OPTIMIZATION OF ULTRASONICATION AND LYSOZYME DISRUPTION METHODS FOR THE RELEASE OF NS1 PROTEIN OF INFLUENZA H5N1 VIRUS FROM *Escherichia coli* NOVABLUE

By

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ABSTRACT

OPTIMIZATION OF ULTRASONICATION AND LYSOZYME DISRUPTION METHODS FOR THE RELEASE OF NS1 PROTEIN OF INFLUENZA H5N1 VIRUS FROM *Escherichia coli* NOVABLUE

Koh Li Jing

Escherichia coli is often used for the production of recombinant proteins. However, it does not excrete the proteins to the medium. Therefore, effective cell disruption methods are needed to release the intracellular proteins. The cell disruption methods can be divided into mechanical and non-mechanical methods. This study aimed to optimize the release of NS1 protein from *Escherichia coli* cells by ultrasonication and lysozyme cell disruption methods. The parameters investigated for ultrasonication were different sonication durations (0, 10, 20, 30, 40, 50, 60 min) and amplitude of sonication (40, 60, 80%). On the other hand, the parameters investigated for lysozyme cell disruption method were incubation duration (20, 40, 60, 120 min), lysozyme concentration (0.01, 0.05, 0.1, 0.2 mg/mL) and temperature (4, 28°C). The yields of NS1 protein obtained from ultrasonication and lysozyme cell disruption method were determined based on the Bradford assay and densitometric analysis. The results showed that the

concentration of NS1 protein released increased with the increase of sonication duration and increased amplitude. It was demonstrated that 60 min of sonication duration with 80% amplitude released the highest concentration of NS1 protein from the *E. coli* cells. In lysozyme method, 120 min of incubation duration with 0.2 mg/mL lysozyme at 4℃ released the highest concentration of NS1 protein from *E. coli* cells. NS1 protein recovered from the cell lysate, resulted from ultrasonication was 1.2 times higher than that obtained from the lysozyme cell disruption method. Western blotting and enzyme-linked immnosorbent assay confirmed that both disruption methods did not affect the structure of the expressed protein so the His-tag was shown to be still intact with the protein. In conclusion, ultrasonication was found to be more effective than lysozyme disruption method in terms of the concentration of NS1 protein released and the overall duration required to disrupt the cells.

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Moreover, a great thank to Mr. Gee and Mr. Tie, our responsible and helpful laboratory officers. Last but not least, emotional supports and unconditional loves from my precious family members are deeply appreciated too.

DECLARATION

I hereby declare that the project is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman or other institutions.

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KOH LI JING

APPROVAL SHEET

This project report entitled "OPTIMIZATION OF ULTRASONICATION AND LYSOZYME DISRUPTION METHODS FOR THE RELEASE OF NS1 PROTEIN OF INFLUENZA H5N1 VIRUS FROM *Escherichia coli* NOVABLUE" was prepared by KOH LI JING and submitted as partial fulfillment requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **KOH LI JING** (ID No: **12ADB04255**) has completed this final year project entitled "OPTIMIZATION OF ULTRASONICATION AND LYSOZYME DISRUPTION METHODS FOR THE RELEASE OF NS1 PROTEIN OF INFLUENZA H5N1 VIRUS FROM *Escherichia coli* NOVABLUE" supervised by Dr. Michelle Ng Yeen Tan (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(KOH LI JING)

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BCIP-NBT	5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium
BSA	Bovine serum albumin
CPSF30	Cleavage and polyadenylation-specific factor 30
DIVA	Differentiation of Infected from Vaccinate Animals
E. coli	*Escherichia coli*
eIF4GI	eukaryotic initiation factor 4GI
g	gram
G6PDH	Glucose-6-phosphate dehydrogenase
HA	Hemagglutinin
HBcAg	Hepatitis B core antigen
HCl	Hydrochloric acid
HPAI	Highly pathogenic avian influenza

h	Hour
IFN	Interferon
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
kHz	Kilo Hertz
LB	Luria Bertani
LPAI	Low pathogenic avian influenza
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride
min	Minutes
NA	Neuraminidase
NaCl	Sodium chloride
NAG	N-acetyl-D-glucosamine
NAM	N-acetyl-muramic acid
NS	Nonstructural protein
NP	Nucleocapsid protein
OD	Optical density

PABP II	Poly-A binding protein II
PAGE	Polyacrylamide gel electrophoresis
PA	Polymerase acidic
PB	Polymerase basic
pH	Power of hydrogen
p-npp	p-nitrophenyl phosphate
P13K	Phosphoinositide 3-kinase
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TBS-T	Tris-Buffered saline+Tween 20
TEMED	Tetramethylethylenediamine
V	Volts
W	Watts

CHAPTER 1

INTRODUCTION

1.1 Background

Influenza A viruses have been isolated from avian and mammalian species, including horses, wild birds and humans can cause acute respiratory disease due to its high variability (Lin et al., 2007). They are responsible for both seasonal influenza outbreaks and occasional pandemics, affecting millions of people worldwide. They can be classified into two groups, highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). Mortality of HPAI can be as high as 100% whereas LPAI causes milder symptoms (Metreveli, 2006). Furthermore, they are divided into subtypes based on the antigenic variations of the viral surface glycoproteins, neuraminidase (NA) and hemagglutinin (HA). There are 9 NA and 16 HA subtypes that have been identified in poultry and wild birds throughout the world (Fouchier et al., 2005).

Highly pathogenic avian influenza H5N1 virus has caused disease outbreak in poultry in Asia and the spread of the virus to Europe and Africa have raised pandemic concern as it poses a significant challenge to animal and human health (Peiris et al., 2007). Outbreaks of H5N1 since 1997 have been unprecedented with over 303 human deaths and 500 million birds being killed (Abubakar et al., 2011). H5N1 virus first proved lethal in human was disseminated from birds to human in

1997 in Hong Kong in which 18 confirmed infections have resulted in six deaths (Kawaoka, 2012).

H5N1 virus was firstly isolated from geese in Guangdong, China in 1996 and has caused significant outbreaks in Thailand, Vietnam, Indonesia and other East Asian countries since early 2004 (World Health Organizations, 2012). It has also been detected in Malaysia with a total of 16 outbreaks in poultry in 2004, 2006 and 2007. However, there was no human infections reported (World Health Organizations, 2012). In Malaysia, the first ever HPAI outbreak caused by subtype H5N1 was reported in a village in the state of Kelantan (Tee et al., 2009).

NS1 protein plays a critical role in suppressing type 1 interferon expression and current evidence shows that NS1 proteins able to restrict IFN- β induction through pre-transcriptional and post-transcriptional process (Kochs et al., 2007). It is known that a strong interferon response against the influenza virus can be generated by host to attenuate the virus if the function of the NS1 is blocked. Recombinant influenza virus with mutated NS1 proteins has been use to develop live-attenuated virus vaccine (Steel et al., 2009). Antibody, cell-mediated, and mucosal protective immune responses were elicited when the animal were immunize with NS1-truncated viruses. NS1 protein is not synthesized in the virions but it expressed in large amounts in the nucleus of the virus-infected cell (Li et al., 1998). In addition, NS1 protein has been expressed in prokaryotic

bacteria (Sadeghi et al., 2014) and yeast (Ward et al., 1994) for protein interaction study. Cell disruption method such as sonication has been used to extract the NS1 protein for the study (Allonso et al., 2011).

The cell disruption techniques can be divided into mechanical and non-mechanical methods. Examples of mechanical method are sonication, high pressure homogenization and bead milling whereas non-mechanical methods involve chemical, enzymatic and physical (osmotic) method. Mechanical methods enable a high recovery of the product. However, these methods suffer from several drawbacks such as high operating cost (Bangaru, 2004). On the other hand, the benefits of non-mechanical method such as enzymatic method are low energy consumption and the reaction is specific. Nevertheless, it has been restricted to laboratory scale due to the high cost of enzyme needed for large scale disruption (Balasundaram et al., 2009).

The main objective of this project was to compare two different cell disruption methods (ultrasonication and enzymatic method) for the release of NS1 protein of H5N1 virus from *E. coli* Novablue. In this study, several parameters such as sonication duration, amplitude of ultrasonic processor, incubation duration, lysozyme concentration and temperature were investigated to optimize the release of NS1 protein from the cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Influenza A Virus H5N1

2.1.1 Structure of the Virus

H5N1 is a subtype of influenza A virus and classifiesd under the family of *Orthomyxoviridae*. It is a RNA virus with envelope, which consists eight segments of single-stranded and negative-sense genome. There are three forms of the virus particle that can be seen under the electron microscopy. They can be present as a spherical form with diameters ranging from 80 to 120 nm, a rod-shaped form with a length ranging from 120 to 300 nm and a filamentous form, with length longer than 300 nm. However, it is usually present in spherical shape with glycoprotein spikes on the surface. There are approximately 500 glycoprotein spikes on the surface and these spikes represent the envelope glycoprotein hemagglutinin, which has a rod-like shape and neuraminidase, which is mushroom-shaped (Louisirirotchanakul et al., 2013).

2.1.2 Genome of the Virus

It has eight gene segments which encode 10 proteins as shown in Figure 2.1. They are nonstructural protein 1 (NS1) and nonstructural protein 2 (NS2), hemagglutinin (HA), neuraminidase (NA), matrix proteins M1 and M2,

nucleocapsid protein (NP), and three polymerases, namely polymerase basic (PB) 1, PB2, and polymerase acidic (PA) proteins (Peiris et al., 2007). The three largest segments encoding for PB1, PB2 and PA are essential for RNA synthesis. The fourth segment and sixth segment encoding for HA and NA, respectively are involved in attachment, fusion between the viral envelope and host cellular membrane, and release of virus particles. Fifth segment encoding for NP plays a crucial role for the nuclear transport of viral RNA. The seventh segment encodes for M1 and M2 proteins. M1 matrix protein covers the inner surface of the viral membrane and M2 forms an ion-channel essential for pH regulation. The eighth segment encodes for both NS1 and NS2. NS1 and NS2 are involved in various aspects in the process of taking over the host cell and they only expressed in infected cells, but they are not integrated into virions (Metreveli, 2006).

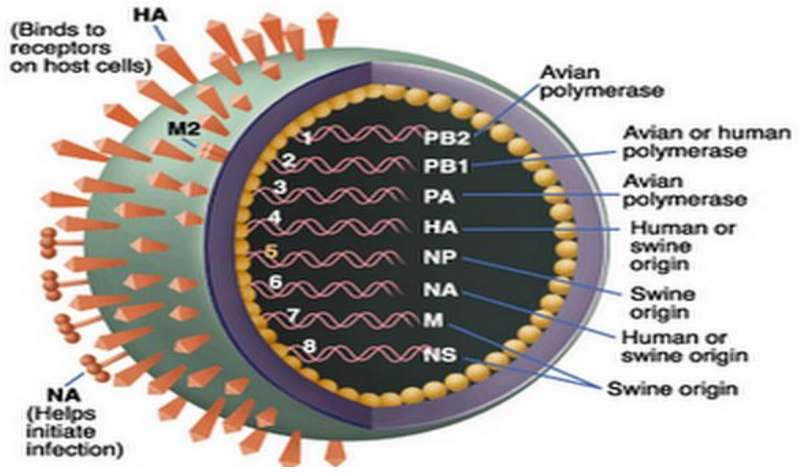


Figure 2.1: Schematic diagram of the structure of influenza A virus H5N1. (Adapted from WordPress 2009)

2.2 NS1 Protein

NS1 protein is conceptually classified into two well characterized functional domains. An N-terminal RNA-binding domain (amino acid residues 1-73), that binds with low affinity to several RNA species and a C-terminal effector domain (amino acid residues 74-230), which mainly mediates interactions with host cell proteins, but also functionally maintains the N-terminal RNA-binding domain (Hale et al., 2008). NS1 protein of most influenza A virus has an average length of 230 amino acids (Li et al., 2010) and a molecular weight of 26 kDa. N-terminal of NS1 protein possess all of the dsRNA binding activities of the full-length protein. It forms a symmetric homodimer and each polypeptide chain of the RNA-binding domain consists of three α-helices: residues Asn4-Asp24, Pro31-Leu50 and Ile54-Lys70 (Chien et al., 2004). In contrast, the C-terminus of the NS1 protein primarily contains three functional domains: eukaryotic initiation factor 4GI (eIF4GI), poly-A binding protein II (PABP II) and cleavage and polyadenylation-specific factor 30 kDa subunit (CPSF30). It consists of three α-helices and seven β-strands (Bomholdt and Prasad, 2006).

NS1 protein is a significant virulence factor of the H5N1 influenza virus and plays a major role in countering host cell antiviral defenses. It predominantly exerts its biological activity via the following strategies: (i) inhibition of cellular pre-mRNA splicing and polyadenylation; (ii) enhancement of viral mRNA translation; (iii) repression of host immune response; (iv) activation of the

phosphoinositide 3-kinase (PI3K) signaling pathway; and (v) involvement in apoptosis of host cells (Hale et al., 2008). NS1 protein is an interferon (IFN) antagonist which induced abundant of chemokines and conferred resistance to the antiviral responses of IFN (Tsai et al., 2013). For instance, NS1 proteins from the A/VN/1203/04 (H5N1) and A/Hongkong/483/97(H5N1) strains were found to exhibit different interactions with CPSF30, a cellular factor needed for the processing of cellular pre-mRNAs (Twu et al., 2007). When NS1 protein binds to the cellular CPSF, it inhibits the formation of 3' end cellular pre-mRNA and therefore is pathogenic.

Spontaneous deletion of amino acid residues in NS1 protein is common in influenza A virus H5N1. The deletion of five residues at position 80–84 has been reported in several studies (Li et al., 2004). The deletion can be observed in almost all H5N1 influenza virus strains isolated during 2001–2009 and it has been implicated in enhancing the virulence of H5N1 in mice and chickens (Long et al., 2008). A substitution of an aspartic acid to glutamic acid at position 92 in NS1 is one important contributing factor to the high virulence of H5N1/1997 and it has been shown to be responsible for countering antiviral cytokine responses in pigs (Seo et al., 2002). In addition, a PDZ domain ligand is constituted by the last four C-terminal residues of the 230 amino acid long NS1 proteins. The ligand binding motif is a possible virulence factor and appears to increase the viral pathogenicity in mouse model. This ligand binding motif binds cellular proteins which contains

PDZ ligand, hence leading to disruption of a range of cellular signaling pathways (Jackson et al., 2008).

NS1 protein has been investigated as a potential candidate for use in Differentiation of Infected from Vaccinated Animals (DIVA) strategies in the control of avian influenza (Capua et al., 2003). In addition, vaccination with a single dose of a selected virus encoding the NS1 1-99 protein completely protects chickens from lethal challenge with homologous HPAI virus A/Viet Nam/1203/04 (H5N1) and provided a high level of protection from a heterologous virus, A/egret/Egypt/01/06 (H5N1) (Steel et al., 2009). It is known that NS1 protein plays an important role in the virulence of influenza A virus H5N1. Besides, NS1 is expressed only in infected cells and not incorporated into virions. Therefore, it can be applied as vaccine and diagnostic tool for the detection of the avian influenza virus (Manasatienkij et al., 2008).

In a comparative study done by Metreveli (2006) on NS1 gene extracted from H7N7, H4N6, H12N5, H6N8 and H5N1 subtypes, it has been observed that, mutant influenza A viruses only exhibit high pathogenicity in mice deficient of antiviral mediators. The study showed that the main activity of NS1 is to antagonize IFN-mediated antiviral responses (Hale et al., 2008). In another study, influenza A virus deficient in NS1 protein is generated and it showed the important role for NS1 protein in resisting the host IFN responses (Egorov et al.,

1998). Direct activation of mRNA translation through interactions between NS1 protein and eukaryotic initiation factor 4GI at effector domain enhances viral replication (Hale et al., 2009). Therefore, deletions in this domain hindered replication of influenza viruses. Zhu and coworkers (2008) showed in their study that deletions of CPSF 30 binding domain (amino acids 191-195) attenuate virus in antagonize IFN production. Moreover, the study showed that binding site for PAB II is at amino acids 215-237 of NS1 protein. A study done by Chen and coworkers (1999) to clone and express NS1 protein in *E. coli* BL21, aimed to investigate the relationship between NS1 protein and the poly(A)-binding protein II. After NS1 binds PAB II, oligo(A) tails elongate during the formation of the 3' poly(A) ends of mRNA. This stops PAB II from elongating the poly-A tails properly in the host cell nucleus (Chen et al., 1999). Thus, successive elongation of this poly(A) tails is arrested because NS1 protein inhibits the function of PAB II. In addition, Chen and coworkers found out that it also prevent these pre-mRNAs from exporting out of nucleus, thus causes accumulation of cellular pre-mRNAs which contains poly(A) tails in nucleus .

2.3 The Need for Bacterial Cell Disruption

The first step in the downstream processing of intracellular proteins is the disruption of bacterial cell (Benov and Al-Ibraheem, 2002). For the purpose of recovering and purifying the proteins efficiently, the proteins have to first release from their host in soluble form. Hence, the significance of cell disruption is

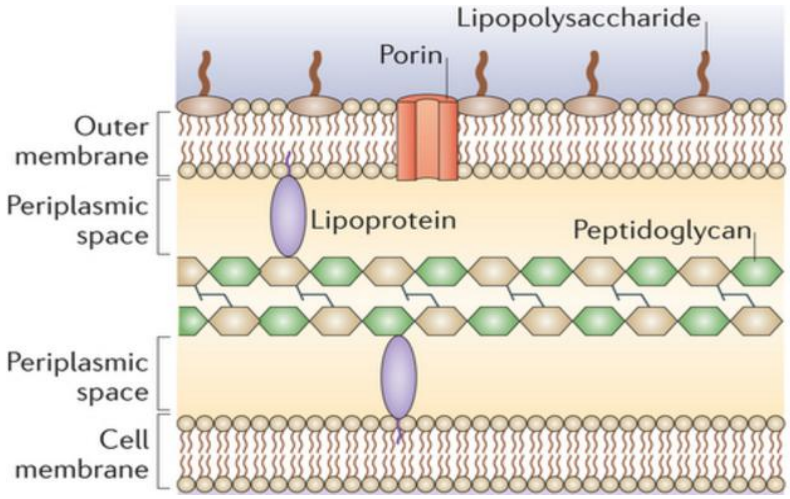
increasing with the increase in the use of genetic engineering techniques which use bacterial cells as host for the production of proteins.

In this study, *Escherichia coli* is the organism of choice for expressing the recombinant NS1 protein. The rationale of using *E. coli* as expression host was due to its ability to grow in cheap media, rapid doubling time and has been proved to work successfully with many expression vectors (Tey et al., 2004). In addition, it is easy to be genetically manipulated and able to carry high amount of plasmids. However, *E. coli* usually does not secrete the product of interest into the culture medium. For this reason, a signal sequence like a poly-histidine tail, maltose-binding proteins and β-galactosidase which are frequently attached to recombinant proteins is essential for the proteins to be transported from the cytoplasm to periplasm. The signal sequence is cleaved either during translation or post translationally (Bangaru, 2004). Due to the bulky nature of the enzyme, it may not cross the cell wall barrier and therefore is retained in the periplasmic region of *E. coli* (Bangaru, 2004). Thus, cell disruption is necessary for the release of the expressed protein.

2.4 Bacterial Cell Structure

Almost all bacterial cell walls contain a basic peptidoglycan network. There are two classes of bacteria defined on the basis of their staining characteristics of the cell wall, which are Gram-positive and Gram-negative bacteria. The difference

between the cell envelope of Gram-negative and Gram-positive bacteria is shown in Figure 2.2. The cell wall of Gram-negative bacteria comprises of a thinner peptidoglycan layer (1.5 - 2.0 nm), which itself is surrounded by an outer membrane containing lipopolysacharide (Silhavy et al., 2010). In another word, it is made up of outer cell wall and inner cytoplasmic membrane separated by periplasm. The peptidoglycan of Gram-positive bacteria and Gram-negative bacteria is comprised of linear polysaccharide chains of alternating N-acetyl-muramic acid (NAM) and N-acetyl-D- glucosamine (NAG) residues joined by β-1,4-glycosidic bonds. The chains are cross-linked by a tetra peptide of basic structure L-alanyl-D-glutamyl-L-R_3-D-alanine attached to the C_3 lactic acid side chains of the NAM residues (Suvorov et al., 2008). The branches of the peptide of the parallel chains are cross-linked. The resulting rigid structure composes a single macromolecular network to give the tensile strength and shape. The cytoplasmic membrane is comprised mainly of phospholipids and protein. The cell wall is readily dissociable by Triton X-100 and other detergents (Bangaru, 2004). Disruption of bacterial cell to release protein seems to be more difficult than disruption of mammalian cells because of their firm cell wall that is stronger than the plasma membrane (Aderiye and Oluwole, 2014). Hence, effort must be centered on cell disruption strategies to release highest amount of protein, particularly when the production yield and also the preservation of the activity and structure of the recombinant protein are concerned (Feliu et al., 1998).



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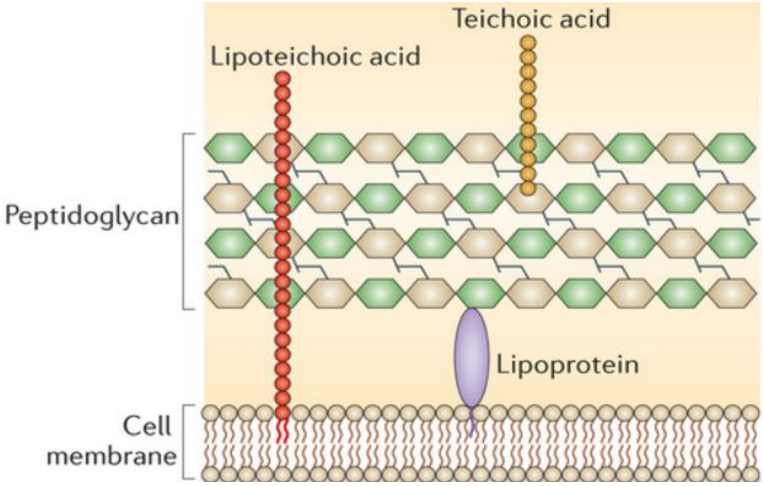

B

Figure 2.2: Cell wall envelope of (A) Gram-negative bacteria, (B) Gram-positive bacteria (Brown et al., 2015).

2.5 Cell Disruption

Different cell disruption methods have been developed to establish a low-priced and effective release of intracellular contents and this can be carried out either by mechanical and non-mechanical cell disruption methods. Mechanical cell disruption techniques such as ultrasonication, French Press, high-pressure homogenization, high-speed homogenization and bead milling are the common methods used in cell disruption. On the other hand, examples of non-mechanical cell disruption methods that are commonly used are enzymatic cell lysis and chemical treatment (Gunerken et al., 2015). Although there are various techniques available for intracellular contents release, mechanical methods found to have greater commercial application than non-mechanical methods because it is deduced that the latter have economic and operational limitations at process scale. Difficulty in transmitting adequate power to large volumes of cell has become an obstacle for mechanical method such as ultrasonication in large-scale cell disruption (Kalumuck et al., 2003). Non-mechanical methods such as enzymatic and chemicals method are limited to laboratory scale because it is expensive in large scale (Baldwin and Robinson, 1994).

2.5.1 Mechanical Method: Ultrasonication

Ultrasonication is one of the most frequently used mechanical cells disruption method. This method based on liquid shear generated by ultrasound (above 16 kHz) is produced electronically and transmitted through a metallic tip to a

concentrated cellular suspension (Chemat et al., 2011). A combination of formation, growth and implosive collapse of the vapour bubbles, known as cavitation process are created by the motion of strong sound waves. Cavitation phenomenon occurs in short time intervals, usually in microseconds to milliseconds (Gunerken et al., 2015). During the collapse of the cavitation bubbles, a high amount of sonic energy was changed to mechanical energy in the form of intense elastic waves. Very high energy densities are obtained locally and the kinetic energy content of the bacterial cells exceeds the cell wall strength which eventually leads to cell wall disintegration (Tangtua, 2014).

There are several factors that affect cell disruption using ultrasonication. The factors are acoustic power input, initial cell concentration and sonication duration. Literatures on the effects of process variables on the microbial cell disruption by ultrasonication are summarized in Table 2.1. The protein release constant during the disruption of *E. coli* was found to increase linearly with the increase in the acoustic power over the range of 20 to 80 W (Kuboi et al., 1995) and in the range of 100 to 200 W (Fonseca and Cabral, 2002). Besides, the disruption rate for Brewers' yeast was found to increase linearly in the range of 67 to 187 W (James et al., 1972). For the initial cell concentration parameter, James et al. (1972) found that there was no detectable dependence of cell concentration on the disruption rate of Brewers' yeast over the range 20 – 60% w/v. Moreover, temperature increase from 17 to 30 ℃, increased the total soluble protein release from 52 to 63% (James et al., 1972). Nevertheless, while high temperature is

known to promote cell disruption, however it may cause protein denaturation and therefore should be avoided, if possible.

Ultrasonication is not suitable for large-scale disruption due to the difficulty in processing large volumes of cell suspension and its high operation cost for large-scale disruption. The high operation cost is owing to its small processing volume per operation, long operation period and energy-intensive (Ho et al., 2008). In a study done by Ho and coworkers (2008), the operation price was reported to be 2.8- and 3.4-fold greater than that of bead milling and high pressure homogenization, respectively. Besides, ultrasonication was found to be similar to bead milling with respect to the amount of Hepatitis B core antigen released. Nonetheless, it was reported that ultrasonication is less efficient compared to high-pressure homogenization.

Table 2.1: Summary of the effects of process variables on microbial cell disruption by ultrasonication.

Microorganism	**Acoustic power (W)**	**Initial cell concentration**	**Analysis**	**References**
Acetobacter peroxydans	30 – 100 (20 kHz)	60% w/v (wet wt)	Soluble protein	Kapucu, 2000
Brewers' yeast	67 – 195 (20 kHz)	20 – 60% w/v (wet wt)	Soluble protein	James et al., 1972
E. coli, *S. cerevisiae*, *B. subtilis*	20 – 30 (20 kHz)	35 – 200% w/v (wet wt)	Soluble protein, acid phosphatase, G6PDH, fumerase	Kuboi et al., 1995
E. coli	100 – 250 (20 kHz)	130% w/v (dry wt)	Soluble protein, penicillin acylase	Fonseca and Cabral, 2002

2.5.2 Non-mechanical Method: Enzymatic Disruption

Non-mechanical cell disruption method such as enzymatic disruption is an excessively studied cell disruption method because it is gentle (prevention of aggressive physical conditions), easy to scale-up and has a high selectivity. Parameters such as lysozyme concentration, incubation time and incubation temperature can lead to changes in the disruption efficiency. Heat production due to conversion of mechanical energy to heat energy during cell disruption process can be avoided. The main enzyme classes that have been studied for cell lysis of different organisms are glycosidases, glucanases, peptidases and lipases (Gunerken et al., 2015). Enzymes will bind to specific molecules in the cell wall or cytoplasmic membrane to hydrolyze the bonds, which result in cell wall or cytoplasmic membrane disintegration. In glycosidases class, lysozyme is the most prominent member which hydrolyzes β-1,4-glycosidic bond from N-acetylmuramic acid to N-acetylglucosamine in bacterial cell wall peptidoglycan. In a study by Knorr et al. (1979), lysozyme was observed not to have any effect on Brewers' yeast cells. Instead, combination of lysozyme and zymolase were able to release 44% more carbohydrates than zymolase alone.

Enzymatic cell disruption method is advantageous in terms of specific reaction and low energy consumption. In a study by Malamy and Horecker (1964), lysozyme was used to digest the peptidoglycan cell wall for spheroplast formation. It was found that 93% of the periplasmic enzyme, alkaline phosphatase was

released. Nevertheless, it has been limited to laboratory scale because of the high cost of enzyme required for disruption at large scale. In a study done by Ho and coworkers (2008), enzymatic lysis was found to be an expensive technique. Even though it was 31% cheaper than ultrasonication, the operation cost per unit volume of feedstock required was 1.0-, 1.4-, and 5.6-fold higher than that of batch-mode bead milling, high-pressure homogenization and continuous-recycling bead milling, respectively. Besides, the added lytic enzyme in the cell lysate may complicate downstream product purification steps (Balasundaram et al., 2009).

In this study, the performance of ultrasonication was compared to enzymatic (lysozyme) disruption method for the release of intracellular NS1 protein of H5N1 virus in *E. coli*. Besides, several parameters such as sonication duration, amplitude of ultrasonic processor, incubation duration, lysozyme concentration and temperature were investigated to optimize the release of NS1 protein from the cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 *E. coli* clone

E. coli clone, NNS1 harboring pRSETB vector containing the gene NS1 from influenza A virus H5N1 (A/Chicken/Malaysia/5858/2004) was obtained from Dr. Yap Wei Boon, Universiti Kebangsaan Malaysia. The pRSETB vector has T7 promoter, 6x His tag and was ampicilin resistant.

3.2 Reagents, Chemicals and Equipment

The list of reagents and chemicals used throughout this study are listed in Table 3.1.

Table 3.1: List of reagents and chemicals used.

Reagents/Chemicals	Manufacturers, Country
0.1% Coomassie Brilliant Blue R-250	Bio Basic Canada Inc., Canada
5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP-NBT)	Millipore, USA
95% Ethanol	HmbG Chemicals, Germany
Acetic glacial acid	QR ëc, Malaysia
Acrylamide	Amersham Biosciences, U.K.

Table 3.1: List of reagents and chemicals used (continued).

Reagents/Chemicals	**Manufacturers. Country**
Albumin fraction V	Merck, Germany
Ammonium persulfate	Sigma Aldrich, U.K.
Ampicillin	Bio Basic Canada Inc., Canada
Benchmark protein ladder	Thermo Fisher Scientific, USA
Bis-acrylamide	Amresco, USA
Bromophenol blue	Sigma Aldrich, USA
Butanol	Merck, Germany
Coomassie Blue G-250	Bio Basic Canada Inc., Canada
Diethanolamine (DEA)	Merck, Germany
Glycerol	Thermo Fisher Scientific, U.K.
Glycine	Thermo Fisher Scientific, U.K.
Hydrochloric acid	VWR Prolabo, Singapore
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Bio Basic Canada Inc., Canada
Luria Bertani agar	Sigma Aldrich, U.K.
Luria Bertani broth	Sigma Aldrich, U.K.
Methanol	Merck, Germany
Milk diluent	Kirkegaard & Perry Laboratories Inc., USA
Phosphoric acid	QR ëc^{TM}, New Zealand
p-nitrophenyl phosphate (p-npp)	Bio Basic Canada Inc., Canada

Table 3.1: List of reagents and chemicals used (continued).

Reagents/Chemicals	**Manufacturers. Country**
Sodium chloride	Bio Basic Canada Inc., Canada
Sodium dodecyl sulphate (SDS)	Bio Basic Canada Inc., Canada
Tetramethylethylenediamine (TEMED)	Thermo Fisher Scientific, U.K.
Tris	Bio Basic Inc., Malaysia
Triton X-100	Sigma Aldrich, U.K.
Tween 20	Merck, Germany

The list of instruments, apparatus and laboratory wares used throughout this study are listed in Table 3.2.

Table 3.2: List of instruments, apparatus and laboratory wares used.

Instruments/Apparatus/Laboratory wares	**Brand/Model, Country**
Autoclave machine	HIRAYAMA, Japan
Cellulose acetate 0.45 µm syringe filter	Pall Corporation, USA
Centrifuge machine	Eppendorf, Germany
Centrifuge tubes (15 ml and 50 ml)	AXYGEN Scientific, USA
ChemiDoc™ MP imaging system	Bio-Rad, USA
Flat bottom 96-well microplate	Greiner bio-one, Germany
Infinite® 200 PRO multimode reader	TECAN, Switzerland
Microcentrifuge machine	Thermo Scientific, USA

Table 3.2: List of instruments, apparatus and laboratory wares used (continued).

Instruments/Apparatus/Laboratory wares	**Brand/Model, Country**
Microcentrifuge tubes (200 µl and 1.5 ml)	AXYGEN Scientific, USA
Mini-PROTEAN Tetra System	Bio-Rad, USA
Nitrocellulose membrane	Pall Corporation, USA
Petri dishes	Labmart, Pakistan
pH meter	Sartorius, Germany
Shaking incubator	Straits Scientific (M) Sdn. Bhd., Malaysia
Spectrophotometer and cuvettes	Thermo Scientific, USA
SSL4 see-saw rocker	Stuart, U.K.
Syringe (10 ml/cc)	Terumo, Philippines
Trans-Blot SD semi-dry electrophoretic transfer cell	Bio-Rad, USA
U-bottom 96 well microplate	Greiner bio-one, Germany
Ultrasonic probe (6 mm diameter)	Cole-Parmer, USA
Ultrasonic processor	Cole-Parmer, USA
Weighing balance	Sartorius, Germany
Whatman filter paper	Sigma Aldrich, U.K.

3.3 Methodology

3.3.1 Growth of *E. coli* cells

3.3.1.1 Preparation of Luria Bertani Agar

Luria Bertani (LB) agar was prepared in a 1 L blue-capped bottle by adding 17.5 g of LB agar powder and topped up with 500 mL of distilled water. The mixture was mixed well and autoclaved. Then, 500 µL of 100 mg/mL ampicillin was added into the medium to obtain a final concentration of 100 µg/mL ampicillin. The medium was then poured into sterile Petri dishes and allowed to solidify at room temperature. The prepared agar plates were then stored at 4 ℃.

3.3.1.2 Preparation of LB Broth

For the preparation of overnight cultures, LB broth (pH 7) was prepared in a conical flask by adding 1 g of LB broth powder and topped up with 50 mL of distilled water. The mixture was mixed well and autoclaved.

In order to grow the *E. coli* cells, 2 L of LB broth (pH 7) were prepared in four different 1 L conical flasks, each containing 500 mL broth. Ten grams of LB broth powder were added into each conical flask and topped up with 500 mL of distilled water. The mixture was mixed well and autoclaved.

3.3.1.3 Streaking of Bacteria

The bacterial glycerol stock was streaked onto a LB agar containing 100 µg/mL ampicillin and incubated at 37 ℃ for 16-18 h. Single colonies of the bacterial cell were observed after 16-18 h of incubation.

3.3.1.4 Preparation of Overnight Culture

A volume of 50 µL of 100 µg/mL ampicillin was added into 50 mL of LB broth. Next, a single bacterial colony was inoculated into the LB broth. The culture was then incubated at 37 ℃ under vigorous shaking at 250 rpm for 16-18 h in a shaking incubator.

3.3.1.5 Preparation of Log-phase Bacteria

The overnight culture was inoculated into 2 L fresh LB broth supplemented with 100 µg/mL ampicillin at a ratio of 1:50. A volume of ten millilitres of overnight bacterial culture was added into each conical flask and incubated at 37 ℃ under vigorous shaking at 250 rpm. IPTG was then added into the culture with a final concentration of 0.5 mM when log-phase of the culture (OD_{600} = 0.6 - 0.8) was attained to induce protein expression.

3.3.2 Collection of Cell Pellets

The culture was then further incubated at 37 ℃ under vigorous shaking at 250 rpm for another 16-18 h. The next day, the 2 L culture was poured into 50 mL centrifuge tubes and the tubes were centrifuged at 7,000 rpm for 15 min at 4 ℃. After centrifugation, the supernatants were discarded and the harvested pellets were kept at -20 ℃ until further use.

3.3.3 Ultrasonication Bacterial Cell Disruption

3.3.3.1 Preparation of 50 mM Tris-HCl, 150 mM NaCl Tris-Buffered Saline (TBS) (pH 7.6)

A weight of 6.05 g Tris and 8.76 g of NaCl were dissolved in 800 mL of distilled water. The pH of the mixture was adjusted to pH 7.6 using 37% hydrochloric acid (HCl). Then, the volume of the mixture was topped up to 1 L and autoclaved.

3.3.3.2 Ultrasonication

The harvested cell pellet was resuspended in TBS (pH 7.6) to obtain a 25 mL of 2.5% biomass concentration of *E. coli* cell suspension. Disruption of cells was performed using an ultrasonic processor at 20 kHz, 130 W and 40% amplitude equipped with a needle titanium probe of 6 mm in diameter. The sonication duration was 60 min with 20 s pulse on and 20 s pulse off. One millilitre of cell suspension was sampled from the suspension every 10 min up to 60 min into

different 1.5 mL microcentrifuge tubes. The cell suspension was kept in an ice bath during the cell disruption process to prevent overheating. The cell disruption process was repeated by changing the amplitude parameter to 60% and 80%. The collected samples were then centrifuged at 3,000 rpm for 3 min. Then, the supernatants were separated from the pellets into another microcentrifuge tubes. Both of the pellets and supernatants were kept at 4 ℃ for further analyses.

3.3.4 Enzymatic Bacterial Cell Disruption

3.3.4.1 Preparation of 1 M Magnesium Chloride

One molar of magnesium chloride ($MgCl_2$) was prepared in a universal bottle by adding 0.952 g of $MgCl_2$ powder and topped up with distilled water to 10 mL. The solution was then autoclaved.

3.3.4.2 Lysozyme Bacterial Cell Disruption

The harvested cell pellet was resuspended in TBS in the presence of 0.1% triton X-100 to obtain 25 mL final volume of 2.5% biomass concentration of *E. coli* cell suspension. Then, the cell pellets were lysed with lysozyme in the presence of 4 mM $MgCl_2$. The parameters used were different incubation durations (20 min, 40 min, 60 min, 120 min), different lysozyme concentrations (0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL) and temperature at 4 ℃ and 28 ℃). The collected samples were centrifuged at 3,000 rpm for 3 min. Then, the supernatants were

separated from the pellets by transferring them into another microcentrifuge tubes. Both of the pellets and supernatants were kept at 4 ℃ until use.

3.3.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.5.1 Preparation of 10% Sodium Dodecyl Sulphate (SDS)

Ten grams of SDS was mixed with 100 mL of autoclaved distilled water. The solution was filtered with a 0.45 µm cellulose acetate syringe filter into a blue-capped bottle and stored at room temperature.

3.3.5.2 Preparation of 10X Running Buffer

Thirty grams of Tris-base and 144 g of glycine were added into 800 mL of distilled water. The mixture was topped up to 1000 mL with distilled water and mixed well.

3.3.5.3 Preparation of 4X Lower Buffer for Resolving Gel (pH 8.8)

To prepare 100 mL of 4X lower buffer, 1.5 M of Tris-base and 4 mL of 10% SDS were added into 80 mL of autoclaved distilled water and mixed well. The pH of the mixture was adjusted to pH 8.8 using 37% HCl**.** The mixture was topped up to

100 mL with distilled water and mixed well. The mixture was filtered with a 0.45 µm cellulose acetate syringe filter and stored at 4 ℃ until use.

3.3.5.4 Preparation of 4X Upper Buffer for Stacking Gel (pH 6.8)

To prepare 100 mL of 4X upper buffer, 0.5 M of Tris-base and 4 mL of 10% SDS were added into 80 mL of distilled water and mixed well. The pH of the mixture was adjusted to pH 6.8 using 37% HCl**.** The mixture was topped up to 100 mL with autoclaved distilled water and mixed well. The mixture was filtered with a 0.45 µm cellulose acetate syringe filter and stored at 4 ℃ until use.

3.3.5.5 Preparation of 10% Ammonium Persulfate (APS) Stock Solution

To prepare 1 mL of 10% APS stock solution, 100 mg of APS was dissolved in 1 mL of autoclaved distilled water and stored at -20 ℃ until use.

3.3.5.6 Preparation of Staining Solution

To prepare 1000 mL of staining solution, 0.5 g of 0.1% Coomassie Blue R-250, 40% methanol and 1% acetic acid were added into a blue-capped bottle which has been wrapped with aluminium foil. The mixture was then topped up to 1000 mL with distilled water and mixed well.

3.3.5.7 Preparation of Destaining Solution

To prepare 1000 mL of destaining solution, 40% methanol and 10% acetic acid were added into a blue-capped bottle which has been wrapped with aluminium foil. The mixture was then topped up to 1000 mL with distilled water and mixed well.

3.3.5.8 Preparation of 6X SDS Sample buffer

Seven millilitres of 4X upper buffer, 3 mL of glycerol, 1 g of 10% SDS and 12 mg of bromophenol blue were added into a universal bottle and mixed well. A volume of 0.5 mL of 5% β-mercaptoethanol was added when required.

3.3.5.9 Casting of Gels and Electrophoresis

A spacer plate and a short plate were cleaned with 70% ethanol. The glass plates were then inserted into a gel-casting apparatus and tightened. Table 3.3 shows the compositions of SDS-PAGE resolving gel and stacking gel.

Table 3.3: Compositions of resolving and stacking Gels for SDS-PAGE.

Gel Components	**Resolving Gel (µL)**	**Stacking Gel (µL)**
4 x upper buffer (pH 6.8) [0.5 M Tris-base, 0.4% SDS]	-	496.0
4 x lower buffer (pH 8.8) [1.5 M Tris-base, 0.4% SDS]	1243	-
30% acrylamide/bis (30% T, 2.67% C)	2479	332
Autoclave distilled water	1243	1159
10% APS	31	13.2
TEMED	5	2.8

Resolving gel casting solution was prepared and mixed thoroughly and aspirated into the space between the assembled spacer plate and short plate. The gel was overlaid with 1 mL of 1-butanol to prevent air bubbles formation. The resolving gel was allowed to polymerize for 40 min. A line would become visible at the top of the resolving gel as it polymerized. Butanol was removed by inverting the gel and rinsing the top of the gel with distilled water. The residual liquid was drained using filter paper. Stacking gel solution was prepared and pipetted slowly on top of the solidified resolving gel. The comb was then inserted and the stacking gel was left to polymerize for 30 min. For sample preparation, 20 µL of 10X diluted samples (pellets) and samples without dilution (supernatants) were aliquot into separate centrifuge tubes and mixed with 4 µL of 6X SDS-PAGE gel loading

buffer supplemented with 5% β-mercaptoethanol. The mixtures were then boiled for 10 min. As the stacking gel has polymerized, the comb was removed. The gel-casting apparatus with the gels were transferred to the electrophoresis tank. The wells were rinsed with 1X SDS-PAGE running buffer to remove residual unsolidified polymers and the inner and outer chamber were filled with running buffer. Subsequently, 6 µL of the boiled samples were loaded into the wells alongside with 4 µL protein marker. Electrophoresis was conducted at 16 mA/gel and 100 V power supply (MSMP-300V, USA) for 1 h and 30 min or until the dye front ran off. Protein bands were then detected by staining the gel with staining solution for 20 min and the gel was destained with destaining solution until a clear background was obtained.

3.3.6 Quantification of Protein

3.3.6.1 Bradford Assay

Bovine serum albumin (BSA) standards were prepared at concentrations ranging from 0-10 µg/µL. The Greiner brand medium binding flat bottom 96-well plate was filled with 15 µL of TBS. Five microlitres of 10X diluted samples (pellets) and 5 µL of samples without dilution (supernatants) were then added into their respective wells. As for BSA standard, 20 µL, 18 µL, 16 µL, 14 µL, 12 µL and 10 µL of TBS were added into six different wells separately. Then, 2 µL, 4 µL, 6 µL, 8 µL and 10 µL of BSA were added accordingly. Subsequently, 200 µL of the Bradford dye was added into each well and the 96-well plate was agitated for 5

min to ensure thorough mixing of the samples with the Bradford dye. The plate was read at OD_{595nm} using Infinite® 200 PRO multimode reader and the sample concentrations were calculated from the standard curve generated (refer to Appendix A) using Microsoft Excel.

3.3.6.2 Gel image software analysis

The bands on the gel were visualized using ChemiDoc™ MP imaging system and the image was acquired using ImageLab™ version 5.1. Software window was clicked > new protocol > gel imaging > protein gels > coomassie blue > position gel > run protocol. Lanes and bands were created automatically by selecting the appropriate button in the Navigator. The lanes were edited by resizing or bending the lanes as per the band positions. The analysis table generated from the software was exported to Excel. In the Excel files, the "band %" of band of interest was highlighted. The band % obtained was multiplied with the concentration of total protein obtained from the Bradford assay to obtain the concentration of NS1 protein.

3.3.7 Western Blotting

3.3.7.1 Preparation of Transfer Buffer

To prepare 1000 mL transfer buffer, 14.5 g of glycine, 3 g of Tris and 200 mL of methanol were added into a blue-capped bottle. The mixture was then topped up to 1000 mL with distilled water and mixed well.

3.3.7.2 Preparation of 10X Tris Buffer Saline (pH 7.4)

To prepare 1000 mL 10X TBS, 12.1 g of Tris and 116.9 g of NaCl were added into 800 mL of distilled water and mixed well. The pH of the mixture was adjusted to pH 7.4. The mixture was then topped up to 1000 mL with distilled water and mixed well.

3.3.7.3 Preparation of Blocking Buffer (Tris Buffered-Saline-Tween 20 (TBS-T))

To prepare 1000 mL blocking buffer, 100 mL of 10X TBS (pH 7.4) and 1 mL of 0.1% Tween 20 were added into a blue-capped bottle. The mixture was then topped up to 1000 mL with distilled water and mixed well.

3.3.7.4 Semi-dry Transfer

Following SDS-PAGE, the gel was removed from the glass plates and the stacking gel was cut away. Nitrocellulose membrane and six sheets of filter papers were cut into the desired size. The gel, filter papers and nitrocellulose membrane were then immersed into the transfer buffer for 10 min. Trans-Blot SD semi-dry electrophoretic transfer cell was assembled. Three of the pre-soaked filter papers were placed onto the platinum anode. A roller was rolled over the surface of the filter paper to remove air bubbles. The nitrocellulose membrane was then placed on top of the filter papers. Air bubbles were rolled out again. Next, the gel was placed on top of the nitrocellulose membrane. Air bubbles were rolled out. The other three sheets of filter papers were placed on top of the gel and air bubbles were rolled out again. A safety cover was placed on the semi-dry electrophoretic transfer cell. The gel was then transferred at 15 V for 30 min.

3.3.7.5 Blotting of Nitrocellulose Membrane

After the transfer process was completed, the membrane where the protein ladder located was cut from the nitrocellulose membrane. The protein ladder was dipped into the staining solution for 2 s and followed by destaining with destaining solution until the bands could be seen clearly. The membrane with the transferred proteins was incubated with blocking buffer for 1 h at room temperature with agitation using Stuart-SSL4 see-saw rocker. Then, the blocking buffer was removed and followed by incubation with primary antibody, anti-Histidine

monoclonal antibody (GeneTex, USA) diluted in TBS-T in the ratio of 1:2500 for 1 h 30 min. Subsequently, the membrane was washed four times with TBS-T on the rocker for 10 min each before incubation with secondary antibody, anti-mouse IgG (GeneTex, USA) diluted in TBS-T with the ratio of 1:2500 for 1 h at room temperature. Then, the membrane was washed four times with TBS-T on the rocker for 10 min each to remove any unbound antibodies.

3.3.7.6 Blot Development

During the final wash, TBS-T was discarded and 1 mL of substrate 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP-NBT) was added onto the membrane surface. Colorimetric reaction (dark blue colour) was then detected.

3.3.8 Enzyme-linked Immunosorbent Assay (ELISA)

U-shaped high binding microplate wells were coated with different amount of NS1 antigen (10-1000 ng; 100 µL) overnight at 4℃. His-tagged Hepatitis B core antigen (HBcAg) (10-1000 ng; 100 µL) as positive control and Tris Buffer Saline (TBS) (100 µL) as negative control were also coated in the microplate wells. The coated wells were then blocked with 10% milk diluent for 2 h at room temperature. Next, the wells were washed three times with TBS-T for 20 s each and the primary antibody (anti-Histidine monoclonal antibody) (1:5000, 100 µL) was added into each well and incubated for 1 h at room temperature. The wells

were washed with TBS-T three times for 20 s each and followed by addition of anti-mouse antibody conjugated with alkaline phosphatase (1:5000, 100 µL) and incubated for 1 h at room temperature. The unbound antibodies were washed off with TBS-T two times for 20 s each and the NS1 antigen was detected by adding p-nitrophenyl phosphate (1 mg/mL, 200 µL). The absorbance was read at $OD_{405\ nm}$ using a microtiter plate reader. The assay was performed in triplicates.

CHAPTER 4

RESULTS

4.1 Optimization of Cell Disruption Using Ultrasonication

Ultrasonication is a mechanical cell disruption method which created high shear force by high frequency ultrasound that is produced electronically and transported to a concentrated cellular suspension through the metallic tip. The common parameters investigated to optimize cell disruption are acoustic power, suspension volume and sonication duration. In this study, the effects of amplitude and sonication duration were investigated to optimize the release of NS1 protein from *E. coli* cells.

4.1.1 Duration and Amplitude

Sonication duration is the duration that a cell suspension is subjected to sonication. In this study, sonication duration of 0, 10, 20, 30, 40, 50 and 60 min were investigated. The concentration of NS1 protein released from 0 min to 20 min was in the range of 8-13 μg/μL when 40, 60 and 80% amplitude were used. However, the concentration of the protein did not increase significantly from 20 min to 60 min as shown in Figure 4.1 as there was only 1.28-, 1.13- and 1.25-fold higher at 60 min compared to that at 20 min when 40, 60 and 80% amplitude were used, respectively.

The next parameter investigated was amplitude. The term amplitude refers to the maximum distance in which an individual air molecule will move from its starting point (Dubbs, 1996). The amplitude of the ultrasonic source is proportional to the intensity of sonication (Ho et al., 2006). In this study, three amplitudes (40, 60 and 80%) were investigated. Figure 4.1 shows that higher concentration of NS1 protein was released when higher amplitude was used. When 80% amplitude was used, it was observed that the concentration of NS1 protein released was 2-fold higher than the one obtained at 40% amplitude. Figure 4.2 shows the NS1 protein obtained in the supernatant after sonication at 40, 60 and 80% amplitude for 60 min. There was a gradual increase in thickness of the band, which supported that more NS1 protein was released into the supernatant when 80% amplitude was used compared to those obtained at 40 and 60% amplitude.

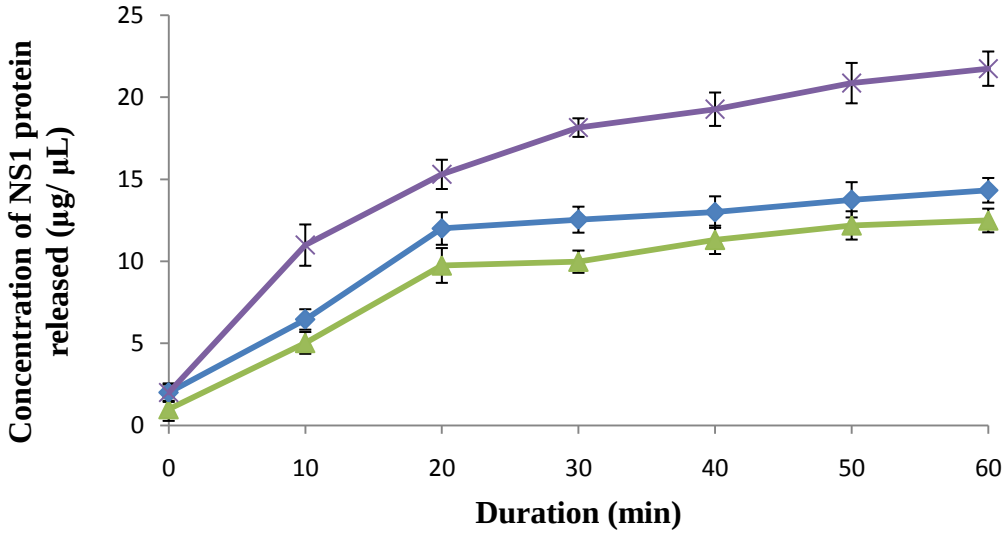

Figure 4.1: Concentration of NS1 protein release from *E. coli* cells in amplitude of 40, 60 and 80% over the period of 60 min. ▲ represents 40% amplitude, ♦ shape represents 60% and × represents 80% amplitude. The data represents the mean ±S.D. of duplicate determinations.

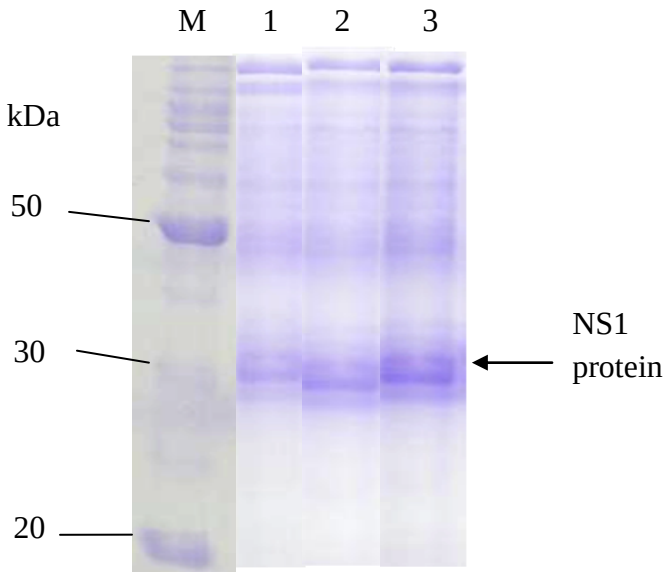

Figure 4.2: SDS-PAGE gel image after sonication at 40%, 60% and 80% amplitude for 60 min. M: Protein ladder; Lane 1: Protein obtained in supernatant after sonication at 40% amplitude; Lane 2: Protein obtained in supernatant after sonication at 60% amplitude; Lane 3: Protein obtained in supernatant after sonication at 80% amplitude.

4.2 Optimization of Cell Disruption Using Lysozyme

Enzymes are used to digest cell wall or cell membrane to cause cell disruption with the purpose of releasing intracellular expressed protein. In this study, four parameters were investigated such as different durations of incubation, lysozyme concentrations and temperatures.

4.2.1 Duration of Incubation

In this study, the cell lysate obtained was incubated with lysozyme for 20, 40, 60 and 120 min duration. The lysozyme concentration and temperature were fixed at 0.10 mg/mL and 4℃, respectively. The concentration of NS1 protein released into the supernatant was the highest at 120 min (15.14 μg/μL) and the lowest at 20 min (13.21 μg/μL) of incubation as shown in Figure 4.3. However, there was only 12.7% increment of concentration of NS1 protein released into supernatant from 20 min to 120 min. Besides, it was observed that the concentration of NS1 protein released was only approximately 1-fold higher between each duration tested (20, 40, 60 and 120 min). The SDS-PAGE gel image shown in Figure 4.4 demonstrated that the concentration of NS1 protein released did not increase significantly from 20 to 120 min as the protein bands obtained were similar in thickness.

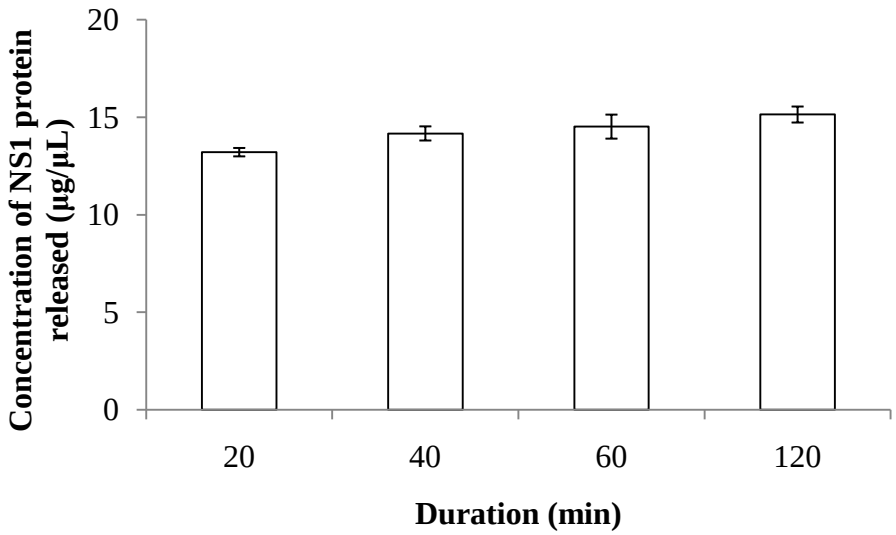

Figure 4.3: The released of NS1 protein from *E. coli* cells at different durations. The data represents the mean ± S.D. of duplicate determinations.

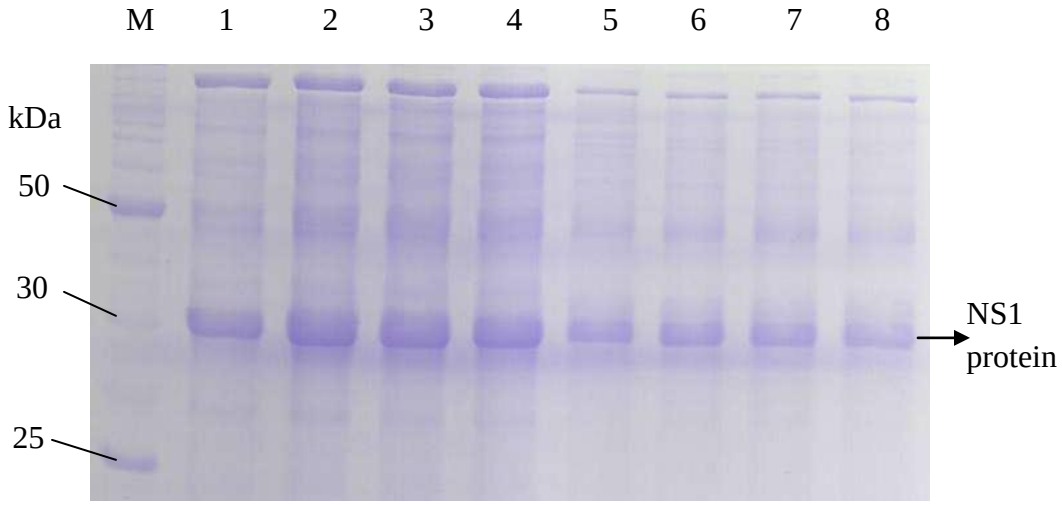

Figure 4.4: SDS-PAGE gel image of lysozyme cell disruption with different durations. M: Protein ladder; Lane 1 to lane 4: Protein obtained in the pellet at 20 min, 40 min, 60 min and 120 min, respectively; Lane 5 to lane 8: : Protein obtained in the supernatant at 20 min, 40 min, 60 min and 120 min, respectively.

4.2.2 Lysozyme Concentrations

The concentration of lysozyme investigated were 0.01 mg/mL, 0.05 mg/mL, 0.10 mg/mL and 0.20 mg/mL. The duration of cell disruption and temperature were fixed at 120 min and 4 ℃, respectively. Among the four lysozyme concentrations tested, the highest concentration of NS1 protein released was observed with 0.20 mg/mL of lysozyme was used, which released 17.57 µg/µL of NS1 protein (Figure 4.5). When 0.01 µg/µL of lysozyme was used, 14.21 µg/µL of NS1 protein was released. There was a 19.1% difference of NS1 protein concentration released when 0.01 and 0.20 mg/mL of lysozyme were used. However, the concentration of NS1 protein released did not increase significantly from one lysozyme concentration to another lysozyme concentration as there was only 8.2, 6.8 and 5.4% increment of concentration of NS1 protein released when the four lysozyme concentration (0.01, 0.05, 0.10, 0.20 mg/mL) were used.

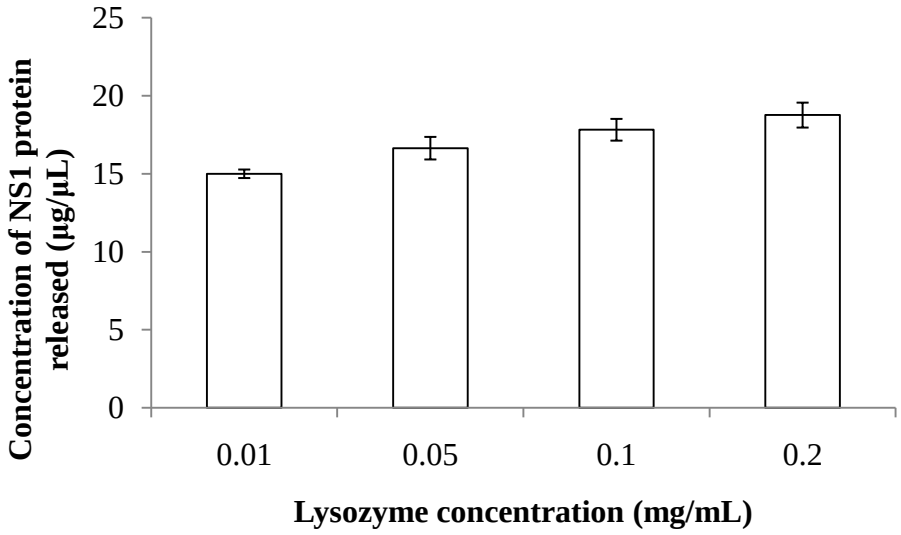

Figure 4.5: The released of NS1 protein from *E. coli* cells at different lysozyme concentrations. The data represents the mean ± S.D. of duplicate determinations.

4.2.3 Temperature

The temperatures chosen in this study were 4 ℃ and 28 ℃. The duration of cell disruption was fixed at 120 min and the lysozyme concentration used was at 0.20 mg/mL. As shown in Figure 4.6, the concentration of NS1 protein released when the cell suspension was incubated at 4 ℃ was higher than that obtained when the cell suspension was incubated at 28 ℃. When the cell suspension was incubated at 4 ℃, the concentration of NS1 protein released was 18.77 µg/µL. On the other hand, when the cell suspension was incubated at 28ºC, the concentration of NS1 protein released was 16.68 µg/µL. This showed that lysozyme works better at 4 ℃. However, the concentration of NS1 protein released at 4ºC was only 1.13-fold higher than that obtained at 28ºC.

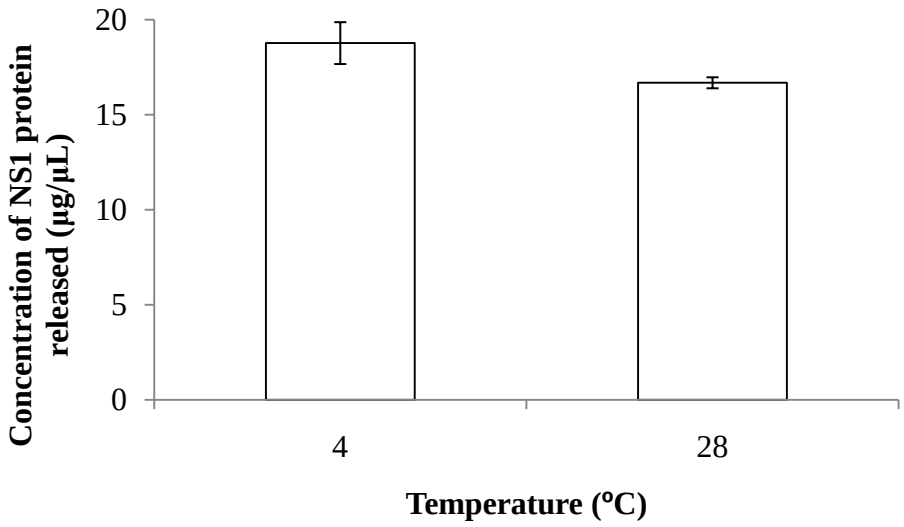

Figure 4.6: The released of NS1 protein from *E. coli* cells at 4 ℃ and 28 ℃. The data represents the mean ± S.D. of duplicate determinations.

4.3 Comparison between Ultrasonication and Lysozyme Methods

The optimum sonication duration and amplitude used for ultrasonication were 60 min and at 80% amplitude, respectively. On the other hand, the optimum duration of incubation, lysozyme concentration and incubation temperature used were 120 min, 0.2 mg/mL and 4 ℃, respectively. Figure 4.7 shows the comparison between the concentration of NS1 protein released by the ultrasonication and lysozyme cell disruption method at their optimal conditions. Cell disruption by ultrasonication released 21.7 µg/µL of NS1 protein whereas lysozyme cell disruption released 18.7 µg/µL of NS1 protein as shown in Figure 4.7. It was observed that NS1 protein recovered from the cell lysate obtained using

ultrasonication was approximately 1.2-fold higher than the one obtained using the lysozyme method. In addition, there was 13.7% difference in the percentages of NS1 protein released between the two cell disruption methods. Besides, in terms of the duration used in both methods, it was observed that ultrasonication only required 60 min whereas lysozyme disruption method required incubation time of 120 min as shown earlier. Therefore, ultrasonication was better than lysozyme cell disruption method in disrupting *E. coli* cells to release NS1 protein in terms of concentration of NS1 protein released and the duration used.

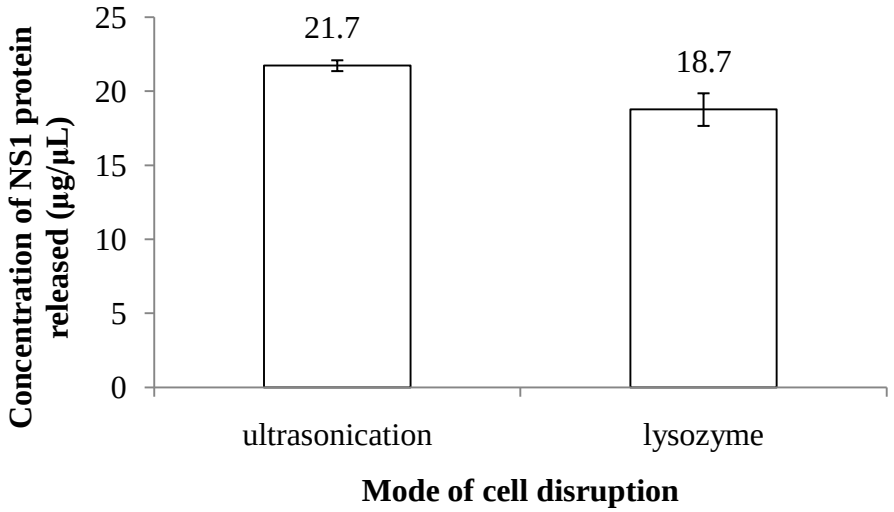

Figure 4.7: Comparison between the concentration of NS1 protein released by the ultrasonication and lysozyme cell disruption method at optimal conditions. The data represents the mean ± S.D. of duplicate determination.

4.4 Western Blot Analysis

The objective to run this analysis was to specifically detect the NS1 protein band with intact His tag. Based on the results obtained, the target protein (NS1 protein) bands were observed in all lane with molecular weight of approximately 30 kDa (Figure 4.8). NS1 protein bound specifically to anti-His monoclonal antibody. This confirmed that the 6x His tag remained intact to the protein. This proved that ultrasonication and lysozyme disruption methods did not affect the structure of NS1 protein expressed.

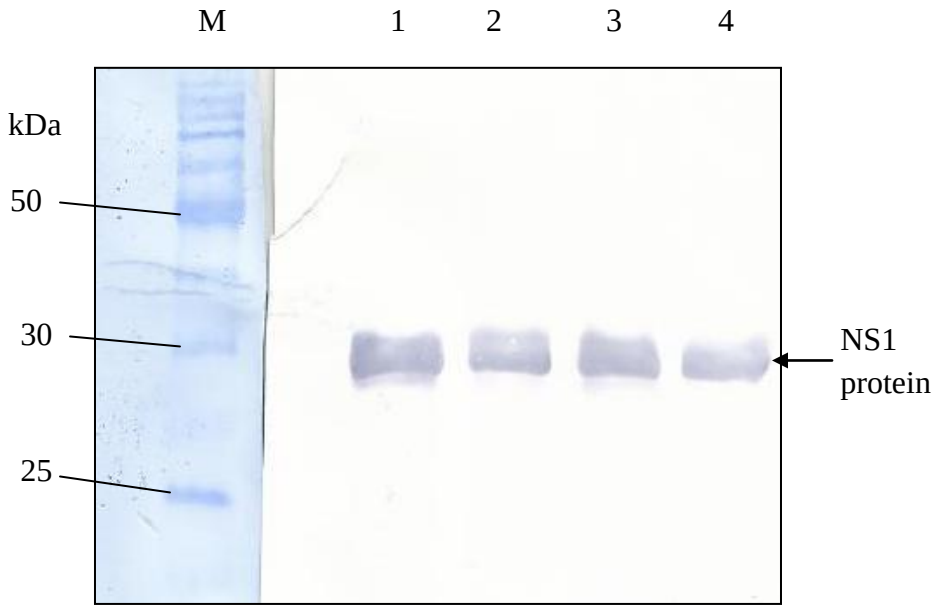

Figure 4.8: Western blot analysis of the released NS1 protein. M: Protein ladder; Lane 1: Protein obtained in the pellet by ultrasonication; Protein obtained in the supernatant by ultrasonication; Lane 3: Protein obtained in the pellet with lysozyme disruption method; Lane 4: Protein obtained in the supernatant with lysozyme disruption method.

4.5 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay using an anti-His monoclonal antibody (primary antibody) was performed to detect both denatured and non-denatured forms of the protein. Figure 4.9 shows the antigenic study of NS1 protein with an anti-His monoclonal antibody. Hepatitis B core antigen with His-tag was used as positive control whereas the negative control used was Tris-Buffered saline. The results showed that the antibody could detect NS1 protein as low as 10 ng. As shown in Figure 4.9, NS1 protein's signals were similar to the positive control. This indicated that the 6x His-tag remained intact to the NS1 protein. Besides, it also verified that the NS1 protein released using both ultrasonication and lysozyme disruption methods did not affect the structure of NS1 protein.

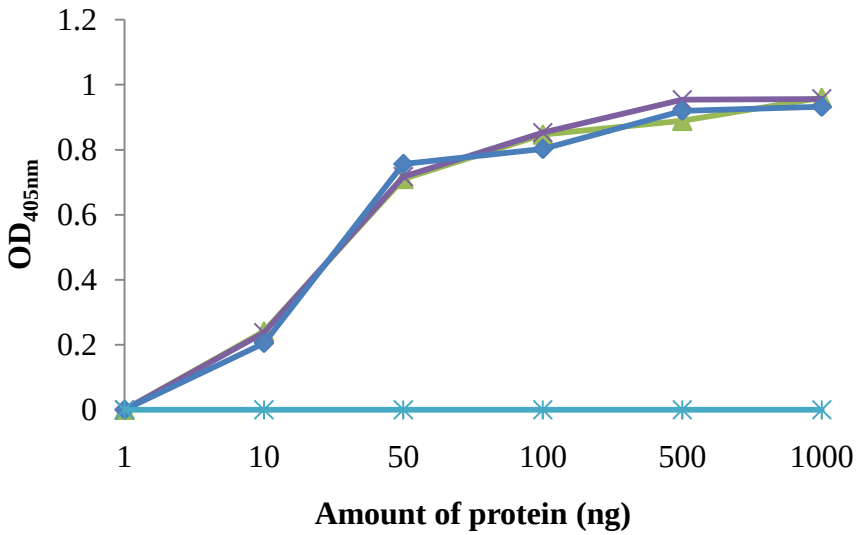

Figure 4.9: Antigenic study of NS1 protein with an anti-His monoclonal antibody via ELISA. The bound NS1 protein was detected by anti-His monoclonal antibody (1:5000 dilution) and an anti-mouse antibody conjugated to alkaline phosphatase (1:5000 dilution). ▲ represents anti-His monoclonal antibody (lysozyme disruption). × represents anti-His monoclonal antibody (ultrasonication). ♦ represents the positive control. * represents the negative control.

CHAPTER 5

DISCUSSION

5.1 Optimizations of Cell Disruption Using Ultrasonication

Ultrasound is any sound of frequency higher than that 15-20 kHz which is inaudible to the human ear. It is known to cause inactivation of microbial cells, and with higher power input, it could lead to disruption of the microbial cells in suspension (Bangaru, 2004). Ultrasonication is a mechanical cell disruption method based on liquid shear created by ultrasound that is produced electronically and transported to a concentrated cellular suspension through a metallic tip. The mechanism of disintegration was associated with the phenomenon of cavitation (Vargas et al., 2004). Cavitation was the combination of formation, growth, and collapse of vapor-filled bubbles, created by the action of strong sound waves (Gogate, 2011). The pressure reached as high as thousands of atmosphere near the point of collapse. As a result, local intense shock waves were generated by the implosion of the vapor phase bubbles, thus cause cell wall disruption (Ho et al., 2006). In brief, shear from the imploding cavitation bubbles and whirlpools induced by the vibrating ultrasonic probe disrupt the cells.

Several studies have been done on ultrasonication using different cell types, such as *E. coli* (Feliu et al., 1998), *Acetobacter peroxydans* (Kapucu et al., 2000) and

yeast (Liu et al., 2013). The common parameters used to optimize cell disruption are acoustic power, sonication duration and sample volume. In a study done by Kapucu and colleagues (2000), the effects of acoustic power on the survival percent of Gram-negative bacteria, *Acetobacter peroxydans* was investigated. It was found that when the acoustic power was increased from 30 to 100 W, the survival percent (number of intact cells) decreased from 50 to 36% in a period of 1 h. Besides, Feliu and colleagues (1998) reported a similar finding and suggested that the rate of intracellular β-galactosidase protein released in *E. coli* was linearly related to acoustic power in the interval of 35-95 W.

NS1 protein has been expressed in various host cells such as *E. coli* strain DH5α (Manasatienkij et al., 2008) *E.coli* strain BL21 (Sadeghi et al., 2014) and *Pichia pastoris* (Abubakar et al, 2011) to investigate the suitability of the host cells as expression system for NS1 protein. However, so far no consideration has been made to optimize the release of recombinant NS1 protein using different cell disruption methods. Therefore, the finding from this current study is important, particularly when downstream processing is concerned.

In the current study, the effect of amplitude of ultrasonication on the released of NS1 protein from *E. coli* was investigated. The amplitude investigated was 40, 60 and 80%. Based on the results obtained, the yield of NS1 protein obtained increased when higher percentage of amplitude was used as the increase in

amplitude caused a higher disruption rate. The amplitude of the ultrasonication is proportional to the intensity of sonication. Therefore, the higher the amplitude, the higher the sonication intensity generated (Herran et al., 2010). Microstreaming near the bubble surface generate stresses caused by velocity gradients, which are strong enough to disrupt cells. In the collapse phase of cavitation bubbles, a large quantity of sonic energy is released as mechanical energy in the form of elastic waves (Doulah, 1977). According to Ho and colleagues (2008), the degree of cell disruption is linked with the shear stress promoted by the dissipative whirlpools caused by shock waves, which is generated by imploding cavitation bubbles. If the whirlpools created by the shear force are smaller than the cells, the cells are disrupted rather than move around. Increasing the amplitude of the ultrasonic processor will produce smaller whirlpools, thus increase the number of disruptive whirlpools. At a constant power input into the cavitation field, the elastic waves will work as continuous source of whirpools (Doulah, 1977). As a consequence, a greater number of cells will be disrupted.

In the current study, the effects of sonication duration (0, 10, 20, 30, 40, 50 and 60 min) on the release of NS1 protein from *E. coli* cells were investigated. In the present study, it was observed that the longer the sonication duration, the higher the concentration of NS1 protein was released from the cells as observed in Figure 4.1. In addition, sonication duration of 60 min released the highest amount of NS1 protein. The overall trend of the amount of NS1 protein release increased with time. This is in good agreement with the findings by Ho and colleagues

(2006), which demonstrated that the amount of HBcAg released from *E. coli* cells was proportionate to the sonication duration.

In the present study, NS1 proteins remained in the pellet and low concentration of protein was released into the supernatant (Figure 4.4). This could be due to some of the ultrasonication factors that may lead to aggregation of the NS1 protein. Sonication produces gas bubbles which collapse, in a process known as cavitation, creating high shear forces and high local temperatures. As a result, thermolysis and sonolysis of water around the bubbles forms highly reactive free radicals such as hydrogen and hydroxyl radical (Riesz et al., 1985). Proteins may be destabilized at the air-liquid interface of sonication-induced bubbles (Satheeshkumar and Jayakumar, 2002). The reactive oxygen species react with chemical moieties on the protein, producing protein radicals, which then undergo further reactions such as oxidation and cross-linking, which is likely to cause protein instability (Hawkins and Davies, 2001). Example of a destabilizing chemical modification that may cause protein aggregation is formation of nonnative disulphide bonds (Senistera et al., 1997). The recombinant NS1 protein used in the current study has a total of 264 amino acids with two cysteine amino acids, one at position 28 and the other one at position 146. It is hypothesized that the two cysteine amino acids undergo oxidation caused by the reactive oxygen species and formed a disulphide bond. The expression of disulphide bond containing proteins in *E. coli* results in the production of insoluble protein (due to misfolding) sequestered into inclusion bodies (Chen and Leong, 2009). Besides,

another possibility to consider is the association of NS1 protein with insoluble components of cell debris which resulted it to be remained in the pellet. In a study by Feliu and Villaverde (1994), sonication was done at 100 W and 20 kHz on *E. coli* cell for 15 min. It was reported that about 30% of recombinant protein in *E. coli* present in the sample still remained associated to cell debris and it could not be removed even with prolonged sonication duration. After a second sonication treatment of the resuspended cell debris, only a 70% of the remaining recombinant protein was released (Feliu and Villaverde, 1994). Therefore, it was hypothesized that most of the NS1 protein associates with insoluble cell debris during the sonication process. Hence, longer sonication duration showed no significant effect in releasing more NS1 protein and caused most of the NS1 protein to be remained in the pellet as seen in this study.

5.2 Optimizations of Cell Disruption Using Lysozyme

Enzymatic lysis is the application of enzymes to bind to specific molecules in the cell wall or cell membrane and hydrolyze the bonds. As a result, cell wall or cell membrane disintegrates and releases the intracellular proteins (McKenzie and White, 1991). Lysozyme was used in the present study as non-mechanical cell disruption method. Lysozyme is a single chain polypeptide of 129 amino acid cross-linked with four disulfide bridges. It is used for lysing bacterial cells by hydrolyzing β-1,4-glycosidic bonds between N-acetyl-muramic acid (NAM) and N-acetyl-D- glucosamine (NAG) residues, and may therefore be used to disrupt

cell walls containing peptidoglycan (White and Marcus, 1988). So far, there are no broad studies investigating the optimal conditions for cell disruption to release intracellular proteins using lysozyme. Therefore, the findings from this current study are important.

In the current study, different parameters such as incubation duration, lysozyme concentration and temperature were investigated. Based on the different parameters tested, the concentration of NS1 protein released was the highest with 120 min incubation duration, 0.2 mg/mL lysozyme concentration and at 4℃. In the present study, it was demonstrated that the longer the incubation duration, more time is available for lysozyme to react with *E. coli* cells. Therefore, more NS1 protein will be released compared to shorter incubation time. This is in good agreement with a study by Voss (1964), which reported that there was a decrease in the optical density of suspensions of *E. coli* and increase in the amount of liberated proteins during 10 min incubation with 0.02 mg/mL of lysozyme. Futhermore, Knorr and colleagues (1979) also reported the similar finding and demonstrated that there was an increase in the amount of carbohydrates released from yeast cells with 120 min incubation using 0.5 mg/mL of lysozyme.

The common concentration of lysozyme used ranges from 0.02 to 0.2 mg/mL (Voss, 1964). In the current study, 0.01, 0.05, 0.1 and 0.2 mg/mL of lysozyme concentration tested on the release of NS1 protein from *E. coli* were investigated.

Previous studies also showed that higher concentration of lysozyme enable more products release from the cells such as *Chlorella vulgaris,* Brewer yeast and *Amycolatopsis sp.* cells (Zheng et al., 2011; Knorr et al., 1979; Babu and Choudhury, 2013). In comparison to these studies, this demonstrated a similar outcome, whereby with higher concentration of lysozyme used, the higher the concentration of NS1 protein would be released (Figure 4.5).

In a study by Hamaguchi and colleagues (1960), the optimum temperature for lysozyme activity was found to be at 50ºC. The hydrophobicity of lysozyme will be increased at high temperature. It has been shown previously that increasing lysozyme hydrophobicity by engineering a hydrophobic pentapeptide to its C terminus enhanced lysozyme activity against Gram-negative bacteria (Ibrahim et al., 1994). The hydrophobic region is able to disrupt the inner cell membrane, leading to a loss of electrochemical potential and increased lysis. However, increased bactericidal activity is not accompanied by a substantial increase in the rate of protein release (Middelberg, 1995). Hence, with the purpose of optimizing the release of NS1 protein from *E. coli* cells, the incubation temperature investigated was at 4ºC and 28ºC instead of 50ºC because the rate of protein release will not increase as it might cause protein degradation. Based on the result obtained, the concentration of NS1 protein released was higher at 4ºC. However, there was no significant difference observed in the concentration of NS1 protein released at 4ºC (18.77 µg/µL) and 28ºC (16.68 µg/µL) (Figure 4.6). Several studies that researched on the recovery of proteins, the temperature for lysozyme

incubation was at 4ºC (Zaful et al., 2015). In contrast, the optimum temperature for the recovery of non-protein product such as lipids was at higher temperature such as at 55ºC (Zheng et al., 2011). Therefore, it was hypothesized that the higher amount of protein released at 4ºC compared to at 28ºC presumably due to protein recovery reaction stable at 4ºC than at 28ºC.

In a study by Babu and Choudhury (2013), *Amycolatopsis sp.* cells, which is a Gram-positive bacterium was incubated with lysozyme concentration ranging from 0.5 to 5.0 mg/mL. It was reported that 85% of the nitrile metabolizing enzyme was recovered in the supernatant by incubating the cells with 2 mg/mL lysozyme for 1 h. In this current study, only 7.7% of NS1 protein was released using the optimized conditions. This was most probably due to the difference between cell wall structure of Gram-positive and Gram-negative bacteria. The cell wall of Gram-negative bacteria mainly composed of two layers. They are the inner peptidoglycan layer, which is the structural component for the rigid, rod-like appearance of the bacterial cell and an outer lipopolysaccharide (LPS) layer, which is held together by weaker bonds and contains surface antigens and receptors (Kohn, 1960). This conception is well supported by the morphological studies by Kellenberger and Ryter (1958). Gram-negative bacteria are less susceptible to lysozyme than Gram-positive bacteria due to the presence of an outer LPS layer that shields the peptidoglycan from the enzyme. The LPS layer should be permeabilized first to expose the peptidoglycan cell wall for lysozyme digestion (Middelberg, 1995). Besides, the lower protein yield obtained in the

current study was most probably due to the higher concentration of NS1 protein expressed in insoluble protein. Therefore, longer incubation period and higher lysozyme concentration used did not result in significant increase of NS1 protein released.

5.3 Comparison between Ultrasonication and Lysozyme

This study showed that ultrasonication is better than the lysozyme cell disruption method in terms of concentration of NS1 protein released and the duration used. It was observed that NS1 protein recovered from the cell lysate obtained with ultrasonication at the optimized conditions was approximately 1.2-fold higher than the lysozyme method. The yield obtained in ultrasonication was 21.74 μg/μL whereas lysozyme cell disruption method released 18.77 μg/μL of NS1 protein. This is in good agreement with the finding by Ho and colleagues (2006), which demonstrated that ultrasonication method was better compared to lysozyme cell disruption method. It was reported that HBcAg recovered from the cell lysate obtained in ultrasonication was 22 times higher than the one obtained using lysozyme cell disruption method (Ho et al., 2006). Besides, similar result was reported by Tan and colleagues (2008). It was demonstrated that the protein released by lysozyme cell disruption method was 43% lower compared to ultrasonication.

Besides, in the current study, the duration used for ultrasonication was shorter than the duration used in lysozyme cell disruption method. The duration used in ultrasonication was 60 min and NS1 protein released at that duration was 21.74 μg/μL. In contrast, lysozyme cell disruption method released 18.77 μg/μL of NS1 protein with 120 min of incubation period, which was lesser than ultrasonication, which required only 60 min to release 21.74 μg/μL of protein. This is in good agreement with the finding by Tan and colleagues (2008), which reported that the duration used in enzymatic lysis (120 min) for protein recovery from *E. coli* was longer than the ultrasonication method (90 min).

5.4 Limitation of Study

Most of the NS1 protein retained in the pellet although longer sonication duration and longer lysozyme incubation period was used as it did not result in significant increase of NS1 protein released.

5.5 Future Studies

In the current study, most of the NS1 protein retain in the pellet as insoluble proteins. Therefore, in the future study, the growth conditions for *E. coli* Novablue cells can be optimized to obtain more soluble proteins.

Besides, other mechanical cell disruption method to disrupt *E. coli* cells for the recovery of NS1 protein such as bead milling and high-pressure homogenization can be used instead of ultrasonication method. Lastly, mechanical such as ultrasonication and non-mechanical method (lysozyme, detergent) can be combined to disrupt *E. coli* cells for better recovery of NS1 protein.

CHAPTER 6

CONCLUSIONS

It is likely that *E. coli* cells will continue to receive widespread use as hosts for the production of biological products. *E. coli* cells are incapable of secreting their products into the medium, hence most will remain intracellularly. Therefore, effective and well characterized methods applicable for cell disruption are required. In this study, ultrasonication and lysozyme cell disruption methods to release NS1 protein from *E. coli* Novablue have been studied. In ultrasonication, the results of the present study showed that NS1 protein release increased with longer sonication duration and high sonication amplitude. On the other hand, conditions for the highest amount of NS1 protein released from *E. coli* cells with lysozyme method was 120 min of incubation period, 0.2 mg/mL lysozyme concentration and temperature at 4 ℃.

The findings from this study also showed that there was a 13.7% difference between the two methods in terms of concentration of NS1 protein released with ultrasonication better than lysozyme cell disruption method. In addition, ultrasonication is more efficient than lysozyme cell disruption in terms of duration where ultrasonication only required 60 min whereas lysozyme method required 120 min. Qualitative analysis using Western blot and ELISA revealed that NS1 protein obtained from the cell disruption methods was detected. However, the

result of the present study showed that most of the NS1 protein was retained in the pellet and this was most probably due to the insoluble NS1 protein produced. The disruption methods used are feasible to use as it did not affect the structure of the protein or lead to denaturation of the protein.

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Appendix A

The picture below shows the standard curve of different concentration of BSA. The concentration of the NS1 protein released was calculated from the standard curve below.