A PRELIMINARY STUDY OF *BACILLUS LICHENIFORMIS* SPORE COAT PROTEINS DETECTION BY SURFACE PLASMON RESONANCE

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ABSTRACT

Food poisoning is mainly caused by pathogenic microorganisms and is now a severe problem worldwide. Therefore, rapid and sensitive methods are required to detect foodborne pathogens. A locally isolated bacterium, *Bacillus licheniformis* B38 was selected for this study. The spores of *B. licheniformis* B38 were induced by Schaeffer's sporulation medium containing KCl, $MgSO_4.7H_2O$, $Ca(NO_3)_4$, $MnCl_2$ and $FeSO_4$. Schaeffer-Fulton endospore staining was used to differentiate spores and vegetative cells, where spores were stained green and vegetative cells were stained red. In order to separate the spores from the cells, a two-phase system was used to obtain pure spore suspension for following experiments. Spore coat proteins were extracted by SDS-8 M urea sample buffer and visualized by two different types of coomassie brilliant blue staining solutions. One of the staining solutions was more suitable for gel elution by diffusion. An ~10 kDa spore coat protein was selected for protein purification. Based on the given results, the protein purification by liquid chromatography was less convincing than using gel elution by diffusion technique. The two hypothetical protein sequences, P06552 and P45693, from the ~10 kDa spore coat protein were identified. In the preliminary study of *B. licheniformis* B38 spores detection by surface plasmon resonance, several binding parameters were studied. Dot blot was done to verify the reaction between the *Bacillus* spores polyclonal antibody against the *B. licheniformis* B38 spore coat protein. The most promising result was the binding of 0.1 mg/mL polyclonal antibody (analyte) to the 0.2 mg/mL spore coat protein at pH 2 (ligand) which showed 5.74 RU. The differences between a dot blot and a SPR detection techniques are described.

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~	approximately equal
CFU	colony forming unit
DPA	dipicolinic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
g	gravitational acceleration
kDA	kilo Dalton
mAU	milli absorbance unit
nm	nanometre
OD_{600}	optical density at 600 nm
PEG	polyethylene glycol
RU	response unit
SASP	small acid soluble protein
SDS	sodium dodecyl sulphate
SPR	surface plasmon resonance
w/v	weight per volume
σ^H	sigma H factor

CHAPTER 1

INTRODUCTION

1.1 *Bacillus*

The general characteristics of the *Bacillus* genus are spore-forming Gram positive or Gram-variable bacteria that can grow aerobically or facultative anaerobically in various harsh environments (Fekete, 2009). Most of the research focuses on two main groups of *Bacillus* species: the *Bacillus cereus* group comprises of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*; and the *Bacillus subtilis* group comprising *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus mojavensis* and *Bacillus vallismortis* (Qin & Driks, 2013). Extensive studies on these two groups include, for example, *B. cereus* causation of foodborne illnesses (Tewari & Abdullah, 2014); *B. thuringiensis* for the production of a bacterial type insecticide (Kumari et al., 2014); *B. anthracis* possible role in biological warfare and the cause anthrax (Spencer, 2003); *B. subtilis* as a model for studying sporulation and germination processes (McKenney et al., 2013) and *B. licheniformis* for the production of an enzyme used as a detergent (Nadeem et al., 2013).

The genus *Bacillus* comprises more than 60 species which are found in soil and water, in food products and in eukaryotic organisms (Dahl, 2000). Spore formation is one of the significant characteristics for identifying *Bacillus* species (Dahl, 2000). The presence of spore-forming bacteria in food industry remains a major problem because of: (i) the abundance of spore formers and the difficulty in eliminating them in raw foods and food ingredients; (ii) the failure of existing processes e.g. pasteurization to kill spores and all vegetative cells; (iii) the characteristic of adhesion which allows spores to remain attached to industrial food equipment and (iv) the ability of microorganisms to adapt the changes in their environment e.g. the extreme conditions that are applied in food processing and

packaging technologies (Postollec et al., 2012; Scheldeman et al., 2006). Besides of causing detrimental food poisoning and infections (**Table 1.1**), enzymes produced by *Bacillus* species are commercialized for different purposes such as insecticides, laundries and dishwasher detergents (Fekete, 2009). In addition, *Bacillus* species are used to produce heterologous proteins, antibiotics, purine nucleotides, vitamins, poly-γ-glutamic acids and $_D$-riboses for industries purposes (Schallmey et al., 2004).

1.2 *Bacillus licheniformis*

B. licheniformis is one of the closest relatives to *B. subtilis* (Qin & Driks, 2013). The general features of *B. licheniformis* are Gram positive, motile rods and its cells are 0.6 - 0.8 by 1.5 - 3.0 μm (Vos et al., 2011). This facultative anaerobic spore-forming organism has ellipsoidal to cylindrical spores in unswollen sporangia observed in the sporulation stage (Vos et al., 2011). Taxonomic studies such as 16S rDNA and 16S-23S internal transcribed spacer (ITS) nucleotide sequence suggest that *B. licheniformis* is closely related to *B. subtilis* and *B. amyloliquefaciens* (Xu & Côté, 2003). Qin and Driks (2013) also support the idea that *B. licheniformis* is one of the closest relatives to *B. subtilis* which is identified by 16S rRNA and multi-locus approaches. Rey et al. (2004) stated that the similarity between *B. licheniformis* ATCC 14580 and *B. subtilis* 168 at nucleotide level was approximately 84.6%. In addition, *B. licheniformis* and *B. subtilis* share a total of 139 sporulation genes (Kunst et al., 1997), except for 6 genes (e.g. *spsABCEFG*) which important in the synthesis of spore coat polysaccharide in *B. subtilis* (Rey et al., 2004).

B. licheniformis is usually found in natural habitats such as soil (Ghani et al., 2013; Voigt et al., 2014) and bird feathers (Burtt & Ichida, 1999). This bacterium is capable of growing at between 15 ^{o}C and 55 ^{o}C (Vos et al., 2011), and can also be found in the extreme environments (Llarch, 1997). On the other hand, *B. licheniformis* is commonly found in the dairy industry (Burgess et al., 2014; Masiello et al., 2014; Watterson et al., 2014) and its spores can survive at high temperature (Lücking et al., 2013). A recent study shows that the *B. licheniformis* spores can be inactivated efficiently using a combination of pressure-heat treatment and low pH (Tola & Ramaswamy, 2014a). *B. licheniformis* can cause food poisoning in different contaminated foods which will be discussed at later section.

Despite the fact that *B. licheniformis* can cause foodborne illnesses and food spoilage, it also secretes many useful enzymes. For example, thermostable α-amylase is a starch degrading enzyme (Siddique et al., 2014); keratinase is a feather degrading enzyme (Ni et al., 2011); tannase which is mainly used for food industry (Das Mohapatra et al., 2009); and thermostable phytase which is used as an animal feed supplement (Fasimoye et al., 2014).

Table 1.1 *The characteristics of food-borne illnesses caused by Bacillus species (Beattie & Williams, 2000).*

	B. cereus emetic syndrome	*B. cereus* diarrhoeal syndrome		*B. thuringiensis*	*B. subtilis*	*B. licheniformis*	*B. pumilus*	*B. brevis*
		Type I	Type II					
Symptoms	Malaise, nausea, vomiting	Abdominal pain, watery diarrhoea	Gastroenteritis	As for *B. cereus* diarrhoeal syndrome	Vomiting, diarrhoea, nausea, abdominal pain, pyrexia, headaches	Diarrhoea, vomiting, abdominal pain	Diarrhoea, vomiting, nausea	Nausea, vomiting, abdominal pain
Implicated food vehicle	Farinaceous rice, pasta, noodles, pastry	Proteinaceous dairy products, meats, sauces, desserts	Proteinaceous dairy products, meats, sauces, desserts	As *B. cereus* unwashed sprayed vegetables	Meat, seafood, pastry, rice	Cooked meat and vegetable dishes	Meat products	Meat
Infective dose (CFU/g)	10^5-10^8	$> 10^4$	$> 10^4$	$> 10^4$	$> 10^5$-10^9	$> 10^6$	$> 10^6$	$> 10^8$
Incubation period (h)	0.5-5	8-16	8-16	8-16	< 1-14	2-14	< 1-11	1-9.5 ($\bar{x} = 4$)
Duration of illness (h)	6-24	12-24	12-24	12-24	1.5-8	6-24	Unknown	Unknown
Nature of toxin	Heat-stable, cyclic dodecadepsipeptide, ingested in food	Haemolytic, dermonecrotic, heat-labile complex of 3 peptides. Formed *in situ* in gut	Non-haemolytic heat-labile complex of 3 peptides	Enterotoxin, structure not determined but reacts with antibodies to *B. cereus* enterotoxin	Unknown	Unknown, but culture supernatants of some strains react with antibodies to *B. cereus* enterotoxin	Unknown, but culture supernatant of some strains react with antibody in Oxoid RPLA assay for *B. cereus* enterotoxin detection	Heat-labile enterotoxin

1.3 Endospore Structure

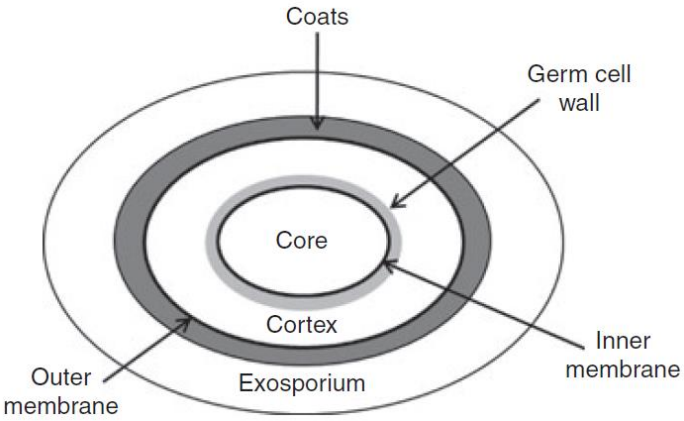


Figure 1.1 *The general structure of a bacterial spore. The exosporium layer may not be presented in all Bacillus species (Leggett et al., 2012).*

The exosporium is a layer that consists of glycoprotein located at the surface of the spore which separated the outer coat by a gap called the interspace (McKenney et al., 2013). An exosporium can be found in *B. cereus*, *B. anthracis* and *B. thuringiensis* but not in *B. licheniformis*, *B. pumilus*, *B. sporothermodurans* and *B. subtilis* (Faille et al., 2010). Those *Bacillus* species that have an exosporium possess hair-like projections on the surface of the exosporium (McKenney et al., 2013). This projection structure consists of few collagen-like glycoproteins that have 3 domains: an N-terminal domain, a collagen-like region and a proximal C-terminal domain (Todd et al., 2003). The exosporium has low or lack of amino acids such as cysteine, methionine, histidine and tyrosine; lipids (e.g. diphosphatidylglycerol) and polysaccharides (Leggett et al., 2012). However, the ability adhere to stainless steel surfaces might not be entirely explained by the presence of an exosporium (Faille et al., 2010) and the presence of an exosporium did not significantly provide any protection for spores from biocide attack (Leggett et al., 2012).

The general spore coat structure consists of a series of thin and concentric layers which vary from species to species, for instance, *B. cereus* has a simple coat but *B. sphaericus* has a much more complex coat (Driks, 1999). Based on observations of Kim et al. (2009), *B. licheniformis* spores had a general structure as described above (e.g. a lamellar inner coat and a thick outer coat without exosporium). A contradictory opinion was given by Holt and Leadbetter (1969) who stated that the spore of *B.*

licheniformis had an exosporium with juxtaposed strands lying underneath this structure. Monosaccharides such as glucosamine ($GlcNH_2$) and galactosamine ($GalNH_2$) were found on *B. licheniformis* spore coat (Faille et al., 2010). Snoke (1964) suggested that tyrosine and glycine were the major amino acids in the *B. licheniformis* spore coat. On the other hand, Aronson (2012) stated that *B. licheniformis* and *B. subtilis* shared some spore coat proteins, for example, YuzC, YtrI, YheD, YknT, CotR and CotO. The spore coat is a flexible structure that can respond to variation in environmental conditions such as fully hydrated to air-dried state (Plomp et al., 2005) and germination (Plomp et al., 2007). These characteristics play roles in protection and sustaining dormancy for the spores (Aronson, 2012). Another function is acting as a protective barrier to oxidizing agents such as peroxynitrite (Genest et al., 2002), hydrogen peroxide (Young & Setlow, 2004a), and ozone (Young & Setlow, 2004b) but does not provide protection from low-molecular-weight alkylating agents (Setlow et al., 1998). In fact, the structural component of a coat and/or coat/exosporium is influenced by the differences of available nutrients, the presence of solvents (Nicholson et al., 2000), temperatures (Melly et al., 2002) and types of media (Rose et al., 2007).

Leggett et al. (2012) in a review stated that 50 - 80% of the protein fraction was mainly from the spore coat and could be divided into 70% soluble fraction and 30% insoluble fraction. The combination of denaturing and reducing agents in alkaline conditions is required for isolating soluble protein fractions (Pandey & Aronson, 1979). The insoluble character of protein fractions is due to high cysteine content by disulphide bond formation (Goldman & Tipper, 1978; Pandey & Aronson, 1979). Goldman and co-worker found that the amino acid residues such as cysteine and methionine were relatively high after the two-step extraction with sodium dodecyl sulphate (SDS), dithiothreitol (DTT) and β-mercaptoethanol (Goldman & Tipper, 1978).

The outer spore membrane is located under the spore coat (Leggett et al., 2012). This structure is vital for spore formation (Popham, 2002). The outer spore membrane is believed not to have any protective effects against radiation, heat and chemicals (Nicholson et al., 2000). It is agreed that this structure has a lower permeability barrier than the inner spore membrane in dormant spores (Setlow, 2003).

Although no recent evidence has been reported for *B. licheniformis* outer spore coats, the outer spore membrane of *B. megaterium* has been shown to prevent the uptake of methacrylate unless disturbed by potassium permanganate ($KMnO_4$) fixation (Rode et al., 1962). Other molecules such as methylamine, small uncharged, lipophilic molecules and glucose can permeate through the outer spore membrane (Gerhardt et al., 1982; Setlow & Setlow, 1980; Swerdlow et al., 1981). The outer spore coat layer plays a main role in environmental adaptation, and the protein composition of this layer is easier to alter than the inner layer spore coat (Qin & Driks, 2013).

A thicker structure under the spore outer membrane is called the cortex (Setlow, 2013). The spore cortex has its own spore-specific properties in spite of being similar to the peptidoglycan layer of vegetative cells (Popham, 2002). The main difference is the replacement of approximately 50% of peptide side chain muramic acid residues with muramic acid lactam in the spore cortex (Warth & Strominger, 1972). This modified structure is believed to contribute to the resistance properties and spore dormancy (Popham, 2002). The roles of cortex are to degrade and allow for spore core expansion and outgrowth (Setlow, 2003). A germ cell wall is located under the cortex and has a similar chemical structure to the peptidoglycan of vegetative cells (Setlow, 2006). This structure will become cell wall during germination and outgrowth but does not possess any of the resistance properties of the spore (Leggett et al., 2012; Setlow, 2006).

The next structure is inner membrane. Cortezzo and Setlow (2005) explained that the low permeability of inner membrane to different DNA damaging chemicals showed spore protection. Another study showed that the rate of water passing through the inner membrane to the spore core is low due to its low permeability (Sunde et al., 2009), which was also supported by a previous publication (Swerdlow et al., 1981). According to the above studies, the main factor contributing to spore resistance to DNA damaging agents and low core water maintenance is the inner membrane permeability (Aronson, 2012). Although the spore inner membrane has a similar structure to the vegetative cell plasma membrane in terms of lipid molecules, the former has largely immobile lipids during dormant state (Cowan et al., 2004). However, the immobile lipid molecules will become mobile and the inner membrane permeability will increase during germination (Cowan et al., 2004).

The spore core is located at the centre of a spore and has: (i) lower water content; (ii) slightly lower pH than vegetative cells; (iii) α/β-type SASP (small acid-soluble protein) and (iv) a large amount of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] (Setlow, 2007). The main contributing factors for spore DNA resistance to different conditions are the Ca-DPA and the α/β-type SASP (Setlow, 2007). It is thought that the low water content or the slightly lower pH does not contribute to the spores DNA resistance (Setlow, 2007). Although water is not important in spore DNA resistance, as reported by Setlow (2007), it has been indicated that the low core water content does contribute to the spore enzymatic dormancy and heat resistance (Cowan et al., 2003; Setlow, 1994). In other words, the lower the water content in spore core, the greater the resistance of spore to moist heat (Setlow, 2006). Besides that, low spore core water content also contributes to γ-radiation resistance (Setlow, 2006). *Bacillus* spore inactivation by γ-radiation is somehow related to the spore core water content (Blatchley III et al., 2005). However, how spore core water relates to γ-radiation resistance is remained unclear (Setlow, 2006). On the other hand, a recent study shows that either the DNA-binding α/β-type and γ-type SASP alone or in a combination with other protective elements (e.g. low core water content and DPA) contributes to the spore resistance to ionizing radiation (Moeller et al. 2014). Moeller et al. (2014) suggested that the α/β-type SASP was the most crucial element for spore protection from ionizing radiation exposure, followed by core dehydration and DPA content. The mineral compositions are also crucial in spore resistance (Nicholson et al., 2000).

1.4 Endospore Formation

Endospores are formed in mother cells and are released into environment once the conditions are unfavourable to vegetative cells (Traag et al., 2013). Endospores are able to survive conditions the vegetative cells cannot, for example, heat, radiation, chemicals and desiccation (Setlow, 2006). Formation of endospore can be categorized in different stages where starting from Stage 0 - I (pre-asymmetric division), Stage II (asymmetric division), Stage III (engulfment), Stage IV (cortex formation), Stage V (spore coat formation) and Stage VI - VII (maturation, mother cell lysis and spore release) (**Figure 1.2**) (González-Pastor, 2011). Each stage of sporulation is temporally controlled by five specific sigma factors: σ^H (Stage 0 - I), σ^F (Stage II), σ^E (Stage III),

σ^G (Stage IV - V) and σ^K (Stage V - VII) (Robleto et al., 2012). More specifically, these sigma factors regulate the gene expressions in different cell stages: σ^F (early forespore), σ^E (early mother cell), σ^G (late forespore) and σ^K (late mother cell) (Losick & Stragier, 1992).

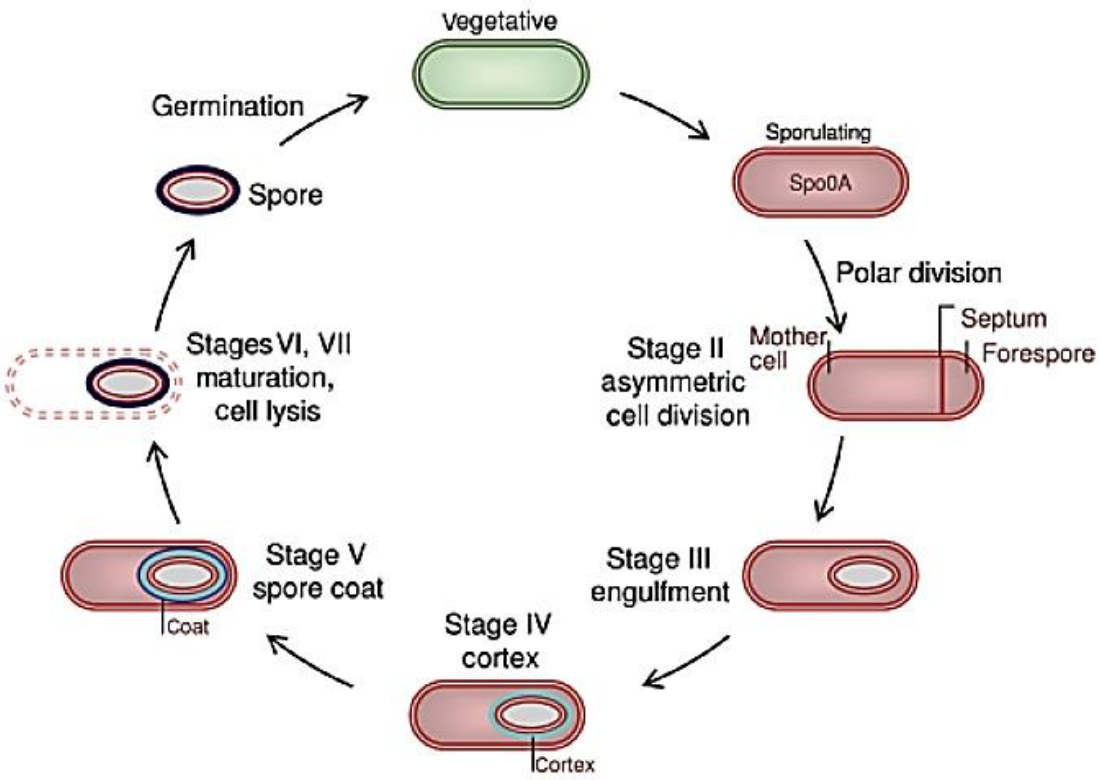


Figure 1.2 *The sporulation stages of B. subtilis (González-Pastor, 2011).*

The following descriptions of sigma factors regulations and the expression of its genes in sporulation stages are summarized in **Figure 1.3**. The Spo0A~P is the master sporulation regulator activated by a phosphorelay to initiate sporulation (Burbulys et al., 1991). First of all, RNA polymerase consists of σ^A that transcribes Spo0A at a low level at the early stage of sporulation (Burbulys et al., 1991). After that, a group of histidine kinases are auto-phosphorylated when environmental changes have been detected (Burbulys et al., 1991). The histidine kinases then transport the phosphate group to Spo0B, Spo0F and Spo0A to initiate sporulation (Hoch, 1993).

On the other hand, the sigma factor σ^H is important in the predivisional cell process (Carter & Moran 1986). The σ^H uses a specific sporulation promoter to

increase the transcription of Spo0A and the gene *sigH* is indirectly regulated by Spo0A~P (de Hoon et al., 2010). This sigma factor also transcribes *spoIIAA-spoIIAB-sigF* operon to encode the anti-anti-sigma factor SpoIIAA, the anti-sigma factor SpoIIAB and the early forespore-specific sigma factor σ^F (de Hoon et al., 2010; Wu et al., 1989).

The asymmetrical division of the cell into two uneven compartments is then regulated by σ^F (Robleto et al., 2012). Meanwhile, σ^F activation results in transcription of about 48 genes at the forespore forming stage (Wang et al., 2006). For instance, the genes are *rsfA* (Wu & Errington, 2000), *spoIIR* (Karow et al., 1995) and *spoIIQ* (Londoño-Vallejo et al., 1997). The expression of *spoIIR* in the early stage of a forespore compartment facilitates the communication between a mother cell and a forespore (Karow et al., 1995). On the other hand, proteolysis of the inactive form of σ^E, pro-σ^E is mediated by SpoIIR (Rubio & Pogliano, 2004). Meanwhile, RsfA acts as both activator and repressor to regulate gene expressions (de Hoon et al., 2010).

The formation of early mother cell sigma factor σ^E relies on the activation of σ^A by Spo0A to produce *spoIIGA-sigE* operon which encodes SpoIIGA (de Hoon et al., 2010). However, SpoIIGA must be working in conjunction with SpoIIR repressed by RsfA in order to switch on σ^E in the early mother cell phase (Robleto et al., 2012). Based on Eichenberger et al. (2004), sigma factor σ^E switches on approximately 270 genes including *spoIIID* and *gerR*. These two genes encode SpoIIID and GerR and form coherent and incoherent feed-forward-loops with σ^E (Eichenberger et al., 2004). One of the expressed genes from SpoIIID transcription is *sigK* that encodes sigma factor σ^K in mother cell (Kunkel et al., 1989). Some processes such as engulfment, coat and cortex formation and mother cell metabolism are influenced by the σ^E gene expressions (Errington, 1993). The proteins involved in engulfment of the forespore by the mother cell are SpoIID (Lopez-Diaz et al., 1986), SpoIIB (Margolis et al., 1993), SpoIIM (Smith et al., 1993), SpoIIP (Frandsen & Stragier, 1995), SpoIIQ (Londoño-Vallejo et al., 1997) and SpoIIIE (Sharp & Pogliano, 1999). On the other hand, SpoIIID suppresses many genes involved in spore cortex formation (Eichenberger et al., 2003; Piggot & Losick, 2002). The σ^E-dependent gene *spoIIIA* (Stragier & Losick 1996) which encodes the SpoIIIAH protein (Meisner et al., 2008). The SpoIIIAH protein (mother cell) forms a channel between a mother cell and a

forespore and interacts with the SpoIIQ protein (forespore) to activate sigma factor σ^G in the forespore (Blaylock et al., 2004; Meisner et al., 2008). The transcriptions of *bofA* and *spoIVF* operon are regulated by σ^E (Ricca et al., 1992). The *spoIVF* operon consists of promoter-proximal (*spoIVFA*) and promoter-distal (*spoIVFB*) cistrons (Cutting et al., 1991). These two cistron operons are integral membrane proteins (Cutting et al., 1991) forming a heterotrimetric complex with BofA in the mother cell membrane (Ricca et al., 1992) or/and in the outer forespore membrane (Rudner & Losick 2002).

As stated earlier, RsfA forms coherent and incoherent feed-forward loops with the early forespore sigma factor σ^F and activates approximately 50 genes including *sigG* (de Hoon et al., 2010). The sigma factor σ^G is encoded by *sigG* in the forespore compartment (Sun et al., 1991). Besides the encoding of the gene *sigG* transcribed by σ^F, the expression of σ^G also requires the early mother cell sigma factor σ^E (Evans et al., 2004). According to Wang et al. (2006), σ^G activates more than 90 genes including *spoVT* gene. The product of this gene, SpoVT protein regulates σ^G-dependent gene expressions (Igarashi & Setlow, 2006). One of the genes, *spoIVB* is stimulated by SpoVT (Wang et al., 2006) and its expression subsequently cleaves pro-σ^K to σ^K through the interaction between SpoIVFB, SpoIVFA and BofA in the outer forespore membrane (Hoa et al., 2002). The σ^G coordinates the gene expressions for later stages of forespore development, for example, SASPs encoded by six *ssp* genes for spore germination and UV protection (Bagyan et al., 1996).

A late mother cell sigma factor σ^K coordinates the gene expressions in different stages (Steil et al., 2005) including spore germination (Setlow, 2003) and mother-cell lysis (Nugroho et al., 1999). The σ^K triggers the activation of GerE (Eichenberger et al., 2004) in formation of spore coat, lysozyme resistance properties and germination (Cutting et al., 1989). GerE is a small protein (74 amino acid residues) can either be an activator or a repressor in conjunction with σ^K (Ducros et al., 2001). The activation and repression of coat genes by GerE have been reviewed (Ducros et al., 2001). Besides of spore coat formation, GerE also negatively regulates the transcription of genes involved in DPA synthesis and accumulation (Daniel & Errington, 1993).

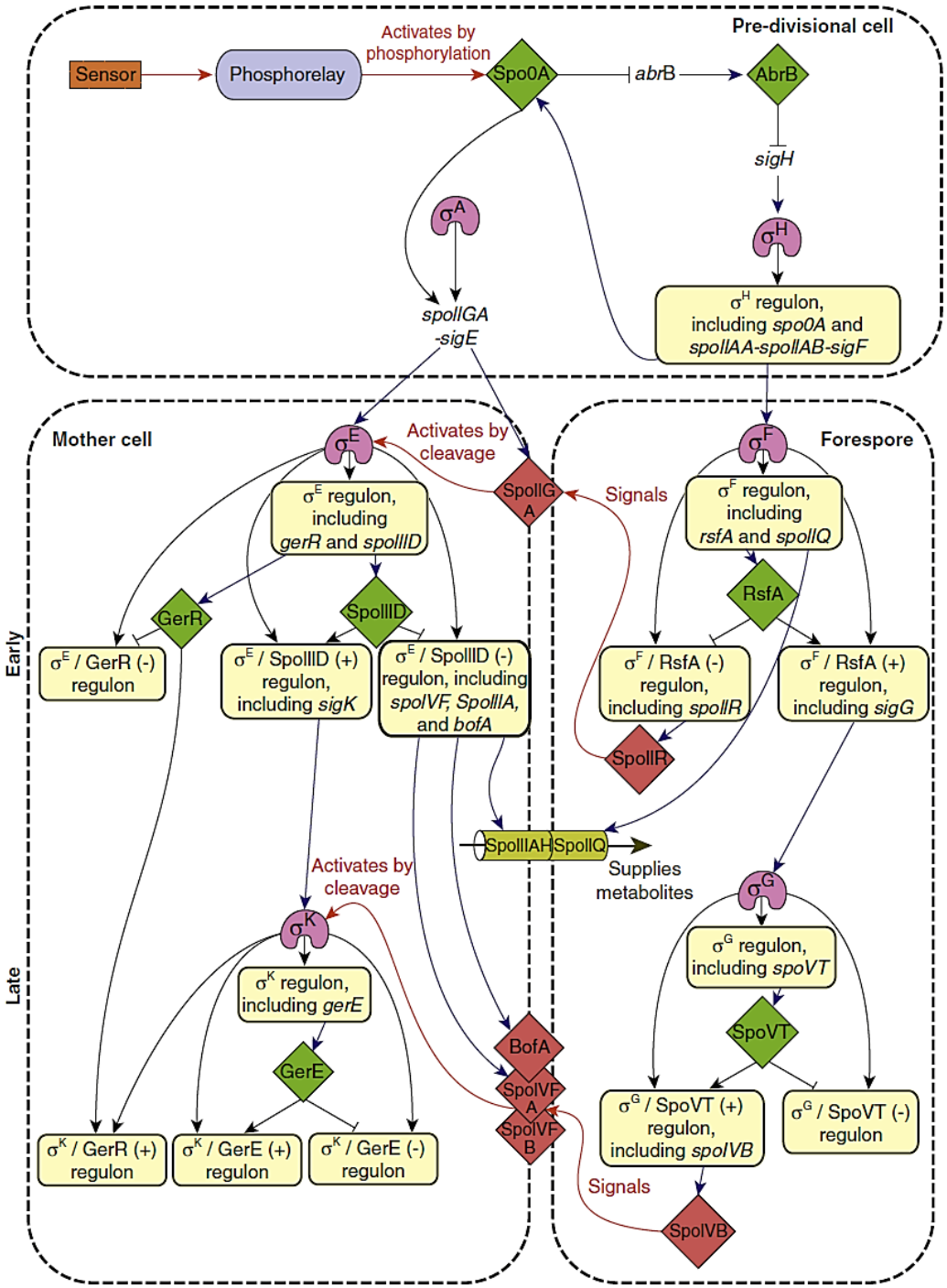


Figure 1.3 *The sigma factors and its gene expressions during different stages of sporulation in B. subtilis (de Hoon et al., 2010).*

1.5 Food Poisoning Outbreaks and Food Safety Regulations

Spore-forming bacteria (e.g. *Bacillus* and *Clostridium*) have been identified in a wide range of food products including dairy products (Postollec et al., 2012). Endospores are an issue in clinical environment, biotechnology processes and food

production due to their special characteristics such as resistance to heat, radiation, disinfectants, and the ability to form biofilms on processing equipment (Logan, 2011).

In August 2013, the dairy products from a New Zealand dairy company were recalled due to the possibly contamination with *Clostridium botulinum* although no public health issues had been observed [International Commission on the Microbiological Specifications for Foods (ICMSF), 2014]. Australia, China, Hong Kong, Malaysia, Saudi Arabia, Thailand and Vietnam were affected by this incident but no cases of *C. botulinum* were reported (Hon & Leung, 2014). In January 2014, the same dairy company recalled almost 9000 bottles of fresh cream products due to the contamination of *Escherichia coli* (Dougan, 2014). Besides that, other spore-forming bacteria such as psychrotolerant, mesophilic and thermophilic organisms are also a potential problem in dairy products (Watterson et al., 2014).

B. licheniformis is a foodborne pathogen that can cause food poisoning, bacteremia, septicemia, peritonitis, ophthalmitis and bovine toxemia (Turnbull & Kramer, 1995). In 1976, a food poisoning outbreak caused by *B. licheniformis* occurred in the United Kingdom (Jephcott et al., 1977). A total of 48 persons were infected by food poisoning and one died after having a meals-on-wheels lunch (Jephcott et al., 1977). Toxigenic strains of *B. licheniformis* involved in food poisoning cases were also isolated from different sources including dairy products in the United Kingdom, Norway and Finland (Salkinoja-Salonen et al., 1999). The collected data showed that the *B. licheniformis* infections had symptoms such as stomach cramps, vomiting, diarrhoea, nausea, abdominal pain and oral burning (Salkinoja-Salonen et al., 1999). Unexpectedly, plate count for the contaminated food was as much as 1.1×10^8 CFU/g (Salkinoja-Salonen et al., 1999).

A food poisoning outbreak caused by *B. licheniformis* had also been reported in Split, Croatia in 2005 (Pavić et al., 2005). Pavić et al. isolated $10 - 11 \times 10^4$ CFU/g of *B. licheniformis* from milk powder and this number increased to $3 - 4 \times 10^5$ after 2 hours incubation at room temperature. The observed symptoms were nausea, headache and vomiting without fever (Pavić et al., 2005). Another case associated with *B. licheniformis* was studied in one of the hospitals in Turkey (Ozkocaman et al., 2006). The patients with neutropenia were infected by *B. licheniformis* from the

contaminated non-sterile cotton wool used for medical purposes (Ozkocaman et al., 2006). According to the same study, antibiotics such as clindamycin, penicillin and other β-lactam agents did not have any effect on *B. licheniformis* although aztreonam, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, ofloxacin, tetracycline and vancomycin were cidal (Ozkocaman et al., 2006). A recent study shows that one *B. licheniformis* strain was isolated in evaporators of a milk powder manufacturing plant in New Zealand (Burgess et al., 2014). Although further investigation for the *B. licheniformis* was omitted, this study showed that the mesophile could form biofilms and spores in the dairy processing plant (Burgess et al., 2014). Formation of biofilms is an issue in the food industry because it is hard to remove with sanitizing agents because of the strong adhesion and thus allowing vegetative cells and spores survive in food processing facilities (Watterson et al., 2014).

In order to determine the spoilage potential, several biochemical tests have been done for aerobic spore-forming organisms including *B. licheniformis* isolated from raw milk (De Jonghe et al., 2010). The findings showed that some *B. licheniformis* strains had intermediate but significant proteolytic activity (1.84 - 12.06 Gly/mL); very low lipolytic activity (0.05 - 0.11% oleic acid); positive for lactose fermentation; but no nitrate reduction (De Jonghe et al., 2010). The lychenysin synthase genes *lchAA*, *lchAB* and *lchAC* of *B. licheniformis* strains were isolated from mastitic milk (Nieminen et al., 2007). Nieminen et al. (2007) stated that these toxin synthase genes were characterized as non-proteinaceous products because of its heat-stability and solubility in methanol. These toxin substances could cause food poisoning because they had similar characteristics compared to the previous reported journal (Nieminen et al., 2007). Vyletělová and co-workers found that the *B. licheniformis* strains were able to survive at ultra-high temperature (UHT) treatment (138 ^{o}C, 4 seconds) with up to 10 CFU/mL detected after the treatment (Vyletělová et al., 2002).

B. licheniformis can cause spoilage in many types of food, for example, in silage (Te Giffel et al., 2002), ropy bread (Pepe et al., 2003), gelatin (De Clerck et al., 2004), raw milk (Banyk ó & Vyletělová, 2009), fermented foods (Falegan, 2011), fish-based products (Coton et al., 2011), egg-based products (Postollec et al., 2012), milk powder (Dhakal et al., 2013), and canned foods (Andr é et al., 2013). According to

New Zealand Food Safety Authority (NZFSA), (2005), equal and/or more than 1×10^3 CFU/g of *B. cereus* and other pathogenic *Bacillus* species isolated from ready-to-eat foods is considered unsatisfactory and potentially hazardous for human consumption. On the other hand, the EU regulation states that colony forming unit of UHT milk should not be more than 100 per mL [European Economic Community (EEC), 1985]. Based on the US Dairy Export Council, the colony count for thermophilic and mesophilic spores in dairy powders must be in the range of < 500 to < 1000 CFU/g (Watterson et al., 2014).

1.6 Biosensors for Detecting Foodborne Pathogens

Many issues caused by foodborne pathogens have been reported to have negative impacts on human health safety (**Table 1.1**). Therefore, rapid detection methods are required to diagnose pathogenic bacterial strains, so that, immediate actions can be taken to cure the infections such as giving antibiotic treatments (Gopinath et al., 2014). Polymerase chain reaction (PCR), enzyme-linked immunosorbent assays (ELISAs) and microarray-based techniques are generally used for foodborne bacteria detection but these techniques are time consuming (Arora et al., 2013).

Biosensors can provide sensitive, rapid and reliable ways to detect foodborne pathogens from contaminated food (Guerrero & González-Aguilar, 2013). Electrochemical biosensors (e.g. amperometric and impedimetric biosensors) and optical biosensors (e.g. fibre optic biosensor) are the prevalent biosensors used for whole bacteria detection (Ahmed et al., 2014). For example, an evanescent fibre-optic biosensor could detect 3.2×10^5 spores/mg of *B. anthracis* spores in spiked powders within 1 hour (Tims & Lim, 2004). Hao and co-workers found that quartz crystal microbalance (QCM) could be used to detect *B. anthracis* spores as low as 1×10^3 spores/mL (Hao et al., 2009).

1.6.1 Optical Biosensor - Surface Plasmon Resonance

A general principle of optical biosensors is to observe the changes of refractive index (RI) caused by the interactions between ligands and analytes (Akdoğan & Mutlu,

2010). Optical biosensors e.g. SPR is more commonly used than mechanical biosensors [e.g. QCM and microcantilever sensor] for whole bacterial detection (Ahmed et al., 2014). Many foodborne bacteria have been detected using SPR (Velusamy et al., 2010). For example, the detection limit for SPR detection of *Listeria monocytogenes* is 3.5×10^3 CFU/mL (Taylor et al., 2006); *Campylobacter jejuni* is 1×10^3 CFU/mL (Wei et al., 2007); *Salmonella* is 1×10^2 - 10^{10} CFU/mL (Zhang et al., 2012) and *E. coli* O157:H7 is 3×10^3 CFU/mL (Wang et al., 2013). SPR can also be used to detect *B. anthracis* and *B. atrophaeus* spores (Farka et al., 2013; Wang et al., 2009). Wang and co-workers were the first to demonstrate the detection of *B. anthracis* spores at concentration as low as 1×10^4 CFU/mL by a SPR biosensor (Wang et al., 2009). Farka et al. (2013) showed that 1×10^5 CFU/mL of *B. atrophaeus* spores could be detected by a SPR biosensor.

1.7 Aims of the Thesis

The aim of this thesis is to examine a microorganism isolated from soil in New Zealand, namely *B. licheniformis* B38. Detection of spores in *Bacillus* species has been widely studied and published for a couple of decades, including *B. cereus*, *B. subtilis*, *B. anthracis* and *B. atrophaeus*. The research emphasis for *B. licheniformis* has focused on the extraction of useful enzymes and inactivation of spores. However, no study has been reported for *B. licheniformis* spore coat proteins detection using antibodies with a SPR biosensor. Therefore, the focus of this research is a preliminary study of developing a biosensor for spore coat proteins detection by SPR. Chapter 2 describes the growth and importance of minerals in *B. licheniformis* B38 spore induction. Pure spores are required for subsequent studies. Chapter 3 assesses the spore coat protein extraction methods using chemical and physical treatments. Chapter 4 focuses on protein purification of a selected spore coat protein using different methods. The identified hypothetical protein sequences might be useful in immobilization to a SPR chip for spore coat proteins detection. Chapter 5 examines the detection of *B. licheniformis* B38 spore coat proteins by SPR. Different binding parameters are tested to observe the signal generated by SPR. Some suggestions and recommendations are given for future experiments.

CHAPTER 2

THE GROWTH AND SPORULATION OF *BACILLUS LICHENIFORMIS* B38

2.1 Introduction

In this chapter, the growth and sporulation of *B. licheniformis* B38 isolated in New Zealand were investigated. The general characteristics of *B. licheniformis* (Vos et al., 2011) and some details were previously discussed in **Section 1.2**. Bacterial growth and sporulation are mainly driven by abiotic factors such as pH, temperature, oxygen availability and the composition of the medium (Black, 2008; Knyasi, 1945). Therefore, the listed abiotic factors had been investigated to induce and increase the spore yield of *B. licheniformis* B38 isolated from soil. Spore purification and staining were done to differentiate and obtain pure spores for later experiments.

2.2 Methods

2.2.1 Recovery of Pure Culture

The glycerol stock (20% glycerol + 80% culture) of *B. licheniformis* B38 from -80 ^{o}C freezer was thawed and 100 μL of inoculum was pipetted into a universal bottle containing 10 mL of sporulation broth. After 18 - 24 hours incubation, one loop of culture was streaked on sporulation agar plates. After 1 - 2 days incubation, a single colony was picked and transferred to 10 mL of sporulation broth. After 18 - 24 hours culture induction, 1% of the broth was used as an inoculum and transferred into conical flasks containing a larger volume (e.g. 100 mL or 200 mL) of sporulation broth. The flasks were agitated and incubated for up to 4 days at 30 ^{o}C for spore production.

2.2.2 Induction of Sporulation

The preparation of spore induction media was adapted from Schaeffer et al. (1965) and Nicholson & Setlow (1990). Briefly, the composition of a spore induction medium was 8 g/L of nutrient broth (Oxoid), 10 mL of 10% (w/v) KCl, 10 mL of 1.2% (w/v) $MgSO_4.7H_2O$ with the addition of 17 g/L bacteriological agar (Oxoid) for agar plates. The pH was adjusted to pH 7.6 using 1 M NaOH and topped up to the specified volume prior to sterilization. The sterile medium was cooled to approximately 50 ^oC before adding 1 mL of each of sterile solution: 1 M $Ca(NO_3)_2$ (autoclaved), 0.01 M $MnCl_2$ (autoclaved) and 1 mM $FeSO_4$ (filter-sterilized).

2.2.3 Spores Purification

The spores were harvested after incubating in a sporulation medium for 4 days as described previously. The spore purification procedures were adapted from Seale et al. (2008). The culture containing a mixture of vegetative cells and spores was centrifuged at 10,000 $\times g$ for 10 minutes at 4 ^oC. The pellet was then washed 3 times with cold sterile Milli-Q ultrapure water.

Polyethylene glycol 4000 (PEG 4000) was used to purify spores from the mixture. 5.6 g of PEG 4000 was dissolved in 17 mL of 3 M phosphate buffer (pH 7.4) and gently heated to completely dissolve the PEG to create a two-phase system.

The pellet was resuspended with cold sterile Milli-Q ultrapure water before layering on PEG two-phase system to make up a total volume of 50 mL. This mixture was vortexed vigorously and the phases were allowed to separate before centrifugation at 1,500 $\times g$ for 3 minutes at 20 ^oC using swinging buckets. The spores were located in upper layer (PEG 4000 rich phase) while the vegetative cells were migrated to lower layer (phosphate rich phase) during centrifugation. The upper layer containing the spores was carefully transferred to a new tube and washed with sterile Milli-Q ultrapure water and centrifuged at 10,000 $\times g$, 20 ^oC for 10 minutes. This procedure was repeated for 5 times. The effectiveness of pure spore separation was determined by Schaeffer-Fulton endospore staining. The pure spore suspension was kept in a microcentrifuge tube at 4 ^oC.

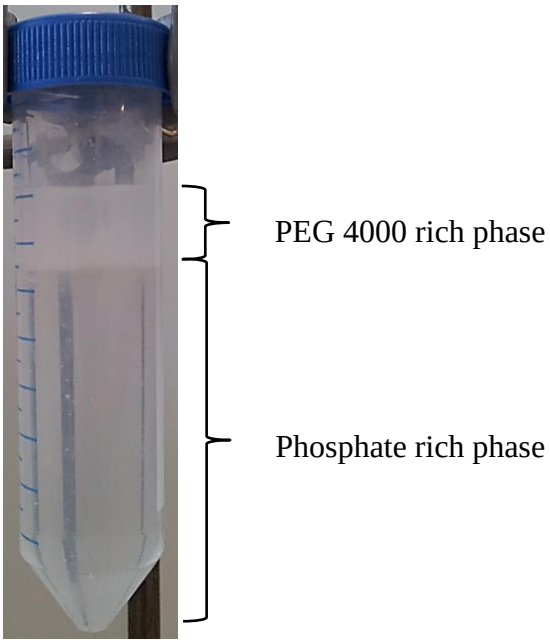


Figure 2.1 *A PEG two-phase system.*

2.2.4 Schaeffer-Fulton Endospore Staining

The staining technique was based on Schaeffer and Fulton (1933). 0.5% (w/v) malachite green and 2.5% (w/v) safranin O stock were prepared using Milli-Q ultrapure water and 95% ethanol. Malachite green staining solution was filtered using a filter paper grade 1 (Whatman) prior to be used. Safranin counterstain working solution was prepared by adding 10 mL of stock solution to 90 mL of Milli-Q ultrapure water and mixing thoroughly.

The sample to be stained was air-dried and heat-fixed on a glass slide. The slide was later covered with a blotting paper that fitted to the glass slide (e.g. 75 × 25 mm). The blotting paper was saturated with malachite green staining solution while being steamed over a beaker filled with boiling water for 5 minutes. The staining solution was added to the blotting paper to keep it moist throughout the staining process. The blotting paper was removed and the slide was raised with tap water to remove excessive dye. After that, the slide was counterstained with safranin for 2 minutes and washed with tap water. The slide was blotted dry before examining under TCS SP5 Confocal Microscope (Leica) using oil immersion lens (100 x magnification). The endospores were stained green and vegetative cells were stained red.

2.3 Results and Discussion

2.3.1 The Factors Affect the Growth and Sporulation of *Bacillus licheniformis* B38

The *B. licheniformis* B38 culture was from Microbiology Culture Collection of School of Biological Sciences, University of Canterbury, New Zealand. This organism was previously isolated from soil and identified by BBL™ Crystal™ Gram-Positive ID Kit (BD) and 16S rRNA (data not shown) to confirm the identity of the organism.

The *B. licheniformis* B38 growth curve (**Figure 2.2**) is a typical bacterial growth curve, which has four phases: (1) lag phase, (2) log phase, (3) stationary phase and (4) death phase (Black, 2008). Briefly, lag phase is the stage where organisms start adapting themselves in a new environment or a medium (Black, 2008). Once the organisms have adapted well in the environment, they start growing exponentially, which is called log phase (Black, 2008). Stationary phase is achieved when the rates of cell division and cell death are the same (Black, 2008). When it comes to death phase, many cells die because of the nutrient limitation (Black, 2008). Additionally, more spores can be observed at death phase for spore-forming organisms (Black, 2008).

The first 4 hours was the lag phase (**Figure 2.3**) and the subsequent hours to about 16 hours were the log phase of *B. licheniformis* B38. Formation of *B. licheniformis* B38 endospore started at approximately 16 hours (stationary phase) (**Figure 2.4**). *B. licheniformis* B38 has started forming endospores to adapt to unfavourable environmental conditions due to nutrient depletion and toxic accumulation in the medium in stationary phase (Black, 2008). At the stage of spore formation, α-amylase, proteases, nucleases and extracellular antibiotics are synthesized by sporulating cells, and alkaline phosphatase, DNase, SASP and DPA are synthesized at the later stages of spore formation (Nicholson & Setlow, 1990). The *B. licheniformis* B38 growth curve did not extent for after 20 hours because absorbance more than 2.5 could not be measured by Novaspec III spectrophotometer (Amersham Biosciences) at OD_{600}. The death phase of *B. licheniformis* B38 would be predicted after 24 hours since increase number of spores could be observed after that

period of time. Apparently, more endospores had been released from mother cells after 24 hours (**Figure 2.5**).

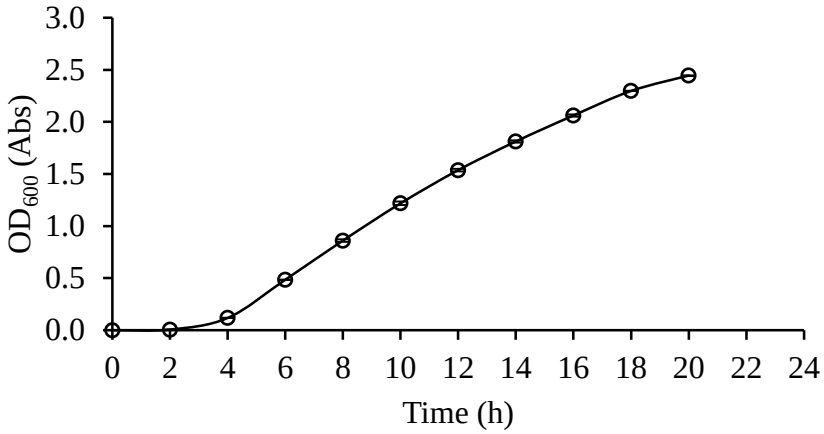


Figure 2.2 *The growth curve of B. licheniformis B38. Data are mean ± standard error, n = 3.*

There are many factors that contribute to sporulation of *Bacillus* species including pH, incubation temperature, composition of the medium (e.g. cations) and others (Knyasi, 1945). These factors are equally necessary for endospore formation. Knaysi (1945) found that endospores were formed at pH between 6.0 and 8.0. Induction of endospore at pH 7.0 was also reported by Schaeffer (1986). Minh et al. (2011) showed that pH affected sporulation of *Bacillus* species because certain enzymes took part in the endospore forming processes relied on pH changes. More recently, Baril et al. (2012a) studied the effect of temperature and pH on sporulation abilities in a *B. licheniformis* strain isolated from raw dairy ingredients. This study showed that the endospore concentration of *B. licheniformis* was higher at pH 7.0 - 8.5 than pH 6.3 and no endospores could be observed at pH between 5.2 and 5.8 (Baril et al., 2012a). The current study used pH 7.6 to produce endospores over a 4-day incubation period is compromise with the previous reported results. The pH changes, particularly at low pH lead to endospore demineralization and stimulate endospore germination (Tola & Ramaswamy, 2014b).

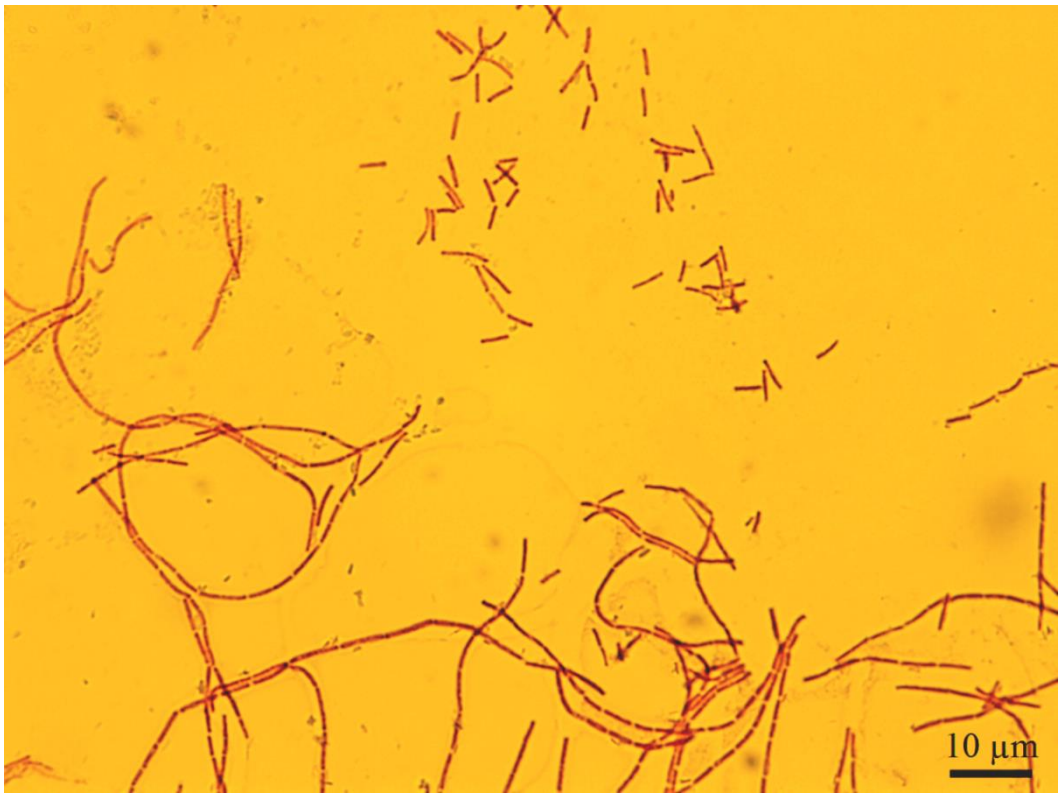


Figure 2.3 *B. licheniformis B38 at 4-hour incubation.*

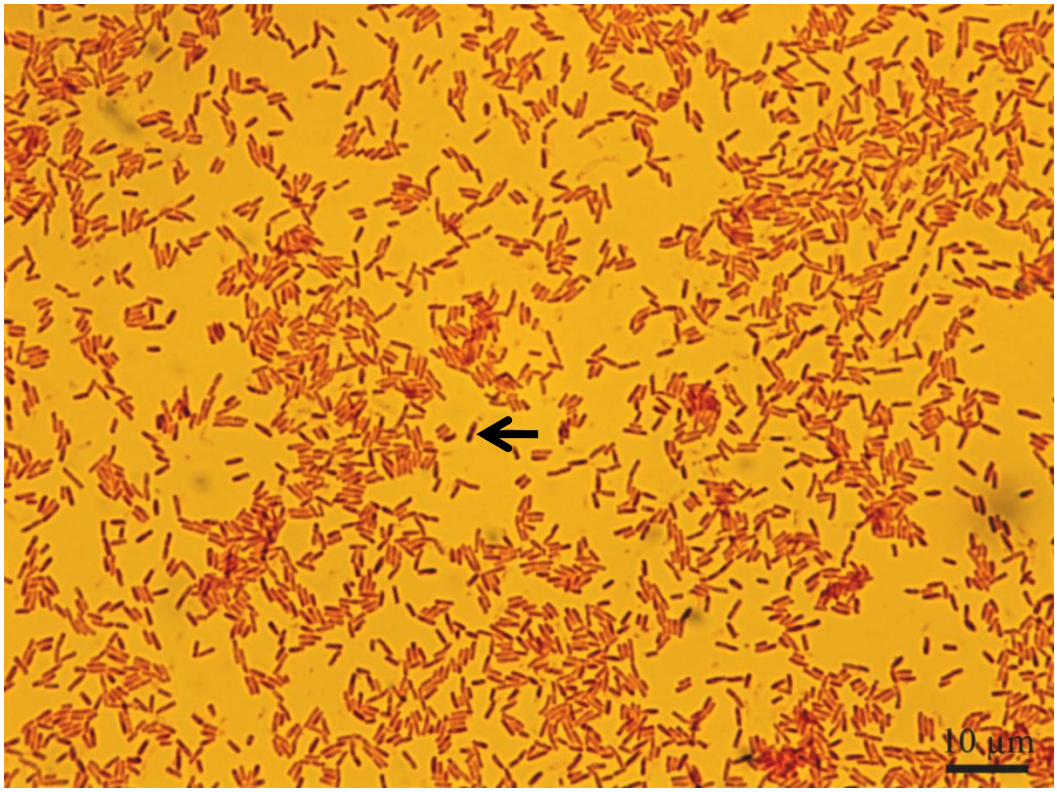


Figure 2.4 *B. licheniformis B38 at 16-hour incubation. A green stained encapsulated endospore by a mother cell is indicated with an arrow.*

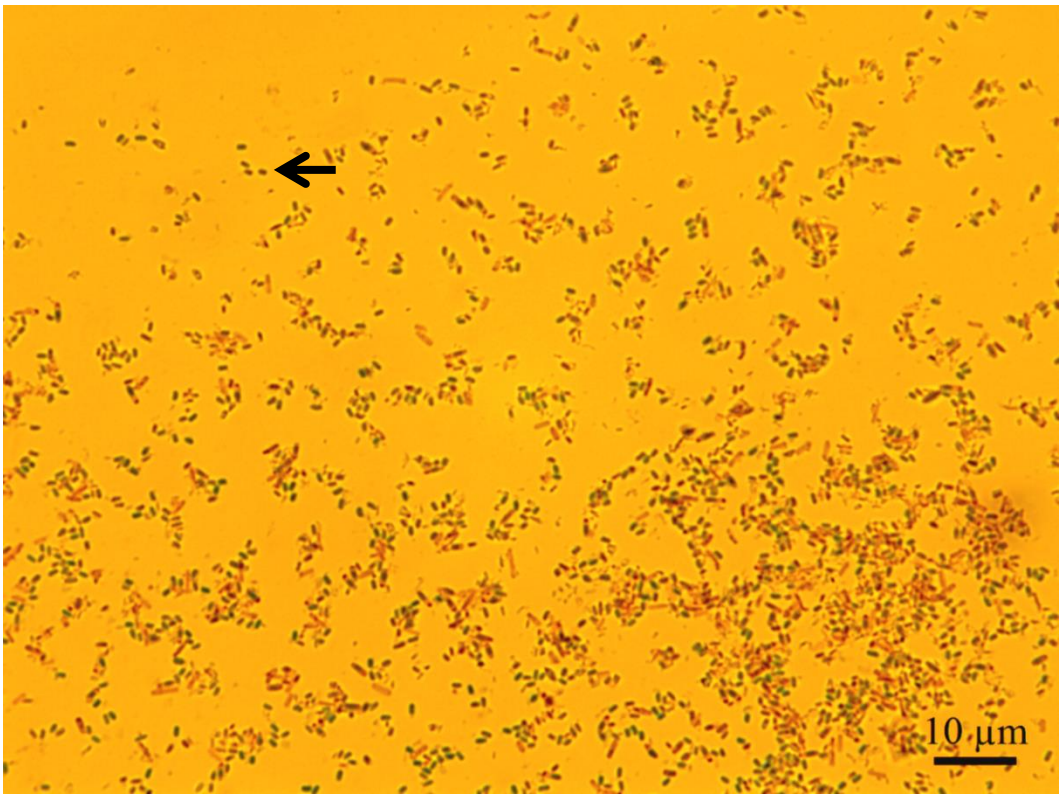


Figure 2.5 *B. licheniformis B38 at 96-hour incubation. A green stained free endospore is indicated with an arrow.*

B. licheniformis has a wide growth temperature range from a minimum 15 ^{o}C to maximum 50 - 55 ^{o}C (Vos et al., 2011). Llarch (1997) reported that *B. licheniformis* isolated from a geothermal environment could grow at 68 ^{o}C. Baril (2012a) showed that *B. licheniformis* isolated from raw dairy products was able to form endospores at 20 - 50 ^{o}C but endospores did not form between 5 - 15 ^{o}C. The temperature 30 ^{o}C was chosen to induce *B. licheniformis* B38 sporulation which was also the culture reviving protocol of Microbiology Culture Collection, School of Biological Sciences, University of Canterbury, New Zealand. A higher temperature of 55 ^{o}C was tried to induce sporulation based on a method reported by Seale et al. (2008) with minor modifications. Briefly, the same organism was grown in a sporulation medium (30 g/L of Bacto tryptone or tryptic soy broth, 0.125 g/L of $CaCl_2$, 0.15 g/L of $MnSO_4$, 0.155 g/L of $FeSO_4$ and 0.55 g/L of $MgCl_2$) without agitation for 3 days and checked for the sporulation by Schaeffer-Fulton endospore staining. However, endospores were not observed using this method (data not shown). It suggests that 30 ^{o}C with agitation is necessary for *B. licheniformis* B38 sporulation. Tanaka et al. (2012) reported that growth temperature affected the heat resistance of *B. licheniformis* endospores.

Lindsay et al. (1990) also showed that the physical (e.g. spore wet density) and chemical (e.g. DPA and cations contents) characteristics of *Bacillus* endospores were influenced by different temperatures. In addition, temperature may alter the endospore structure especially the proteins that are responsible for the coat formation (Melly et al., 2002).

Sporulation was low when the cells were grown in flasks covered with aluminium foil and sealed with parafilm although it was with agitation. Therefore a suitable blue cap or cotton bung covered with aluminium foils should be selected during spore induction. The main reason for doing this is to allow good aeration during spore induction while avoiding any contamination. Liquid media with agitation is suitable to induce sporulation (Foster, 1956). This is because these two conditions will provide a homogenous nutritional environment and promote the availability of oxygen during sporulation (Foster, 1956). Several publications (e.g. Aronson et al., 2014; Baril et al., 2012a; Baril et al., 2012b; Seale et al., 2008) also showed that agitation was necessary to induce sporulation of *Bacillus* species. *B. licheniformis* is a facultative anaerobic organism that can grow with or without oxygen (Vos et al., 2011). However, agitation is the best option to induce sporulation. Agar media are used to check the purity of the culture and obtaining single colonies for further experiments.

2.3.2 The Importance of Minerals for Sporulation

Unfavourable environmental conditions such as exhaustion of nutrients can lead vegetative cells to form endospores in order to adapt to the environment (Gould, 2000). Using exhausted nutrient media or transferring the growing cells from a rich to a poor media has been suggested to induce sporulation of *Bacillus* species (Nicholson & Setlow, 1990). A previous study showed that sporulation could be induced by transferring mature vegetative cells to distilled water (Knaysi, 1945). In contrast, Foster (1956) stated that sporulation process could not occur unless either potassium (K), magnesium (Mg), manganese (Mn) or calcium (Ca) was included in the media. Most of the studies (e.g. Han & Wilson, 2013; Lončar et al., 2013; Lu et al., 2012) used Schaeffer's sporulation media to induce sporulation of *Bacillus* species.

Potassium

One of the important minerals for *Bacillus* endospore induction is potassium. Potassium is needed for protein synthesis in growing cells and sporulating cells (Eisenstadt, 1972). It is generally assumed that adding of manganese can stimulate the accumulation of potassium in sporulating cells and activate all membrane transport systems (Eisenstadt, 1972). Mazas et al. (2009) explained that the potassium ion (K^+) was required for microbial growth and only a small amount of this cation was needed in sporulation medium because higher concentrations did not increase the rate of sporulation for *Bacillus* species. They found that K^+ did not play a role in heat resistance which was in contrast to a number of other publications (Mazas et al., 2009).

Magnesium

Magnesium is required in sporulation and for nucleic acid, enzymes and protein synthesis, however, not for heat resistance (Bassi et al., 2012). Grimshaw et al. (1998) showed that magnesium ion (Mg^{2+}) was required for both activation (K_{act} = 7 mM) and inhibition (K_i = 24 mM) of KinA-mediated Spo0F~P formation in sporulation process. Regulation of this phosphorylation mechanism to initiate sporulation process was described previously in **Section 1.4** (**Figure 2.6**). The presence of a Mg^{2+}-ATP chelate complex was to be suggested to act as a substrate in an auto-phosphorylation reaction (Grimshaw et al., 1998).

Signal transduction

$$ATP + KinA \leftrightarrow ADP + KinA\sim P$$

Phosphotransfer

$$KinA\sim P + Spo0F \leftrightarrow KinA + Spo0F\sim P$$

Phosphotransfer

$$Spo0F\sim P + Spo0B \leftrightarrow Spo0F + Spo0B\sim P$$

Phosphotransfer

$$Spo0B\sim P + Spo0A \leftrightarrow Spo0B + Spo0A\sim P$$

Figure 2.6 *The sporulation process is first regulated by a Spo0A~P response regulator of phosphorelay pathways (Grimshaw et al., 1998).*

On the other hand, Hanlon et al. (1982) proved that the higher the concentration (0.05 mM - 1 mM) of magnesium, the higher the rate of sporulation in *B. licheniformis*. The rate of sporulation was affected by the magnesium-exhausted medium and magnesium only in the nitrogen-exhausted medium but not by the glucose-exhausted medium (Hanlon et al., 1982).

Calcium

Calcium is important in endospore maturation and endospore activation process (Burgess et al., 2010). Minerals, mainly calcium, are largely accumulated in endospore core (Burgess et al., 2010). *Bacillus* endospores can survive for a long period in the dormant state because the 5 - 15% dry weight of DPA is chelated with calcium ions (Ca^{2+}) to form Ca-DPAs in a 1:1 ratio particularly located in the spore core (Huang et al., 2007; Setlow, 2006). The concentration of Ca-DPA is more than 800 mM and its variation can be due to the different sizes of individual endospores (Huang et al., 2007). Oomes et al. (2009) reported that a total of 305 genes was affected by calcium and 41 genes among the total number were playing the role in sporulation (e.g. *spoVFA* and *spoVFB*) in *Bacillus* species. Minh et al. (2011) described that the sporulation media without Ca^{2+} affected endospore formation.

Manganese

Manganese is necessary for sporulation in *Bacillus* species (Charney et al., 1951). Opheim and co-worker found that the manganese ion (Mn^{2+}) was essential to stabilize fructose-1,6-bisphosphatase in formation of cortex and membrane biosynthesis by increasing phosphoenolpyruvate enhanced hexose phosphate production during sporulation in *B. licheniformis* (Opheim & Bernlohr, 1975). Manganese is also required by phosphoglycerate phosphomutase (EC 5.4.2.1) for the interconvertion of 3-phosphoglyceric acid (3PGA) and 2-phosphoglyceric acid (2PGA) during growth and sporulation in *Bacillus* species (Oh & Freese, 1976). According to Oh and Freese (1976), cell growth and sporulation (formation of prespore septation) were resumed when accumulation of 3PGA inside the cells disappeared upon adding the manganese. Later, Vasantha and Freese (1979) proved that Mn^{2+} was specific to the enzyme phosphoglycerate phosphomutase in *Bacillus* species. Oh and Freese

(1976) also showed that Mn^{2+} was required for full utilization of carbohydrates in sporulation. Manganese is required for septum development in forespore formation stage regulated by σ^F and then accumulated in new endospores (Bassi et al., 2012). Lack of $MnCl_2$ in sporulation medium led to poor sporulation (Granger et al., 2011). Mn^{2+} alone is not adequate to protect the endospores themselves from ionizing radiation, therefore, it must be coupled with DPA to increase the effectiveness of ionizing radiation protection (Granger et al., 2011).

Iron

Iron is hypothesized to induce sporulation by stressing the bacteria in the *Bacillus* species (Purohit et al., 2010). Purohit and co-workers found that the sporulation rate was ≥ 98% after 40 hours incubation for the *Bacillus* species grown in a medium that contained iron (Purohit et al., 2010). Aconitase is an iron dependent enzyme and transcriptional regulator which plays a role in late sporulation gene expression (e.g. the binding of aconitase to *gerE* mRNA) in *Bacillus* species (Serio et al., 2006). Iron may necessary to induce and increase the rate of sporulation in different *Bacillus* species (Kolodziej & Slepecky, 1964). Accumulation of iron can be found in *Bacillus* species during sporulation (Powell & Strange, 1956). Lundgren and Cooney (1962) showed that iron was taken up by *Bacillus* species during cell growth and the uptake level was fallen at sporulation stage.

2.3.3 Spore Purification by Polyethylene Glycol

PEG is a type of polymer and has many uses in pharmaceutical (Knop et al., 2010) and protein purification (Walker, 2010b). Sacks and Alderton (1961) showed that PEG could be used for endospore purification. Although some characteristics of the endospore surface have been described (McKenney et al., 2013), there is no specific explanation to elucidate the preference of endospores for PEG rich phase. It is believed that endospore surface has a similar nonpolar characteristic to polystyrene which can be attracted by a PEG rich phase (Sacks & Alderton, 1961). The PEG rich phase in a PEG two-phase system is highly selective for bacterial endospores (Sacks & Alderton, 1961). Therefore, it is recommended for separating endospores from vegetative cells. This mild chemical separation method does not alter spore coat

proteins or spore viability (Harrold et al., 2011) compared to harsh methods such as lysozyme (Jenkinson et al., 1981), SDS (Jenkinson et al., 1981) and EDTA (He & Tebo, 1998). Other endospore purification methods have been demonstrated, i.e. sucrose density gradients (Prentice et al., 1972), urografin density gradients (Nicholson & Setlow, 1990), water washing (Nicholson & Setlow, 1990) and French press (Jenkinson et al., 1981). Some possible problems have been discussed for the above methods (Nicholson & Setlow, 1990). Harrold et al. (2011) explored the effectiveness of this spore separating technique based on the work of Sacks and Alderton (1961) with a slight modification. As a result, a high purity of spore could be obtained by a PEG two-phase system endospore extraction method (Harrold et al., 2011).

Different *Bacillus* species behave differently when a PEG two-phase system is applied for spore purification. Seale et al. (2008) found that *Geobacillus* species concentrated in the PEG rich layer whereas debris concentrated at the interface between the PEG and the phosphate rich layers. Seale et al. (2008) however reported that the phase where accumulation of endospores was slightly different compared to the finding of Sacks and Alderton (1961). Presumably, different *Bacillus* species have different endospore surface characteristics (Seale et al., 2008). Based on the observation in this study, the endospores of *B. licheniformis* B38 were accumulated in the PEG rich phase while the cellular debris and vegetative cells accumulated in the phosphate rich phase. After purification, the green stained endospores could be observed under the microscope (**Figure 2.7**). Extensive washing is required to remove the PEG on the endospore surface because it may affect the spore surface properties (Seale et al., 2008).

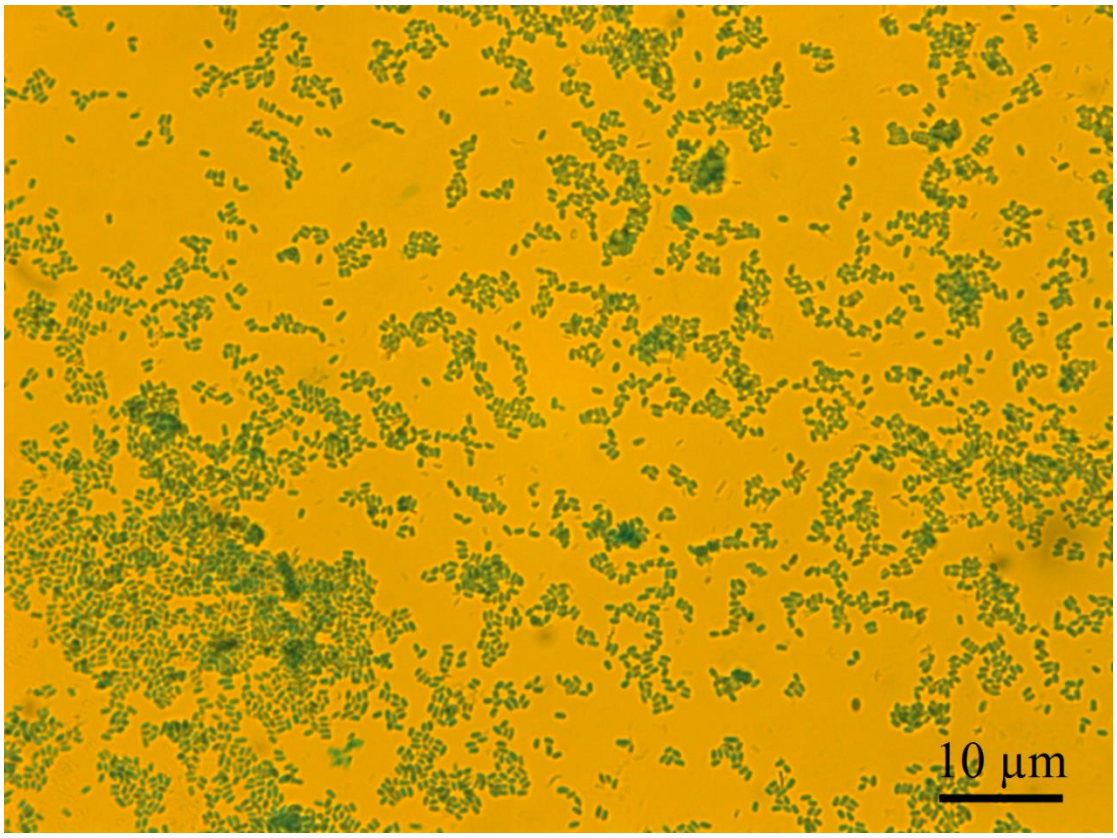


Figure 2.7 *The pure endospores of B. licheniformis B38.*

2.3.4 Differentiating the Vegetative Cells and the Endospores by Staining

Although phase-contrast microscopy can be applied to determine phase-bright endospores (e.g. Olivier et al., 2012; Traag et al., 2013), Schaeffer-Fulton endospore staining can also be used to differentiate between endospores and vegetative cells (e.g. Harrold et al., 2011; Smitha et al., 2013; Tola & Ramaswamy, 2014b). Black (2008) also suggested that endospores were much more easily seen by Schaeffer-Fulton endospore staining. Steaming makes endospore walls are more permeable to malachite green dye (Black, 2008). The green dye is later washed off using water and safranin counterstains the whole vegetative cells except the endospores (Black, 2008). **Figure 2.5** shows that the vegetative cells stained red while the endospores stained green. **Figure 2.7** shows that only green stained endospores after the purification step has been done.

CHAPTER 3

EXTRACTION OF *BACILLUS LICHENIFORMIS* B38 SPORE COAT PROTEINS

3.1 Introduction

Laemmli buffer system (Laemmli, 1970) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are the proteomic technologies commonly applied in analysis of spore coat proteins of *Bacillus*, *Clostridium* and other spore-forming organisms (Abhyankar et al., 2014). Coomassie brilliant blue (CBB) staining is used to visualize proteins in gels after separation (Abhyankar et al., 2014). Denaturing and reducing agents in alkaline conditions are required for extraction of soluble spore coat proteins (Pandey & Aronson, 1979). Urea is a common strong chaotropic agent for extracting spore coat proteins (Venir et al., 2014) and solubilizing inclusion bodies (Yang et al., 2011). Physical treatments alone may or may not help in removing outer spore coat proteins (Basu et al., 2007; Thompson et al., 2011). However, chemical treatments alone are strong enough to solubilize spore coat proteins which can be visualized by CBB staining (Thompson et al., 2011).

3.2 Methods

3.2.1 Gel Electrophoresis for Chemical Treatments

The pure spore suspension was centrifuged at 10,000 × *g*, 4 ^{o}C for 10 minutes to obtain a pellet. The following methods were adapted from Thompson et al. (2011). Briefly, the pellet was resuspended in 1 mL of SDS-8 M urea sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 8 M urea, 2% β-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue) in a microcentrifuge tube. The mixture was then boiled at 100 ^{o}C for 10 minutes and spun down to obtain the supernatant for gel electrophoresis. 5 μL of PageRuler Plus Prestained Protein Ladder (Fermentas) was used as a protein reference. 20 μL of supernatant was loaded into each well of NuPAGE 4 - 12% Bis-

Tris Gel (Life Technologies). The XCell SureLock^{TM} Mini-Cell Electrophoresis System (Life Technologies) was filled with NuPAGE MOPS SDS Running Buffer (Life Technologies) and the gel was run at 200 V for 50 minutes as recommended by the instructions of the product.

The gel was stained with CBB stain (50% methanol, 10% acetic acid and 0.05% Brilliant Blue R-250) for 1 hour and destained (50% methanol, 10% acetic acid and 40% Mili-Q ultrapure water) for overnight. The gel pictures were taken using GeneGenius Gel BioImaging System (Syngene).

3.2.2 Preparation of Coomassie Brilliant Blue Staining Without Methanol and Acetic Acid

The following procedures were adapted from Dong et al. (2011) with slight modifications. 500 mg of Brilliant Blue R-250 was dissolved in 1000 mL of purified water and stirred for 2 - 4 hours. The solution was filtered, if necessary.

3.3 Results and Discussion

3.3.1 Extraction of Spore Coat Proteins by Chemical Treatments

Some publications (e.g. Li et al., 2013; Mundra et al., 2013; Venir et al., 2014) suggest that SDS, DTT, β-mercaptoethanol and urea are able to extract spore coat proteins. Basically, these detergents and/or chaotropic agents are crucial in spore coat protein extraction. For instance, SDS is an anionic detergent which binds and provides negative charge around proteins as well as denaturing the protein structure (Walker, 2010a). DTT and mercaptoethanol reduce disulphide bridges found in protein tertiary structure (Walker, 2010a; Walker, 2010b). Urea is used to solubilize the spore coat proteins (Pandey and Aronson, 1979). As mentioned earlier, the denaturing and reducing agents were required to extract spore coat proteins due to insoluble and soluble characteristics (Pandey & Aronson, 1979).

Barbosa et al. (2004) showed that *B. licheniformis* 9945A and a *B. licheniformis* natural isolate had different spore coat protein profiles. Faille et al.

(2010) and Giorno et al. (2007) compared the spore coat proteins of a *B. licheniformis* strain and other *Bacillus* species using SDS-PAGE gel. By comparing the result in **Figure 3.1** to previous publications (e.g. Faille et al., 2010; Giorno et al., 2007), it shows that endospores of *Bacillus* species with or without exosporium contains a wide range of glycoproteins or polysaccharides (Faille et al., 2010) and biochemical compositions (Giorno et al., 2007).

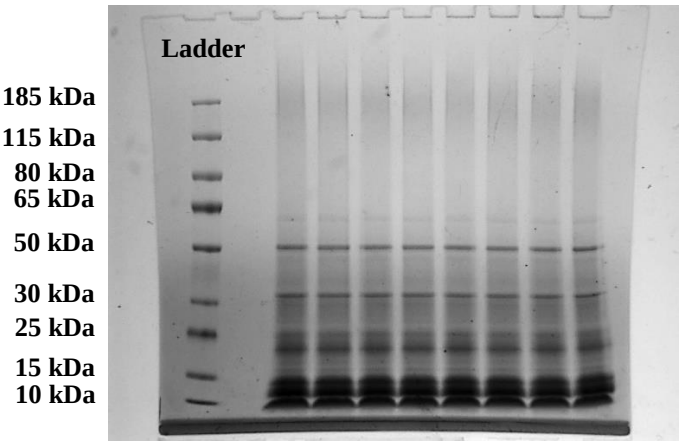


Figure 3.1 *Wells loaded with 20 µL of spore coat proteins digested with a SDS-8 M urea sample buffer. B. licheniformis B38 spore coat proteins range in size between 10 kDa and 65 kDa.*

pH 6.8 and pH 9.0 were also tested for extraction of *B. licheniformis* B38 spore coat protein. There was no difference in results using the same SDS-8 M urea sample buffer at different pHs (data not shown). Some publications (e.g. Pandey & Aronson, 1979; Riesenman & Nicholson, 2000) suggest that using alkaline conditions for spore coat protein extraction. The reason is that about 70% of *Bacillus* spore coat protein can be extracted under alkaline conditions (Pandey & Aronson, 1979) and Driks (1999) also states that alkaline pH is applicable for analysing *Bacillus* spore coat biochemistry. The pH 6.8 of this buffer worked well in this study.

3.3.2 Extraction of Spore Coat Proteins by a Combination of Chemical and Physical Treatments

This study also used PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4), 8 M urea, 0.05% and 0.1% Triton X-100, combined with sonication for

B. licheniformis B38 spore coat protein extraction (data not shown). It showed that sonication twice for approximately 10 seconds each did not help in spore coat protein extraction. Sonication and bead beating are not enough to release spore coat protein unless combined with detergents (Thompson et al., 2011). In contrast, Basu et al. (2007) showed that using sonication could release spore coat protein. The limitations of this method are small scale and heat is produced during sonication (Walker, 2010b). So that, some precautions must be taken such as using ice bath during the treatment (Walker, 2010b). Prolong the sonication time has not been further carried out for this study since the SDS-8 M urea sample buffer worked well for spore coat protein extraction.

3.3.3 Different Techniques for Gel Staining and Denaturing

CBB staining solution is a mixture of methanol and acetic acid that fixes and precipitates proteins in the gels to avoid protein loss during staining (Lei et al., 2007; Walker, 2010a). Nonetheless, this staining solution can reduce protein recovery from gel electroelution (Lei et al., 2007). Therefore, two different staining solutions were tested for using in gel elution by diffusion in **Section 4.2.4**.

Two types of staining solutions: (a) a CBB staining with methanol and acetic acid (CBB Staining Solution A) and (b) a CBB staining without methanol and acetic acid (CBB Staining Solution B) (Dong et al., 2011) were used to perform gel staining. These two staining solutions were able to stain the bovine serum albumin (BSA) at concentration of 2 - 16 μg (data not shown). The CBB Staining Solution A is able to stain the protein concentration at as low as 0.1 μg (Walker, 2010a). However, the CBB Staining Solution B can stain even lower protein concentrations at 0.01 μg (Dong et al., 2011). The staining process with the CBB Staining Solution B can be done within 30 minutes (Dong et al., 2011) compared to the traditional methods (Walker, 2010a) which required a longer time to stain and destain the gel. The approach of Dong et al. (2011) possesses two advantages in that the staining solution without methanol and acetic acid is suitable for gel elution by simple diffusion and the staining process can be done in a short time.

These two staining solutions were also used to test on *B. licheniformis* B38 spore coat proteins treated with the SDS-8 M urea sample buffer. **Figure 3.2** shows a gel staining using two types of CBB staining solutions. It shows less clear bands (Lanes 7 - 9) in the gel for the CBB Staining Solution B compared to the bands (Lanes 1 - 3) stained with the CBB Staining Solution A. The results showed that the CBB Staining Solution B was suitable for gel elution in the next section. The protein bands at between 10 and 15 kDa (e.g. **Figure 3.1**) were always consistently and clearly shown on the gels. Hence, an ~10 kDa band was chosen for the next study.

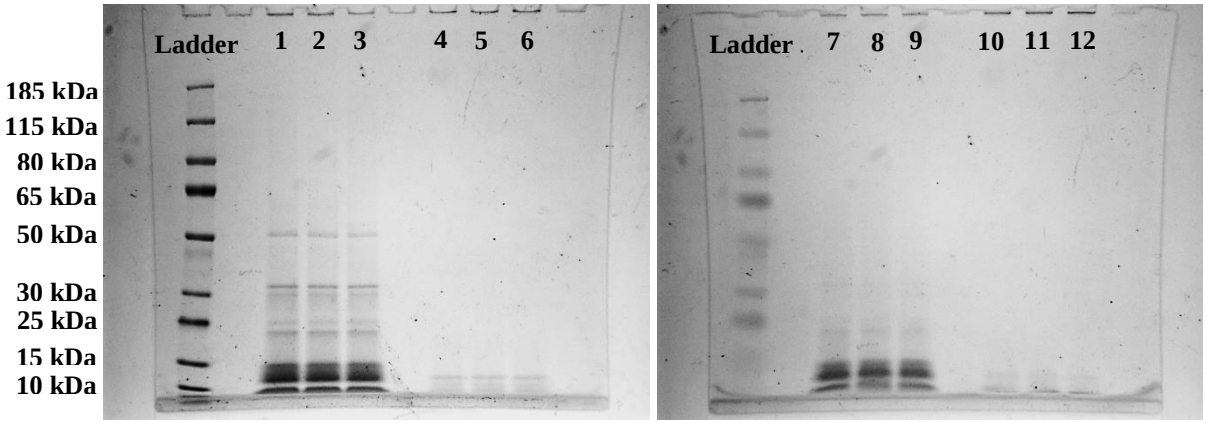


Figure 3.2 *Lanes 1 - 3 represent a SDS-8 M urea treatment with boiling at 100 ^{o}C for 10 min before loading to gel. Lanes 4 - 6 represent a SDS-8 M urea treatment with incubation at room temperature for 10 min. Subsequently, the gel was stained with CBB Staining Solution A. Lanes 7 - 9 and Lanes 10 - 12 are the same conditions as Lanes 1 - 3 and Lanes 4 - 6. However, the gel is stained with CBB Staining Solution B.*

Protein samples are boiled with sample buffers containing detergent and reducing agents in order to denature and open up the polypeptide chain into rod shape structures for gel electrophoresis (Walker, 2010a). A study was performed to incubate the *B. licheniformis* B38 spore pellet with the SDS-8 M urea sample buffer at room temperature (~20 ^{o}C) for 10 minutes before loading to the gel. Some faint bands (**Figure 3.2**: Lanes 4 - 6 and Lanes 10 - 12) at between 10 and 15 kDa could still be observed with this treatment. These faint bands were visible up after staining with both CBB staining solutions. In short, the SDS-8 M urea sample buffer is strong enough to denature *B. licheniformis* B38 spore coat protein without boiling.

CHAPTER 4

PURIFICATION AND IDENTIFICATION OF A SELECTED SPORE COAT PROTEIN OF *BACILLUS LICHENIFORMIS* B38

4.1 Introduction

Liquid chromatography is used to separate and analyse organic components from complicated samples (Pan et al., 2014). For example, size exclusion chromatography, ion-exchange chromatography, hydrophobic interaction chromatography and affinity chromatography (Janson & Jönsson, 2011). Liquid chromatography is still a leading technology in biopharmaceutical purification (Hanke & Ottens, 2014). For example, production of monoclonal antibodies and related proteins (e.g. Orthoclone OKT3 and ReoPro) for biopharmaceutical requires chromatographic polishing steps in the downstream processes (Shukla & Thömmes, 2010).

Liquid chromatography has also been used to analyse *Bacillus* spore coat proteins (Abhyankar et al., 2011; Krajčíková et al., 2009). Ichikawa et al. (1982) analysed the spore coat protein of *B. megaterium* strain by purifying supernatant fractions using liquid chromatography. This study performed a similar approach to an Ichikawa et al. (1982) method to purify a selected *B. licheniformis* B38 spore coat protein. The reason for doing this was that the purified spore coat protein could be ideally combined with an OB-fold and then immobilized to a chip for *B. licheniformis* spores detection using SPR. Characteristics of the selected spore coat protein are also discussed in this chapter.

4.2 Methods

4.2.1 Protein Purification by Size Exclusion Chromatography

A GE Äkta Explorer 10 (Amersham Pharmacia) fast protein liquid chromatography (FPLC) system was coupled with different columns (GE Healthcare) to perform all protein purification work unless otherwise stated. A size exclusion column (Superdex 75 10/300) was pre-equilibrated with a running buffer containing 50 mM Tris-HCl, 8 M urea, 2% SDS and 2% β-mercaptoethanol at pH 6.8. The buffer needed to be filtered through a 0.22 μm filter, sonicated and degassed before use. The collected spore suspension from PEG separation system was treated with the SDS-8 M urea sample buffer without 10% glycerol and 0.02% bromophenol blue before injecting to the system. The system was run at 0.5 mL/min flow rate.

4.2.2 Protein Purification by Desalting and Ion-Exchange Chromatography

A HiTrap Desalting Column, an anion exchanger (HiTrap Q Sepharose HP) and a cation exchanger (HiTrap SP Sepharose HP) were pre-equilibrated with two running buffers: 10 mM Tris-HCl, pH 7.0 and 10 mM Tris-HCl, pH 7.0 with 1 M NaCl. The buffers were filtered through a 0.22 μm filter, sonicated and degassed before use. The collected spore suspension from PEG separation system was treated by 8 M urea, 2% SDS and 5 mM DTT before injecting to the system. The flow rate of each run is given with the figures in **Section 4.3.2**.

4.2.3 Removing Detergent and Reducing Agents by Dialysis

A suitable length of SnakeSkin Dialysis tubing with 3.5 kDa molecular weight cutoff (MWCO) (Thermo Scientific) was measured and cut. The tubing was washed and rubbed for few times in Milli-Q ultrapure water and 10 mM of Tris-HCl buffer at pH 7.0. The fractions from the desalting column were then dialyzed with 10 mM of Tris-HCl buffer at pH 7.0. The buffer was changed after 3 - 4 hours and continued dialyzing for overnight at 4 ^{o}C. The dialysate was concentrated using Savant^{TM} SPD131DDA SpeedVac^{TM} Concentrator (Thermo Scienctific), overnight, if necessary.

The dry material was then dissolved in 3 mL of 10 mM Tris-HCl buffer at pH 7.0 and injected in the system with cation and anion exchangers.

4.2.4 Protein Purification Using Gel Elution by Diffusion

The *B. licheniformis* B38 spore coat proteins were digested with the SDS-8 M urea sample buffer as stated previously. The following steps were adapted from Burgess (2009a). The selected bands were cut and ground using a micropestle with 1 mL elution buffer (50 mM Tris, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 0.15 M NaCl and 0.1% SDS) immediately after the gel electrophoresis, staining and destaining. The ground gel matrices with the elution buffer were incubated for overnight at room temperature with agitation. After that, the gel matrices were centrifuged at 10,000 $\times g$ for 10 minutes at room temperature. The supernatant was carefully transferred into a new microcentrifuge tube.

4.2.5 Protein Precipitation

The 1 volume of the supernatant from gel elution was mixed with 9 volumes of ice cold ethanol (100%) (1:9) and incubated at least one hour at -20 ^{o}C (Evans et al., 2009). The mixture was spun down at 13,000 $\times g$ for 15 minutes at 4 ^{o}C to obtain a pellet. The supernatant was discarded and the pellet was washed with 90% of ice-cold ethanol. The suspension was spun down again using the same conditions. The supernatant was discarded and the pellet was resuspended with PBS buffer.

16.5 µL of supernatant was mixed with 2.5 µL of NuPAGE LDS Sample Buffer (4X) (Life Technologies) and 1.0 µL of NuPAGE Reducing Agent (10X) (Life Technologies) to make up a total volume of 20 µL. The mixture was then heated at 70 ^{o}C for 10 minutes and loaded into a well in a NuPAGE 4 - 12% Bis-Tris Gel (Life Technologies). The gel was run, stained, destained and recorded as stated previously.

4.2.6 Protein Concentration Assay

Pierce^{TM} BCA Protein Assay Kit (Thermo Scientific) was used to determine the protein concentration. A BSA standard curve at concentrations from 0 - 2000 µg

was generated according to the protein assay kit instructions. Briefly, BSA stock (2000 µg) was diluted accordingly to obtain a range of concentrations. A volume of 0.1 mL of each concentrations and unknown protein samples were pipetted into labelled test tubes. A volume of 2.0 mL of the working reagent (50:1, Reagent A:B) was pipetted into each tube and mixed well. A ready-made BCA Reagent A containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide and BCA Reagent B containing 4% cupric sulphate. Test tubes were covered and incubated at 37 ^{o}C for 30 minutes. Then, all test tubes were allowed to cool to room temperature before measuring the protein content using Novaspec III spectrophotometer (Amersham Biosciences) at 562 nm with a plastic cuvette.

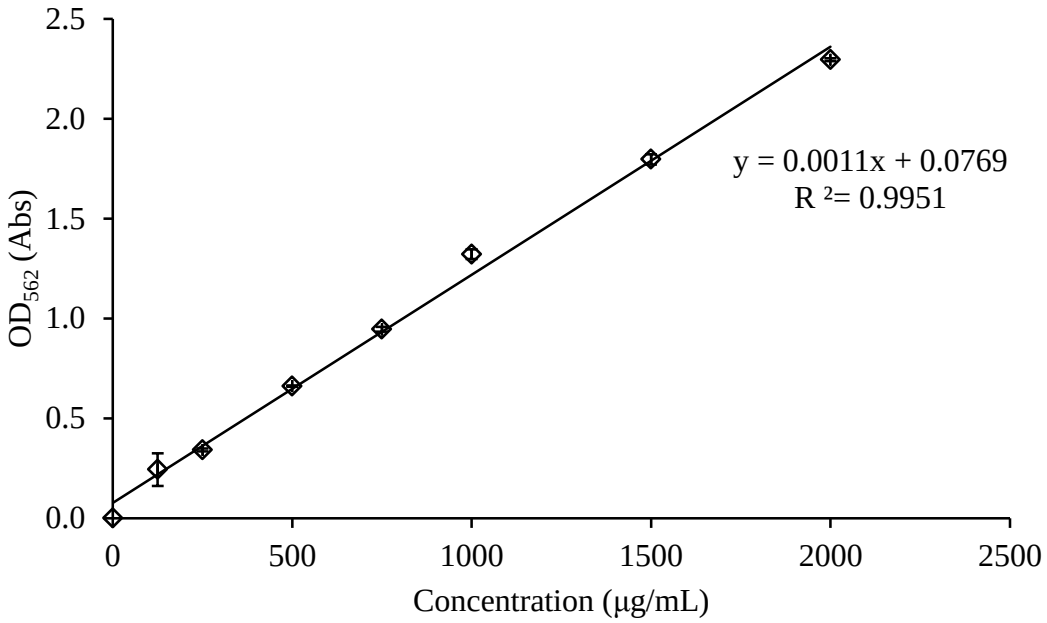


Figure 4.1 *A BSA standard curve range from 0 - 2000 µg/mL. Data are mean ± standard error, n = 3.*

4.2.7 Protein Sequencing

A selected protein band at ~10 kDa was carefully excised on a light box. The excised band was put into a microcentrifuge tube and sent to the Centre for Protein Research (CPR), Department of Biochemistry, University of Otago, New Zealand for

protein sequencing. The predicted protein sequences results were based on Sequest and Mascot search engines.

4.3 Results and Discussion

4.3.1 Purification of a Selected Spore Coat Protein by Size Exclusion Chromatography

The Superdex 75 10/300 is a size exclusion column applied for spore coat protein separation based on different sizes. The column is compatible with all aqueous buffers from pH 3 - 12, up to 8 M urea, ionic and non-ionic detergents and can be used to separate globular proteins between 3 kDa and 70 kDa (GE Healthcare, n.d.). Therefore, this column was selected to separate an ~10 kDa spore coat protein of *B. licheniformis* B38. The running buffer containing 8 M urea, 2% SDS and 2% β-mercaptoethanol are compatible with the column. The fractions were collected to coincide with the peaks shown on the chromatogram (**Figure 4.2**) and then were subjected to gel electrophoresis (**Figure 4.3**) to determine the purity of spore coat proteins.

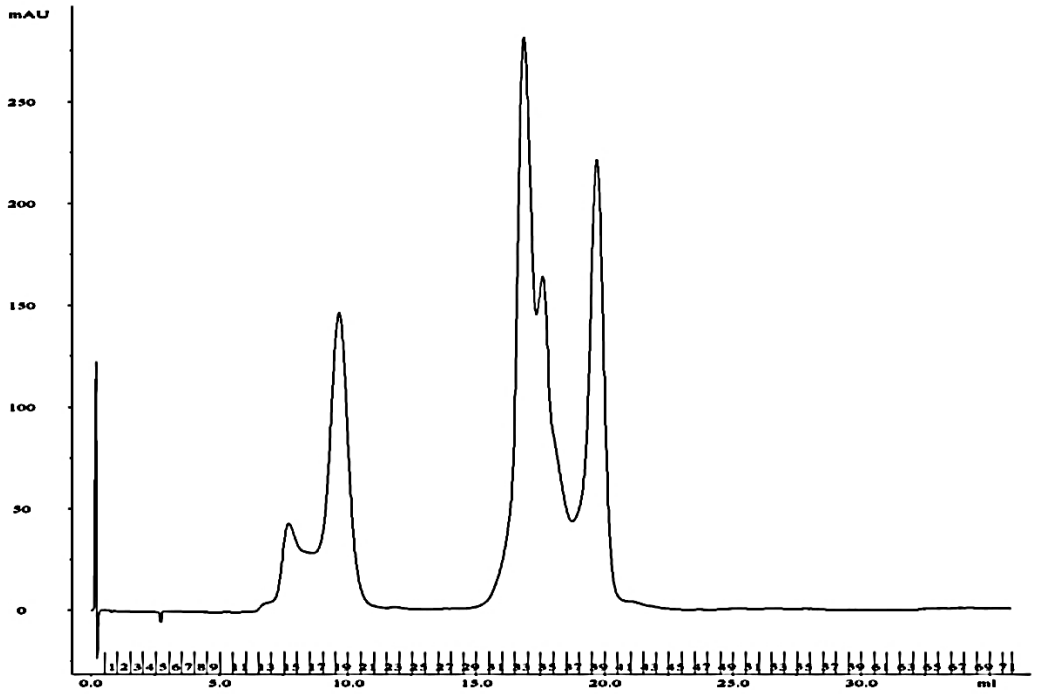


Figure 4.2 *A size exclusion chromatogram profile of B. licheniformis B38 spore coat protein.*

The processes of a size exclusion chromatography is large molecules are excluded by the small pores in the column and therefore eluted first (Hong et al., 2012). This is followed by the smaller molecules to be eluted off the column (Hong et al., 2012). 8 M urea is always the choice for spore coat protein extraction (e.g. Li et al., 2013b; Mundra et al., 2013; Venir et al., 2014) as it is important in solubilizing the spore coat proteins (Pandey and Aronson, 1979). Furthermore, a high concentration of urea can unfold and maintain protein solubility (Tsumoto et al., 2003) and to prevent protein aggregation (Yamaguchi & Miyazaki, 2014). Protein aggregation formation was not observed because the column was pre-equilibrated with the running buffer containing high concentration of urea, SDS and β-mercaptoethanol to maintain the protein solubility. The peaks shown in **Figure 4.2** do not represent a well-separated single band protein (**Figure 4.3:** Fractions 14 - 21). This may mean that the spore coat proteins cannot be separated well under the stated conditions. On the other hand, a protein band was not observed from Fractions 31 - 41 (data not shown) although the peaks showed in the chromatogram. The possibility is the sample buffer is a part of the eluent which gives some absorbance readings at 280 nm for the Fractions 31 - 41. The compatibility of these substances at 280 nm for SDS, β-mercaptoethanol and urea were 0.1%, 10 mM and > 1.0 M (Noble & Bailey, 2009). More than these values may interfere with the absorbance readings at 280 nm in the FPLC system. Thus, ion exchange column (IEC) was tried to separate the spore coat proteins.

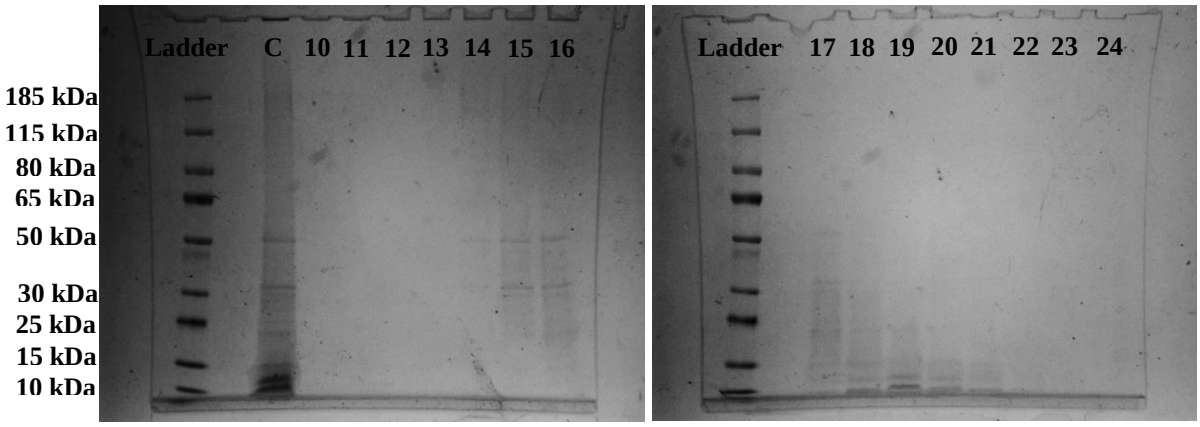


Figure 4.3 *Lanes 10 - 24 are the fractions of the size exclusion chromatogram (**Figure 4.2**). C is the reference of B. lichenformis B38 spore coat protein digested with the SDS-8 M urea sample buffer without passing through the column.*

4.3.2 Purification of a Selected Spore Coat Protein by Desalting and Ion-Exchange Chromatography

As mentioned earlier, the detergent and reducing agents in the sample buffer might affect in protein purification when using size exclusion chromatography. Therefore, a desalting column was selected to remove the detergent and reducing agents (data not shown). Two runs were performed to confirm the detergent and reducing agents were completely removed from the spore coat proteins before proceeding to the anion exchange column (**Figure 4.4**). The principle of ion-exchange chromatograph is that opposite charges between the stationary phase and the analyte are attracted to each other to achieve the separation and purification (Wilson, 2010).

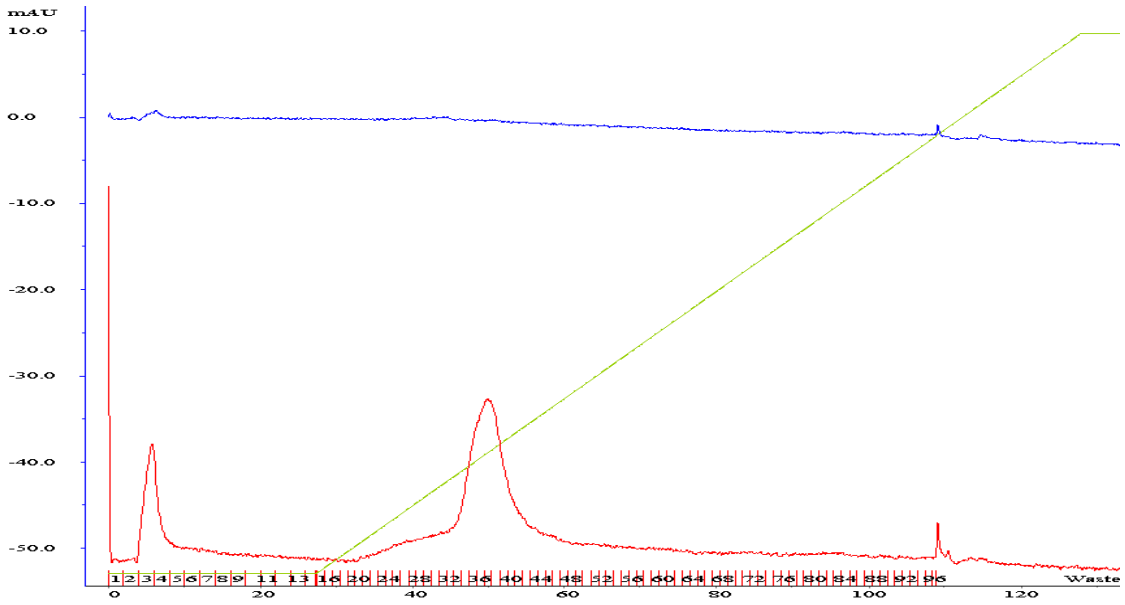


Figure 4.4 *An anion exchange chromatogram profile of B. licheniformis B38 spore coat protein after desalting. Blue line is 280 nm, red line is 215 nm and green line is NaCl concentration. The flow rate is 2.0 mL/min.*

Smears could be seen for Lanes C, D1 and D2 after the two runs of desalting (**Figure 4.5**). Some protein bands especially an ~10 kDa band could barely be seen for Lanes C and D2. In addition, the control treated with the sample buffer containing 8 M urea, 2% SDS and 5 mM DTT did not have clear bands compared to the spore coat protein digested with the SDS-8 M urea sample buffer (**Figure 4.3**). Unexpectedly,

the anion exchange chromatogram profile did not show any readings (~0 mAU) at 280 nm. A suspected possibility is the spore coat proteins are being diluted while running through the column. This was confirmed by the **Figure 4.5** where Lanes 2 - 42 did not show any protein bands in the gel.

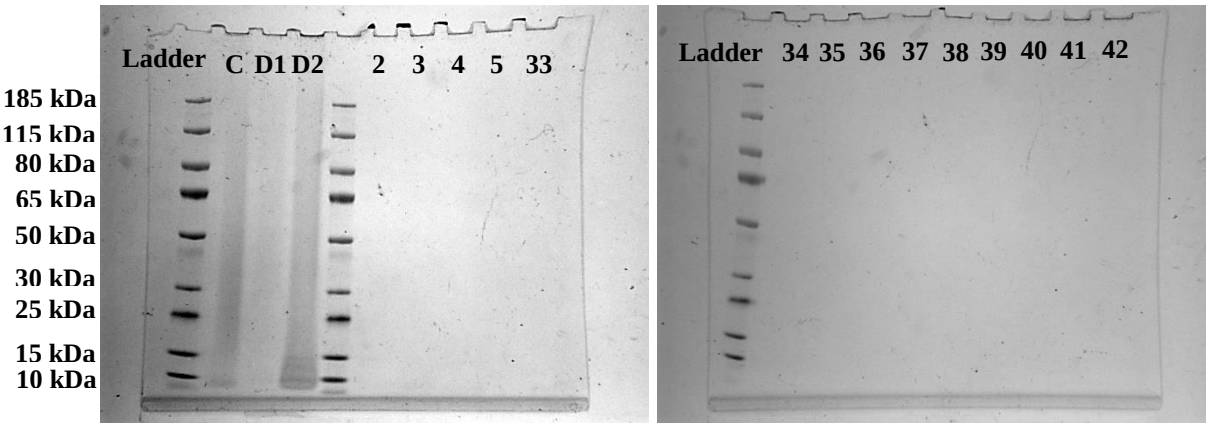


Figure 4.5 *Lanes 2 - 42 are the fractions of the anion exchange chromatogram profile (**Figure 4.4**). C is the reference of B. lichenformis B38 spore coat protein digested with the sample buffer containing 8 M urea, 2% SDS and 5 mM DTT without passing through the column. D1 and D2 are the fractions from first and second runs of desalting column.*

The previous experiment only used one spore pellet for the anion exchange chromatography. Another experiment was conducted using 10 spore pellets for the anion and cation exchange columns. It was thought that an increase in the number of spores would increase the absorbance readings at 280 nm. Hong et al. (2012) suggested that the process could be optimized by changing sample load and flow rate. Therefore, the sample load was increased and the flow rate was decreased.

The desalting result for 10 spore coat pellets showed the peaks (**Figure 4.6**) were unexpectedly odd and not normally seen in other published results (e.g. Murko et al., 2011). Therefore, only the sample buffer containing 8 M urea, 2% SDS and 5 mM DTT was run through the desalting column to compare the differences of the 10 spore coat pellet trial. However, there were no differences between both chromatograms (data not shown). The suspected reason is the compatibility values for detergent and reducing agents may influence the absorbance readings at 280 nm in the FPLC system as mentioned above (Noble & Bailey, 2009).

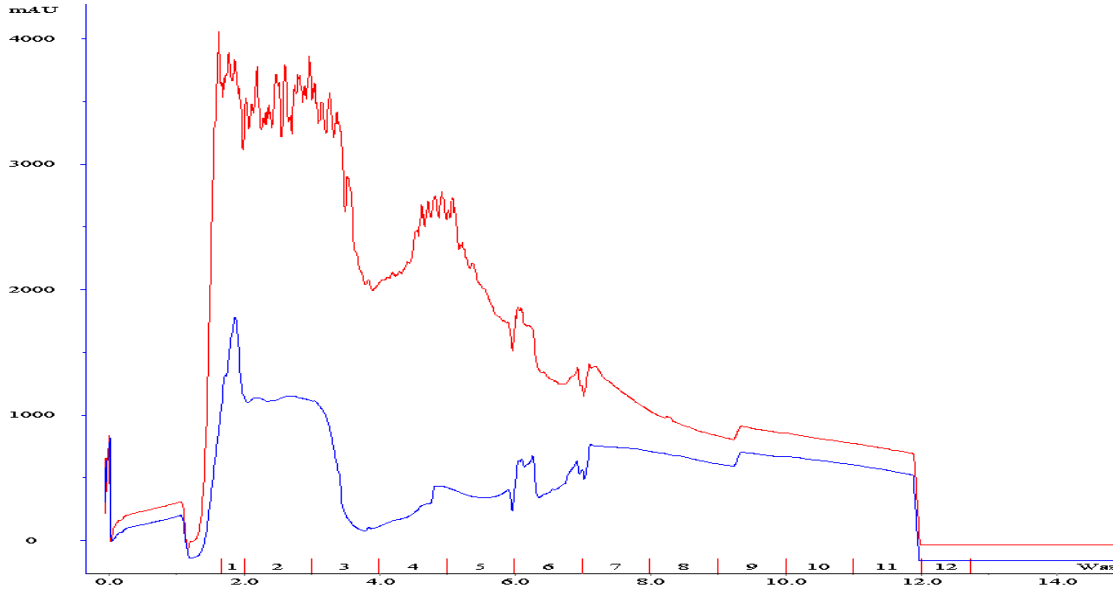


Figure 4.6 *A desalting chromatogram profile of 2.5 mL B. licheniformis B38 spore coat protein. Blue line is 280 nm and red line is 215 nm. The flow rate is 1.0 mL/min.*

Besides using a desalting column to remove detergent and reducing agents, dialysis was also tried to purify the spore coat proteins. This technique is used to separate small solutes from large protein molecules (Evans et al., 2009). Dialysis tubing with 3.5 kDa MWCO was used to retain the spore coat proteins larger than 10 kDa before purification by cation and anion exchange columns. The cation and anion fractions were collected according to the peaks at 280 nm. A sharp peak was observed with the cation exchange column before the salt concentration was increased (**Figure 4.7**). It is believed that in low salt conditions the spore coat proteins pass through the column without binding to the stationary phase. This phenomenon is called negative chromatography (Lee et al., 2014). The anion exchange column chromatogram (**Figure 4.9**) showed a similar profile and had lower mAU readings at 280 nm than the cation exchange chromatogram.

The 10 spore pellets preparation (Lane C) showed clearer bands in **Figure 4.8** than the only one spore pellet in **Figure 4.5**. A single ~10 kDa protein band could not be clearly observed by either using anion or cation exchange column (**Figure 4.8** & **Figure 4.10**). The protein concentrations of Lane 2 and Lane 3 were 146.45 µg/mL and 211.91 µg/mL in **Figure 4.8**. The absorbance readings at 280 nm of cation

exchange column were much higher than the anion exchange column. The cation exchange column may be a better choice to separate the ~10 kDa protein at a high concentration. Unfortunately, the ~10 kDa spore coat protein could not be separated by the techniques stated above.

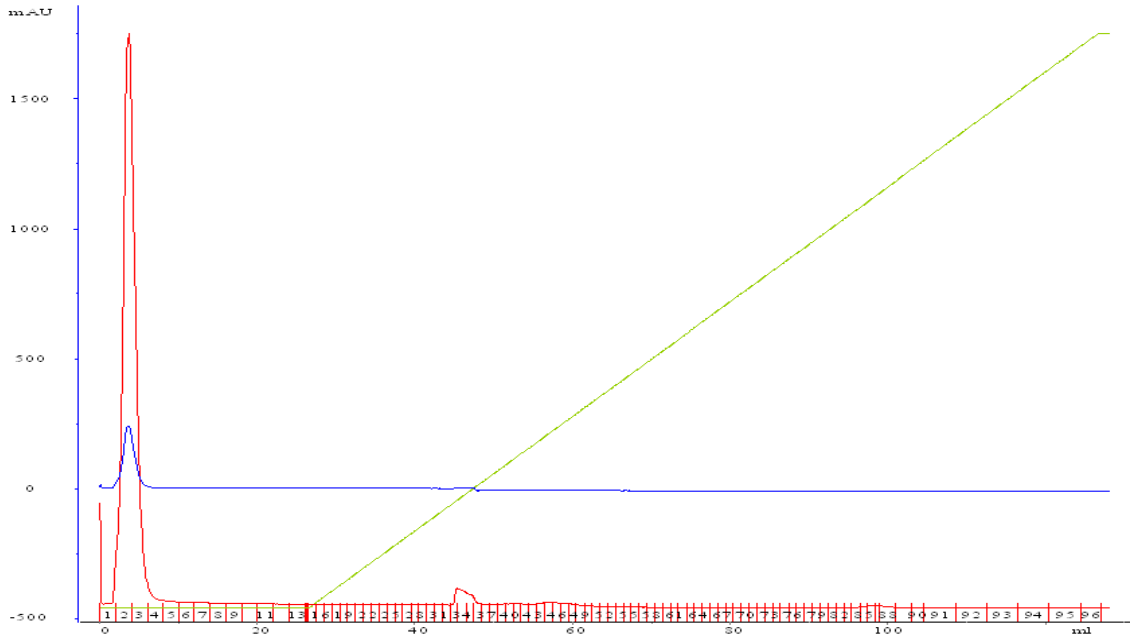


Figure 4.7 *A cation exchange chromatogram profile of B. licheniformis B38 spore coat protein after dialysis. Blue line is 280 nm, red line is 215 nm and green line is NaCl concentration. The flow rate is 2.0 mL/min.*

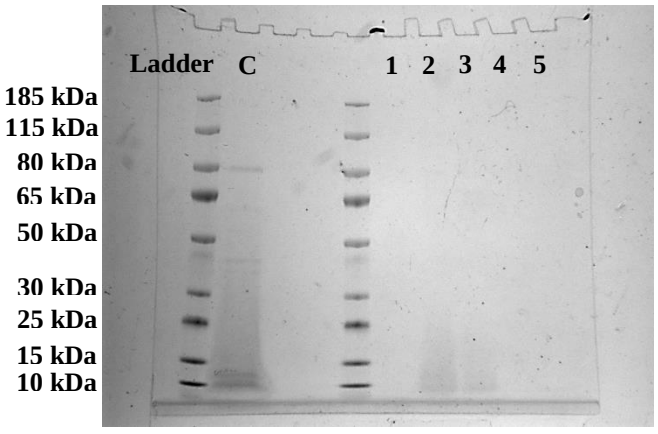


Figure 4.8 *Lanes 1 - 5 are the fractions of the cation exchange chromatogram profile (**Figure 4.7**). C is the reference of B. lichenformis B38 spore coat protein digested with the sample buffer containing 8 M urea, 2% SDS and 5 mM DTT without passing through the column*

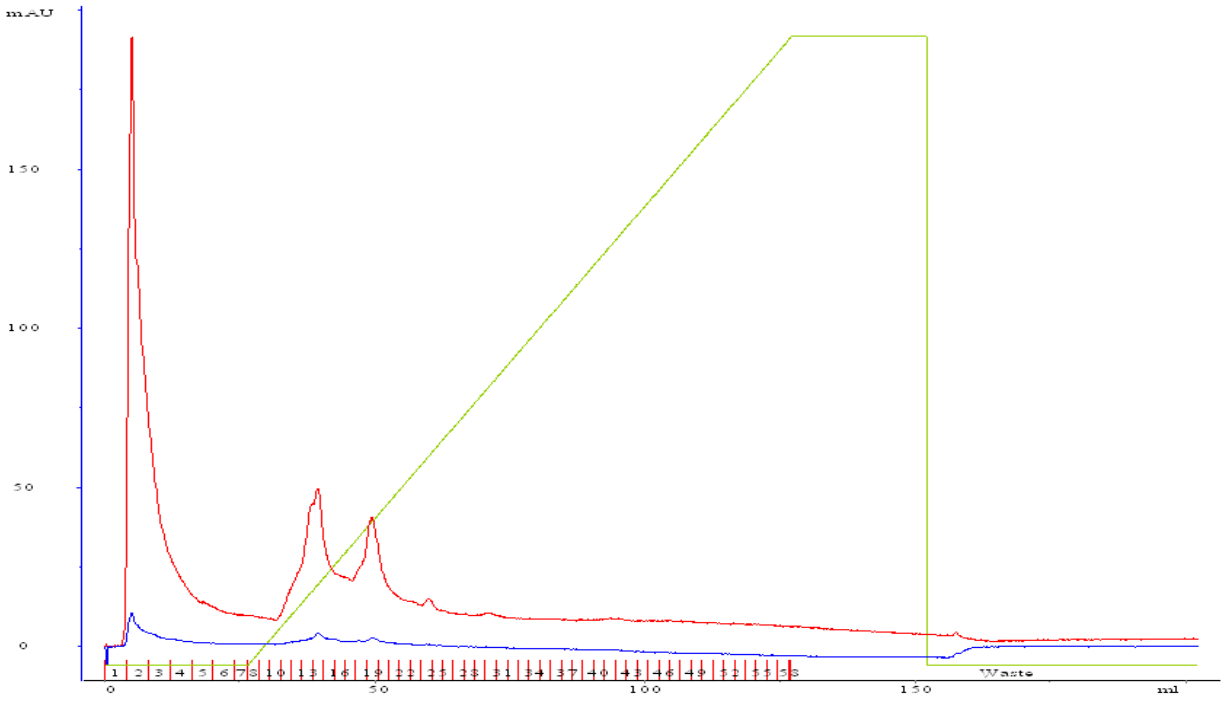


Figure 4.9 *An anion exchange chromatogram profile of B. licheniformis B38 spore coat protein after dialysis. Blue line is 280 nm, red line is 215 nm and green line is NaCl concentration. The flow rate is 2.0 mL/min.*

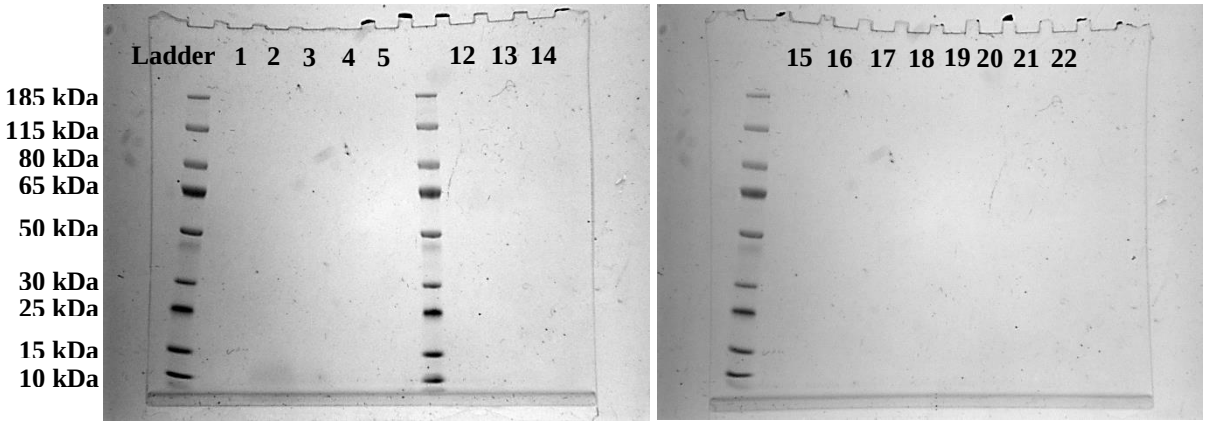


Figure 4.10 *Lanes 1 - 22 are the fractions of the anion exchange chromatogram profile (**Figure 4.9**).*

Instead of using CBB stain, SYPRO® Ruby Protein Gel Staining was also used to stain the spore coat proteins from the cation exchange column (**Figure 4.7**). This is a fluorescent staining which increases the detection range by 10 - 100 fold than colorimetric methods (Sundaram et al., 2012). A different sample buffer was also

tested to digest the spore coat protein before loading to the gel. A stock was prepared for the Sample Buffer A (10 µL of 100% β-mercaptoethanol, 25 µL of NuPAGE LDS Sample Buffer and 65 µL of purified water) and the Sample Buffer B (10 µL of NuPAGE Reducing Agent, 25 µL of NuPAGE LDS Sample Buffer and 65 µL of purified water). Unfortunately, the results did not show any differences between these sample buffers (data not shown). Also, the results for staining the protein fractions of the cation exchange column did not show any differences between the CBB Staining Solution A and the SYPRO® Ruby Protein Gel Staining.

4.3.3 Purification of a Selected Spore Coat Protein Using Gel Elution by Diffusion

Gel elution by diffusion has been reviewed (e.g. Burgess, 2009a; Kurien & Scofield, 2012; Seelert & Krause, 2008) and applied (e.g. Cohen & Chait, 1997; Petrotchenko et al., 2014) by many publications. This technique is applied before the downstream processes such as mass spectrometric detection (e.g. Cohen & Chait, 1997; Petrotchenko et al., 2014). Strong detergent such as SDS is required for the elution process (Kurien and Scofield, 2012; Seelert and Krause, 2008; Shoji et al., 1995). This is believed that SDS can solubilize the proteins fixed in the gel (Shoji et al., 1995). Therefore, SDS and DTT are suggested to add to the elution buffer (Burgess, 2009a). This passive elution process is recommended for molecules smaller than 60 kDa (Seelert and Krause, 2008). Protein bands in gels were usually fixed and precipitated by CBB Staining Solution A (Walker, 2010a), however, this will reduce the protein recovery (Lei et al., 2007). Thus, CBB Staining Solution B was selected to stain the gel prior to proceeding to gel elution and protein precipitation for the ~10 kDa spore coat protein. Lane 1 in **Figure 4.11** shows that a purified ~10 kDa spore coat protein using passive elution. The same spore coat protein (Lane 2) did not degrade after 3 days incubation with the elution buffer at room temperature. This may mean that the ~10 kDa spore coat protein is stable at room temperature for at least 3 days. By comparison with the purification methods previously attempted, gel elution by simple diffusion is a suitable method to purify the ~10 kDa spore coat protein of *B. licheniformis* B38 spore.

The protein is separated from a solution by altering the solubility of a protein, which is called protein precipitation (Evans et al., 2009). In order to avoid protein denaturation, ethanol and acetone precipitations must be done at very cold temperature (Burgess, 2009b). Centrifuge and filtration are performed to separate a protein from the solution once it is precipitated (Evans et al., 2009). Precipitation for the ~10 kDa spore coat protein was done after the gel elution (Lane 3). Protein precipitation using ethanol is to purify and remove excess detergent and reducing agents which bind to the ~10 kDa spore coat protein as well as concentrates the protein (Burgess, 2009a).

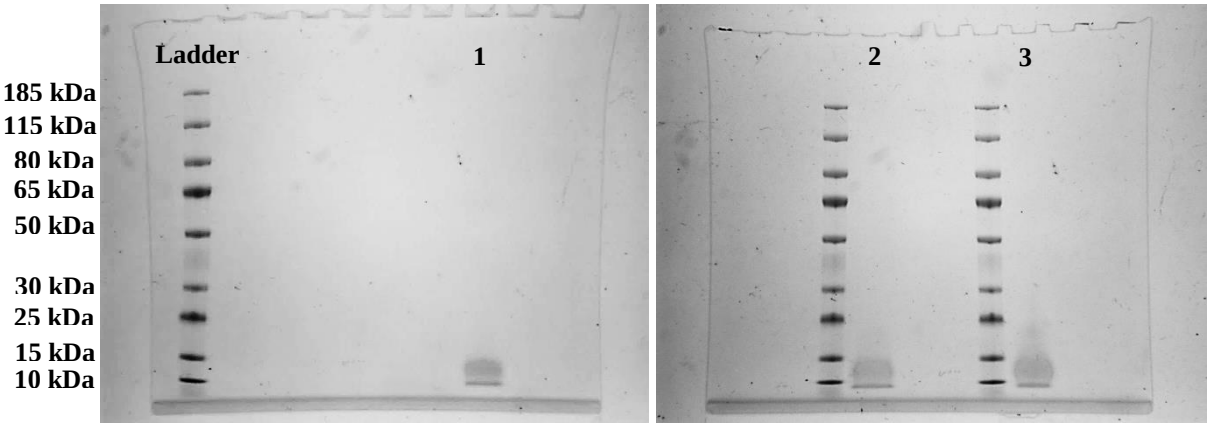


Figure 4.11 *Lane 1 shows the selected spore coat protein at ~10 kDa. Lanes 2 and 3 show the same spore coat protein after 3 days incubation in the elution buffer. Lane 3 shows the spore coat protein precipitated by ethanol.*

4.3.4 Protein Sequence Analysis of a Selected Spore Coat Protein

Kuwana et al. (2002) analysed the *B. subtilis* spore coat protein by cutting the gel into separate fragments and analysing each of the fragment by liquid chromatography-mass spectrometry (LC-MS/MS). This study was done using the method of Kuwana et al. (2002) with slight modifications. The protein sequencing for the selected spore coat protein was done by CPR, University of Otago using Orbitrap LC-MS.

The total of 10 predicted protein sequences of a selected spore coat protein band were identified (data not shown). However, only 2 protein sequences were selected based on the information given by the protein sequencing results. Both identified hypothetical protein sequences are P06552 and P45693 and its information

refers to Uniprot (http://www.uniprot.org/) (**Table 4.1**). The SASP (P06552) plays a role in spore resistance by changing the spores DNA conformation (Sella et al., 2014). This protein is highly conserved across spore-forming species and abundant in spores (Loshon et al., 1986; Pedraza-Reyes et al., 2012; Setlow, 1988). On the other hand, gene *spoVS* is induced early and controlled by σ^H during sporulation to encode Stage V sporulation protein (P45693) (Resnekov et al., 1995). The main reasons to select these two protein is because their molecular weights are ~10 kDa and they are part of the spore coat proteins from Bacillaceae family (data not shown).

Table 4.1 *The general information for two selected protein sequences of an ~10 kDa protein band.*

Accession	Description	Number of Amino Acids	Molecular Weight (kDa)	Isoelectric point (pI)
P06552	SASP 1 from *G. stearothermophilus*	70	7.2	5.02
P45693	Stage V sporulation protein S from *B. subtilis*	86	8.8	6.62

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to compare two selected protein sequences (**Figure 4.12**). These two protein sequences do not share many conserved residues but do possess many similar functions. This is supported by previous explanations stating these two proteins are in different roles although they are originated from a Bacillaceae family.

```
sp|P06552|SAS1_GEOSE      --MPNQSGSNSSNQLL--VPGAAQVIDQMKFEIASEFGVNLGAETTSRANGSVG--GEIT 54
sp|P45693|SP5S_BACSU      MEILKVSAKSSPNSVAGALAGVLRERGAAEIQAIGAGALNQAVKAVAIARGFVAPSGVDL 60
                            : : *...*.*.:   :.*. :  .  ::: .  .:* ..::.: *.* *.  *

sp|P06552|SAS1_GEOSE      KRLVSFAQQQMGG----GVQ------ 70
sp|P45693|SP5S_BACSU      ICIPAFTDIQIDGEERTAIKLIVEPR 86
                            : :*:: *:.*    .::
```

Figure 4.12 *A comparison between two protein sequences: P06552, a SASP 1 of G. stearothermophilus and P45693, a stage V sporulation protein S of B. subtilis. "*" represents both amino acid residues are fully conserved; ":" represents both residues have strongly similar properties and; "." represents both residues have weakly similar properties.*

These two protein sequences cannot be detected by UV and/or at 280 nm wavelength because they are lack of tryptophan and tyrosine residues (**Table 4.2**) (Boyer, 2000; Hofmann, 2010). This suggests that why the protein content of *B. licheniformis* B38, which lack of these two residues, was low at 280 nm detection (e.g. **Figure 4.4**). Cysteine and tryptophan cannot be found in the major SASPs but tryptophan can be found in the minor SASPs (Setlow, 1988). Based on the previous results, the protein fractions from ion exchangers were collected first before the salt gradient increased (e.g. **Figure 4.7**). This phenomenon can be explained by the principle of negative chromatography (Lee et al., 2014). This also can be explained by referring the pI values of these two protein sequences: P06552 and P45693 have isoelectric points near to 7.0. This means that the amino acid residues do not contribute net charges at isoelectric points (Walker, 2010b) and suggests that a substance with this isoelectric point will not bind to the stationary phase of either type of ion exchanger (Boyer, 2000).

Table 4.2 *The amino acid compositions of two selected protein sequences.*

P06552			P45693		
Amino Acid	Number of Amino Acid	Percentage of Amino Acid (%)	Amino Acid	Number of Amino Acid	Percentage of Amino Acid (%)
Ala (A)	6	8.6	Ala (A)	16	18.6
Arg (R)	2	2.9	Arg (R)	5	5.8
Asn (N)	5	7.1	Asn (N)	2	2.3
Asp (D)	1	1.4	Asp (D)	3	3.5
Cys (C)	0	0.0	Cys (C)	1	1.2
Gln (Q)	8	11.4	Gln (Q)	3	3.5
Glu (E)	4	5.7	Glu (E)	6	7.0
Gly (G)	10	14.3	Gly (G)	8	9.3
His (H)	0	0.0	His (H)	0	0.0
Ile (I)	3	4.3	Ile (I)	10	11.6
Leu (L)	4	5.7	Leu (L)	6	7.0
Lys (K)	2	2.9	Lys (K)	4	4.7
Met (M)	3	4.3	Met (M)	1	1.2
Phe (F)	3	4.3	Phe (F)	2	2.3
Pro (P)	2	2.9	Pro (P)	4	4.7
Ser (S)	8	11.4	Ser (S)	5	5.8
Thr (T)	3	4.3	Thr (T)	2	2.3
Trp (W)	0	0.0	Trp (W)	0	0.0
Tyr (Y)	0	0.0	Tyr (Y)	0	0.0
Val (V)	6	8.6	Val (V)	8	9.3

The *G. stearothermophilus* (P06552) and *B. subtilis* (P45693) protein sequences identified from the ~10 kDa spore coat protein of *B. licheniformis* B38

were selected to compare to the complete genome of *B. licheniformis* ATCC 14580 (http://www.genome.jp/kegg-bin/show_organism?org=T00197). Firstly, the three selected protein sequences (e.g. BL00430, BL02834 and BL03629) of the *B. licheniformis* ATCC 14580 were compared to P06552 (**Figure 4.13**). These 4 protein sequences share many conserved amino acid residues indicating that P06552 of *G. stearothermophilus* is closely related to the other 3 protein sequences of *B. licheniformis* ATCC 14580. The higher the score, the closer between two protein sequences. The highest score was achieved by the BL00430 protein sequence was 85.71 (data not shown). It shows that this is the closest protein sequence to P06552.

```
sp|P06552|SAS1_GEOSE      MPNQSGSNSSNQLLVPGAAQVIDQMKFEIASEFGVNLGAETTSRANGSVGGEITKRLVSF
bli_BL00430               MAQNNRQSSSNQLLVPGAAQAIDQMKFEIASEFGVNLGAETTSRANGSVGGEITKRLVSF
bli_BL02834               ---MANQNSSNQLLVPGAAQAIEQMKFEIASEFGVNLGADTTSRANGSVGGEITKRLVSQ
bli_BL03629               ------MARTNKLLVPGAEQVLDQFKYEIAQEFGVQLGSDSVARSNGSVGGEMTKRLVQQ
                                     :*:****** *.::*:*:***.****:**::::.:*:*******:*****.

sp|P06552|SAS1_GEOSE      AQQQMGGGVQ-
bli_BL00430               AQQQMGGTQQ-
bli_BL02834               AQASMGGQQF-
bli_BL03629               AQAQLNGHNDK
                          ** .:.*
```

Figure 4.13 *A comparison between four protein sequences: P06552, a SASP 1 of G. stearothermophilus; BL00430, a major β-type SASP B; BL02834, a major β-type SASP B and BL03629, a minor α/β-type SASP D. The last three protein sequences are from B. licheniformis ATCC 14580.*

Secondly, five protein sequences of *B. licheniformis* ATCC 14580 were closely related to P45693 of *B. subtilis* (data not shown). The highest score was 98.84 by the BL03654 protein sequence. BL03654 shared many conserved amino acid residues with P45693 (**Figure 4.14**). This shows that BL03654 is the closest protein sequence to the P45693. The scores for the rest of the protein sequences were less than 50.

```
sp|P45693|SP5S_BACSU      MEILKVSAKSSPNSVAGALAGVLRERGAAEIQAIGAGALNQAVKAVAIARGFVAPSGVDL
bli_BL03654               MEILKVSAKSNPNSVAGALAGVLRERGAAEIQAIGAGALNQAVKAVAIARGFVAPSGVDL
                          **********.**************************************************

sp|P45693|SP5S_BACSU      ICIPAFTDIQIDGEERTAIKLIVEPR
bli_BL03654               ICIPAFTDIQIDGEERTAIKLIVEPR
                          *************************
```

Figure 4.14 *A comparison between two sporulation protein sequences: P45693, a stage V protein S of B. subtilis and BL03654, a stage V protein S of B. licheniformis ATCC 14580.*

CHAPTER 5

DETECTION OF *BACILLUS LICHENIFORMIS* B38 SPORE COAT PROTEINS BY SURFACE PLASMON RESONANCE

5.1 Introduction

The SPR biosensor techniques have been gradually developed over the past 30 years (Abadian et al., 2014). The signal measurements of a SPR biosensor are based on the changes of refractive index when binding occurs on a gold surface (Ahmed et al., 2014). SPR biosensors are applied in many ways including detection of foodborne pathogens (Dostálek et al., 2006). This preliminary study used the SPR biosensor ProteOn XPR36 from Bio-Rad to demonstrate the binding of *B. licheniformis* B38 spores to a *Bacillus* spores polyclonal antibody. This multi-channel system is capable of performing up to a 36 interaction between six ligands and six analytes in a single injection stage (Abdiche et al., 2011; Schasfoort & McWhirter, 2008). The advantages can be made used of studying a broad range of biomolecular interactions (Schasfoort & McWhirter, 2008). For instance, a ProteOn XPR36 system was used with therapeutic antibodies (Bronner et al., 2010). Literature on ProteOn XPR36 has also been extensively discussed by Rich and Myszka (2010).

5.2 Methods

5.2.1 Spore Coat Protein Extraction

A pure spore suspension was collected as indicated previously. The subsequent extraction steps were adapted from Ragkousi and Setlow (2004) with slight modifications. Briefly, the pure spore suspension was decoated by incubating in 1 mL of decoating buffer (0.1 M NaCl, 0.1 M NaOH, 1% SDS and 0.1 M DTT) for 30 minutes at 70 ^{o}C. The coat extract was then centrifuged at 10,000 $\times g$ for 5 minutes at 4 ^{o}C to obtain supernatant. Ethanol precipitation was done by referring to **Section 4.2.5**.

5.2.2 Dot Blot Assay

A grid was drawn on a Sequi-Blot™ polyvinylidene fluoride (PVDF) membrane (Bio-Rad) before pre-wetting in 100% methanol for a few seconds until it became translucent. All the following steps were done by shaking on a rocker platform at room temperature. The membrane was equilibrated with TBS-T (2.4 g/L Tris base, 8.8 g/L NaCl, 0.05% Tween-20 at pH 7.6 adjusted by a concentrated HCl) for 3 minutes. 2 μL of protein samples were spotted onto the membrane. The dots were allowed to air dry to avoid forming white spots. The membrane was soaked in 5% non-fat trim milk in TBS-T for 1 hour to block non-specific sites. The membrane was later incubated with 10 μg/mL rabbit anti *Bacillus* spores (GenWay: GWB-E8782F) as a primary antibody in 0.1% non-fat trim milk in TBS-T for 30 minutes. The membrane was then washed 3 times for 5 minutes each with TBS-T. The membrane was incubated with 1:5000 goat anti-rabbit IgG-HRP (Santa Cruz Biotechology: sc-2054) as a secondary antibody in 0.1% non-fat trim milk in TBS-T for 30 minutes. After that, the membrane was washed with TBS-T for 15 minutes once and 5 minutes twice followed by TBS for 5 minutes once. Finally, the membrane was incubated with Enhanced Chemiluminescence (ECL) Prime Western Blotting Reagent (GE Healthcare) for 5 minutes and ImageQuant LAS 500 (GE Healthcare) was used to visualize the protein signals on the membrane.

5.2.3 Spore Coat Protein Detection using Surface Plasmon Resonance

5.2.3.1 Chip Initialization

A ProteOn XPR36 protein interaction array system (Bio-Rad) and a GLM chip (Bio-Rad) were used throughout the SPR studies. All the solutions for the studies were filtered with 0.22 μm filters, sonicated and degassed before use. The system was set at 25 ^{o}C during the studies. The chip was inserted into the system and normalized with 50% glycerol. The chip was then pre-conditioned accordingly with 0.25% Tween-20, 10 mM NaOH and 100 mM HCl at a horizontal orientation (the analyte flow direction) and a vertical orientation (the ligand flow direction) at 30 μL/min for 60 seconds. The system was replenished with fresh PBS-T (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4 and 0.005% Tween-20) running buffer. Then the chip

was washed with PBS-T for 3 times horizontally and vertically at 100 μL/min for 60 seconds.

5.2.3.2 Ligand Immobilization via Amine Coupling

Activation, ligand immobilization and deactivation were done at 30 μL/min for 300 seconds. The chip was activated first before immobilizing the ligands to the surface. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Bio-Rad) and N-hydroxysulfosuccinimide (NHS) (Bio-Rad) were mixed freshly in a 1:1 ratio to activate carboxyl groups on the chip. The ligands 0.1 mg/mL rabbit anti *Bacillus* spores (GenWay: GWB-E8782F), an estimated 0.2 mg/mL B38 spores and an estimated 0.2 mg/mL spore coat protein at pH 2 were prepared in 10 mM acetate buffer at pH 4 (Bio-Rad). Later the chip was deactivated by 1 M ethanolamine HCl at pH 8.5 (Bio-Rad).

5.2.3.3 Ligand-Analyte Interactions and Regeneration

The chip was again washed with PBS-T 3 times at the analyte flow direction at 100 μL/min for 60 seconds until the baseline became stable. Analytes with different concentrations were prepared by diluting with PBS-T. The flow rate and contact time were adjusted based on the quantity of samples. The chip was regenerated by 10 mM glycine-HCl at pH 2 at 100 μL/min for 18 seconds.

5.3 Results and Discussion

5.3.1 Ligand Immobilization

According to the manufacturer, the GLM chip is designed with a medium density of alginate polymer layer to create a medium binding capacity (~12 kRU) ligand surfaces through amine coupling (Bio-Rad, n.d.). This sensor chip is suitable for protein-protein interactions and protein-small molecule interactions as recommended (Bio-Rad, n.d.). Amine coupling is a common approach for immobilizing ligands to a solid surface through reactive esters (Fischer, 2010; Gedig, 2008). The chip has to be preconditioned to eradicate contaminants and to rewet the

polymer layer, so that, a stable baseline can be obtained and ready for amine coupling (Fischer, 2010). Amine coupling involves in two steps (**Figure 5.1**). Firstly, water-soluble EDC compounds react with carboxylic groups on the surface of the chip to form reactive *O*-acylisourea intermediates that reactive to the nucleophiles (Gedig, 2008). These intermediates will immediately transform back to carboxylic acids in aqueous environment if it is not reacted with other nucleophiles (Löfås & McWhirter, 2006). Therefore, secondly, they must react with water-soluble NHS compounds to form stable NHS ester intermediates that have a long half-life (Gedig, 2008). An acidic coupling buffer is favoured for immobilizing the ligands *Bacillus* spores antibody and *B. licheniformis* B38 spore coat protein at pH 2 to the NHS-activated carboxylated surface (Löfås & McWhirter, 2006). A large fraction of the proteins (ligands) now have positive charges and are electrostatically attracted to the negatively charged surface (Löfås & McWhirter, 2006). One of the advantages for N-terminal amines protein coupling in acidic conditions is to inhibit multisite binding and maintain the proteins activity (Gedig, 2008). The last stage is to deactivate the remaining active carboxyl groups to avoid nonspecific binding of analyte to the surface and also remove non-covalent bound ligand using ethanolamine (Biacore, n.d.; Bio-Rad, n.d.).

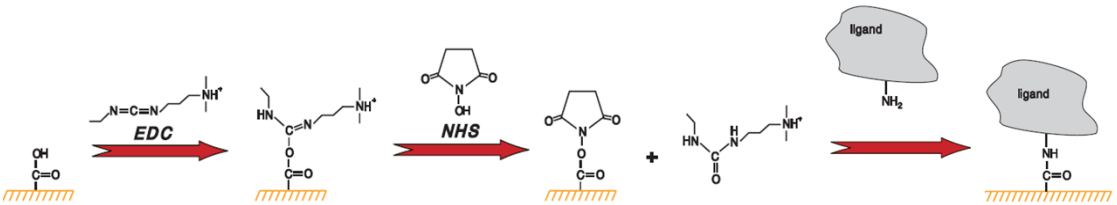


Figure 5.1 *An amine coupling process of a ligand to a sensor surface (Biacore, n.d.).*

The sensorgrams below illustrate that the *Bacillus* spores antibody, B38 spores and spore coat protein at pH 2 are immobilized to the GLM chip via amine coupling (**Figures 5.2 - 5.4**). The working concentrations of these ligands were prepared between 0.01 and 0.2 mg/mL in acetate acid buffer adapted from Fischer (2010). Some problems such as aggregation, overcrowding, cross-linking and mass transport could develope if high concentrations of ligands were immobilized to the chip surface (Fischer, 2010). Another reason is random immobilization could happen due to more amine groups found in ligands, and thus, lead to surface heterogeneity (Fischer, 2010). Mass transport limitations can be explained using the association

phase model which the diffusion rate of the analyte from the analyte bulk flow is slower than the rate of ligand to be bound to the surface; and/or dissociation phase model which the retention rate of analyte close to the surface (including rebinding to empty sites) is higher than the diffusion rate back to the analyte bulk flow (Bio-Rad, n.d.; Schuck & Zhao, 2010). Hence, an increase analyte flow rate or a decrease ligand concentration is recommended (Bio-Rad, n.d.).

The issue of surface heterogeneity has also been discussed by Schuck and Zhao (2010). The orientation of ligand on the surface affects the binding of analyte to a part of active sites, nonspecific sites and multiple independent sites on ligands (Schuck and Zhao, 2010). Some suggestions are to try a different capture technique and/or bind with protecting agents or cofactors (Bio-Rad, n.d.). However, no further binding kinetic was examined. In a broader sense, mass transport limitation and surface heterogeneity may have occurred in this study and this should be taken into account in the future optimization experiments.

SPR signals are expressed in response units (RU) in real time on the sensorgrams. The signals obtained from the chip activation by EDC-NHS reaction for the 3 sensorgrams were ~7100 RU, which meant the preparation for the chip activation was identical for all these 3 ligands (**Figures 5.2 - 5.4**). Nevertheless, the RU responses of these 3 ligands for later stages were different because of the protein conformations and different extraction methods. As mentioned earlier, the concentration of 0.1 mg/mL *Bacillus* spores antibody was selected because it corresponded to a reasonable range of ligand concentrations. The amount of bound *Bacillus* spores antibody on the chip surface was 18450.14 RU at the immobilization stage and was reduced to 16541.75 RU at the deactivation stage (**Figure 5.2**). Some of the loosely bound *Bacillus* spores antibodies were washed away by ethanolamine. The curves of activation, ligand immobilization and deactivation of this sensorgram are similar to other publications (e.g. Alfonso et al., 2014; Chou et al., 2006).

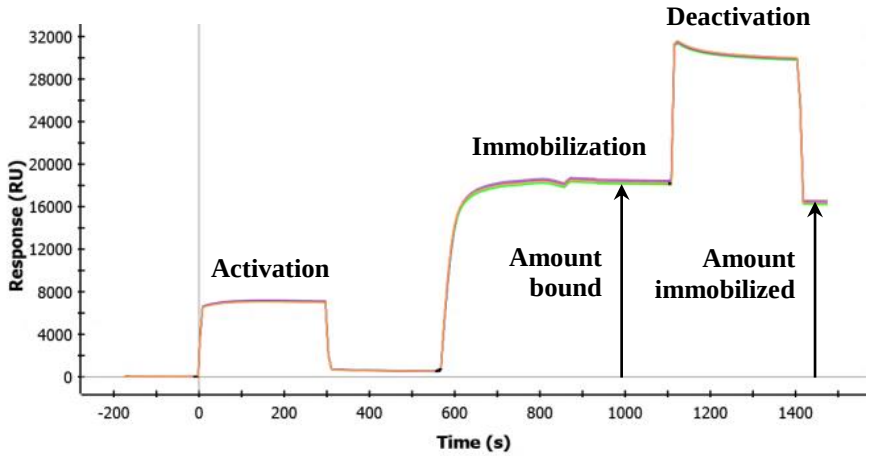


Figure 5.2 *The activation, immobilization and deactivation of 0.1 mg/mL Bacillus spores antibody.*

On the other hand, the immobilization of *B. licheniformis* B38 spores to the chip was not a good practice as advised by the manufacturer (Bio-Rad, n.d.) (**Figure 5.3**). In contrast to *Bacillus* spores antibody, *B. licheniformis* B38 spores had a slightly different immobilization curve. As stated above, SPR signals were generated by a change in the refractive index caused by an angle change when the materials bound to the surface (de Mol & Fischer, 2010). The size of biomolecule affects in ligand immobilization and ligand-analyte interactions. The molecular weights of *Bacillus* spores antibody are ~50 kDa (heavy chain) and ~25 kDa (light chain) (data not shown) and the *B. licheniformis* spores are oval in shape and size approximately 1 - 1.8 μm by 0.6 - 0.9 μm (Bradley & Franklin, 1958) and have variety of protein bands as shown in **Figure 5.5**.

The *B. licheniformis* B38 spore size is bigger than the *Bacillus* spores antibody and the immobilization of the spores to the chip surface is random and probably exceeds the evanescent wave field range. A change in the SPR signal will not be observed if the changes of refractive index do not occur within the evanescent wave field range from the surface (Skottrup et al., 2008). Only a small portion of spore surfaces are interacting with the activated carboxyl groups which may generate a small or even a very low RU signal (Abdulhalim et al., 2008; Ahmed et al., 2014; Shilpakala et al., 2012; Skottrup et al., 2008; Torun et al., 2012). The responses for the binding of *B. licheniformis* B38 spores to the chip were 417.17 RU at the end of

immobilization and 212.72 RU at the end of deactivation. The amount of bound B38 spores to the chip was very low for the subsequent interactions.

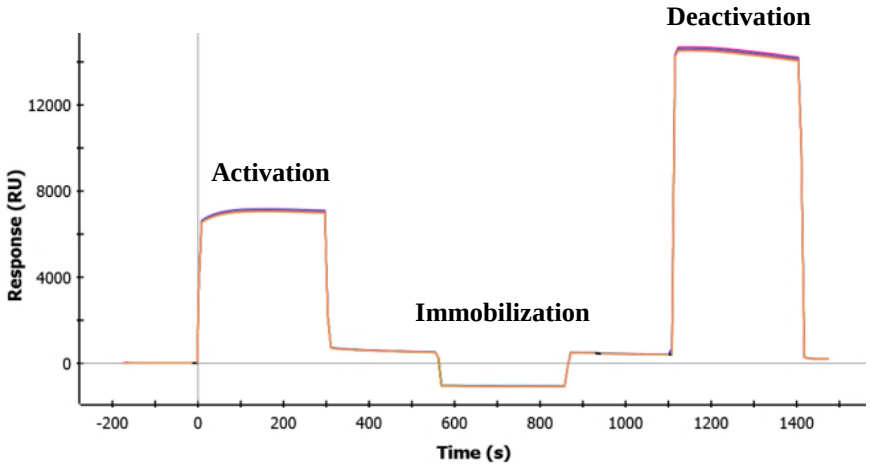


Figure 5.3 *The activation, immobilization and deactivation of an estimated 0.2 mg/mL of B. licheniformis B38 spores.*

Immobilization of spore coat protein at pH 2 to the chip generated a good signal although there was a slightly increased in signal at 850 seconds (**Figure 5.4**). The signal for bound spore coat protein at pH 2 at the end of immobilization was 4086.14 RU and remained stable on the chip after the deactivation (4094.66 RU). The response of immobilized spore coat protein at pH 2 was lesser 4 times than the immobilization of *Bacillus* spores antibody to the chip.

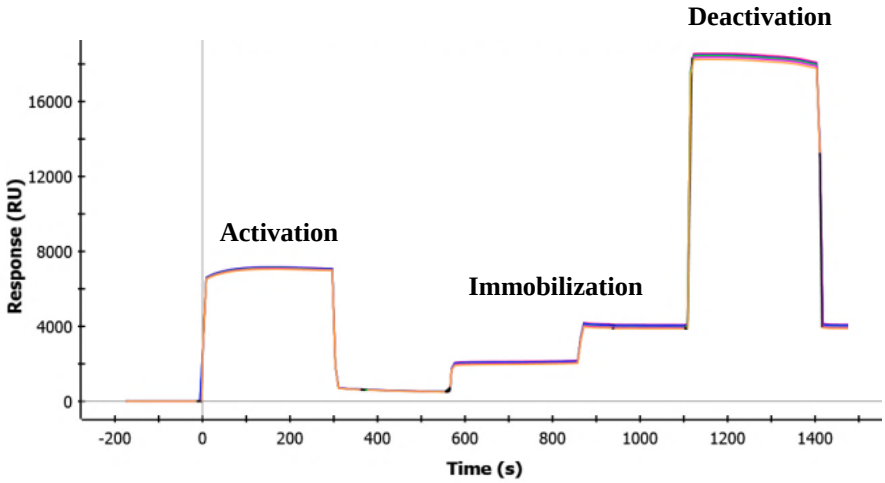


Figure 5.4 *The activation, immobilization and deactivation of an estimated 0.2 mg/mL of B. licheniformis B38 spore coat protein at pH 2.*

The reason for choosing the spore coat protein at pH 2 is because the protein is more soluble in glycine-HCl buffer at pH 2 than in PBS buffer at pH 7.4. A protein has a minimum solubility at its isoelectric point (Walker, 2010b). The spore coat protein did not dissolve completely in PBS buffer indicating that the sequenced spore coat proteins had isoelectric points near to 7 (**Table 4.1**). An amino acid carries positive charges at low pH (Walker, 2010b) which results in more spore coat protein dissolved in the buffer at pH 2. The positive charge facilitates immobilization to the chip as the positively charged proteins covalently bind to the negatively charged chip surface via amine coupling.

In order to substantiate the above statements, qualitative assays were done to estimate and the amount of spore coat proteins dissolved at pH 2 and pH 7.4. The estimated protein concentrations for spore coat proteins at pH 2 and pH 7 were 2.02 mg/mL and 0.22 mg/mL respectively at 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific). This was also confirmed by SDS-PAGE where the bands of the spore coat proteins at pH 2 were darker than at pH 7.4 (**Figure 5.5**). Therefore, the spore coat protein at pH 2 was chosen as the ligand for the immobilization.

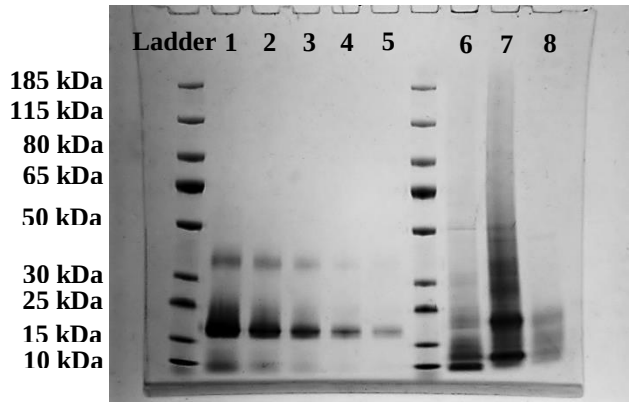


Figure 5.5 *The total volume of each well is 20 µL. Lanes 1 - 5 are β-lactoglobulin as a reference standard, with concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL, respectively. Lane 6 is the B. licheniformis B38 spores, lanes 7 and 8 are the B38 spore coat proteins extracted at pH 2 and pH 7.4, respectively.*

5.3.2 Immunoblotting Assay for Antibody-Protein Interactions

A Dot Blot was used as a qualitative test for examining the binding of *B. licheniformis* B38 spore coat proteins against the primary antibody before performing the ligand-analyte interactions by SPR. The primary antibody *Bacillus* spores antibody is a polyclonal antibody which has advantages for immunoassay. For instance, this type of antibody can recognize several antigen epitopes and bind to the denaturation-resistance epitopes (Dunbar & Schwoebel, 1990; Harlow & Lane, 1988). The test showed that signals were developed by the *B. licheniformis* B38 spore coat proteins against the antibody via ECL (**Figure 5.6**). This verifies that the *Bacillus* spores antibody is able to detect the *B. licheniformis* B38 whole spores and, spore coat proteins at pH 2 and pH 7.4 by dot blot immunoassay. This idea was supported by testing the immunoreactivity of *Bacillus* species by dot blot before the detection assays (Peckham et al., 2013). Although western blot was more common for immunoblotting assay, Putra et al. (2014) mentioned that dot blot could be regularly used to analyse large sample sizes and dealt with different range of protein molecular weights. Dot blot is even used as a rapid detection method to identify foodborne pathogens (Fontanot et al., 2014).

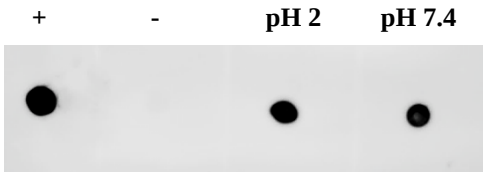


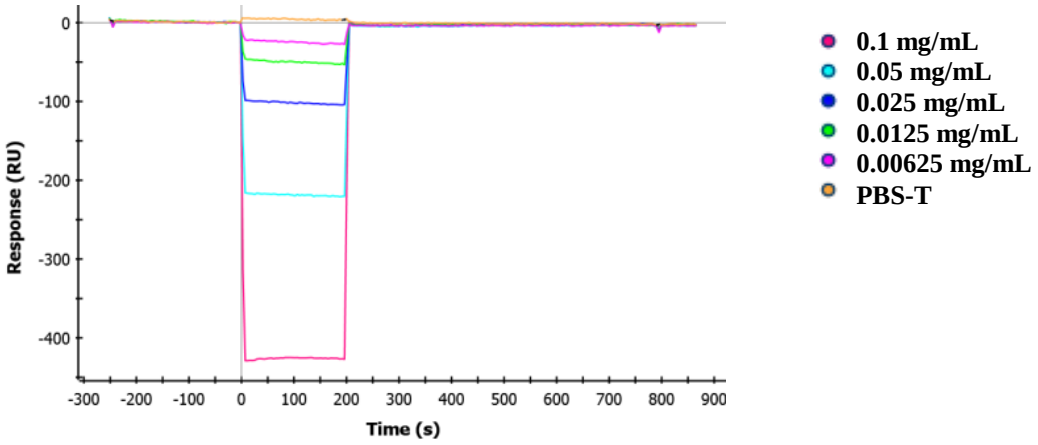
Figure 5.6 *An immunoblotting of B. licheniformis B38 spore coat protein. + is a positive control of B. licheniformis B38 spores, - is a negative control of β-lactoglobulin, pH 2 and pH 7.4 are the B38 spore coat proteins extracted at pH 2 and pH 7.4, respectively.*

5.3.3 Spore-Antibody Interactions

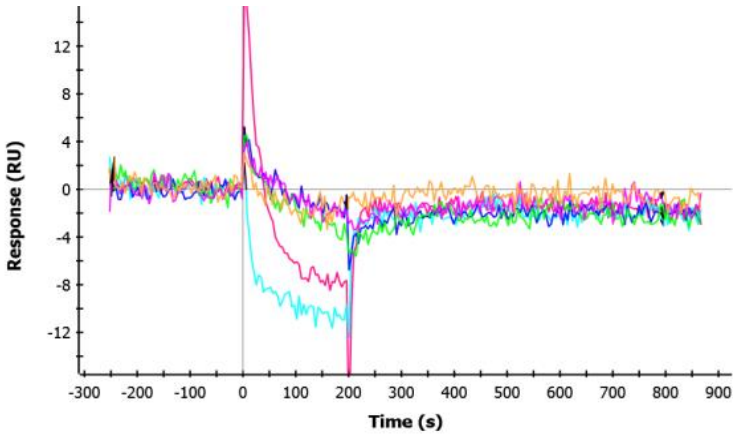
In the beginning, the *B. licheniformis* B38 spores were subjected into SPR where 0.1 mg/mL of *Bacillus* spores antibody was immobilized to the chip for the detection at 50 μL/min for 240 seconds contact time. However, no binding signal was observed for the spores at 1×10^8 CFU/mL against the antibody (data not shown). This

was supported by Perkins and Squirrell (2000) saying that the detection of *B. subtilis* spores at 1×10^7 CFU/mL by a conventional SPR (Biacore 2000) was insignificant, which only had 4.9 RU compared to a combination of light scattering in a SPR system.

The concentrations of *B. licheniformis* B38 spores were estimated by SDS-PAGE gel (data not shown). By using this measurement, five different concentrations of *B. licheniformis* B38 spores were prepared and injected into the channels to measure the detection signal (**Figure 5.7**). The result showed either no or low responses to the *Bacillus* spores antibody. After subtracting the reference channel, the highest peak was observed for an estimated 0.1 mg/mL of *B. licheniformis* B38 spores concentration (channel 1) at 4.5 seconds. The recorded response at that time was 14.31 RU and after that the signal dropped significantly below the baseline until 200 seconds. This phenomenon can be explained by the changes of microfluidic pressure in the SPR system as observed by Farka et al. (2013). Although this method was slightly modified from the previous trial, they still showed unsatisfactory results between these two methods.



(a)



(b)

Figure 5.7 *Five channels are injected with B38 spores as an analyte at estimated concentrations. Channel 6 is the reference channel injected with PBST. The flow rate is 50 µL/min and the contact time is 200 s. (a) is B38 spore-antibody interactions before subtracting the reference channel and (b) is the interactions after subtracting.*

It was interesting to compare the *Bacillus* spores antibody-*B. licheniformis* B38 whole spores interaction could be observed with dot blot assay but the interaction was not readily observable with SPR system. A summary of the differences between these two methods are shown in **Table 5.1**. These differences may explain why the spore detection signal generated by SPR is relatively low compared to dot blot assay. It is known that the limiting factors for low signal of cell detection by SPR are caused by the fluidic forces that affecting the binding stability and the large cell size does not fall into the evanescent wave field (Skottrup et al., 2008).

Table 5.1 *The differences between a SPR and a dot blot detection methods.*

Conditions	Detection Assays	
	SPR	Dot Blot
Types of Measurement	Quantitative	Qualitative
Platform	A gold chip	A PVDF membrane
Microfluidic Pressure	Yes	No
Temperature	25 ^{o}C	Room temperature (~20 ^{o}C)
Buffer	PBS-T	TBS-T
Signals	Detected by a change of refractive index within the evanescent wave field	Detected by a horseradish peroxidase (HRP) conjugated secondary antibody and chemiluminescence reaction
B. licheniformis B38 Spores	As analyte bound to the antibody	Dotted on the membrane and soaked by antibodies
Bacillus Spores Antibody	As ligand immobilized to the chip	Diluted in buffer and bound to the spores

5.3.4 Antibody-Spores and Antibody-Spore Coat Protein at pH 2 Interactions

Due to some unconvincing results as reported above, the system was reversed to test the binding of the antibody to two different ligands. This means that the *B. licheniformis* B38 spore coat protein became a ligand and *Bacillus* spores antibody used as the analyte. The antibody was injected at 0 second and the contact time ended at 200 seconds. This means that the interactions between the antibody and ligands must take place within 200 seconds. After the 200 seconds, 10 mM glycine-HCl at pH 2 was used to regenerate the chip to remove bound antibody. The signal was then back to the baseline after regeneration. Five channels were being injected with *Bacillus* spores antibody at different concentrations and one channel was untreated as a reference channel (**Figure 5.9**). The **Figures 5.9a** and **5.9b** show the interaction between *Bacillus* spores antibody and *B. licheniformis* B38 spores. After subtracting the reference channel, 0.0125 mg/mL *Bacillus* spores antibody (channel 4) showed a high response at 9 seconds and its unit was 4.54 RU. However, it dropped consistently throughout the contact time. This sharp peak is similar to **Figure 5.7b** which the possibility of pressure change affects the signals (Farka et al., 2013). In this case, low detection signal is probably caused by the spore size of *B. licheniformis* B38 as described earlier in **Section 5.3.1**. Besides, steric hindrance effects among the spores themselves would affect the signal response, too (Skottrup et al., 2008). The signal

couldn't be amplified when the antibody bound to the spores due to the spores themselves had already occupying and blocking the surface of the chip. In addition, the small amount of immobilized spores to the chip (**Figure 5.3**) reduces the probability of antibody binding. Presumably, the requirements of a 1:1 interaction model described by Langmuir adsorption model cannot be achieved by this situation (Knoll et al., 2008).

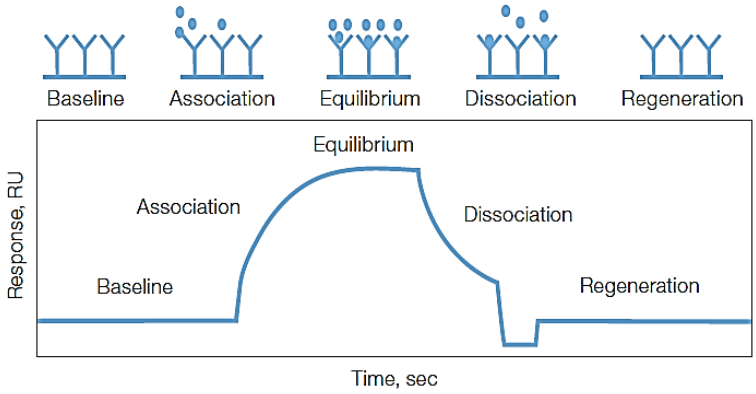


Figure 5.8 *A SPR sensorgram. "Y" is a ligand and "●" is an analyte (Bio-Rad, n.d.).*

Figures 5.9c and **5.9d** show the interaction between *Bacillus* spores antibody and spore coat protein at pH 2. Compared with **Figures 5.9b** and **5.9d**, they had a general signal response which was more similar to a typical sensorgram in **Figure 5.8**, as an example of an idealized sensorgram for comparison with the sensorgrams generated in this study. In the early stage of association, the signal was elevated to ~4 RU and slightly decreased before going up again to the equilibrium stage. The highest recorded response for 0.1 mg/mL *Bacillus* spores antibody over this stage was 5.74 RU (channel 1). Later, the running buffer was injected to the system to remove specific and nonspecific bound antibody from the ligand at dissociation stage. Finally, 10 mM glycine-HCl at pH 2 was injected to remove the remaining bound antibody at the regeneration stage where a strong pH shift was observed (Schasfoort et al., 2008). The result for binding of *Bacillus* spores antibody to spore coat protein at pH 2 is more encouraging than the antibody binding to the spores, although the signal remains low. Farka et al. (2013) reported that the response signal for the detection of *B. atrophaeus* spores by polyclonal antibodies was 10 RU. The spore size is still a limitation and affected the SPR signal (Farka et al., 2013).

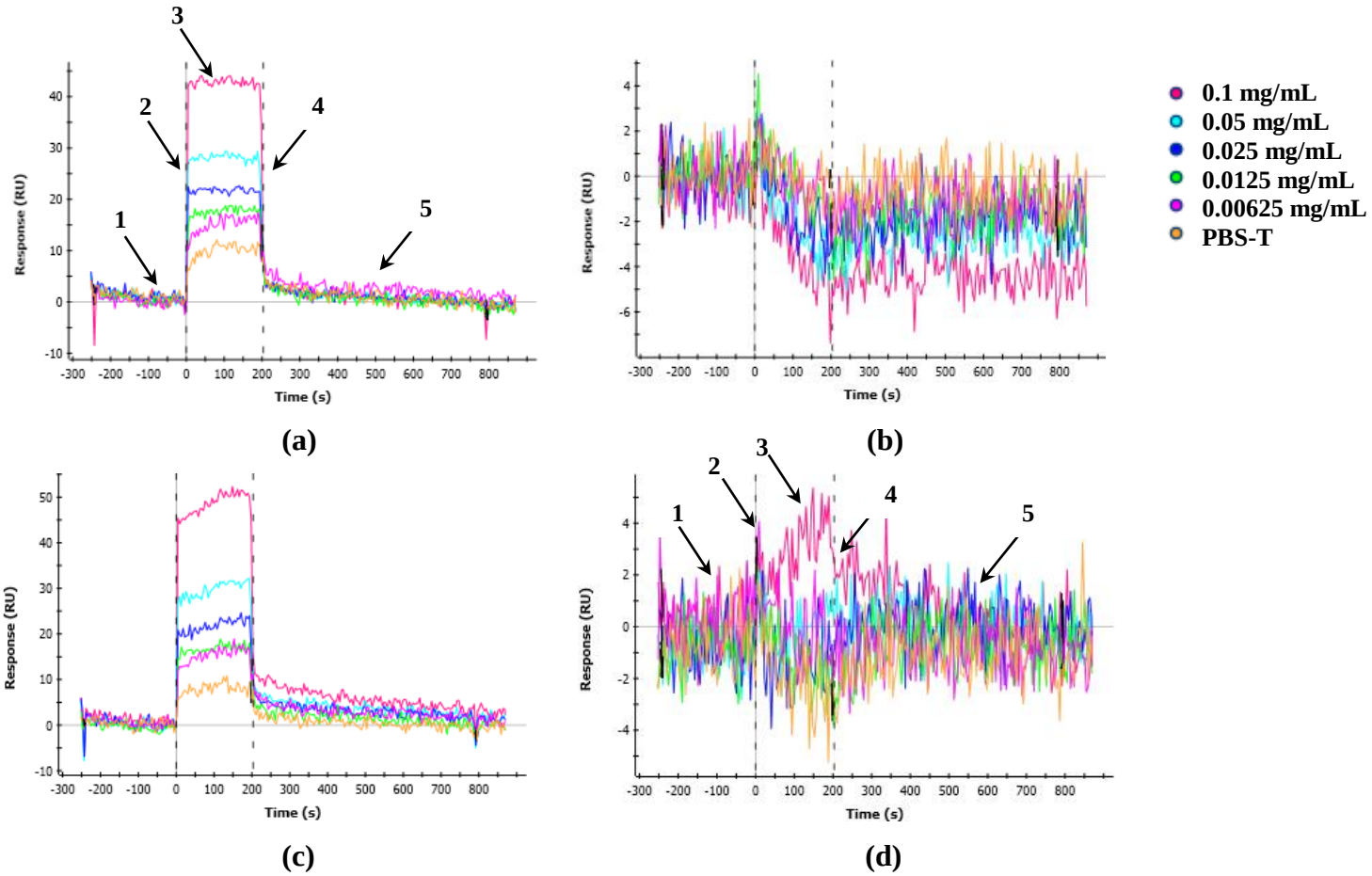


Figure 5.9 *Five channels are injected with Bacillus spores antibody as the analyte at different concentrations. The last channel is the reference channel injected with PBST. The flow rate is 50 µL/min and the contact time is 200 s. (a) is antibody-B38 spore and (c) is antibody-B38 spore coat protein at pH 2 interactions before subtracting the reference channel. (b) and (d) are the interactions after subtracting the reference channel for (a) and (c). The arrows indicate the phases of ligand-analyte interactions where 1 is baseline, 2 is association, 3 is equilibrium, 4 is dissociation and 5 is regeneration.*

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

An easy, fast and economical method of pathogen detection remains a challenge for global biological safety (Singh et al., 2014). For instance, the recent Ebola outbreak is raising awareness about global health issues (Frieden et al., 2014). Food poisoning outbreaks now have the same level of importance and also need to be taken seriously (Khabbaz et al., 2014). Consumers are less confident about food safety due to the high number of food outbreaks and have concerns about how the food is processed (Pividori & Alegret, 2010). These factors are increasing the demand for fast, sensitive and specific methods for the detection of pathogens (Kara et al., 2010). For example, Lawrence Livermore Microbial Detection Array (LLMDA) can detect bacterial and viral pathogens, including the Ebola virus within 1 hour (Thissen et al., 2014). Biosensors such as SPR and QCM are frequently used to detect foodborne pathogens (Ahmed et al., 2014). Although accounts of these biosensors for foodborne bacterial cells and spores detection have been published for years, no publication to date was found that utilized the SPR system to detect *B. licheniformis* spore coat proteins. This study has explored another feasible method to detect the spores coat proteins of locally isolated *B. licheniformis* B38.

The *B. lichenformis* B38 spore preparation was the first step to produce spores for subsequent studies. Some important minerals were involved in the spore induction. However, optimization tests (e.g. length of incubation time and mineral concentration) for spore induction were not further explored since the stated methods worked well for spore preparation. An approximately 8 - 9 day-incubation period was enough to allow endospores to be released from mother cells. With the aid of the PEG separating system, more than 95% free spores could be obtained for the next experiments. Schaeffer-Fulton endospore staining technique was a relatively simple method to differentiate endospores and cells where the cells were stained red and the spores were stained green. Nonetheless, a disadvantage of using this staining method was that artefacts might be present when observed under the microscope and false positives

could possibly occur. A phase-contrast microscope is an optional choice for examining phase-bright spores without using any staining solution. However, the microscope must be handled extreme carefully because this microscopic system is expensive and training is required before using this system.

In order to extract spore coat proteins, the spores were digested with a SDS-8 M urea sample buffer before running gel electrophoresis. Physical treatments such as sonication and bead beating were not adequate to extract the spore coat proteins (Thompson et al., 2011). Hence, reducing and chaotropic agents were used to solubilize the spore coat proteins. If necessary, atomic force microscope observation could be used to examine the structure of the spores after digestion with the SDS-8 M urea sample buffer. This would help to obtain clearer images of the morphology and indicate how the reducing and chaotropic agents work instead of just checking the spores during the spore induction step.

Conventional CBB staining is a common technique for visualizing protein bands on gels. However, CBB staining without methanol and acetic acid (CBB Staining Solution B) offers more advantages than the conventional staining technique (Dong et al., 2011). This provides another choice for those who want to perform gel elution by simple diffusion and stain gels in a short time.

Purification of a selected spore coat protein was done by liquid chromatography. As stated previously, the purpose for purifying an ~10 kDa spore coat protein was that it could be used to combine with an OB-fold and immobilized to a chip for *B. licheniformis* spores detection by SPR. The idea is for an artificial OB-fold is to replace conventional antibodies synthesized by animals which normally used for spore detection. It was initially thought that the liquid chromatography technique could be used to separate each of the protein bands shown on the gel after digesting with the SDS-8 M urea sample buffer (**Figure 3.1**). This was because a protocol similar to an Ichikawa et al. (1992) method, which separated *B. megaterium* spore coat proteins using chromatography, was followed. Unfortunately, this study did not successfully separate the spore coat proteins of *B. licheniformis* B38 by liquid chromatographic methods. The reason for this outcome needs to be investigated further. Krajčíková et al. (2009) and Velásquez et al. (2014) utilized liquid

chromatography to purify recombinant *Bacillus* species spore coat proteins expressed by *E. coli*, i.e. not directly extracted from the spore coat. This suggested that protein purification of the ~10 kDa spore coat protein expressed in *E. coli* should be tried in the future. On the other hand, gel elution by diffusion offered another feasible way to purify the selected spore coat protein.

The reason for checking the protein sequences particularly for the ~10 kDa band was because this protein band consistently appeared in the gels and had the potential to become a target for detection with an OB-fold. Therefore, it was necessary to identify the protein sequences for the ~10 kDa protein band. However, protein sequencing using gel digestion by trypsin was not as accurate and precise as using molecular techniques to extract DNA sequences from *B. licheniformis* B38 spore coat. This was because errors and deviations were possible through codon degeneracy and trypsin contamination when digesting the gels. In order to match the identified protein sequences from *B. licheniformis* B38 spore coat, spore coat DNA extraction needs to be done. Once sequenced, it should be deposited in the *B. licheniformis* genomic database if novel DNA and protein sequences are found.

A preliminary study was done to detect *B. licheniformis* B38 spores by SPR. Different binding assays were tried but the results were disappointing due to the problems stated above. However, a paper was found that had similar results to this study. Some suggestions can be made to improve the detection study in the future. For instance, a GLC chip could be used instead of a GLM chip for whole spore detection because it can mitigate mass transport effects (Bio-Rad, n.d.). This shows that a thinner alginate polymer layer of a GLC chip would increase the sensitivity because the spores would be closer to the evanescent wave field (Skottrup et al., 2008). Different ligand coupling techniques (e.g. aldehyde coupling and sandwich coupling) (Fischer, 2010) and detection assays (e.g. sandwich assay, competitive assay and inhibition assay) (Dostálek et al., 2006) could also be used to increase the sensitivity of the detection conditions. Furthermore, monoclonal antibodies could be used for SPR detection studies because they are more specific and abundant than polyclonal antibodies (Pividori & Alegret, 2010).

In the future, immobilization of a bioreceptor such as an OB-fold-spore coat binding protein complex on a matrix may become a new technique for pathogenic bacterial cells and spore detection by SPR. This newly proposed method could be used extensively in New Zealand dairy companies since the country enforces the safety regulations [e.g. Animal Products (Dairy) Regulations 2005] for dairy productions to avoid contaminations. Furthermore, this method diagnoses the contaminants in a short time and allows a rapid response to the contamination and increased the efficiency in applying appropriate treatments to reduce illnesses.

REFERENCES

Abadian, P. N., Kelley, C. P., & Goluch, E. D. (2014). Cellular analysis and detection using surface plasmon resonance techniques. *Analytical Chemistry*, *86*(6), 2799-2812.

Abdiche, Y. N., Lindquist, K. C., Pinkerton, A., Pons, J., & Rajpal, A. (2011). Expanding the ProteOn XPR36 biosensor into a 36-ligand array expedites protein interaction analysis. *Analytical biochemistry*, *411*(1), 139-151.

Abdulhalim, I., Zourob, M., & Lakhtakia, A. (2008). Surface plasmon resonance for biosensing: a mini-review. *Electromagnetics*, *28*(3), 214-242.

Abhyankar, W., Beek, A. T., Dekker, H., Kort, R., Brul, S., & de Koster, C. G. (2011). Gel-free proteomic identification of the *Bacillus subtilis* insoluble spore coat protein fraction. *Proteomics*, *11*(23), 4541-4550.

Abhyankar, W., Koning, L. J., Brul, S., & Koster, C. G. (2014). Spore proteomics: the past, present and the future. *FEMS Microbiology Letters*, 1-8.

Ahmed, A., Rushworth, J. V., Hirst, N. A., & Millner, P. A. (2014). Biosensors for whole-cell bacterial detection. *Clinical Microbiology Reviews*, *27*(3), 631-646.

Akdoğan, E., & Mutlu, M. (2010). Basic principles of optical biosensors in food engineering. In M. Mutlu (Ed.), *Biosensors in food processing, safety, and quality control* (pp. 53-70). The United State of America: Taylor & Francis.

Alfonso, A., Pazos, M. J., Fernández-Araujo, A., Tobio, A., Alfonso, C., Vieytes, M. R., & Botana, L. M. (2014). Surface plasmon resonance biosensor method for palytoxin detection based on Na^+, K^+-ATPase affinity. *Toxins*, *6*(1), 96-107.

André, S., Zuber, F., & Remize, F. (2013). Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey. *International Journal of Food Microbiology, 165*, 134-143.

Animal Products (Dairy) Regulations 2005. Retrieved from http://www.legislation.govt.nz/regulation/public/2005/0104/latest/whole.html?search=ts_regulation_animal+products_resel&p=1#DLM327127

Aronson, A. I. (2012). The structure and composition of the outer layers of the bacterial spore. In E. Abel-Santos (Ed.), *Bacterial spores: current research and applications* (pp. 57-71). United Kingdom: Caister Academic Press.

Aronson, A., Goodman, B., & Smith, Z. (2014). The regulated synthesis of a *Bacillus anthracis* spore coat protein that affects spore surface properties. *Journal of Applied Microbiology, 116*, 1241-1249.

Arora, P., Sindhu, A., Kaur, H., Dilbaghi, N., & Chaudhury, A. (2013). An overview of transducers as platform for the rapid detection of foodborne pathogens. *Applied Microbiology and Biotechnology*, *97*(5), 1829-1840.

Bagyan, I., Hobot, J., & Cutting, S. (1996). A compartmentalized regulator of developmental gene expression in *Bacillus subtilis*. *Journal of Bacteriology, 178*(15), 4500-4507.

Banykó, J., & Vyletělová, M. (2009). Determining the source of *Bacillus cereus* and *Bacillus licheniformis* isolated from raw milk, pasteurized milk and yoghurt. *Letters in Applied Microbiology, 48*, 318-323.

Barbosa, T. M., Serra, C. R., & Henriques, A. O. (2004). Gut sporeformers. In E. Ricca, A. O. Henriques & S. M. Cutting (Eds.), *Bacterial spore formers: probiotics and emerging applications* (pp. 183-191). United Kingdom: Horizon Bioscience.

Baril, E., Coroller, L., Couvert, O., El Jabri, M., Leguerinel, I., Postollec, F., Boulais, C., Carlin, F., & Mafart, P. (2012a). Sporulation boundaries and spore formation kinetics of *Bacillus* spp. as a function of temperature, pH and a_w. *Food Microbiology*, *32*(1), 79-86.

Baril, E., Coroller, L., Couvert, O., Leguérinel, I., Postollec, F., Boulais, C., Carlin, F., & Mafart, P. (2012b). Modeling heat resistance of *Bacillus weihenstephanensis* and *Bacillus licheniformis* spores as function of sporulation temperature and pH. *Food Microbiology, 30*(1), 29-36.

Bassi, D., Cappa, F., & Cocconcelli, P. S. (2012). Water and cations flux during sporulation and germination. In E. Abel-Santos (Ed.), *Bacterial spores: current research and applications* (pp. 143-167). United Kingdom: Caister Academic Press.

Basu, S., Kang, T. J., Chen, W. H., Fenton, M. J., Baillie, L., Hibbs, S., & Cross, A. S. (2007). Role of *Bacillus anthracis* spore structures in macrophage cytokine responses. *Infection and Immunity*, *75*(5), 2351-2358.

Beattie, S. H., & Williams, A. G. (2000). *Bacillus*: detection of toxins. In R. K. Robinson, C. A. Batt & P. D. Patel (Eds.), *Encyclopedia of food microbiology* (Vol. 1, pp. 141-149). London: Academic Press.

Biacore. (n.d.). *Biacore sensor surface handbook*. Retrieved from http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Biacore%20Sensor%20SSurfac%20Handbook.pdf

Bio-Rad. (n.d.). *ProteOn™ XPR36 experimental design and application guide*. Retrieved from http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6414.pdf

Black, J. G. (2008). Microbiology: Principles and explorations (7th ed.). The United State of America: John Wiley & Sons.

Blatchley III, E. R., Meeusen, A., Aronson, A. I., & Brewster, L. (2005). Inactivation of *Bacillus* spores by ultraviolet or gamma radiation. *Journal of Environmental Engineering*, *131*(9), 1245-1252.

Blaylock, B., Jiang, X., Rubio, A., Moran, C. P. Jr., & Pogliano, K. (2004). Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization. *Genes and Development*, *18*(23), 2916-2928.

Boyer, R. (2000). *Modern experimental biochemistry* (3rd ed.). San Francisco: Benjamin Cummings.

Bradley, D. E., & Franklin, J. G. (1958). Electron microscope survey of the surface configuration of spores of the genus *Bacillus*. *Journal of Bacteriology*, 76(6), 618-630.

Bronner, V., Denkberg, G., Peled, M., Elbaz, Y., Zahavi, E., Kasoto, H., Reiter, Y., Notcovich, A., & Bravman, T. (2010). Therapeutic antibodies: discovery and development using the ProteOn XPR36 biosensor interaction array system. *Analytical Biochemistry*, *406*(2), 147-156.

Burbulys, D., Trach, K. A., & Hoch, J. A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell*, *64*(3), 545-552.

Burgess, R. R. (2009a). Elution of proteins from gels. In R. R. Burgess & M. P. Deutscher (Eds.), *Methods in enzymology: guide to protein purification* (2nd ed., Vol. 463, pp. 565-572). The United State of America: Academic Press.

Burgess, R. R. (2009b). Protein precipitation techniques. In R. R. Burgess & M. P. Deutscher (Eds.), *Methods in enzymology: guide to protein purification* (2nd ed., Vol. 463, pp. 331-342). The United State of America: Academic Press.

Burgess, S. A., Flint, S. H., & Lindsay, D. (2014). Characterization of thermophilic bacilli from a milk powder processing plant. *Journal of Applied Microbiology*, *116*(2), 350-359.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology*, *144*(2), 215-225.

Burtt Jr, E. H., & Ichida, J. M. (1999). Occurrence of feather-degrading bacilli in the plumage of birds. *The Auk*, *116*(2), 364-372.

Carter, H. L. III, & Moran, C. P. Jr. (1986). New RNA polymerase σ factor under *spo0* control in *Bacillus subtilis*. *Proceedings of the National Academy of Science, 83,* 9438-9442.

Charney, J., Fisher, W. P., & Hegarty, C. P. (1951). Manganese as an essential element for sporulation in the genus *Bacillus*. *Journal of Bacteriology*, *62*(2), 145-148.

Chou, C., Wu, H. T., Huang, Y. C., Kuo, W. C., & Chen, Y. L. (2006). Characteristics of a paired surface plasma waves biosensor. *Optics Express*, *14*(10), 4307-4315.

Cohen, S. L., & Chait, B. T. (1997). Mass spectrometry of whole proteins eluted from sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels. *Analytical Biochemistry*, *247*(2), 257-267.

Cortezzo, D. E., & Setlow, P. (2005). Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. *Journal of Applied Microbiology*, *98*(3), 606-617.

Coton, M., Denis, C., Cadot, P., & Coton, E. (2011). Biodiversity and characterization of aerobic spore-forming bacteria in surimi seafood products. *Food Microbiology*, *28,* 252-260.

Cowan, A. E., Koppel, D. E., Setlow, B., & Setlow, P. (2003). A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. *Proceedings of the National Academy of Sciences*, *100*(7), 4209-4214.

Cowan, A. E., Olivastro, E. M., Koppel, D. E., Loshon, C. A., Setlow, B., & Setlow, P. (2004). Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile. *Proceedings of the National Academy of Sciences*, *101*(20), 7733-7738.

Cutting, S., Panzer, S., & Losick, R. (1989). Regulatory studies on the promoter for a gene governing synthesis and assembly of the spore coat in *Bacillus subtilis*. *Journal of Molecular Biology*, *207*(2), 393-404.

Cutting, S., Roels, S., & Losick, R. (1991). Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. *Journal of Molecular Biology*, *221*(4), 1237-1256.

Dahl, M. K. (2000). *Bacillus*: introduction. In R. K. Robinson, C. A. Batt & P. D. Patel (Eds.), *Encyclopedia of food microbiology* (Vol. 1, pp. 113-118). London: Academic Press.

Daniel, R. A., & Errington, J. (1993). Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in *Bacillus subtilis*. *Journal of Molecular Biology*, *232*(2), 468-483.

Das Mohapatra, P. K., Maity, C., Rao, R. S., Pati, B. R., & Mondal, K. C. (2009). Tannase production by *Bacillus licheniformis* KBR6: Optimization of submerged culture conditions by Taguchi DOE methodology. *Food Research International*, *42*(4), 430-435.

De Clerck, E., Vanhoutte, T., Hebb, T., Geerinck, J., Devos, J., & De Vos, P. (2004). Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. *Applied and Environmental Microbiology*, *70*(6), 3664-3672.

de Hoon, M. J. L., Eichenberger, P., & Vitkup, D. (2010). Hierarchical evolution of the bacterial sporulation network. *Current Biology*, *20*(17), R735-R745.

De Jonghe, V., Coorevits, A., De Block, J., Van Coillie, E., Grijspeerdt, K., Herman, L., De Vos, P., & Heyndrickx, M. (2010). Toxinogenic and spoilage potential of aerobic spore-formers isolated from raw milk. *International Journal of Food Microbiology, 136*, 318-325.

de Mol, N. J., & Fischer, J. E. (2010). Surface plasmon resonance: a general introduction. In N. J. de Mol & M. J. E. Fischer (Eds.), *Surface plasmon resonance: methods and protocols* (pp. 1-14). New York: Humana Press.

Dhakal, R., Chauhan, K., Seale, R. B., Deeth, H. C., Pillidge, C. J., Powell, I. B., Craven, H., & Turner, M. S. (2013). Genotyping of dairy *Bacillus licheniformis* isolates by high resolution melt analysis of multiple variable number tandem repeat loci. *Food Microbiology*, *34*, 344-351.

Dong, W. H., Wang, T. Y., Wang, F., & Zhang, J. H. (2011). Simple, time-saving dye staining of proteins for sodium dodecyl sulphate-polyacrylamide gel electrophoresis using coomassie blue. *PloS one*, *6*(8), e22394.

Dostálek, J., Ladd, J., Jiang, S., & Homola, J. (2006). SPR biosensors for detection of biological and chemical analytes. In J. Homola (Ed.), *Surface plasmon resonance based sensors* (pp. 177-190). Berlin: Springer.

Dougan, P. (2014, January 14). 9000 bottles in Fonterra cream recall. *The New Zealand Herald*. Retrieved from http://www.nzherald.co.nz/business/news/article.cfm?c_id=3&objectid=11186111

Driks, A. (1999). *Bacillus subtilis* spore coat. *Microbiology and Molecular Biology Reviews*, *63*(1), 1-20.

Ducros, V. M. A., Lewis, R. J., Verma, C. S., Dodson, E. J., Leonard, G., Turkenburg, J. P., Murshudov, G. N., Wilkinson, A. J., & Brannigan, J. A. (2001). Crystal structure of GerE, the ultimate transcriptional regulator of spore formation in *Bacillus subtilis*. *Journal of Molecular Biology*, *306*(4), 759-771.

Dunbar, B. S., & Schwoebel, E. D. (1990). Preparation of polyclonal antibodies. In M. P. Deutscher (Ed.), *Methods in enzymology: guide to protein purification* (Vol. 182, pp. 663-670). London, UK: Academic Press.

Eichenberger, P., Fujita, M., Jensen, S. T., Conlon, E. M., Rudner, D. Z., Wang, S. T., Ferguson, C., Haga, K., Sato, T., Liu, J. S., & Losick, R. (2004). The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis*. *PLoS Biology*, *2*(10), 1664-1683.

Eichenberger, P., Jensen, S. T., Conlon, E. M., van Ooji, C., Silvaggi, J., Gonz âlez-Pastor, J. E., Fujita, M., Ben-Yehuda, S., Stragier, P., Liu, J. S., & Losick, R. (2003). The σ^E regulon and the identification of additional sporulation genes in *Bacillus subtilis*. *Journal of Molecular Biology*, *327*(5), 945-972.

Eisenstadt, E. (1972). Potassium content during growth and sporulation in *Bacillus subtilis*. *Journal of Bacteriology*, *112*(1), 264-267.

Errington, J. (1993). *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiological Reviews*, *57*(1), 1-33.

European Economic Community. (1985). *Council Directive of 5 August 1985 on health and animal-health problems affecting intra-community trade in heat-treated milk* (85/397/EEC). *Official Journal of the European Communities*, *L226*, 13-29. Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1985:226:0013:0029:EN:PDF

Evans, D. R. H., Romero, J. K., & Westoby, M. (2009). Concentration of proteins and removal of solutes. In B. R. Richard & M. P. Deutscher (Eds.), *Methods in enzymology: guide to protein purification* (2nd ed., Vol. 463, pp. 97-120). The United State of America: Academic Press.

Evans, L., Feucht, A., & Errington, J. (2004). Genetic analysis of the *Bacillus subtilis sigG* promoter, which controls the sporulation-specific transcription factor σ^G. *Microbiology*, *150*(7), 2277-2287.

Faille, C., Lequette, Y., Ronse, A., Slomianny, C., Garénaux, E., & Guerardel, Y. (2010). Morphology and physico-chemical properties of *Bacillus* spores surrounded or not with an exosporium: consequences on their ability to adhere to stainless steel. *International Journal of Food Microbiology*, *143*(3), 125-135.

Falegan, C. (2011). Microbiology profile and biochemical characteristics of commercial 'ogiri' samples from south-western, Nigeria. *Journal of Microbiology, Biotechnology and Food Sciences*, *1*(2), 187-203.

Farka, Z., Kovář, D., Přibyl, J., & Skládal, P. (2013). Piezoelectric and surface plasmon resonance biosensors for *Bacillus atrophaeus* spores. *International Journal of Electrochemical Science*, *8*, 100-112.

Fasimoye, F. O., Olajuyigbe, F. M., & Sanni, M. D. (2014). Purification and characterization of a thermostable extracellular phytase from *Bacillus licheniformis* PFBL-03. *Preparative Biochemistry and Biotechnology*, *44*(2), 193-205.

Fekete, T. (2009). *Bacillus* species (not *anthracis*). *Clinical Microbiology Newsletter*, *31*(12), 87-92.

Fischer, M. J. E. (2010). Amine coupling through EDC/NHS: a practical approach. In N. J. de Mol & M. J. E. Fischer (Eds.), *Surface plasmon resonance: methods and protocols* (pp. 55-73). New York: Humana Press.

Fontanot, M., Iacumin, L., Cecchini, F., Comi, G., & Manzano, M. (2014). Rapid detection and differentiation of important *Campylobacter* spp. in poultry samples by dot blot and PCR. *Food Microbiology*, *43*, 28-34.

Foster, J. W. (1956). Morphogenesis in bacteria: some aspects of spore formation. *The Quarterly Review of Biology*, *31*(2), 102-118.

Frandsen, N., & Stragier, P. (1995). Identification and characterization of the *Bacillus subtilis spoIIP* locus. *Journal of Bacteriology*, *177*(3), 716-722.

Frieden, T. R., Damon, I., Bell, B. P., Kenyon, T., & Nichol, S. (2014). Ebola 2014-new challenges, new global response and responsibility. *The New England Journal of Medicine*, 1-3.

GE Healthcare. (n.d.). *Superdex^{TM} 75 10/300 GL and Superdex 200 10/300 GL*. Retrieved from https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314807262343/litdoc71501796_20150114215337.pdf

Gedig, E.T. (2008). Surface chemistry in SPR technology. In R. B. M. Schasfoort & A. J. Tudos (Eds.), *Handbook of surface plasmon resonance* (pp. 173-220). Cambridge, UK: Royal Society of Chemistry.

Genest, P. C., Setlow, B., Melly, E., & Setlow, P. (2002). Killing of spores of *Bacillus subtilis* by peroxynitrite appears to be caused by membrane damage. *Microbiology*, *148*(1), 307-314.

Gerhardt, P., Beaman, T. C., Corner, T. R., Greenamyre, J. T., & Tisa, L. S. (1982). Photometric immersion refractometry of bacterial spores. *Journal of Bacteriology*, *150*(2), 643-648.

Ghani, M., Ansari, A., Aman, A., Zohra, R. R., Siddiqui, N. N., & Qader, S. A. U. (2013). Isolation and characterization of different strains of *Bacillus licheniformis* for the production of commercially significant enzymes. *Pakistan Journal of Pharmaceutical Sciences*, *26*(4), 691-697.

Giorno, R., Bozue, J., Cote, C., Wenzel, T., Moody, K. S., Mallozzi, M., Ryan, M., Wang, R., Zielke, R., Maddock, J. R., Friedlander, A., Welkos, S., & Driks, A. (2007). Morphogenesis of the *Bacillus anthracis* spore. *Journal of Bacteriology*, *189*(3), 691-705.

Goldman, R. C., & Tipper, D. J. (1978). *Bacillus subtilis* spore coats: complexity and purification of a unique polypeptide component. *Journal of Bacteriology*, *135*(3), 1091-1106.

González-Pastor, J. E. (2011). Cannibalism: a social behavior in sporulating *Bacillus subtilis*. *FEMS Microbiology Reviews*, *35*(3), 415-424.

Gopinath, S. C. B., Tang, T. H., Chen, Y., Citartan, M., & Lakshmipriya, T. (2014). Bacterial detection: from microscope to smartphone. *Biosensors and Bioelectronics*, *60*, 332-342.

Gould, G. W. (2000). Bacterial endospores. In R. K. Robinson, C. A. Batt & P. D. Patel (Eds.), *Encyclopedia of food microbiology* (Vol. 1, pp. 168-173). London: Academic Press.

Granger, A. C., Gaidamakova, E. K., Matrosova, V. Y., Daly, M. J., & Setlow, P. (2011). Effects of Mn and Fe levels on *Bacillus subtilis* spore resistance and effects of Mn^{2+}, other divalent cations, orthophosphate, and dipicolinic acid on protein resistance to ionizing radiation. *Applied and Environmental Microbiology*, *77*(1), 32-40.

Grimshaw, C. E., Huang, S., Hanstein, C. G., Strauch, M. A., Burbulys, D., Wang, L., Hoch, J. A., & Whiteley, J. M. (1998). Synergistic kinetic interactions between components of the phosphorelay controlling sporulation in *Bacillus subtilis*. *Biochemistry, 37*(5), 1365-1375.

Guerrero, Y. C., & Gonz áez-Aguilar, G. (2013). Shiga toxin detection methods: a short review. *arXiv preprint arXiv*: 1312.4748.

Han, H., & Wilson, A. C. (2013). The two CcdA proteins of *Bacillus anthracis* differentially affect virulence gene expression and sporulation. *Journal of Bacteriology, 195*(23), 5242-5249.

Hanke, A. T., & Ottens, M. (2014). Purifying biopharmaceuticals: knowledge-based chromatographic process development. *Trends in Biotechnology*, *32*(4), 210-220.

Hanlon, G. W., Hodges, N. A., & Russell, A. D. (1982). The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. *Journal of General Microbiology*, *128*(4), 845-851.

Hao, R., Wang, D., Zhang, X., Zuo, G., Wei, H., Yang, R., Zhang, Z., Cheng, Z., Guo, Y., Cui, Z., & Zhou, Y. (2009). Rapid detection of *Bacillus anthracis* using monoclonal antibody functionalized QCM sensor. *Biosensors and Bioelectronics, 24*(5), 1330-1335.

Harlow, E., & Lane, D. (1988). *Antibodies: A laboratory manual*. The United State of America: Cold Spring Harbor Laboratory.

Harrold, Z. R., Hertel, M. R., & Gorman-Lewis, D. (2011). Optimizing *Bacillus subtilis* spore isolation and quantifying spore harvest purity. *Journal of Microbiological Methods, 87*(3), 325-329.

He, L. M., & Tebo, B. M. (1998). Surface charge properties of and Cu (II) adsorption by spores of the marine *Bacillus* sp. strain SG-1. *Applied and Environmental Microbiology*, *64*(3), 1123-1129.

Hoa, N. T., Brannigan, J. A., & Cutting, S. M. (2002). The *Bacillus subtilis* signaling protein SpoIVB defines a new family of serine peptidases. *Journal of Bacteriology*, *184*(1), 191-199.

Hoch, J. A. (1993). Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annual Review of Microbiology, 47*, 441-465.

Hofmann, A. (2010). Spectroscopic techniques: I spectrophotometric techniques. In K. Wilson & J. Walker (Eds.), *Principles and techniques of biochemistry and molecular biology* (pp. 477-521). United Kingdom: Cambridge University Press.

Holt, S. C., & Leadbetter, E. R. (1969). Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. *Bacteriological Reviews*, *33*(2), 346-378.

Hon, K. L., & Leung, T. F. (2014). Issues associated with formula milk: the Hong Kong's experience. *World Journal of Pediatrics*, *10*(1), 5-6.

Hong, P., Koza, S., & Bouvier, E. S. P. (2012). A review size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. *Journal of Liquid Chromatography & Related Technologies*, *35*(20), 2923-2950.

Huang, S. S., Chen, D., Pelczar, P. L., Vepachedu, V. R., Setlow, P., & Li, Y. Q. (2007). Levels of Ca^{2+}-dipicolinic acid in individual *Bacillus* spores determined using microfluidic raman tweezers. *Journal of Bacteriology*, *189*(13), 4681-4687.

Ichikawa, T., Yamanishi, K., Watabe, K., Nishihara, T., & Kondo, M. (1982). Purification and biosynthesis of a spore coat protein in *Bacillus megaterium* QM B1551. *Journal of General Microbiology*, *128*(9), 2149-2155.

Igarashi, T., & Setlow, P. (2006). Transcription of the *Bacillus subtilis gerK* operon, which encodes a spore germinant receptor, and comparison with that of operons encoding other germinant receptors. *Journal of Bacteriology*, *188*(11), 4131-4136.

International Commission on the Microbiological Specifications for Foods. (2014). *Usefulness of testing for Clostridium botulinum in powdered infant formula and dairy-based ingredients for infant formula.* Retrieved from http://www.icmsf.org/pdf/ICMSF_Infant_Formula_Testing_Revision1-20140117.pdf

Janson, J. C., & Jönsson, J. Å. (2011). Introduction to chromatography. In J. C. Janson (Ed.), *Protein purification: principles, high resolution methods, and applications* (pp. 25-50). New Jersey: John Wiley & Sons.

Jenkinson, H. F., Sawyer, W. D., & Mandelstam, J. (1981). Synthesis and order of assembly of spore coat proteins in *Bacillus subtilis*. *Journal of General Microbiology*, *123*(1), 1-16.

Jephcott, A. E., Barton, B. W., Gilbert, R. J., & Shearer, C. W. (1977). An unusual outbreak of food-poisoning associated with meals-on-wheels. *The Lancet*, *310*(8029), 129-130.

Kara, P., Kiliçkaya, O., & Özsöz, M. Ş. (2010). Electrochemical DNA biosensors in food safety. In M. Mutlu (Ed.), *Biosensors in food processing, safety, and quality control* (pp. 123-134). The United State of America: Taylor & Francis.

Karow, M. L., Glaser, P., & Piggot, P. J. (1995). Identification of a gene, *spoIIR*, that links the activation of σ^E to the transcriptional activity of σ^F during sporulation in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, *92*, 2012-2016.

Khabbaz, R. F., Moseley, R. R., Steiner, R. J., Levitt, A. M., & Bell, B. P. (2014). Challenges of infectious diseases in the USA. *The Lancet*, *384*(9937), 53-63.

Kim, S. Y., Shin, S. J., Song, C. H., Jo, E. K., Kim, H. J., & Park, J. K. (2009). Destruction of *Bacillus licheniformis* spores by microwave irradiation. Journal of Applied Microbiology, *106*(3), 877-885.

Knaysi, G. (1945). A study of some environmental factors which control endospore formation by a strain of *Bacillus mycoides*. *Journal of Bacteriology*, *49*(5), 473-493.

Knoll, W., Kasry, A., Liu, J., Neumann, T., Niu, L., Park, H., Paulsen, H., Robelek, R., Yao, D., & Yu, F. (2008). Surface plasmon fluorescence techniques for bioaffinity studies. In R. B. M. Schasfoort & A. J. Tudos (Eds.), *Handbook of surface plasmon resonance* (pp. 275-312). Cambridge, UK: Royal Society of Chemistry.

Knop, K., Hoogenboom, R., Fischer, D., & Schubert, U. S. (2010). Poly (ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angewandte Chemie International Edition*, *49*(36), 6288-6308.

Kolodziej, B. J., & Slepecky, R. A. (1964). Trace metal requirements for sporulation of *Bacillus megaterium*. *Journal of Bacteriology*, *88*(4), 821-830.

Krajčíková, D., Lukáčová, M., Müllerová, D., Cutting, S. M., & Barák, I. (2009). Searching for protein-protein interactions within the *Bacillus subtilis* spore coat. *Journal of Bacteriology*, *191*(10), 3212-3219.

Kumari, B. R., Vijayabharathi, R., Srinivas, V., & Gopalakrishnan, S. (2014). Microbes as interesting source of novel insecticides: a review. *African Journal of Biotechnology*, *13*(26), 2582-2592.

Kunkel, B., Kroos, L., Poth, H., Youngman, P., & Losick, R. (1989). Temporal and spatial control of the mother-cell regulatory gene *spoIIID* of *Bacillus subtilis*. *Genes and Development*, *3*(11), 1735-1744.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G. O., Azevedo, V., ... & Haga, K. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature*, *390*(6657), 249-256.

Kurien, B. T., & Scofield, R. H. (2012). Extraction of proteins from gels: a brief review. In B. T. Kurien & R. H. Scofield (Eds.), *Protein electrophoresis: methods and protocols* (pp. 403-405). New Jersey: Humana Press.

Kuwana, R., Kasahara, Y., Fujibayashi, M., Takamatsu, H., Ogasawara, N., & Watabe, K. (2002). Proteomics characterization of novel spore proteins of *Bacillus subtilis*. *Microbiology*, *148*(12), 3971-3982.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680-685.

Lee, M. F. X., Chan, E. S., & Tey, B. T. (2014). Negative chromatography: Progress, applications and future perspectives. *Process Biochemistry*, *49*(6), 1005-1011.

Leggett, M. J., McDonnell, G., Denyer, S. P., Setlow, P., & Maillard, J. Y. (2012). Bacterial spore structures and their protective role in biocide resistance. *Journal of Applied Microbiology*, *113*(3), 485-498.

Lei, Z., Anand, A., Mysore, K. S., & Sumner, L. W. (2007). Electroelution of intact proteins from SDS-PAGE gels and their subsequent MALDI-TOF MS analysis. In H. Thiellement, M. Zivy, C. Damerval & V. Méchin (Eds.), *Plant proteomics: methods and protocols* (pp. 353-363). New Jersey: Humana Press.

Li, Y., Butzin, X. Y., Davis, A., Setlow, B., Korza, G., Üstok, F. I., Christie, G., Setlow, P., & Hao, B. (2013). Activity and regulation of various forms of CwlJ, SleB, and YpeB proteins in degrading cortex peptidoglycan of spores of *Bacillus* species *In vitro* and during spore germination. *Journal of Bacteriology*, *195*(11), 2530-2540.

Lindsay, J. A., Barton, L. E., Leinart, A. S., & Pankratz, H. S. (1990). The effect of sporulation temperature on sporal characteristics of *Bacillus subtilis* A. *Current Microbiology*, *21*(1), 75-79.

Llarch, À., Logan, N. A., Castellv í, J., Prieto, M. J., & Guinea, J. (1997). Isolation and characterization of thermophilic *Bacillus* spp. from geothermal environments on Deception Island, South Shetland Archipelago. *Microbial Ecology*, *34*(1), 58-65.

L öf ås, S., & McWhirter, A. (2006). The art of immobilization for SPR sensors. In J. Homola (Ed.), *Surface plasmon resonance based sensors* (pp. 117-151). Berlin: Springer.

Logan, N. A. (2011). *Bacillus* and relatives in foodborne illness. *Journal of Applied Microbiology*, *112*, 417-429.

Lončar, N., Božić, N., Lopez-Santin, J., & Vujčić, Z. (2013). *Bacillus amyloliquefaciens* laccase - from soil bacteria to recombinant enzyme for wastewater decolorization. *Bioresource Technology*, *147*, 177-183.

Londo ño-Vallejo, J. A., Fr échel, C., & Stragier, P. (1997). *spoIIQ*, a forespore-expressed gene required for engulfment in *Bacillus subtilis*. *Molecular Microbiology*, *24*(1), 29-39.

Lopez-Diaz, I., Clarke, S., & Mandelstam, J. (1986). *spoIID* operon of *Bacillus subtilis*: cloning and sequence. *Journal of General Microbiology*, *132*, 341-354.

Loshon, C. A., Fliss, E. R., Setlow, B., Foerster, H. F., & Setlow, P. (1986). Cloning and nucleotide sequencing of genes for small, acid-soluble spore proteins of *Bacillus cereus*, *Bacillus stearothermophilus*, and "*Thermoactinomyces thalpophilus*". *Journal of Bacteriology*, *167*(1), 168-173.

Losick, R., & Stragier, P. (1992). Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. *Nature*, *355*(6361), 601-604.

Lu, L., Zhao, M., Wang, T. N., Zhao, L. Y., Du, M. H., Li, T. L., & Li, D. B. (2012). Characterization and dye decolorization ability of an alkaline resistant and organic solvents tolerant laccase from *Bacillus licheniformis* LS04. Bioresource Technology, *115*, 35-40.

Lücking, G., Stoeckel, M., Atamer, Z., Hinrichs, J., & Ehling-Schulz, M. (2013). Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. *International Journal of Food Microbiology*, *166*(2), 270-279.

Lundgren, D. G., & Cooney, J. J. (1962). Chemical analyses of asporogenic mutants of *Bacillus cereus*. *Journal of Bacteriology*, *83*(6), 1287-1293.

Margolis, P. S., Driks, A., & Losick, R. (1993). Sporulation gene *spoIIB* from *Bacillus subtilis*. *Journal of Bacteriology*, *175*(2), 528-540.

Masiello, S., Martin, N. H., Watters, R. D., Galton, D. M., Schukken, Y. H., Wiedmann, M., & Boor, K. J. (2014). Identification of dairy farm management practices associated with the presence of psychrotolerant sporeformers in bulk tank milk. *Journal of Dairy Science*, *97*(7), 4083-4096.

Mazas, M., Fernández, A., Álvarez, A., López, M., & Bernardo, A. (2009). Effects of phosphate and sodium and potassium chlorides on sporulation and heat resistance of *Bacillus cereus*. *Journal of Food Safety*, *29*(1), 106-117.

McKenney, P. T., Driks, A., & Eichenberger, P. (2013). The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nature Reviews Microbiology*, *11*(1), 33-44.

Meisner, J., Wang, X., Serrano, M., Henriques, A. O., & Moran, C. P. Jr. (2008). A channel connecting the mother cell and forespore during bacterial endospore formation. *Proceedings of the National Academy of Sciences*, *105*(39), 15100-15105.

Melly, E., Genest, P. C., Gilmore, M. E., Little, S., Popham, D. L., Driks, A., & Setlow, P. (2002). Analysis of the properties of spores of *Bacillus subtilis* prepared at different temperatures. *Journal of Applied Microbiology*, *92*(6), 1105-1115.

Minh, H. N. T., Durand, A., Loison, P., Perrier-Cornet, J. M., & Gervais, P. (2011). Effect of sporulation conditions on the resistance of *Bacillus subtilis* spores to heat and high pressure. *Applied Microbiology and Biotechnology*, *90*(4), 1409-1417.

Moeller, R., Raguse, M., Reitz, G., Okayasu, R., Li, Z., Klein, S., Setlow, P., & Nicholson, W. L. (2014). Resistance of *Bacillus subtilis* spore DNA to lethal ionizing radiation damage relies primarily on spore core components and DNA repair, with minor effects of oxygen radical detoxification. *Applied and Environmental Microbiology*, *80*(1), 104-109.

Mundra, R. V., Mehta, K. K., Wu, X., Paskaleva, E. E., Kane, R. S., & Dordick, J. S. (2013). Enzyme-driven *Bacillus* spore coat degradation leading to spore killing. *Biotechnology and Bioengineering*, *111*(4), 654-663.

Murko, S., Ščančar, J., & Milačič, R. (2011). Rapid fractionation of Al in human serum by the use of HiTrap desalting size exclusion column with ICP-MS detection. *Journal of Analytical Atomic Spectrometry*, *26*(1), 86-93.

Nadeem, M., Qazi, J. I., Syed, Q., & Gulsher, M. (2013). Purification and characterization of an alkaline protease from *Bacillus licheniformis* UV-9 for detergent formulations. *Songklanakarin Journal of Science & Technology*, *35*(2), 187-195.

New Zealand Food Safety Authority. (2005). *A guide to calculating the shelf life of foods*. Retrieved from http://www.foodsafety.govt.nz/elibrary/industry/guide_calculating-contains_background.pdf

Ni, H., Chen, Q. H., Chen, F., Fu, M. L., Dong, Y. C., & Cai, H. N. (2011). Improved keratinase production for feather degradation by *Bacillus licheniformis* ZJUEL31410 in submerged cultivation. *African Journal of Biotechnology*, *10*(37), 7236-7244.

Nicholson, W. L., & Setlow, P. (1990). Sporulation, germination and outgrowth. In C. R. Harwood & S. M. Cutting (Eds.), *Molecular biological methods for Bacillus* (pp. 391-450). Chichester: John Wiley.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews*, *64*(3), 548-572.

Nieminen, T., Rintaluoma, N., Andersson, M., Taimisto, A-M., Ali-Vehmas, T., Seppälä, A., Priha, O., & Salkinoja-Salonen, M. (2007). Toxinogenic *Bacillus pumilus* and *Bacillus licheniformis* from mastitic milk. *Veterinary Microbiology*, *124*, 329-339.

Noble, J. E., & Bailey, M. J. A. (2009). Quantitation of protein. In B. R. Richard & M. P. Deutscher (Eds.), *Methods in enzymology: guide to protein purification* (2nd ed., Vol. 463, pp. 73-95). The United State of America: Academic Press.

Nugroho, F. A., Yamamoto, H., Kobayashi, Y., & Sekiguchi, J. (1999). Characterization of a new sigma-K-dependent peptidoglycan hydrolase gene that plays a role in *Bacillus subtilis* mother cell lysis. *Journal of Bacteriology*, *181*(20), 6230-6237.

Oh, Y. K., & Freese, E. (1976). Manganese requirement of phosphoglycerate phosphomutase and its consequences for growth and sporulation of *Bacillus subtilis*. *Journal of Bacteriology*, *127*(2), 739-746.

Olivier, S. A., Bull, M. K., & Chapman, B. (2012). *Bacillus* spp. spores produced at lower temperatures are more resistant to high pressure thermal processes but mineralization does not predict relative resistance. *Innovative Food Science and Emerging Technologies*, *16*, 96-101.

Oomes, S. J. C. M., Jonker, M. J., Wittink, F. R. A., Hehenkamp, J. O., Breit, T. M., & Brul, S. (2009). The effect of calcium on the transcriptome of sporulating *B. subtilis* cells. *International Journal of Food Microbiology*, *133*(3), 234-242.

Opheim, D. J., & Bernlohr, R. W. (1975). Purification and regulation of fructose-1,6-bisphosphatase from *Bacillus licheniformis*. *The Journal of Biological Chemistry*, *250*(8), 3024-3033.

Ozkocaman, V., Ozcelik, T., Ali, R., Ozkalemkas, F., Ozkan, A., Ozakin, C., Akalin, H., Ursavas, A., Coskun, F., Ener, B., & Tunali, A. (2006). *Bacillus* spp. among hospitalized patients with haematological malignancies: clinical features, epidemics and outcomes. *Journal of Hospital Infection*, *64*, 169-176.

Pan, J., Zhang, C., Zhang, Z., & Li, G. (2014). Review of online coupling of sample preparation techniques with liquid chromatography. *Analytica Chimica Acta*, *815*, 1-15.

Pandey, N. K., & Aronson, A. I. (1979). Properties of the *Bacillus subtilis* spore coat. *Journal of Bacteriology*, *137*(3), 1208-1218.

Pavić, S., Brett, M., Petrić, I., Laštre, D., Smoljanović, M., Atkinson, M., Kovačić, A., Cetinić, E., & Ropac, D. (2005), An outbreak of food poisoning in a kindergarten caused by milk powder containing toxigenic *Bacillus subtilis* and *Bacillus licheniformis*. *Archiv für Lebensmittelhygiene, 56*(1), 20-22.

Peckham, G. D., Hew, B. E., Waller, D. F., Holdaway, C., & Jen, M. (2013). Amperometric detection of *Bacillus anthracis* spores: a portable, low-cost approach to the ELISA. *International Journal of Electrochemistry*.

Pedraza-Reyes, M., Ram ́ırez- Ram ́ırez, N., Vidales-Rodr ́iguez, L. E., & Robleto, E. A. (2012). Mechanisms of bacterial spore survival. In E. Abel-Santos (Ed.), *Bacterial spores: current research and applications* (pp. 73-87). United Kingdom: Caister Academic Press.

Pepe, O., Blaiotta, G., Moschetti, G., Greco, T., & Villani, F. (2003). Rope-producing strains of *Bacillus* spp. from wheat bread and strategy for their control by lactic acid bacteria. *Applied and Environmental Microbiology, 69*(4), 2321-2329.

Perkins, E. A., & Squirrell, D. J. (2000). Development of instrumentation to allow the detection of microorganisms using light scattering in combination with surface plasmon resonance. *Biosensors and Bioelectronics, 14*(10), 853-859.

Petrotchenko, E. V., Serpa, J. J., Cabecinha, A. N., Lesperance, M., & Borchers, C. H. (2014). "Out-gel" tryptic digestion procedure for chemical cross-linking studies with mass spectrometric detection. *Journal of Proteome Research, 13*(2), 527-535.

Piggot, P. J., & Losick, R. (2002). Sporulation genes and intercomparmental regulation. In A. L. Sonenshein, J. A. Hoch & R. Losick (Eds.), *Bacillus subtilis and its closest relatives: from genes to cells* (pp. 483-518). Washington D.C.: American Society for Microbiology.

Pividori, M. I., & Alegret, S. (2010). Biosensing for food safety. In M. Mutlu (Ed.), *Biosensors in food processing, safety, and quality control* (pp. 89-122). The United State of America: Taylor & Francis.

Plomp, M., Leighton, T. J., Wheeler, K. E., & Malkin, A. J. (2005). The high-resolution architecture and structural dynamics of *Bacillus* spores. *Biophysical Journal*, *88*(1), 603-608.

Plomp, M., Leighton, T. J., Wheeler, K. E., Hill, H. D., & Malkin, A. J. (2007). *In vitro* high-resolution structural dynamics of single germinating bacterial spores. *Proceedings of the National Academy of Sciences*, *104*(23), 9644-9649.

Popham, D. L. (2002). Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cellular and Molecular Life Sciences*, *59*(3), 426-433.

Postollec, F., Mathot, A. G., Bernard, M., Divanac'h, M. L., Pavan, S., & Sohier, D. (2012). Tracking spore-forming bacteria in food: from natural biodiversity to selection by process. *International Journal of Food Microbiology*, *158*, 1-8.

Powell, J. F., & Strange, R. E. (1956). Biochemical changes occurring during sporulation in *Bacillus* species. *Biochemical Journal*, *63*(4), 661-668.

Prentice, G. A., Wolfe, F. H., & Clegg, L. F. L. (1972). The use of density gradient centrifugation for the separation of germinated from nongerminated spores. *Journal of Applied Microbiology*, *35*(2), 345-349.

Purohit, M., Sassi-Gaha, S., & Rest, R. F. (2010). Rapid sporulation of *Bacillus anthracis* in a high iron, glucose-free medium. *Journal of Microbiological Methods*, *82*(3), 282-287.

Putra, S. E. D., Tsuprykov, O., Von Websky, K., Ritter, T., Reichetzeder, C., & Hocher, B. (2014). Dealing with large sample sizes: comparison of a new one spot dot blot method to western blot. *Clinical Laboratory*, *60*, 1-7.

Qin, H., & Driks, A. (2013). Contrasting evolutionary patterns of spore coat proteins in two *Bacillus* species groups are linked to a difference in cellular structure. *BMC Evolutionary Biology*, *13*(1), 261.

Ragkousi, K., & Setlow, P. (2004). Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis* spores. *Journal of Bacteriology*, *186*(17), 5567-5575.

Resnekov, O., Driks, A., & Losick, R. (1995). Identification and characterization of sporulation gene *spoVS* from *Bacillus subtilis*. *Journal of Bacteriology*, *177*(19), 5628-5635.

Rey, M. W., Ramaiya, P., Nelson, B. A., Brody-Karpin, S. D., Zaretsky, E. J., Tang, M., ... & Berka, R. M. (2004). Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. *Genome Biology*, *5*(10), r77.

Ricca, E., Cutting, S., & Losick, R. (1992). Characterization of *bofA*, a gene involved in the intercompartmental regulation of pro-σ^K processing during sporulation in *Bacillus subtilis*. *Journal of Bacteriology*, *174*(10), 3177-3184.

Rich, R. L., & Myszka, D. G. (2010). Grading the commercial optical biosensor literature - Class of 2008: 'The Mighty Binders'. *Journal of Molecular Recognition*, *23*(1), 1-64.

Riesenman, P. J., & Nicholson, W. L. (2000). Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Applied and Environmental Microbiology*, *66*(2), 620-626.

Robleto, E. A., Martin, H. A., Pepper, A. M., & Pedraza-Reyes, M. (2012). Gene regulation of sporulation in *Bacillus subtilis*. In E. Abel-Santos (Ed.), *Bacterial spores: current research and applications* (pp. 9-17). United Kingdom: Caister Academic Press.

Rode, L. J., Lewis, C. W., & Foster, J. W. (1962). Electron microscopy of spores of *Bacillus megaterium* with special reference to the effects of fixation and thin sectioning. *The Journal of Cell Biology, 13*(3), 423-435.

Rose, R., Setlow, B., Monroe, A., Mallozzi, M., Driks, A., & Setlow, P. (2007). Comparison of the properties of *Bacillus subtilis* spores made in liquid or on agar plates. *Journal of Applied Microbiology, 103*(3), 691-699.

Rubio, A., & Pogliano, K. (2004). Septal localization of forespore membrane proteins during engulfment in *Bacillus subtilis*. *The EMBO Journal, 23*, 1636-1646.

Rudner, D. Z., & Losick, R. (2002). A sporulation membrane protein tethers the pro-σ^K processing enzyme to its inhibitor and dictates its subcellular localization. *Genes and Development*, *16*, 1007-1018.

Sacks, L. E., & Alderton, G. (1961). Behavior of bacterial spores in aqueous polymer two-phase systems. *Journal of Bacteriology*, *82*(3), 331-341.

Salkinoja-Salonen, M. S., Vuorio, R., Andersson, M. A., Kämpfer, P., Andersson, M. C., Honkanen-Buzalski, T., & Scoging, A. C. (1999). Toxigenic strains of *Bacillus licheniformis* related to food poisoning. *Applied and Environmental Microbiology*, *65*(10), 4367-4645.

Schaeffer, A. B., & Fulton, M. C. (1933). A simplified method of staining endospores. *Science, 77*(1990), 194.

Schaeffer, P., Millet, J., & Aubert, J. P. (1965). Catabolic repression of bacterial sporulation. *Proceedings of the National Academy of Sciences of the United States of America, 54*(3), 704-711.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, *50*(1), 1-17.

Schasfoort, R. B. M., & McWhirter, A. (2008). SPR instrumentation. In R. B. M. Schasfoort & A. J. Tudos (Eds.), *Handbook of surface plasmon resonance* (pp. 35-80). Cambridge, UK: Royal Society of Chemistry.

Schasfoort, R. B. M., Lokate, A. M. C., Beusink, J. B., Pruijn, G. J. M., & Engbers, G. H. M. (2008). Measurement of the analysis cycle: scanning SPR microarray imaging of autoimmune diseases. In R. B. M. Schasfoort & A. J. Tudos (Eds.), *Handbook of surface plasmon resonance* (pp. 221-245). Cambridge, UK: Royal Society of Chemistry.

Scheldeman, P., Herman, L., Foster, S., & Heyndrickx, M. (2006). *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *Journal of Applied Microbiology, 101*, 542-555.

Schuck, P., & Zhao, H. (2010). The role of mass transport limitation and surface heterogeneity in the biophysical characterization of macromolecular binding processes by SPR biosensing. In N. J. de Mol & M. J. E. Fischer (Eds.), *Surface plasmon resonance: methods and protocols* (pp. 15-54). New York: Humana Press.

Seale, R. B., Flint, S. H., McQuillan, A. J., & Bremer, P. J. (2008). Recovery of spores from thermophilic dairy bacilli and effects of their surface characteristics on attachment to different surfaces. *Applied and Environmental Microbiology, 74*(3), 731-737.

Seelert, H., & Krause, F. (2008). Preparative isolation of protein complexes and other bioparticles by elution from polyacrylamide gels. *Electrophoresis, 29*(12), 2617-2636.

Sella, S. R. B. R., Vandenberghe, L. P. S., & Soccol, C. R. (2014). Life cycle and spore resistance of spore-forming *Bacillus atrophaeus*. *Microbiological Research*. doi: 10.1016/j.micres.2014.05.001

Serio, A. W., Pechter, K. B., & Sonenshein, A. L. (2006). *Bacillus subtilis* aconitase is required for efficient late-sporulation gene expression. *Journal of Bacteriology*, *188*(17), 6396-6405.

Setlow, B., & Setlow, P. (1980). Measurements of the pH within dormant and germinated bacterial spores. *Proceedings of the National Academy of Sciences*, *77*(5), 2474-2476.

Setlow, B., Tautvydas, K. J., & Setlow, P. (1998). Small, acid-soluble spore proteins of the α/β type do not protect the DNA in *Bacillus subtilis* spores against base alkylation. *Applied and Environmental Microbiology*, *64*(5), 1958-1962.

Setlow, P. (1988). Small, acid-soluble spore proteins of *Bacillus species*: structure, synthesis, genetics, function, and degradation. *Annual Reviews in Microbiology*, *42*(1), 319-338.

Setlow, P. (1994). Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. *Journal of Applied Bacteriology Symposium Supplement*, *76*(S23), 49S-60S.

Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, *6*(6), 550-556.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, *101*(3), 514-525.

Setlow, P. (2007). I will survive: DNA protection in bacterial spores. *Trends in Microbiology*, *15*(4), 172-180.

Setlow, P. (2013). Summer meeting 2013 - when the sleepers wake: the germination of spores of *Bacillus* species. *Journal of Applied Microbiology*, *115*(6), 1251-1268.

Sharp, M. D., & Pogliano, K. (1999). An *in vivo* membrane fusion assay implicates SpoIIIE in the final stages of engulfment during *Bacillus subtilis* sporulation. *Proceedings of the National Academy of Sciences*, *96*(25), 14553-14558.

Shilpakala, S. R., Atreya, C. D., & Ketha, K. M. V. (2012). Detection of bacterial spores: prospects and challenges. In E. Abel-Santos (Ed.), *Bacterial spores: current research and applications* (pp. 215-236). United Kingdom: Caister Academic Press.

Shoji, M., Kato, M., & Hashizume, S. (1995). Electrophoretic recovery of proteins from polyacrylamide gel. *Journal of Chromatography A*, *698*(1), 145-162.

Shukla, A. A., & Thömmes, J. (2010). Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends in Biotechnology*, *28*(5), 253-261.

Siddique, F., Hussain, I., Mahmood, M. S., Ahmed, S. I., & Iqbal, A. (2014). Isolation and characterization of a highly thermostable alpha-amylase enzyme produced by *Bacillus licheniformis*. *Pakistan Journal of Agriculture Sciences*, *51*(2), 309-314.

Singh, R., Mukherjee, M. D., Sumana, G., Gupta, R. K., Sood, S., & Malhotra, B. (2014). Biosensors for pathogen detection: a smart approach towards clinical diagnosis. *Sensors and Actuators B: Chemical*, *197*, 385-404.

Skottrup, P. D., Nicolaisen, M., & Justesen, A. F. (2008). Towards on-site pathogen detection using antibody-based sensors. *Biosensors and Bioelectronics*, *24*(3), 339-348.

Smith, K., Bayer, M. E., & Youngman, P. (1993). Physical and functional characterization of the *Bacillus subtilis spoIIM* gene. *Journal of Bacteriology*, *175*(11), 3607-3617.

Smitha, R. B., Jisha, V. N., Pradeep, S., Josh, M. S., & Benjamin, S. (2013). Potato flour mediated solid-state fermentation for the enhanced production of *Bacillus thuringiensis*-toxin. *Journal of Bioscience and Bioengineering*, *116*(5), 595-601.

Snoke, J. E. (1964). Amino acid composition of *Bacillus licheniformis* spore coat. *Biochemical and Biophysical Research Communications*, *14*(6), 571-574.

Spencer, R. C. (2003). *Bacillus anthracis*. *Journal of Clinical Pathology*, *56*(3), 182-187.

Steil, L., Serrano, M., Henriques, A. O., & Völker, U. (2005). Genome-wide analysis of temporally regulated and compartment-specific gene expression in sporulating cells of *Bacillus subtilis*. *Microbiology*, *151*(2), 399-420.

Stragier, P., & Losick, R. (1996). Molecular genetics of sporulation in *Bacillus subtilis*. *Annual Review of Genetics*, *30*(1), 297-341.

Sun, D. X., Cabrera-Martinez, R. M., & Setlow, P. (1991). Control of transcription of the *Bacillus subtilis spoIIIG* gene, which codes for the forespore-specific transcription factor σ^G. *Journal of Bacteriology*, *173*(9), 2977-2984.

Sundaram, R. K., Balasubramaniyan, N., & Sundaram, P. (2012). Protein stains and applications. In B. T. Kurien & R. H. Scofield (Eds.), *Protein electrophoresis: methods and protocols* (pp. 403-405). New Jersey: Humana Press.

Swerdlow, B. M., Setlow, B., & Setlow, P. (1981). Levels of H^+ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. *Journal of Bacteriology*, *148*(1), 20-29.

Tanaka, T., Ito, A., & Kamimado, H. (2012). Control of *Bacillus licheniformis* spores isolated from dairy materials in yogurt production. *Biocontrol Science*, *17*(4), 169-173.

Taylor, A. D., Ladd, J., Yu, Q., Chen, S., Homola, J., & Jiang, S. (2006). Quantitative and simultaneous detection of four foodborne bacterial pathogens with a multi-channel SPR sensor. *Biosensors and Bioelectronics*, *22*(5), 752-758.

Te Giffel, M. C., Wagendorp, A., Herrewegh, A., & Driehuis, F. (2002). Bacterial spores in silage and raw milk. *Antonie van Leeuwenhoek*, *81*, 625-630.

Tewari, A., & Abdullah, S. (2014). *Bacillus cereus* food poisoning: international and Indian perspective. *Journal of Food Science and Technology*, 1-12.

Thissen, J. B., McLoughlin, K., Gardner, S., Gu, P., Mabery, S., Slezak, T., & Jaing, C. (2014). Analysis of sensitivity and rapid hybridization of a multiplexed Microbial Detection Microarray. *Journal of Virological Methods*, *201*, 73-78.

Thompson, B. M., Binkley, J. M., & Stewart, G. C. (2011). Current physical and SDS extraction methods do not efficiently remove exosporium proteins from *Bacillus anthracis* spores. *Journal of Microbiological Methods*, *85*(2), 143-148.

Tims, T. B., & Lim, D. V. (2004). Rapid detection of *Bacillus anthracis* spores directly from powders with an evanescent wave fiber-optic biosensor. *Journal of Microbiological Methods*, *59*(1), 127-130.

Todd, S. J., Moir, A. J. G., Johnson, M. J., & Moir, A. (2003). Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *Journal of Bacteriology*, *185*(11), 3373-3378.

Tola, Y. B., & Ramaswamy, H. S. (2014a). Combined effects of high pressure, moderate heat and pH on the inactivation kinetics of *Bacillus licheniformis* spores in carrot juice. *Food Research International*, *62*, 50-58.

Tola, Y. B., & Ramaswamy, H. S. (2014b). Thermal destruction kinetics of *Bacillus licheniformis* spores in carrot juice extract as influenced by pH, type of acidifying agent and heating method. *LWT-Food Science and Technology*, *56*(1), 131-137.

Torun, Ö., Boyacı, İ. H., Temür, E., & Tamer, U. (2012). Comparison of sensing strategies in SPR biosensor for rapid and sensitive enumeration of bacteria. *Biosensors and Bioelectronics, 37*(1), 53-60.

Traag, B. A., Pugliese, A., Eisen, J. A., & Losick, R. (2013). Gene conservation among endospore-forming bacteria reveals additional sporulation genes in *Bacillus subtilis*. *Journal of Bacteriology*, *195*(2), 253-260.

Tsumoto, K., Ejima, D., Kumagai, I., & Arakawa, T. (2003). Practical considerations in refolding proteins from inclusion bodies. *Protein Expression and Purification*, *28*(1), 1-8.

Turnbull, P. C. B., & Kramer, J. M. (1995). *Bacillus*. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Yolken (Eds.), *Manual of clinical microbiology* (6th ed., pp. 349-356). Washington D.C.: American Society for Microbiology Press.

Vasantha, N., & Freese, E. (1979). The role of manganese in growth and sporulation of *Bacillus subtilis*. *Journal of General Microbiology*, *112*(2), 329-336.

Velásquez, J., Schuurman-Wolters, G., Birkner, J. P., Abee, T., & Poolman, B. (2014). *Bacillus subtilis* spore protein SpoVAC functions as a mechanosensitive channel. *Molecular Microbiology*, *92*(4), 813-823.

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., & Adley, C. (2010). An overview of foodborne pathogen detection: in the perspective of biosensors. *Biotechnology Advances*, *28*(2), 232-254.

Venir, E., Del Torre, M., Cunsolo, V., Saletti, R., Musetti, R., & Stecchini, M. L. (2014). Involvement of alanine racemase in germination of *Bacillus cereus* spores lacking an intact exosporium. *Archives of Microbiology*, *196*(2), 79-85.

Voigt, B., Schroeter, R., Schweder, T., Jürgen, B., Albrecht, D., Dijl, J. M. v., Maurer, K. H., & Hecker, M. (2014). A proteomic view of cell physiology of the industrial workhorse *Bacillus licheniformis*. *Journal of Biotechnology*. doi: 10.1016/j.jbiotec.2014.06.004

Vos, P. D., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K. H., & Whitman, W. B. (2011). *Bergey's manual of systematic bacteriology (Vol. 3): the Firmicutes*. New York: Springer.

Vyletělová, M., Švec, P., Páčová, Z., Sedláček, I., & Roubal, P. (2002). Occurrence of *Bacillus cereus* and *Bacillus licheniformis* strains in the course of UHT milk production. *Czech Journal of Animal Science*, *47*(5), 200-205.

Walker, J. (2010a). Electrophoretic techniques. In K. Wilson & J. Walker (Eds.), *Principles and techniques of biochemistry and molecular biology* (pp. 399-432). United Kingdom: Cambridge University Press.

Walker, J. (2010b). Protein structure, purification, characteristics and function analysis. In K. Wilson & J. Walker (Eds.), *Principles and techniques of biochemistry and molecular biology* (pp. 300-351). United Kingdom: Cambridge University Press.

Wang, D. B., Bi, L. J., Zhang, Z. P., Chen, Y. Y., Yang, R. F., Wei, H. P., Zhou, Y. F., & Zhang, X. E. (2009). Label-free detection of *B. anthracis* spores using a surface plasmon resonance biosensor. *Analyst*, *134*(4), 738-742.

Wang, S. T., Setlow, B., Conlon, E. M., Lyon, J. L., Imamura, D., Sato, T., Setlow, P., Losick, R., & Eichenberger, P. (2006). The forespore line of gene expression in *Bacillus subtilis*. *Journal of Molecular Biology*, *358*, 16-37.

Wang, Y., Ye, Z., Si, C., & Ying, Y. (2013). Monitoring of *Escherichia coli* O157:H7 in food samples using lectin based surface plasmon resonance biosensor. *Food Chemistry*, *136*, 1303-1308.

Warth, A. D., & Strominger, J. L. (1972). Structure of the peptidoglycan from spores of *Bacillus subtilis*. *Biochemistry*, *11*(8), 1389-1396.

Watterson, M. J., Kent, D. J., Boor, K. J., Wiedmann, M., & Martin, N. H. (2014). Evaluation of dairy powder products implicates thermophilic sporeformers as the primary organisms of interest. *Journal of Dairy Science*, *97*(4), 2487-2497.

Wei, D., Oyarzabal, O. A., Huang, T. S., Balasubramanian, S., Sista, S., & Simonian, A. L. (2007). Development of a surface plasmon resonance biosensor for the identification of *Campylobacter jejuni*. *Journal of Microbiological Methods*, *69*(1), 78-85.

Wilson, K. (2010). Chromatographic techniques. In In K. Wilson & J. Walker (Eds.), *Principles and techniques of biochemistry and molecular biology* (pp. 433-476). United Kingdom: Cambridge University Press.

Wu, J. J., Howard, M. G., & Piggot, P. J. (1989). Regulation of transcription of the *Bacillus subtilis spoIIA* locus. *Journal of Bacteriology*, *171*(2), 692-698.

Wu, L. J., & Errington, J. (2000). Identification and characterization of a new prespore-specific regulatory gene, *rsfA*, of *Bacillus subtilis*. *Journal of Bacteriology*, *182*(2), 418-424.

Xu, D., & Côté, J. C. (2003). Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences. *International Journal of Systematic and Evolutionary Microbiology*, *53*(3), 695-704.

Yamaguchi, H., & Miyazaki, M. (2014). Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. *Biomolecules*, *4*(1), 235-251.

Yang, Z., Zhang, L., Zhang, Y., Zhang, T., Feng, Y., Lu, X., Lan, W., Wang, J., Wu, H., Cao, C., & Wang, X. (2011). Highly efficient production of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. *PloS One*, *6*(7), e22981.

Young, S. B., & Setlow, P. (2004a). Mechanisms of killing of *Bacillus subtilis* spores by Decon and Oxone^{TM}, two general decontaminants for biological agents. *Journal of Applied Microbiology, 96*(2), 289-301.

Young, S. B., & Setlow, P. (2004b). Mechanisms of *Bacillus subtilis* spore resistance to and killing by aqueous ozone. *Journal of Applied Microbiology*, *96*(5), 1133-1142.

Zhang, D., Yan, Y., Li, Q., Yu, T., Cheng, W., Wang, L., Ju, H., & Ding, S. (2012). Label-free and high-sensitive detection of *Salmonella* using a surface plasmon resonance DNA-based biosensor. *Journal of Biotechnology*, *160*(3), 123-128.