it were possible a 0.75 mm bead size should be investigated in future experiments.

It is important to note that the amount of lysis occurring at time 0 min cannot be assumed to be 0. Indeed, Figure 4-14 illustrates that the average amount of fractional cellular lysis of *E. coli* at time 0 was 0.4. This indicates that there is another factor contributing to lysis prior to bead milling. One contributing factor to this pre-lysis may be the freeze-thaw process. When cells are frozen, ice crystals can form which could potentially cause some cells to lyse. This phenomenon is prevalent when freezing cells at higher temperatures such as -20°C compared to lower temperatures such as -80°C. At -20°C, the freezing process is very slow [79] which allows for a higher amount of ice crystals to form within the cells promoting a larger amount of lysis. At lower temperatures such as -80°C, the cells freeze very quickly, which reduces the formation of ice crystals within the cells. Since the cells were stored at -20°C, there is a possibility that the above freezing phenomena could contribute to the lysis seen prior to bead milling as indicated in Figure 4-14 [79].

Another potential explanation for the lysis prior to bead milling could be due to the addition of EDTA. The addition of EDTA was used in this experimentation to prevent protein degradation [106] from proteases. However, the addition of EDTA can also destabilize the outer membrane of gram-negative bacteria like *E. coli* which can lead to cellular lysis. This form of lysis could also possibly contribute to the phenomena demonstrated in Figure 4-14 [41], [42].

4.2.3.2 Summary

In conclusion, results have shown that the bead diameter has an insignificant effect on the lysis of cells such that the bead mixture having an average diameter of 0.3 mm can be used for the efficient lysis of *E. coli* cells. Since the 0.3-mm diameter beads are substantially less expensive than the two more uniform sizes of glass bead ($9.50/kg compared to $79.2/kg), it is recommended to use the 0.3 mm diameter beads when scaling up the bead milling operation. Results also showed that the optimal amount of lysis was achieved after 3 min (0.63 ±0.08, 0.70 ±0.10 and 0.55 ±0.23 for the 0.1 mm, 0.3 mm and 0.5 mm beads, respectively). This result was also seen earlier in the lysis experimentation over time with bead milling at different cellular growth phases. It was also seen that the amount of lysis plateaus for each of the respective bead sizes after 3 min of bead milling. It is recommended to investigate the lysis of *E. coli* cells using 0.75 mm beads since this was the optimal bead size determined in literature [69]. Finally, it was noted that there were non-negligible amounts of lysis prior to bead milling at 0 min. This amount of pre-lysis could be attributed to thawing the cells or the addition of EDTA.

4.3 Sonication

A series of experiments were performed to elucidate the efficiency of sonication on the lysis of *E. coli* cells. Sonication, using a sonic probe to generate sound energy usually within a range of 20-50 kHz, is able to disrupt the structure of cells through the formation of violent

implosions of small bubbles and cavitation within the sample. The energy of the sonic waves is able to cause a disruption of the intramolecular forces that provide the integrity for the cellular wall. Four parameters have been tested in order to determine their potential effect on the cellular lysis via sonication: output control (sonicator power input), duty cycle (% of time the sonicator is on during the experiment), optical density (OD) and lysis time period.

4.3.1 Results and discussion

Results for the fractional cell lysis for each run of the experimental design are presented in Table 4-2.The highest amount of fractional lysis was achieved for Run 13 with a value of 0.628. The settings for each input parameter for Run 13 were as follows: Output Control: 6 (+1 input), Duty Cycle: 100 (+1 input), OD: 20 (-1 input) and Time: 1 min (-1 input). In order to determine how each of the individual parameters contributes to this finding, the experimental design in Table 4-2 was used to determine the interaction effects for each of the input parameters and to potentially find a model to represent the lysis conversion as a function of these input parameters.

Table 4-2: A breakdown of the values of each parameter during the sonication experimentation: A represents Output Control, B represents Duty Cycle, C represents OD and D represents Time (min). Y-exp. represents the average amount of fractional lysis over 3 runs (* indicates only 2 runs used). Y-pred. represents the predicted amount of fractional lysis using Equations (4.1) and (4.2) respectively. ^ indicates trials that were run as centre-points for the experimental design that were unable to be reported due to gel sample loading problem.

Run	**A**	**B**	**C**	**D**	**y-exp.**	**y-pred. Eqn. 1**	**y-pred. Eqn. 2**
1	2	20	20	1	0.126	0.145	0.263
2	2	20	20	5	0.231	0.250	0.178
3	2	20	60	1	0.114	0.133	0.134
4	2	20	60	5	0.115	0.134	0.049
5	2	100	20	1	0.172*	0.153	0.165
6	2	100	20	5	0.509	0.490	0.429
7	2	100	60	1	0.062	0.043	0.036
8	2	100	60	5	0.229	0.210	0.301
9	6	20	20	1	0.228	0.209	0.280
10	6	20	20	5	0.534*	0.515	0.544
11	6	20	60	1	0.041	0.022	-0.019
12	6	20	60	5	0.282	0.263	0.245
13	6	100	20	1	0.628	0.647	0.532
14	6	100	20	5	0.408	0.427	0.446
15	6	100	60	1	0.257	0.275	0.233
16	6	100	60	5	0.028	0.047	0.148
17	4	60	40	3	^	^	^

The plots of the main effects for each of the 4 input parameters: output control (A), duty cycle (B), optical density (C) and lysis time (D) are presented in Figure 4-15.

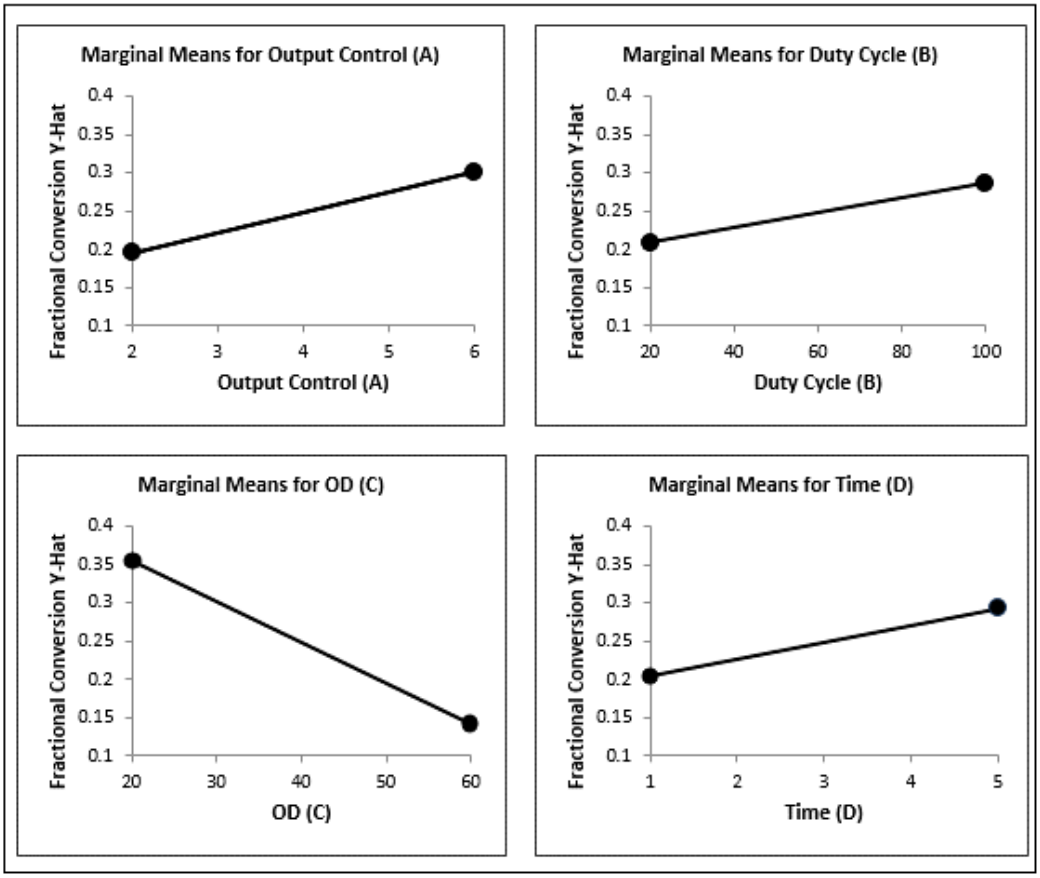

Figure 4-15: The effects output control (A), duty cycle (B), optical density (C) and time (D) [min] on the fractional amount of lysis. Parameter effects are determined through the complete model (Equation (4.1)).

An increase in the output control of the sonicator increases the power of the ultrasonic waves used to disrupt the membrane of the *E. coli* cells. Results obtained in Figure 4-15 confirmed results of previous researchers which showed that an increase in sonication power causes an increase in the amount of cellular lysis [64], [66]–[69]. However, the effect is minor in this case since only a 10 % increase in cellular lysis was observed when increasing the power output from 2 to 6 (80-240 W).

An increase in the duty cycle from 20 to 100 (where 20 indicates that the sonicator emits ultrasonic waves 20 % of the time and 100 indicates 100% of the time) led to an 8 % increase in cellular lysis. This observation makes logical sense since the sample is experiencing five times the

amount of sonication over the allotted amount of time allowing for a higher amount of lysis to occur.

The optical density (OD) or cell concentration of the lysate was also varied. From Figure 4-15, it can be seen that an increase in optical density from 20 to 60 decreases the amount of fractional lysis by about 21 %. It was noted that varying this parameter had the highest effect on the observed amount of lysis. However, in literature it has been determined that the amount of lysis seen during sonication is independent of the OD [39]. Therefore, this result needs to be validated with further experimentation.

Finally, with respect to lysis time, it was demonstrated in literature that an increase in sonication time leads to an increase in the amount of lysis obtained [58], [64], [67], [68]. As seen in Figure 4-15, as the sonication time was increased from 1 to 5 min, the amount of lysis increased by 9 %. It has also been reported in literature that there can be a large variation in the amount of sonication time required to achieve maximum lysis between different batches of *E. coli* cells [65], [71]. It is possible that the amount of lysis time in this experiment was not long enough to achieve the maximum amount of lysis possible with this batch of *E. coli* cells.

4.3.2 Model development

The observations of the main effects seen in Section 4.3.1 were determined using Quantum XL based on the values of the lysis experimental data for each run. The average values for each run are demonstrated in Table 4-2. Unfortunately, the data associated with the central point could not be used because the gel did not allow for proper quantification. Equation (4.1) predicts the fractional lysis as a function of the 4 input parameters: output control (A), duty cycle (B), optical density (C), and time (D). This regression equation is expressed using the coded coefficients

between -1 to +1 represented by the minimum and maximum of each parameter value of Table 4-2. The predicted values of the lysis fraction from Equation (4.1) for all runs are also presented in Table 4-2.

$$y = 0.2477 + 0.053A + 0.0388B - 0.1068C + 0.0444D + 0.0095AB - 0.0421AC - 0.032AD - 0.0358BC - 0.0374BD - 0.0217CD - 0.0032\text{ABC} - 0.0871\text{ABD} + 0.0125\text{ACD} - 0.0006\text{BCD} + 0.0076\text{ABCD} \quad (4.1)$$

Equation (4.1) is not very useful as a predictive model since the number of fitted coefficients is equal to the number of data points used to fit the linear model which obviously gives a perfect prediction. Figure 4-16 presents the lysis fraction predicted by Equation (4.1) as a function of the experimental values of the lysis fraction for the average and individual values for each run. The lysis fraction of each run allows to visually assess the significant variability of the experimental data.

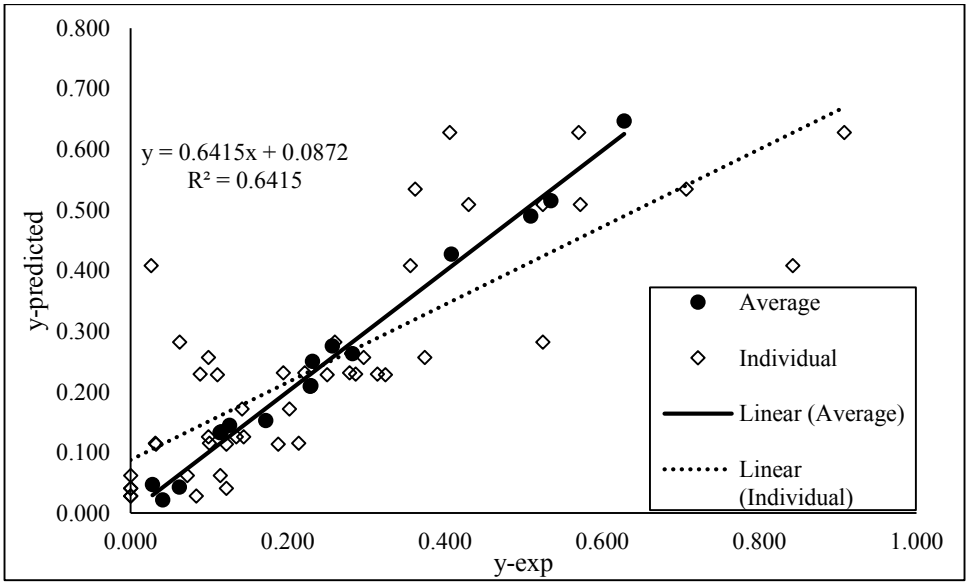

Figure 4-16: A plot of the predicted amount of fractional lysis versus the amount of experimental lysis. Y-predicted values are calculated from Equation (4.1). Dots indicate average y-exp. values taken as an average of 3 samples. Diamonds indicate individual y-exp. values. Linear trend lines have been added for each set of data points. The equation of the trend line shown is for the individual linear trend line (dotted line).

The statistical analysis allows, nevertheless, to observe which coefficient estimates from Equation (4.1) are the most significant to estimate the fractional lysis and at the same time allows to reduce the number of coefficients that are statistically insignificant. Figure 4-17 presents the Pareto plot that can greatly aid in the reduction of the above equation.

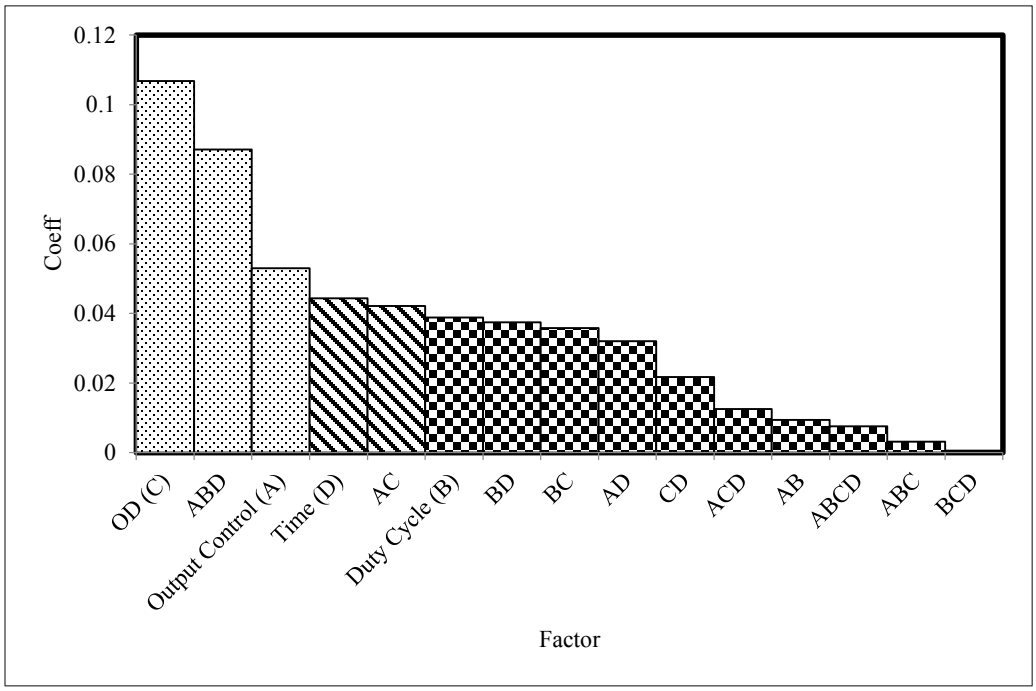

Figure 4-17: Pareto plot for initial model of sonication experimentation. The bars with dots indicate coefficients where the p-value is less than 0.05, striped histogram bars indicate coefficients where the p-value is less than 0.1 but greater than 0.05, the checkered bars indicate coefficients where the p-value is greater than 0.1.

For the purpose of reducing the number of terms in Equation (4.1), coefficients that have a p-value greater than 0.1 were removed from the equation since they have very little significance. The coefficients represented by striped histogram bars (having a p-value between 0.05 and 0.1) were left in the model. Since the duty cycle coefficient (B) had a large impact on the interaction coefficient ABD, it was left in the equation even though it had a p-value over 0.1 (0.115). It should be noted that there would have been great reluctance to remove the duty cycle coefficient based upon the fact that 3 out of the 4 runs containing the greatest amount of lysis (Runs 6, 11 and 14) are experiments that were all run at a duty cycle of 100 (the highest value for the duty cycle

parameter). Another regression was performed on the remaining factors and Equation (4.2) was obtained.

$$y = 0.2478 + 0.0534A + 0.0385B - 0.1069C + 0.0447D - 0.0425AC - 0.0874ABD \quad (4.2)$$

Therefore, the parameters that are left in the lysis model in the case of sonication are the output control (A), the duty cycle (B), optical density (C), the interaction between output control and optical density (AC) and the interaction between output control, duty cycle and time (ABD). Figure 4-18 presents the lysis fraction predicted by the reduced model as a function of the experimental values of the lysis fraction for the average value. Fractional lysis values of individual runs are also plotted in Figure 4-18 which allows to visually assess, once more, the significant variability of the experimental data.

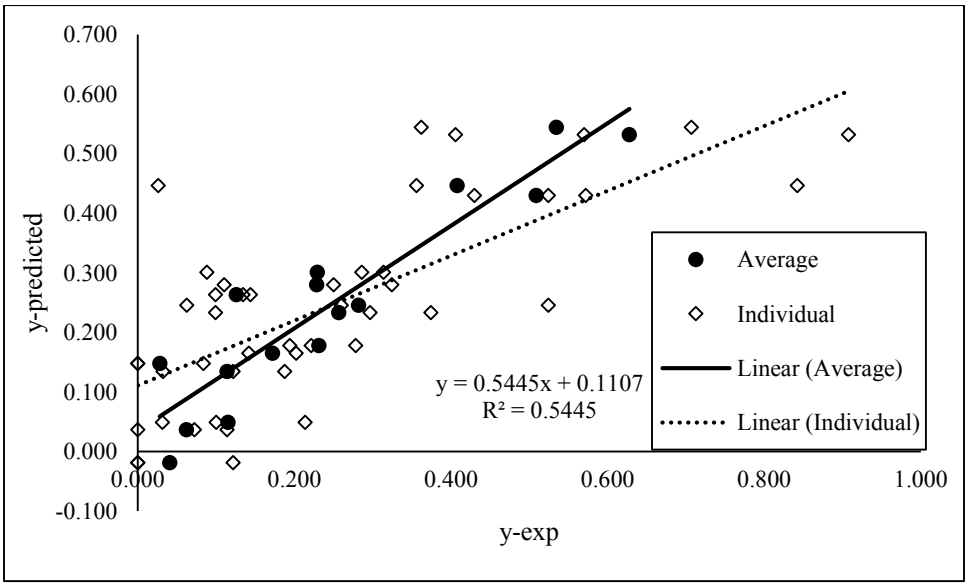

Figure 4-18: A plot of the predicted amount of fractional lysis versus the amount of experimental lysis. Y-predicted values are calculated from Equation (4.2). Dots indicate average y-exp. values taken as an average of 3 samples. Diamonds indicate individual y-exp. values. Linear trend lines have been added for each set of data points. The equation of the trend line shown is for the individual linear trend line.

Given the variability of the experimental data for sonication (R^2 of 0.5445), Equation (4.2) can only be used to provide an overall tendency and to suggest further experiments in the case this

method would be acceptable for lysis. The data obtained in the sonication experiments has significant variability but some preliminary information could still be obtained on the efficiency of this technique. The variability is due to the natural variability of the cell samples and the sonication batch experiments. The variability can also be due to the variation in the positioning of the tip within the sample volume. From trial-to-trial, the positioning of the tip can result in some portions of the sample receiving higher energy intensity compared to other parts of the sample. This variation can be due to operator error since the sonication experiments were done manually. Also, improper mixing may have led to an improper distribution of the sonicator's energy which could introduce variability to the results from run to run. Finally, the gels that were used for the protein analysis varied in staining from gel to gel, thus introducing more potential variation to the results.

4.3.3 Summary

From the above results, it was determined that an increase in the output control from 2 to 6 (80-240 W), an increase in duty cycle from 20 to 100 % and an increase in lysis time from 1 to 5 min led to an increase in lysis of 10, 8 and 9 % respectively. The increase in lysis by increasing each of these parameters is consistent with results reported in literature [58], [64], [66]–[69]. It has also been found in literature that the time required to achieve maximum lysis can vary substantially: from 20 s [65] up to 90 min [71]. One possibility for future work would be to increase the amount of lysis time to see if this would substantially make a difference.

The variable that had the greatest effect on the amount of lysis was the optical density OD). It was determined that increasing the OD from 20 to 60 decreased the amount of fractional lysis by 21 %. This observation is contradictory to what has been previously determined in literature where it has been found that changing the concentration of cells does not have an impact on the

amount of lysis [39], [69]. Therefore, this parameter should be further explored in order to verify this result.

One parameter that was not tested was the effect of volume. It was seen in literature that a decrease in volume leads to an increase in lysis [67], [70]. If the process were to be scaled-up, the challenge of processing large volumes of cell suspension would need to be solved. The sonication power would need to be vastly increased in order to compensate for the increase in volume. Therefore, sonication for the purpose of lysis of *E. coli* cells may not be viable for scale-up. However, this variable would need to be tested for complete verification.

4.4 Synergistic lysis using lysozyme and Triton X-100

A series of experiments was performed to determine the synergistic capabilities of using both lysozymes and Triton X-100 to lyse *E. coli* cells. It is hypothesized that the utilization of these two chemicals will provide a larger level of lysis than what would be obtained if each method was used separately. Lysozymes break down the polysaccharide chains of peptidoglycan which surround the inner membrane of the *E. coli* cell [44], [49]–[52]. Lysozyme achieves this disruption by attacking the β-1,4 linkages of the polysaccharide chains [44], [49]–[52]. However, due to the fact that gram-negative bacteria also have an outer membrane surrounding the peptidoglycan layer, it is more difficult for lysozymes to reach the peptidoglycan layer to achieve complete lysis. Triton X-100 is a non-ionic detergent that is able to solubilize the outer and inner membranes of the *E. coli* cell [37], [38]. However, this is only the case when there are no divalent cations present in the lysis buffer [41]. Therefore, the utilization of Triton X-100 for the solubilisation of the inner and outer membrane coupled with lysozymes and their ability to break down the peptidoglycan layer leads to a higher amount of cellular lysis. Note that for these experiments, 14-2M *E. coli* bacteria was used instead of 2-3P *E. coli*. This was due to the fact that the production of the company

changed in the course of the project. The antibody produced, 14-2M, is a c.a. 16 kDa monomeric form of the antibody of interest which localizes in the periplasmic space of the *E. coli* cell; 2-3P is c.a. 25 kDa pentameric form of the antibody which localizes in the intracellular space.

4.4.1 Results and discussion

Figure 4-19 provides a comparison of SDS-PAGE gels where Gel A represents proteins from *E. coli* cells that have been lysed solely using lysozymes while Gel B represents proteins from *E. coli* cells that have been lysed using both lysozymes and Triton X-100. The "total", "soluble" and "pellet" samples were run on the gel which are denoted in total (T), soluble (S) and insoluble (P).

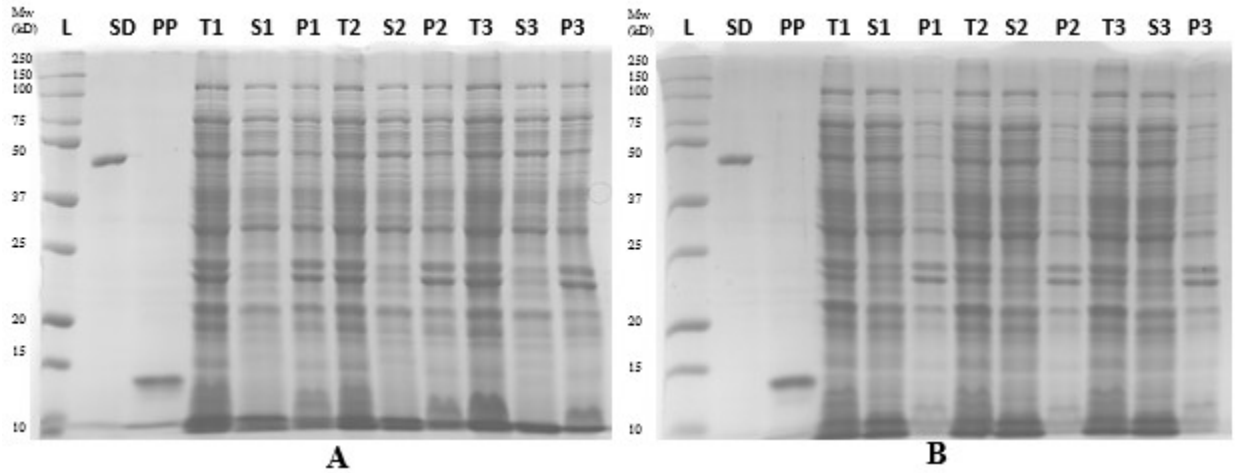

Figure 4-19: SDS-PAGE of *E. coli* proteins. L denotes "protein ladder", SD denotes a BSA standard, PP denotes the purified protein of interest, T denotes total proteins, S denotes soluble proteins, P denotes insoluble proteins. Numerical values denote the cell lysate sample. A: Cells lysed solely in the presence of lysozyme (1350 U per g of wet cell paste). B: Cells lysed in the presence of both lysozymes (1350 U per g of wet cell paste) and Triton X-100 1 % v/v final concentration.

Comparisons were then made between the "total" and "soluble" samples in order to prepare the histogram of Figure 4-20. The visual inspection of each gel reveals that proteins found in each soluble lane are closer in intensity to their corresponding "total lane" in Gel B where synergistic lysis occurred. This observation indicates that more cellular lysis occurs under the conditions performed for the synergistic lysis (Gel B) than with lysis using only lysozymes (Gel A). It can

also be seen from Figure 4-19 that fainter bands are found in the pellet (P) lanes which is a clear indication that a higher amount of lysis and solubilisation occurred. Solubilisation of proteins can also be attributed to the fact that Triton-X 100 has been reported to be able to solubilize proteins [37], [38]. Quantitative analysis of the gels from Figure 4-19 can be observed in Figure 4-20.

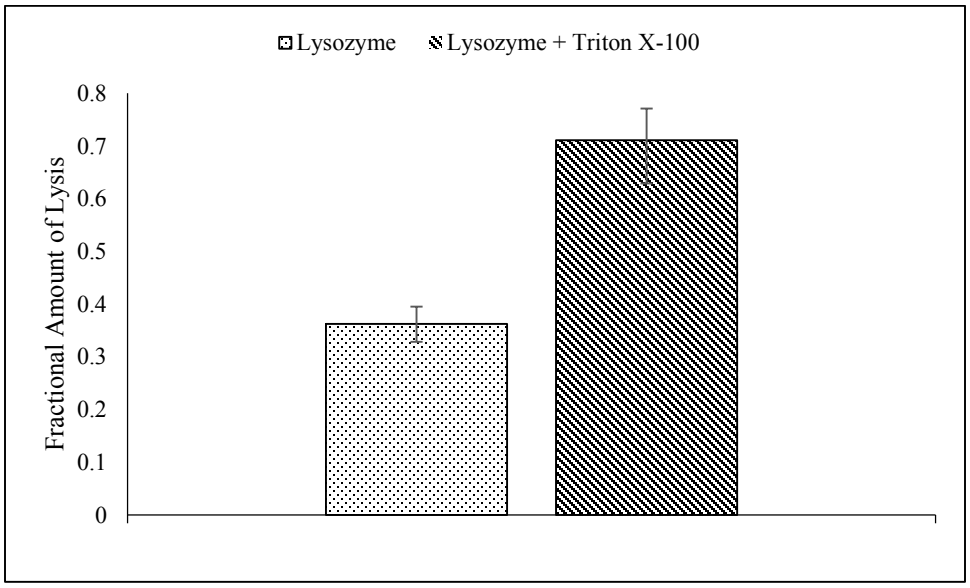

Figure 4-20: A comparison of the amount of fractional lysis of *E. coli* cells using either solely lysozymes (1350 U per g of wet cell paste) or a combination of lysozymes (1350 U per g of wet cell paste) and Triton X-100 1 % v/v final concentration. Samples were run on SDS-PAGE and evaluated through densitometry using ImageJ software to determine the fractional amount of lysis performed. Fractional conversion of lysis was determined by dividing the amount of proteins present in the "soluble" lanes in each gel by the amount of proteins present in each corresponding "total" lane.

Figure 4-20 shows that the amount of cellular lysis obtained from the synergistic lysis is substantially larger than the amount of cellular lysis obtained using only lysozymes with an increase in lysis of approximately 35% (0.71 ± 0.07, 0.36 ± 0.03 for enzymatic lysis with and without Triton X-100 respectively). As stated earlier, Triton X-100 solubilizes the outer membrane of gram-negative cells while lysozymes break apart the peptidoglycan layer. After both the outer membrane and the peptidoglycan layer have been removed from the cell, the cell is then able to lyse and release its intracellular proteins. If only lysozymes were used, the outer membrane would

remain intact and the lysozymes would be unable to attack the peptidoglycan layer thereby limiting the amount of lysis.

4.4.2 Summary

Results have shown that lysing *E. coli* cells in the presence of both lysozymes and Triton X-100 results in 50 % more lysis compared to lysing only with lysozymes (0.71 ± 0.07, 0.36 ± 0.03 for enzymatic lysis with and without Triton X-100 respectively). An experiment where *E. coli* lysis based solely on exposure to Triton X-100 could be performed to better elucidate the effect of Triton X-100. Future experimentation would involve testing multiple concentrations of Triton X-100 or lysozyme to determine an optimal formula for lysis.

4.5 Overall Comparison

Since nearly all proteins can be released using a high pressure homogenizer, results obtained with the high pressure homogenizer were used to demonstrate the amount of lysis that is achievable and for setting a benchmark for comparison between the other forms of lysis used in this investigation. This technique displayed that it is possible to achieve maximum lysis and protein release. However, a high pressure homogenizer can be an expensive piece of equipment for a start-up company with a capital cost of over $22 000. The results of different lysis methods were presented and evaluated to determine which one would provide a cheaper alternative to high pressure homogenization to process *E. coli* cells. The methods that were investigated were bead milling, sonication and chemical/enzymatic lysis.

The experiments involving the bead milling technique did not produce the same amount of lysis that was seen with high pressure homogenization. However, under the optimal conditions that were tested, a very positive fractional lysis of 0.79 was observed (79 % of the intracellular

proteins were released in the soluble fraction). Furthermore, it was demonstrated that cheaper glass beads were available for lysis that did not reduce efficiency. From a capital cost perspective bead milling is a cheaper alternative compared to high pressure homogenization ($700 for the unit used during experimentation with the potential to lyse 175 mL of cell suspension). Finally, there is potential to design and build a large bead milling unit in-house since highly specialized materials are not required (as opposed to building a high pressure homogenizer or a sonicator).

For sonication, the objective of these experiments was to rapidly screen for the most appropriate conditions that would lyse the *E. coli* cells and release large amounts of the specific protein. Compared to other methods, it is clear that sonication does not produce a high level of lysis. When comparing these results with those of bead milling, it can be seen that bead milling generates higher amounts of fractional lysis (0.79 compared to 0.63 which was the highest fractional lysis seen with each technique). Furthermore, the capital costs associated with sonication are quite high (over $4 000 per unit). Therefore, sonication was not selected as the recommended lysis technique.

In regards to the lysozyme/Triton X-100 technique the time required to achieve over 70 % of lysis (30-60 min) was considerably longer than the 3 min it takes to achieve a similar amount of lysis through bead milling. This increase in time can potentially increase significantly the antibody production time if this form of lysis were to be used. Also, the cost of purchasing lysozymes can be a deterrent for opting for chemical lysis ($11.33 per g of 9000 U/mg enzyme). If 1350 U are required to lyse 100 g of cell paste (as described above) the cost to lyse 1 kg of wet cell paste would be $1.70. This operating cost may prove to be unfeasible if the amount of protein produced per kg of cells is determined to be unfavourable. The power input for a bead milling process can be considered to be the major operating cost associated with that form of lysis which

would be much less than for chemical lysis. It should be noted that there would also be some labour costs associated with the bead milling technique. These costs would predominately be due to maintenance of the apparatus and cleaning/replacement of the glass beads. However, since bead milling can be designed in a continuous configuration, these would not be day-to-day costs but rather only seen when shutdown is required. It has been determined that lysing with lysozymes (and Triton X-100) will not be continued based upon the relatively large amount of time required to lyse the cells and the large operating costs associated with the cost of the lysozymes.

Therefore, the lysis technique that has been selected for the purpose of purifying the target protein of interest is bead milling.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The purpose of this research was to make a recommendation to a local start-up company, Ab Biotech Inc., for the selection of the lysis technique for *E. coli* cells for the release a cytoplasmic sdAb. Multiple lysis techniques were tested throughout this thesis including high pressure homogenization, bead milling, sonication, and synergistic lysis with lysozymes and Triton X-100. Since high pressure homogenization is considered to be an industry standard (almost 100 % lysis achieved after a single pass), this form of lysis was used as a benchmark for comparison. Based on the analysis presented, it was recommended that Ab Biotech Inc. implement bead milling for their future large-scale operations. A high extent of fractional lysis (0.7 ± 0.10) was achieved over a short period of lysis time (3 min with 0.3 mm beads). Also, there are many parameters that could be optimized in order to further reduce costs. One example of this was the choice to recommend a 0.3 mm glass bead mixture which could was considerably cheaper ($9.50/kg) compared to 0.1 mm or 0.5 mm uniform bead sizes ($79.2/kg).

5.2 Recommendations

The next logical step would be to see how well bead milling can lyse *E. coli* cells in a continuous bead milling process. It would be pertinent to demonstrate that at least 0.70 fractional lysis could be obtained under 3 min residence time under a continuous configuration with a 0.3 mm mixture of glass beads. A small-scale continuous bead milling unit could be designed and tested prior to designing a large-scale unit. Important parameters to consider when designing a

continuous bead milling unit would be the volume, lysate flow rate and the system to separate the glass beads from the lysate.

Another task that would need to be completed would be an extensive economic analysis. Unfortunately, throughout the entirety of the investigation there was very little expression of the target protein. Therefore, it was very difficult to perform a suitable economic analysis since the profitability of the entire process (from fermentation to purification) is contingent on a high yield of the target protein. Once a sufficient yield is achieved, then a proper economic analysis can be performed.

CHAPTER 6: REFERENCES

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