ANTIBIOFILM ACTIVITIES OF PROTEIN KINASE INHIBITORS AGAINST *Salmonella enterica* SEROVAR TYPHIMURIUM

MOHD FAKHARUL ZAMAN RAJA YAHYA

FACULTY OF SCIENCE
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ANTIBIOFILM ACTIVITIES OF PROTEIN KINASE INHIBITORS AGAINST *Salmonella enterica* SEROVAR TYPHIMURIUM

ABSTRACT

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is an important biofilm producer which causes severe gastroenteritis in humans and other mammals. Many antibiotics have been tested to eradicate *S. typhimurium* infection, however, the gastroenteritis remains a major health problem while the application of known protein kinase inhibitor in combating *S. typhimurium* biofilm is still not well investigated. To address this issue, two protein kinase inhibitors namely dimethyl sulfoxide (DMSO) and afatinib were evaluated against *S. typhimurium* biofilm. It was demonstrated that both 32% DMSO and its combination with 3.2 µg/mL afatinib which was termed DMSO-diluted afatinib (DDA) were effective in killing biofilm cells, reducing biofilm biomass and chemically modifying extracellular polymeric substances (EPS) matrix. These antibiofilm effects were also observed in other biofilm forming bacteria. Of the two protein kinase inhibitors, DMSO was selected as the effective antibiofilm compound. To understand the possible molecular basis underlying the antibiofilm effect of DMSO against *S. typhimurium* biofilm, the protein profiles of whole cells and EPS matrix were investigated. Subtractive protein profile analysis recognized two unique protein bands (25.4 kDa and 51.2 kDa) of whole cells which were present only in control biofilm and not in 32% DMSO-treated biofilm. In turn, 29 and 46 proteins were successfully identified from the protein bands of 25.4 kDa and 51.2 kDa respectively. The protein band of 51.2 kDa was also observed to be uniquely present in 32% DMSO-treated EPS matrix. Three proteins were successfully identified from this EPS protein band. In the next step, protein interaction network analysis identified several biological processes such as glycolysis, PhoP-PhoQ phosphorelay signalling and flagellar biosynthesis to be significantly ($p<0.05$) inhibited by 32% DMSO. In addition, subtractive *in silico*

analysis revealed that 41 out of 75 identified proteins from the whole cells were essential for survival of *S. typhimurium* and were non-homologous to human host, making them ideal therapeutic targets for biofilm control. Collectively, this study demonstrated the remarkable antibiofilm effects of DMSO against *S. typhimurium* biofilm and its molecular basis. These findings develop a new insight into how to combat diseases mediated by *S. typhimurium* biofilm.

Keywords: Biofilm; *Salmonella typhimurium*; protein kinase; extracellular polymeric substances

AKTIVITI ANTIBIOFILEM PERENCAT PROTEIN KINASE KE ATAS *Salmonella enterica* SEROVAR TYPHIMURIUM

ABSTRAK

Salmonella enterica serovar Typhimurium (*S. typhimurium*) merupakan satu bakteria penghasil biofilem yang penting yang menyebabkan gastroenteritis yang serius dalam manusia dan mamalia lain. Banyak antibiotik telah diuji untuk menghapuskan jangkitan *S. typhimurium*, walaubagaimanapun, gastroenteritis masih merupakan masalah kesihatan major manakala penggunaan perencat protein kinase dalam memerangi biofilem *S. typhimurium* masih tidak disiasat dengan baik. Untuk menangani isu ini, dua perencat protein kinase iaitu dimethyl sulfoxide (DMSO) dan afatinib telah diuji ke atas biofilem *S. typhimurium.* 32% DMSO dan kombinasinya dengan 3.2 µg/mL afatinib yang diistilahkan sebagai *DMSO-diluted afatinib (DDA)* adalah efektif dalam membunuh sel biofilem, mengurangkan biojisim biofilem dan mengubahsuai matrik bahan polimerik ekstraselular (EPS) secara kimia. Kesan antibiofilem ini juga telah dilihat dalam bakteria penghasil biofilem yang lain. Antara dua perencat protein kinase ini, DMSO telah dianggap sebagai sebatian antibiofilem yang efektif. Untuk memahami asas molekular bagi kesan antibiofilem DMSO ke atas biofilem *S. typhimurium*, profil protein bagi keseluruhan sel dan matrik EPS telah disiasat. Analisis profil protein subtraktif telah mengenalpasti dua jalur protein unik (25.4 kDa and 51.2 kDa) bagi keseluruhan sel yang hanya hadir dalam biofilem kawalan dan tidak hadir dalam biofilem terawat 32% DMSO. Sebanyak 29 dan 46 protein telah dikenalpasti dari jalur protein 25.4 kDa dan 51.2 kDa masing-masing. Jalur protein 25.4 kDa juga telah didapati hadir secara unik di dalam matrik EPS terawat 32% DMSO. Sebanyak tiga protein telah dikenalpasti dari jalur protein EPS ini. Dalam peringkat seterusnya, analisis jaringan interaksi protein telah mengenalpasti beberapa proses biologi seperti glikolisis, pengisyaratan fosforelai PhoP-PhoQ dan biosintesis flagelar telah terencat

secara signifikan ($p<0.05$) oleh 32% DMSO. Sebagai tambahan, analisis *in silico* subtraktif telah menunjukkan bahawa 41 daripada 75 protein yang telah dikenalpasti merupakan penting untuk kemandirian *S. typhimurium* dan tak homologus dengan manusia, menjadikannya sasaran terapiutik yang ideal untuk kawalan biofilem. Secara keseluruhannya, kajian ini mempamerkan kesan antibiofilem DMSO ke atas *S. typhimurium* yang nyata dan asas molekularnya. Hasil kajian ini membina satu pemahaman baru berkenaan bagaimana memerangi penyakit yang berperantarakan biofilem *S. typhimurium.*

Kata kunci: Biofilm; *Salmonella typhimurium*; protein kinase; extracellular polymeric substances

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Mohd Fakharul Zaman Raja Yahya

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LIST OF SYMBOLS AND ABBREVIATIONS

µ	Micro
%	Perecentage
;	Semicolon
*	Asterisk
±	Plus-minus
AHL	N-Acyl Homoserine Lactones
AI	autoinducer
AMP	antimicrobial peptide
ATP	adenosine triphosphate
ATR-FTIR	attenuated total reflection fourier transform infrared
BAP	biomass-associated products
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CFU	colon forming unit
CID	collision-induced dissociation
CLSM	confocal laser scan microscopy
DDA	DMSO diluted afatinib
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
$DMSO_2$	dimethyl sulfone
DNA	deoxyribonucleic acid
DPD	4,5-dihydroxy-2,3-pentanedione
ELISA	enzyme-linked immunosorbent assay
EPS	extracellular polymeric substance
ESI	electrospray ionization

FDA	food and drug act
FDR	false discovery rate
FTICR	fourier transform ion cyclotron resonance
GFP	green fluorescent protein
GMP	guanosine monophosphate
GO	gene ontology
HPLC	high performance liquid chromatography
IDA	information dependent acquisition
IFM	infinite focus microscope
iTRAQ	isobaric tags for relative and absolute quantitation
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCM	laser capture microdissection
LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
MALDI	matrix assisted laser desorption ionization
MAPK	mitogen-activated protein kinase
MHSA	mannose-sensitive haemagglutinin type IV pilus
MIC	minimum inhibitory concentration
MLST	multi locus sequence typing schemes
MRM	multiple reaction monitoring
MS	mass spectrometry
MudPIT	multidimensional protein identification technology
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PFGE	pulsed-field gel electrophoresis
PKC	protein kinase C

PMF	peptide mass fingerprinting
QAS	quaternary ammonium salt
QTOF	quadrupole time of flight
RAPD-PCR	random amplified polymorphic DNA PCR
REP-PCR	repetitive extragenic palindromic PCR
RNA	ribonucleic acid
ROCK	Rho-associated kinase
RP	reversed-phase chromatography
SCV	salmonella-containing vacuole
SCX	strong cation exchange
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SID	surface-induced dissociation
SILAC	stable isotope labeling with amino acids in cell culture
SMART	simple modular architecture research tool
SMP	soluble microbial products
SRH	*S*-ribosylhomocysteine
SRM	selective reaction monitoring
STRING	search tool for the retrieval of interacting genes/proteins
TA	toxin-antitoxin
TBT	tri-n-butyltin
TMHMM	transmembrane hidden markov model
TOF	time of flight
TTSS	type three secretion system
UAP	utilization-associated products
UPEC	uropathogenic *E. coli*

CHAPTER 1: INTRODUCTION

1.1 Introduction

Biofilm, a sessile and densely packed microbial community is the primary mode of microbial existence. The biofilm which is encapsulated in extracellular polymeric substance (EPS) matrix can form on biotic surfaces which include plants, animals and other microbes. It can also grow on abiotic surfaces such as minerals, steel, the chitinous covering of dead organisms and air-liquid interfaces. The biofilm fraction differs from suspended fraction in the gene and protein expression patterns conferring greater protection against antibiotics and the human immune system (Costerton et al., 1999; Sauer, 2002). These differences are a consequence of the heterogeneous mixture of flat-cell monolayer biofilm and three-dimensional mushroom-shaped biofilm (Entcheva-Dimitrov & Spormann, 2004; Karatan & Watnick, 2009). According to Mah and O'Toole (2001), a heterogeneous biofilm is often dependent on the gradients of nutrients, waste products and signalling molecules. Based on a 3D multiphasic hydrodynamic model which is calibrated against confocal laser scan microscopy (CLSM) data, the heterogeneous biofilm is less susceptible to antimicrobial treatment correlating with higher volume fractions of the persister and EPS (Zhao et al., 2016). One of the important biofilm-forming bacteria is *Salmonella typhimurium*.

Salmonella, a common food-borne pathogenic bacterium can cause a broad range of illness. General symptoms of salmonellosis may include fever, vomiting, bloody diarrhoea, body aches and headache whilst the most common clinical manifestation is gastroenteritis. The incidence of non-typhoidal salmonellosis typically arises from consumption of contaminated food-stuffs (Andreoletti, 2008) causing many human death every year. *S. typhimurium* is a non-typhoidal salmonella serovar. It is host generalist that occur in human and many other mammalian species while other serovar such as *S. typhi* is host specialist that infects only humans (Anonymous, 2005). In

Malaysia, *S. typhimurium* is regarded as one of the most common non-typhoidal serovars and the national disease surveillance for this serovar mainly involve phage typing by pulsed-field gel electrophoresis (PFGE) (Anonymous, 2005). Recently, a total of 43 *S. enterica* isolates including *S. typhimurium* were isolated from catfish (*Clarias gariepinus*) and tilapia (*Tilapia mossambica*) and were extensively studied for the genetic relatedness using random amplified polymorphic DNA PCR (RAPD-PCR), repetitive extragenic palindromic PCR (REP-PCR), and pulsed-field gel electrophoresis (PFGE) (Titik et al., 2016). Their results suggested the influence of several factors such as the quality of the water, density of fish, and size of ponds on the observed close genetic relationship. In recent years, protein kinase inhibitors have received increasing attention in the control of pathogenic bacteria.

Protein kinase is an enzyme that functions in modifying other proteins by phosphorylation and its activity could be reversed by protein phosphatase. The phosphorylation process catalyzed by the protein kinases may take place on serine, threonine, tyrosine and histidine residues and is crucial for protein activation, protein-protein interactions and subcellular localization of proteins. In general, protein kinase plays an important role in transducing signals from the cell membrane into the interior of the cells that participate in normal cellular physiology (Hunter, 1995), infectious diseases (Hale et al., 2006) and also non-infectious diseases (Force et al., 2004). Over the past few decades, protein kinase inhibitors have been frequently used in cancer treatment (Zhang et al., 1997; Anderson et al., 1998; Meyer et al., 2006; Zhang & Munster, 2014). This phenomenon has gradually led to application of the protein kinase inhibitors in antimicrobial treatment because the progress of both infectious and non-infectious disease is greatly dependent on the protein kinase activities (Force et al., 2004; Hale et al., 2006). Many protein kinase inhibitors such as walkmycin C,

palmitoyl-DL-carnitine chloride, hypericin, staurosporine, tryphostin and sphingosine have been demonstrated to impede the biofilm development (Eguchi et al., 2011; Wenderska et al., 2011; Nguyen et al., 2011). A recent study has successfully shown the killing effects of WalK-histidine kinase inhibitor (NH125) on methicillin-resistant *Staphylococcus aureus* (MRSA) persisters, however, no standard antibiofilm screening has been performed (Kim et al., 2016). This, in part, implies that the application of protein kinase inhibitors possesses high potential to modulate the biofilms although the protein kinases are much less widespread in bacterial metabolism. A potent protein kinase inhibitor which is often neglected in the signal transduction research due to its wide range of pharmacological application is dimethyl sulfoxide (DMSO).

DMSO is a naturally occurring substance which is also a commercially manufactured dipolar aprotic solvent. An *in vivo* study has revealed that it is a relatively low-toxicity organic solvent with LD_{50} values of 6.2 ml/kg and 9.9 ml/kg in mice and rats respectively (Bartsch et al., 1976). DMSO is also known to produce no or minor cytotoxic effects on the pulp tissue repair-related activity of odontoblast-like cells (Hebling et al., 2015). As evidenced by an *in vivo* model and firefly luciferase assay, DMSO does not show any potential to induce cell apoptosis (Wang et al., 2016) which is one of the mechanisms of action of known cytotoxic agents. The safe application of DMSO for topical and oral treatments typically involves the concentration up to 40% (Morton, 1993; Zuurmond, 1996) whilst the DMSO concentration up to 50% DMSO solution has been approved by US FDA for dermatological application (Capriotti & Capriotti, 2012). One possible explanation for the use of high dosage of DMSO *in vivo* is that DMSO is rapidly metabolized to $DMSO_2$ and DMS and excreted, primarily through urinary excretion or in expired air, respectively (Elisia et al., 2016). The antibacterial effects of DMSO against a wide spectrum of microorganisms has long

been evidenced (Basch, 1968). On the other hand, DMSO is an important modulator of protein kinase C (PKC) during cell differentiation. While low concentration of DMSO (<0.6%) stimulates the activity of PKC, higher concentrations of DMSO causes a dose-dependent decrease in PKC activity (Balazovich et al., 1987). It also specifically inhibits enzymatic activity of pyruvate kinase in proliferating rat basophilic leukemia 2H3 (RBL-2H3) cells and FcepsilonRI-mediated tyrosine phosphorylation of pyruvate kinase in allergenic activated RBL-2H3 cells (Koo & Kim, 2009). Additionally, phosphorylation of Akt, p38, JNK and ERK1/2 in human monocytes was attenuated in the presence of 2% DMSO (Elisia et al., 2016). Although DMSO is known to inhibit gene expression of OmpR/ ToxR-type transcriptional regulator HilA in *S. typhimurium* and host cell invasion (Antunes et al., 2010), the impact of DMSO on other molecular aspects such as whole-cell protein expression over the course of biofilm formation and protein secretion in EPS matrix remains not investigated. Considering its safe application and the kinase inhibitory properties, DMSO deserve further attention in the signal transduction research.

Over the past few decades, signal transduction research has employed antibody-based methods such as Western blotting and enzyme-linked immunosorbent assay (ELISA) to investigate the molecular mechanisms of protein kinase inhibitors. These methods have limited the identification of previously unknown protein kinase targets and are unable to elucidate the signaling pathway interactions. To address those issues, many researchers have shifted their approaches from the traditional antibody-based methods to the high throughput tandem mass spectrometric strategies. For example, Godl et al. (2003) successfully identified the novel protein kinase targets of p38 kinase inhibitor SB203580, an anti-inflammatory drug namely RICK [Rip-like interacting caspase-like apoptosis-regulatory protein (CLARP) kinase/Rip2/CARDIAK] whilst Gharechahi et al.,

(2014) discovered the signaling pathway interaction between Rho-associated kinase (ROCK) and epigenetic mechanism of some chromatin modifying proteins. These studies show that the mass spectrometric strategy offers remarkable experimental advantages over the antibody-based methods. Thus, the tandem mass spectrometry-based proteomics may substantially contribute to elucidation of pathways associated with the biofilm inhibitory mechanisms.

1.1.1 Problem statement

Biofilms are an important cause of chronic infection and persistent public health problem worldwide. Formation of biofilm begins with attachment of microbial cells to the surface and continues with chemical signalling-mediated recruitment of adjacent cells to the attachment site and encapsulation of aggregated cells in hydrated EPS. These sequential processes of biofilm formation would lead to the establishment of heterogeneous multilayer biofilm which possesses an extreme capacity for evading the host defences.

In the last few decades, multiple lines of works have revealed the potential use of protein kinase inhibitor in controlling cancer, malaria and autoimmune diseases. The same strategy has been shown to effectively hinder biofilm formation and thus revolutionize the existing antimicrobial treatment plans to combat antimicrobial resistance. *S. typhimurium* is an important biofilm-forming pathogen that confers resistance to various antibiotics. While many stress factors such as heat, low pH and disinfectants have been shown to control *S. typhimurium* infection, application of protein kinase inhibitor such as DMSO and afatinib in combating *S. typhimurium* biofilm remains not well investigated.

Insights into the molecular basis of the antibiofilm treatment would substantially improve the fundamental knowledge regarding biofilm survival and therapeutic strategy. Considering numerous experimental evidences that the exposure of microorganisms to the potential antimicrobial agents could result in differential protein expression, inhibition of *S. typhimurium* biofilm by the protein kinase inhibitor may associate with differential protein expression in the whole cells and EPS matrix.

1.1.2 Objectives of study

1. To evaluate antibiofilm activity of DMSO and afatinib on *S. typhimurium.*

2. To understand the molecular basis of antibiofilm activity of selected protein kinase inhibitor.

3. To identify the proteins associated with the biofilm inhibitory effects of the selected protein kinase inhibitor.

4. To investigate the functional linkages among QTOF-identified proteins.

5. To evaluate the potential of QTOF-identified proteins as antibiofilm targets.

1.1.3 Significance of study

The complexity of biofilm physiology warrants further attention and requires research contributions from diverse disciplines such as microbiology, biochemistry and analytical chemistry. In general, this study raises the awareness among people about the negative impacts of biofilms on the overall quality of life and points to more effective strategies to control antimicrobial resistance. These are in line with the National Key

Areas to improve healthcare and serve as a hub for medical tourism. For the future researchers, this study provides the baseline information on antibiofilm properties of known protein kinase inhibitor and an insight into its molecular mechanism which may be helpful in improving the existing antimicrobial treatment plans. This study also highlights the gel-based subtractive protein profiling as a novel approach to study the heterogeneous protein expression in *S. typhimurium* biofilm.

1.1.4 Thesis outline

This thesis is divided into six chapters. Chapter 1 explains the basic concept of key elements of this work such as biofilm and protein kinase, the main objectives, motivations for the development of this work and limitations of work. Chapter 2 encloses the literature review which expand the discussion on the previously explained key elements. The literature review also addresses the potential strategies to make the MS/MS-based proteomic analysis of heterogeneous biofilm more accurate and reliable. In Chapter 3, the materials and methodologies used to perform antibiofilm screening and MS/MS-based proteomic analysis are fully described. Special attention is given to the MS/MS-identified proteins whose functional characteristics are intensively studied using a series of publicly available bioinformatics tools. The results are presented in a well-structured manner in Chapter 4. This chapter provides justification for each analytical approach used herein and highlights the correlation between the main data. In Chapter 5, the findings from this work were interpreted, clearly linked to the published works and evaluated for contributions to the research area. Chapter 6 summarizes all the research findings and presents some ideas and suggestions for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Biofilm

Biofilm is a microbial community that tend to attach to either biotic or abiotic surface. For many decades, it has been the major focus in the study of antibiotic resistances. Protected by a matrix of extracellular polymeric substance (EPS), the biofilm differs from its planktonic counterparts in the gene and protein expression patterns resulting in distinct phenotypes including altered resistance to antibiotics and the human immune system (Costerton et al., 1999; Sauer, 2003). In most cases, the difficulties in combating the biofilm-associated diseases are due to the presence of persisters, the microbial cells that neither grow nor die when exposed to bactericidal agents (Lewis, 2005).

There are several fundamental aspects of biofilm that warrant special attentions, in the search for antibiofilm agents namely biofilm formation and its negative impacts, EPS, persisters in biofilm, and quorum sensing signals. Among the common approaches for biofilm studies are microtitre plate system and flow cell assay system. These assay systems significantly facilitate a wide spectrum of biofilm studies.

2.1.1 Formation of biofilm

In general, formation of mature bacterial biofilm involves five sequential stages as follows: (i) an initial reversible attachment to a pre-conditioned surface, (ii) transition from reversible to irreversible attachment, (iii) development of biofilm architecture, (iv) development of microcolonies into a mature biofilm, and (v) dispersion of cells from the biofilm into the surrounding environment (Stoodley et al., 2002; Van Houdt & Michiels, 2005; Monroe, 2007). This general model of biofilm formation is illustrated in Figure 2.1 while Figure 2.2 shows the representative biofilm-forming bacteria.

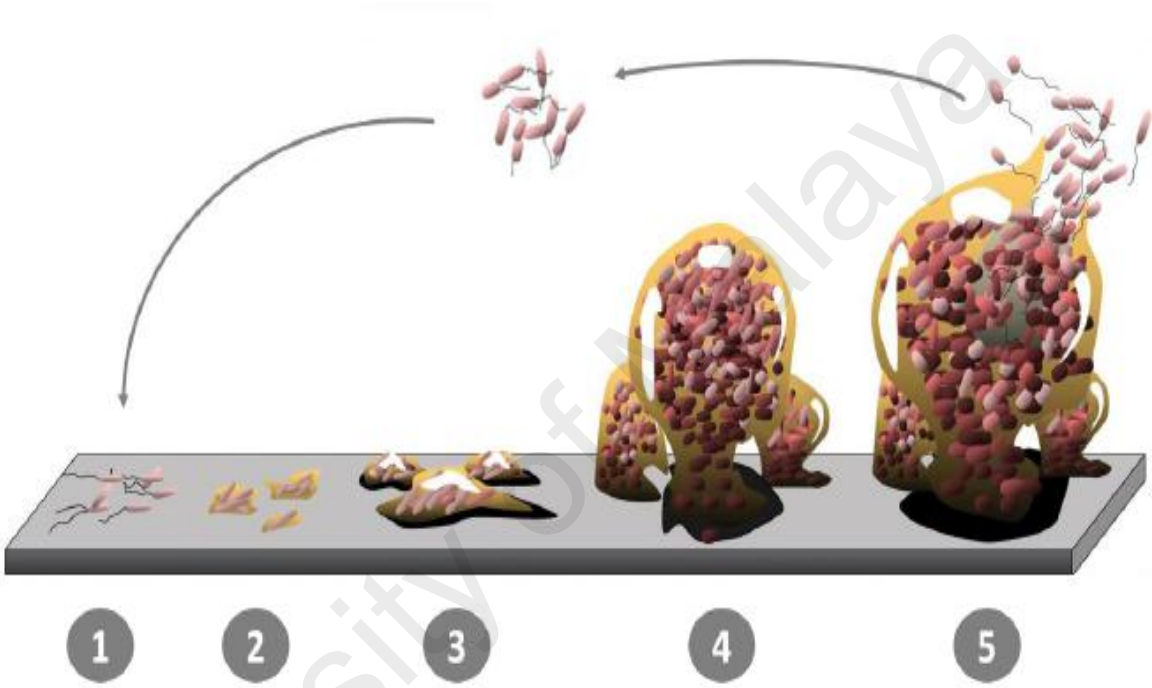


Figure 2.1: Biofilm formation process. 1: initial reversible attachment; 2: irreversible attachment; 3: development of biofilm architecture; 4: maturation; 5: biofilm dispersal (Monroe, 2007)

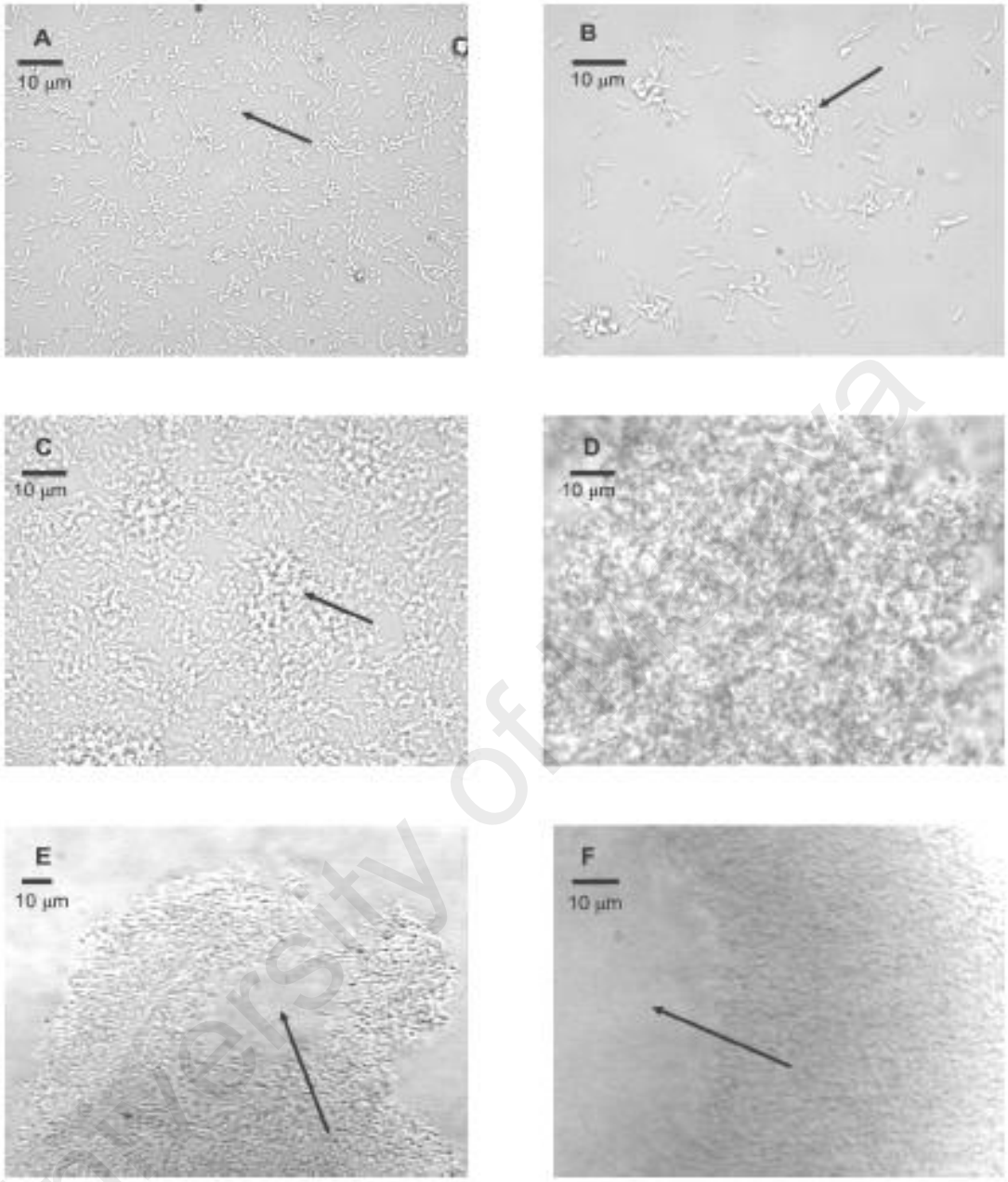


Figure 2.2: Transitional stages in biofilm development by *P. aeruginosa* under light microscope (Sauer et al., 2002). A: reversible attachment; B: irreversible attachment which is characterized by cell clusters (arrow); C: early matured biofilm which is characterized by matured cell clusters embedded in EPS matrix; D: matured cell cluster embedded in EPS matrix with maximum thickness; E and F: dispersion which is characterized by evacuation of cells forming void spaces.

During initial attachment of microbial cells, the flagellum is the operative structure in surface sensing by motile bacteria (Karatan & Watnick, 2009). It helps the bacterial cells to make a decision between the sessile and free-living lifestyle. According to Absalon et al. (2011), the two matrix-associated proteins, Bap1 and RbmA, are also crucial for intercellular adhesion and recruitment of free-floating bacterial cells to the surface respectively. In the context of transcriptional programme, expression of flagellar genes is repressed whereas expression of a large number of methyl-accepting chemotaxis genes is upregulated during biofilm formation (Moorthy & Watnick, 2004). Additionally, there is an inverse regulation between flagellar gene expression and synthesis of biofilm EPS (Moorthy & Watnick, 2004). Basically, biofilms can be classified into monolayer biofilm and multilayer biofilm.

2.1.1.1 Monolayer and multilayer biofilms

Attachment of microbial cells to a surface is either as single cells or clusters of cells would lead to formation of monolayer (immature) or multilayer (mature) biofilm respectively. In particular, the formation of monolayer biofilm begins when the cell-surface interaction is greater than the cell-cell interaction. In turn, the transient attachment of monolayer biofilm undergoes transition to the permanent attachment upon changes in the membrane potential (Van Dellen et al., 2008). The production of adhesive EPS matrix facilitates intercellular adhesion leading to the formation of multilayer of biofilm. The structural differences between the monolayer and multilayer biofilms have been studied by Karatan and Watnick (2009) using confocal scanning laser microscopy as shown Figure 2.3. It was apparent that monolayer and multilayer biofilms are different in the degree of heterogeneity.

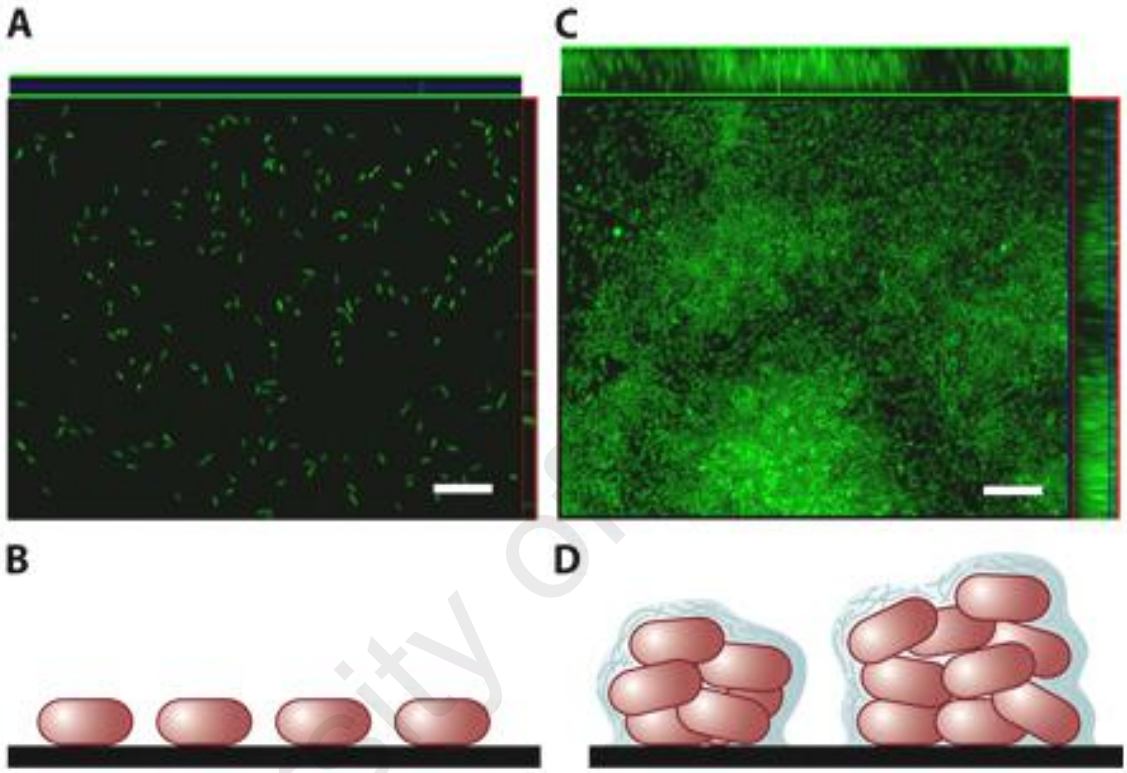


Figure 2.3: Transverse and vertical cross sections of monolayer (A and B) and multilayer(C and D) biofilms (Karatan & Watnick, 2009).

2.1.1.2 Heterogeneity of biofilms

The bacterial cells within biofilm become physiologically heterogeneous due to spatial location and chemical gradient of oxygen, nutrients, waste products and also signalling molecules (Xu et al., 1998; Stewart & Franklin, 2008). The nature of these phenomena typically results from the formation of a biphasic biofilm consisting a flat-cell monolayer biofilm (a more homogeneous collection of surface-attached cells) intermixed with three-dimensional mushroom-shaped biofilm which are different in biomass, surface coverage and average thickness (Entcheva-Dimitrov & Spormann, 2004; Karatan & Watnick, 2009). According to Moorthy and Watnick (2004), the monolayer biofilm is a transient stage in biofilm development, making it difficult to differentiate from the mature biofilm. Furthermore, the heterogeneous biofilms show greater adhesion and higher biomass than homogeneous biofilms due to lower zeta potential and electrostatic repulsion (Merode et al., 2006). Basically, zeta potential is the cell surface charges whilst electrostatic repulsion is the force between bacterial cells and the polystyrene surface repelling each other as both of them are negatively charged (Merode et al., 2006). A work by Entcheva-Dimitrov and Spormann (2004) has shown that the biofilm heterogeneity increases with incubation time and is reflected in the large standard deviation of the biomass and average thickness parameters. These findings are in agreement with Gomes et al. (2014) and Sherry et al. (2014) demonstrating the diverse spatial location and varying biomass of heterogeneous biofilms based on crystal violet assay. The microbial cells normally form a biofilm in response to various environmental factors.

2.1.1.3 Roles of environmental factors in biofilm formation

Environmental factors such as specific or non-specific attachment sites on surface, nutritional cues, temperature, oxygen level, salinity, pH and antibiotics often trigger the

biofilm formation. In other words, the environmental factors modulate microbial gene expression which in turn determine the decision of cells to form or leave a biofilm. For example, nutrient depletion would induce development of multilayer biofilm of *S. typhimurium* (Gerstel & Romling, 2001). In contrast, nutrient-rich environments promote formation of *V. cholera* biofilm (Yildiz et al., 2004). Also, Hoffman et al., (2005) reported that the treatment with tobramycin at subinhibitory concentrations induced the biofilm formation in *P. aeruginosa.* Since many environmental factors can influence biofilm formation dynamics, limiting the negative impacts of biofilm remains a great challenge.

2.1.2 Negative impacts of biofilm

Accumulation of microorganisms and deposition of their metabolic by-products would normally cause a wide spectrum of negative impacts such as food contamination, poor fuel consumption in ships, equipment corrosion and chronic infections in humans.

2.1.2.1 Food contamination

Salmonella species is the common pathogen causing food contamination. It can form biofilm on various food contact surface during food processing such as glass (Armon et al., 1997), stainless steel (Joseph et al., 2001), a variety of synthetic plastic (Joseph et al., 2001) and egg conveyor belt (Stocki, 2007). Interestingly, food contamination by *S. typhimurium* is mediated by quorum sensing (QS), a cell-to-cell communication system (Skandamis & Nychas, 2012). The quorum sensing system is generally known to regulate several proteolytic, lipolytic, chitinolytic, and pectinolytic activities associated with the food spoilage. According to Tarver (2009), cantaloupe melons, apples, and leafy greens are the prevalent biofilm carriers and cyclical causes of foodborne illness

outbreaks. Other biofilm-forming foodborne pathogens include *E. coli, Vibrio vulnificus* and *Listeria monocytogenes.*

2.1.2.2 Biofouling

Accumulation of water borne microorganisms on surface of man-made structures such as boat hull or buoy is referred to as biofouling, which has become an economic burden. Biofouling is known to potentially reduce the vessel speed by 10-16% which in turn increases the fuel consumption and emissions of exhaust fumes causing environmental problems (Schultz, 2007). This occurs when the formation of a slimy layer at all depths and temperatures causes an increase in surface roughness that lead to increase hydrodynamic fluid resistance. The biofouling is also responsible for the spreading of non-indigenous marine species (Piola et al., 2009) which may become invasive and disturb the ecological balances in their new habitats (Mack et al., 2000). Examples of non-indigenous marine species are clam, oyster, sea squirt and mussels.

2.1.2.3 Corrosion

Deterioration of metals may be induced by microbial activities. This often happens when specific microbial metabolic products becomes reactants in the electrochemical corrosion process and alter the physicochemical environment at the reaction sites. The microorganisms mainly related to microbial corrosion are sulfate-reducing bacteria (for example, *Desulfovibrio vulgaris*) which can survive in a wide range of pH and temperature. Since this kind of bacteria reduce sulfate in large amount to obtain energy, their metabolic activity on the surface of metallic materials frequently affect the kinetics of cathodic and anodic reactions (Jones & Amy, 2002) and chemistry of any protective layers (Little & Ray, 2002) which then potentially accelerate of equipment corrosion. In particular, the deposition of extracellular polymeric substances (such as calcium

alginates) on the metal surface interrupts oxygen transport to the metal surface leading to formation of differential aeration cell between the areas covered by biofilm (anode) and uncolonized area exposed to maximal oxygen centration (cathode) (Hamilton, 2003). This in turn facilitates the flow of electrons between the anode and the cathode which determines the rate of corrosion.

2.1.2.4 Chronic infections

Biofilms are responsible for more than 60% of all microbial infections in humans (Shunmugaperumal, 2010). It general, these infections are resulting from the failure of antibiotics to penetrate through the proteinaceous EPS matrix formed on the outer layer of the biofilm, diffuse and interact with the viable cells. The dynamic environment of EPS matrix contributes to biofilm heterogeneity (Sutherland, 2001) which typically forms the top-to-bottom gradient of decreasing antibiotic susceptibility (Fux et al., 2005). Example of chronic infections associated with the microbial biofilms are cystic fibrosis (*Pseudomonas aeruginosa*), periodontitis (*Porphyromonas gingivalis*), ear infection (*Haemophilus influenzae*), urethral infections (*Neisseria gonorhoeae*) and burn wound infections (*Pseudomonas aeruginosa*).

2.1.3 Extracellular polymeric substance of biofilm

Extracellular polymeric substances (EPS) are high-molecular mass organic molecules secreted by the microorganisms to form slime around microbial cells. EPS are mostly composed of polysaccharides and proteins, but also include other macromolecules such as DNA, lipids and humic substances. The forms of EPS that exist and protect the microbial cells against antibiotic attack can be categorized into bound EPS (sheaths, capsular polymers and condensed gels) and soluble EPS (colloids and slimes) (Laspidou & Rittmann, 2002). Bound EPS are strongly bound with cells, while soluble EPS are

weakly bound with cells or dissolved into the solution. Technically, these two types of EPS can be separated by centrifugation by which the supernatant being soluble EPS and the pellets being bound EPS (Sheng et al., 2010). The difference between bound EPS and soluble EPS is shown by Figure 2.4.

EPS matrix contain many functional groups such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups which contribute to its high binding capacity (Ha et al., 2010). Many studies have evidenced that EPS matrix plays important roles in cell aggregation (Burdman et al., 2000), biofilm formation (Decho, 2000) and protection of cells from unfavorable environments (Looijesteijn et al., 2001). Moreover, Flemming et al. (2000) has reported that EPS provides mechanical stability to biofilm architecture and form a three dimensional, gel-like, highly hydrated and negatively charged environment in which the cells are immobilized. According to Laspidou and Rittmann (2002), the relationship between EPS production and biomass growth rate is dependent on the type of microorganisms and the system conditions. They showed that the cells utilized electrons from the electron donor substrate to build active biomass and produce bound EPS and utilization-associated products (UAP). The bound EPS were then hydrolysed to biomass-associated products (BAP) whilst active biomass experiences endogenous decay to form residual dead cells. Finally, UAP and BAP were utilized by active biomass as recycled electron donors. The sum UAP and BAP is known to be soluble microbial products (SMP) or soluble EPS which are released during cell lysis, diffuse through the cell membrane, or are excreted for some purpose.

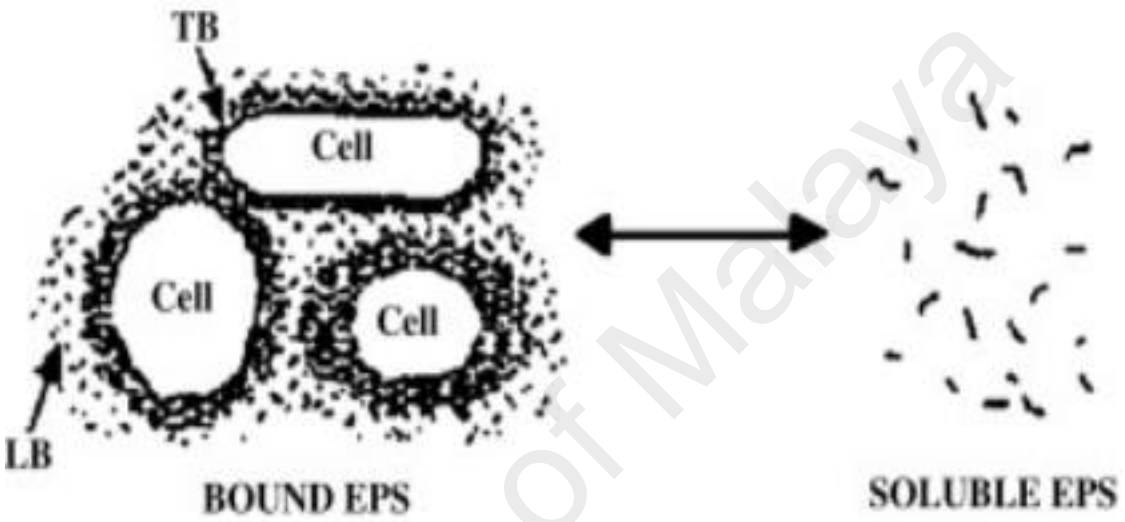


Figure 2.4: Bound and soluble EPS (Nielsen, 1999). LB: loosely bound EPS; TB: tightly bound EPS.

In 1994, Suci et al., studied the composition of EPS matrix of *P. aeruginosa* biofilm using attenuated total reflection fourier transform infrared (ATR-FTIR). They found that the treatment with ciproflaxin caused a change in the bacterial IR spectrum in the range between 1700 cm^{-1} and 1500 cm^{-1} which corresponded to amide I and II of proteins. This alteration associated with a reduction in CFU count of biofilm cells after exposure to the antibiotics for 50 hours. In addition, an investigation by Kondoh and Hashiba (1998) demonstrated a dose-dependent decrease in biofilm proteins by erythromycin at the concentrations of 10 μg mL^{-1} and higher. Considering the results that the effective test concentration of 10 μg mL^{-1} can be achieved in tissues, nasal discharge and sputum with actual clinical doses, they proposed that the erythromycin 10 μg mL^{-1} could be used to treat *P. aeruginosa* biofilm in the field of otolaryngology (Kondoh & Hashiba, 1998). On the other hand, reduction in the composition of biofilm EPS leads to weakening of biofilm and thus facilitating the entry of drugs (Baillie & Douglas, 2000). Taken together, the changes in chemical composition of biofilm EPS is an important indication in estimating the biofilm viability. In addition to EPS matrix, dormant state of persisters also plays an important role in the biofilm viability.

2.1.4 Persisters in biofilm

Antibiotic resistances are also attributed to the persisters, which are microbial cells that neither grow nor die in the presence of bactericidal agents. Inoculation of isolated persisters would regenerate the original sensitive population (Wiuff et al., 2005). There have been studies reporting the stochastic formation of persisters prior to addition of antibiotics (Keren et al., 2004); Balaban et al., 2004). However, the formation of persisters may also result from exposure to the antibiotic and is dependent on the SOS DNA damage response (Dorr et al., 2009). This shows that there are diverse persistence mechanisms for protection against antibiotics. The resistance mechanism by persister

cells in human body has been explicitly described by Lewis (2001). In brief, an initial treatment with antibiotic generally kills the majority of planktonic and biofilm cells while the immune system is responsible to kill the planktonic persisters, but the biofilm persister cells are protected from the host defences by the EPS matrix. After the antibiotic concentration drops, persisters resurrect the biofilm and the infection relapses. The formation of persisters is usually triggered by quorum sensing system.

2.1.5 Quorum sensing in biofilm

Quorum sensing is an integrated communication system that responses to changes in cell-population density. Many bacterial species utilize the quorum sensing to coordinate their behaviours and gene expression by producing and releasing chemical signal molecules called autoinducers which increase in concentration as a function of cell density. Examples for autoinducers are oligopeptides in Gram-positive bacteria, N-Acyl Homoserine Lactones (AHL) in Gram-negative bacteria, and autoinducer-2 (AI-2) in both Gram-negative and Gram-positive bacteria (Miller & Bassler, 2001). Both Gram negative and Gram positive bacteria employ quorum sensing to control a wide range of physiological activities such as symbiosis, virulence, motility, antibiotic resistance and biofilm regulation.

According to Sifri (2008), quorum sensing consists of four stages as follows: 1) production of signal molecules; 2) release of the signal molecules; 3) recognition of signal molecules by specific receptor at a threshold concentration and 4) changes in gene expression. Not only involved in the biofilm formation, the quorum sensing cascade also plays role in the biofilm dispersion. In particular, quorum sensing induces the release of extracellular DNA to stabilize the biofilm structure during developmental stage (Allesen-Holm et al., 2006) and stimulates degradation of EPS upon biofilm

dispersion (Boles & Horswill, 2008). The common bacterial quorum sensing system involved in the biofilm formation and dispersion are LasI/R RhlI/R PQS (*P. aeruginosa*), DSF (*X. campestris*), CAI1 AI2 (*V. cholera*) and Agr (*S. aureus*) (Solano et al., 2014).

Interconnection between the quorum sensing system and intracellular signaling has increasingly been investigated over the past few years. One of the common intracellular signaling molecules in a wide variety of bacteria is cyclic di-GMP (Tamayo et al., 2007). The cyclic di-GMP signalling has been proposed to integrate with the quorum sensing system to enable bacterial cells assimilate the information about the local bacterial population density and other physicochemical environmental signals (Srivastava et al., 2011). This is based on the fact that, in *V. cholera*, the cyclic di-GMP induces expression of *aphA*, a low-cell-density transcriptional quorum sensing regulator (Srivastava et al., 2011). Subsequently, the cyclic di-GMP binds with the transcriptional activator VpsR to promote gene expression during formation of *V. cholera* biofilm (Srivastava et al., 2011). Other transcriptional activators that have been demonstrated to bind to c-di-GMP and modulate the gene expression are FleQ (*P. aeruginosa*) and Clp (*X. campestris*) (Chin et al., 2010; Hickman & Harwood 2008). Measurement of quorum sensing signal for studying biofilm formation usually involves microplate assay system.

2.1.6 Approaches for biofilm assay

Most biofilm studies employ the microplate system due to its simple, rapid and high throughput natures. The standard biofilm assay begins with incubation of test microorganisms in a microplate at optimal temperature in the presence of a nutrition source (such as Tryptic Soy Broth or Luria-Bertani Broth) for 6-72 hours. Following

biofilm formation on the surface, density of cellular attachment is quantified by measurement of optical density after indicator dye has been added (such as crystal violet). This biofilm assay protocol is static and has no consistent flow of nutrients. Therefore it may not reflect real-life conditions of biofilm in industry whereby a biofilms are usually in contact with flow of liquids and nutrients. The surface properties of the polystyrene microplate systems may also not reflect the stainless steel, glass and concrete surfaces usually which are found in many industrial food processing areas (Hyde et al., 1997). Modification on the existing microplate system is essential to enable more analytical techniques.

2.1.6.1 Calgary biofilm device

The Calgary biofilm device is a modified microplate system in which the lid of the plate is set with 96 individual plugs that can fit into the each well of a 96 well microplate (Ceri et al., 1999). The plugs can be coated with any material such as concrete and aseptically removed from the lid of the microplate after biofilm formation. The biofilms formed on plugs are then analyzed by various techniques such as plate counts, scanning electron microscopy and confocal laser scanning microscopy (CLSM). An illustration of the Calgary biofilm device is shown in Figure 2.5. Another modified microplate system is microfluidic device.

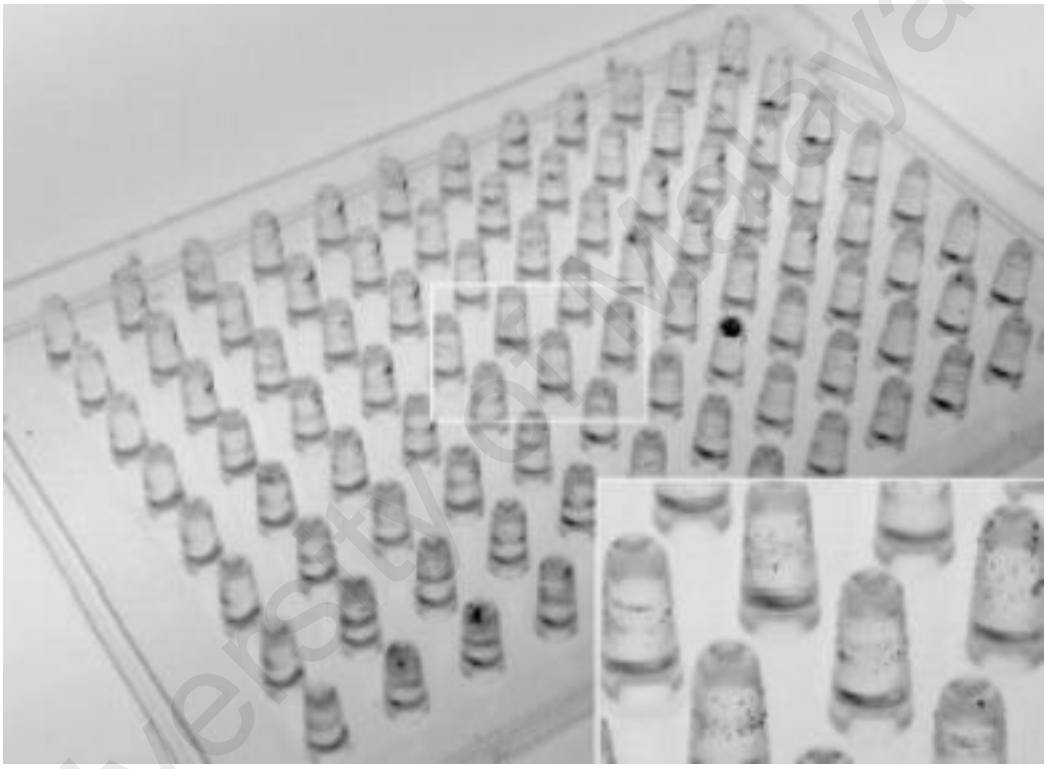


Figure 2.5: Calgary biofilm device (Wei et al., 2006).

2.1.6.2 Microfluidic device

A flow cell assay system cultivates and evaluates biofilm under hydrodynamic conditions of flow. BioFlux, for example, is a microfluidic device with distributed pneumatic pump that controls continuous or intermittent fluid flow to 96 individual biofilms (Benoit et al., 2010). The sides and roofs of the microfluidic channels are made from photolithography-etched micro patterned silicon wafers whilst the bottom of the channels consists of standard 180 μm coverslip glass allowing microscopic examination (Benoit et al., 2010). This device allows high throughput screening of biofilm viability simply by measuring fluorescence with a microplate reader and reduces the need for labour-intensive analyses such as plate count (Benoit et al., 2010). Bioflux system is shown in Figure 2.6. The remarkable advantage of this microfluidic device offers a promising potential in exploring new strategies for biofilm control.

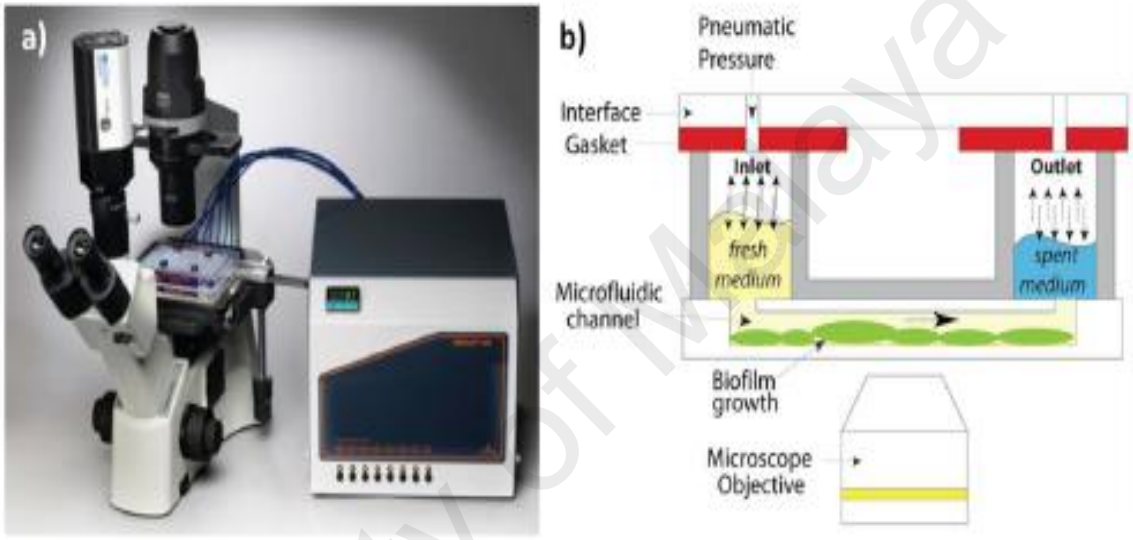


Figure 2.6: BioFlux, a flow cell assay system for biofilm analysis (Benoit et al., 2010).

2.1.7 Strategies for biofilm control

In the last few decades, a wide range of strategies have been researched to overcome the biofilm-associated problems which include the use of polymer coating, antifouling biocides and electrical field. Biomedicine, marine and food industries are the major sectors that actively develop antibiofilm strategies.

2.1.7.1 Biomedicine industry

One of the existing strategy to control biofilm formation is blocking of bacterial adhesion using passive and active polymer coatings. A passive polymer coating limits bacterial attachment to surfaces by altering the surface chemistry without the release of bactericidal agents (Hetrik & Scoenfisch, 2006). This approach is commonly used for medical devices, however, limited success has been achieved so far due to attachment variability between different strains (Hetrik & Scoenfisch, 2006). Quaternary ammonium salt (QAS) (Majumdar et al., 2008) and polyurethanes (Nagel et al., 1996) have previously been used as passive polymer coatings. An active polymer coating has two mode of actions namely alteration of surface chemistry of a device and release of selected antimicrobial substances chemically linked to the polymer (DiTizio et al., 1998; Erdmann & Uhrich, 2000; Curtin & Donlan, 2006). These antimicrobial substances are then free to interact with both planktonic and biofilm cells. Active polymer coatings can be designed to release a variety of antimicrobials such as antibiotics, metal ions, plant extracts or small molecule protein kinase inhibitors. Example for active polymer coatings are ciproflaxin-loaded liposomal hydrogels (DiTizio et al., 1998) and hydrogels coated with bacteriophage (Curtin & Donlan, 2006). The active polymer coating presented by DiTizio et al. (1998) and Curtin and Donlan (2006) do not deliver a targeted release as they provide short bursts of extremely high concentrations of the antimicrobial agents which can be hundreds of times higher than the microbial

minimum inhibitory concentration (MIC). Therefore, further improvement leads to development of targeted active polymer coating which provides antimicrobial release at critical time-points during or under specific parameters (temperature, ultrasound pulsation, pH and ion concentration) during biofilm development (Balasubramanian et al., 2009). Distinct approaches have been taken to control biofilm in marine environment.

2.1.7.2 Marine environment

Other strategies for control of microbial colonization are the release of biocides from the painted ship hull (Dafforn et al., 2011), slippery surfaces with physico-chemical properties (Callow & Callow, 2011), ultrasound that generates ultrasonic cavitation (Guo et al., 2011) and electrochemical coatings that regularly changes the pH at the boat surface (Fraunhofer-Gesellschaft, 2012). On the other hand, antifouling biocide is also commonly used to control biofouling of ships' hulls. For many decades, tri-n-butyltin (TBT) has been widely used as antifouling biocide although its high toxicity to aquatic organisms has been documented (Dahl & Blanck, 1996). Examples of marine organisms successfully controlled by all these strategies are barnacle, microalgae and mussel. The strategies for biofilm control in food industries seem to be more comprehensive because it covers all physical, biological and chemical methods.

2.1.7.3 Food industries

In food industries, biofilms remain the persistent challenges. Typically, biofilm removal in this industry include physical, biological and chemical methods. Physically, biofilm can be eliminated by super high magnetic field (Okuno et al., 1993) and high pulsed electrical field (Liu et al., 1997), heat treatment and scrubbing. Meanwhile, the biological method commonly used to control biofilm includes adsorption of bacteriosin

onto food contact surfaces (Kumar & Anand, 1998). Chemical sanitizer has become a popular approach in the food industries due to its high efficiency and cost effectiveness. Some of the common chemical sanitizers are chlorine compounds such as liquid chlorine, hypochlorite and chlorine dioxide. Chlorine compounds which are temperature tolerant, may act through disruption of protein synthesis, creation of chromosomal aberrations and induction of DNA lesion (Marriott & Gravani, 2006). One of the common biofilm-forming bacteria in causing food contamination is *Salmonella typhimurium*.

2.1.8 Biofilm of *Salmonella typhimurium*

Biofilm is an important survival strategy in all stages of Salmonella infection, from transmission to development of chronic infection. In human, *S. enterica* is known to form biofilm on gallstones and cholesterol-coated surfaces (Prouty et al., 2002) and intestinal epithelial cell layers (Pace et al., 1993). Formation of *S. typhimurium* biofilm has been shown to associate with differential protein expression. Hamilton et al. (2009) developed biofilm of *S. typhimurium* on a borosilicate glass flow cell and proteomic analysis revealed that surface-associated growth of *S. typhimurium* caused upregulation of 59 proteins and downregulation of 65 proteins. Also, at least 175 unique proteins which were present in biofilm fraction and absent in planktonic fraction were identified. Majority of these unique proteins were found to participate in carbohydrate metabolism. Understanding the fundamental aspects of *S. typhimurium* is crucial to fight against its biofilm phenotype.

2.2 *Salmonella typhimurium*

Salmonellosis is a type of food poisoning which can happen in various ways such as poor food handling, poor hand washing and feaces of some pets. Severe salmonellosis (gastroenteritis) in humans and other mammals are mainly caused by *Salmonella*

typhimurium, an aerobic facultative microorganism. It is regarded as the most important serotype of salmonellosis transmitted from animals to humans in most parts of the world. This bacteria has the capacity to grow under either aerobic or anaerobic conditions and is non-encapsulated microorganism. In the study of *S. typhimurium*, there are several important molecular aspects which have been linked to its virulence, namely genomic composition, type III secretion system, phosphorelay signalling and intracellular changes in its hosts. Epidemiological surveillance of *S. typhimurium* typically involves bacteriophage, pulsed field gel electrophoresis (PFGE) and Multi Locus Sequence Typing schemes (MLST).

2.2.1 Genome of *Salmonella typhimurium*

The genome of *S. typhimurium* has been completely sequenced.. The size of chromosome and virulence plasmid are 4857 kb and 94 kb respectively whilst the G+C content of each is 53% (McClelland et al., 2001) (Figure 2.7). Of all coding sequences and pseudogenes, at least 55% have a close homologue in other microorganisms such as *S. typhi, S. paratyphi, E. coli* and *K. pneumonia* (McClelland et al., 2001).

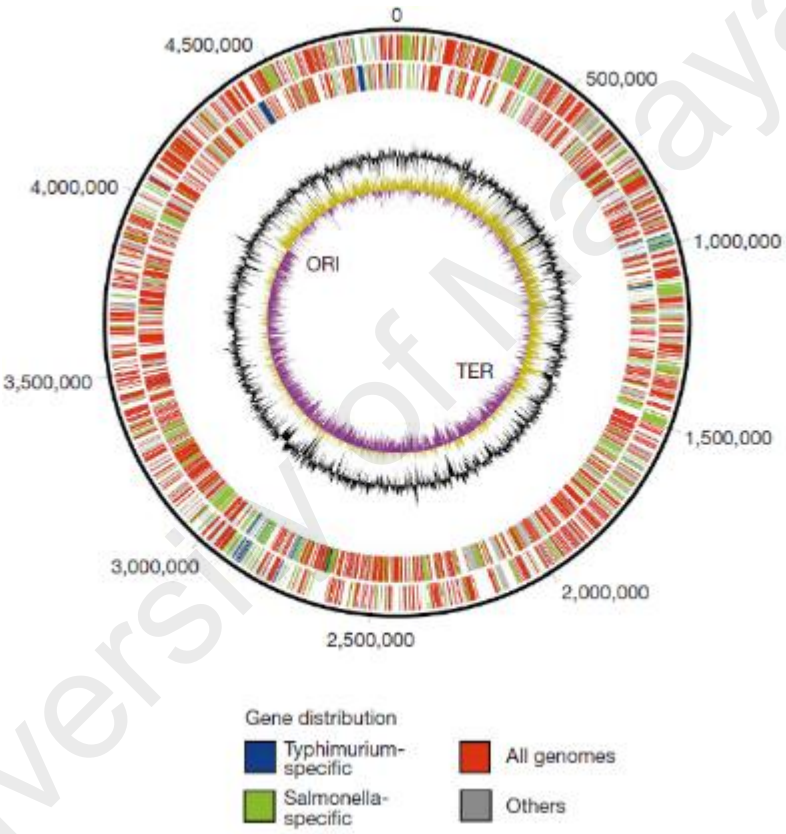


Figure 2.7: Complete genome of *S. typhimurium* (McClelland et al., 2001).

The whole-cell protein reference map of *S. typhimurium* has been established. The major protein bands in Salmonella serovars appear to be 78.1, 51.2, 41.5, 37.3, 30.7, 27.6, 25.4, and 24 kDa (Aksakal, 2010) (Figure 2.8). The presence of these major protein bands in Salmonella serovars has also been reported by Nakamura et al. (2002), Acik et al. (2005), Ngwai et al. (2005) and Begum et al. (2008).

2.2.2 Type three secretion system of *Salmonella typhimurium*

Type three secretion system (T3SS) is a molecular machine used by *S. typhimurium* to inject proteins directly into eukaryotic host cells. There are two virulence-related T3SS namely T3SS1 and T3SS2 which are encoded on Salmonella pathogenicity islands 1 (SPI-1) and 2 (SPI-2) respectively (Hueck, 1998). A flagellum-like injectisome which is projected from the cytoplasm of the bacterial cell to the cytoplasm of a eukaryotic host cell is essential to inject proteins which are known as effectors. In general, the injected proteins are required for pathogenicity, biofilm formation and nutrient acquisition (Ramos-Morales, 2012). Proteins injected by the T3SS also enable Salmonella to grow within and eventually kill host macrophages which then suppresses the innate immune response of the host (Hueck, 1998). Figure 2.9 illustrates T3SS in *S. typhimurium.*

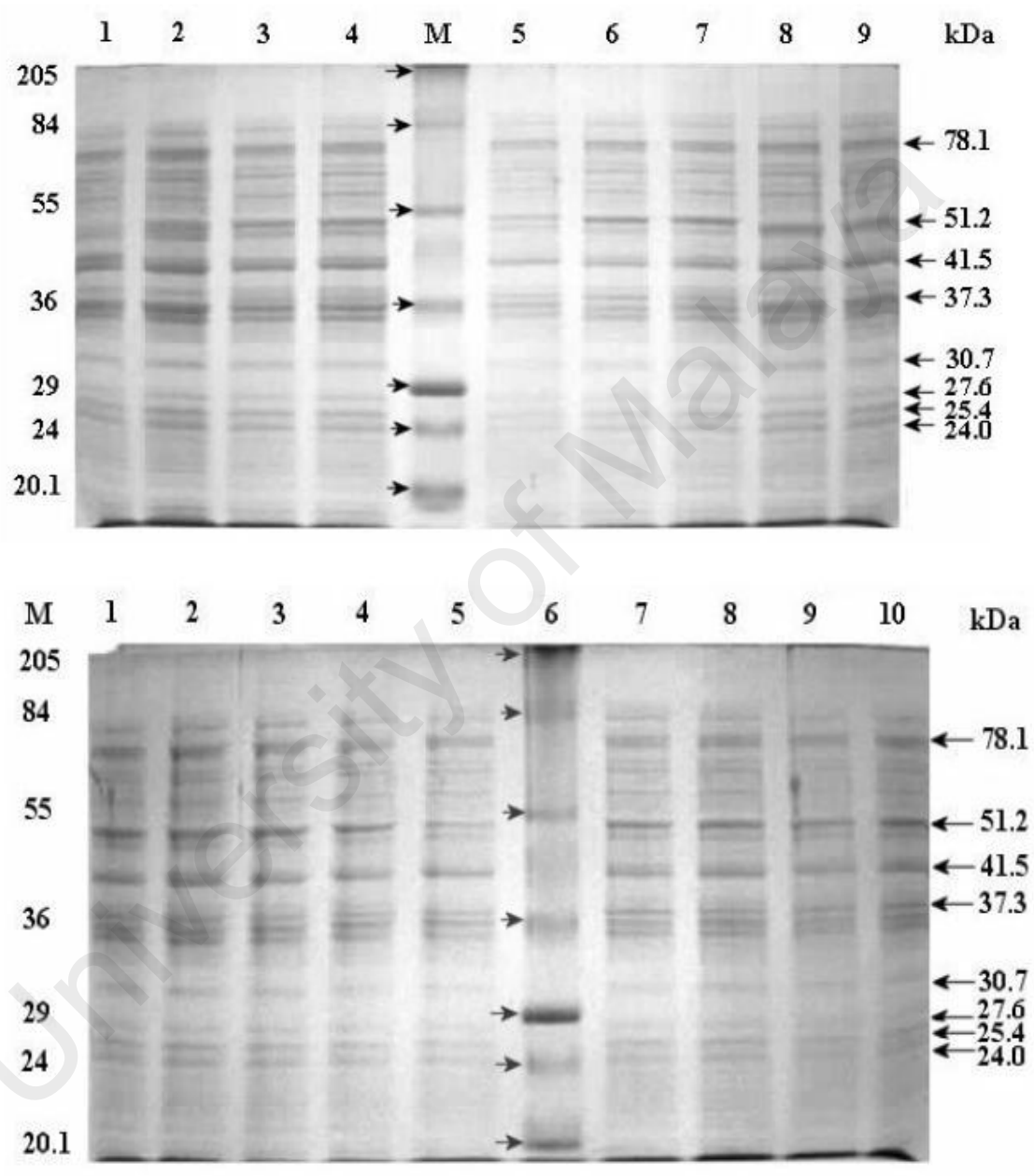


Figure 2.8: Whole-cell protein profiles of Salmonella serovars (Aksakal, 2010). Upper panel, lane 1: *S. corvallis*, lane 2: *S. augustenborg*, lane 3: *S. typhimurium,* lane 4: *S. agona*, lane 5: protein markers, lane 6-8: *S. enteriditis*, lane 9: *S. cambridge*, lane 10: *S. anatum*. Lower panel, lane 1-5 and 7-9: *S. enteritidis* of different isolates, lane 6: protein markers.

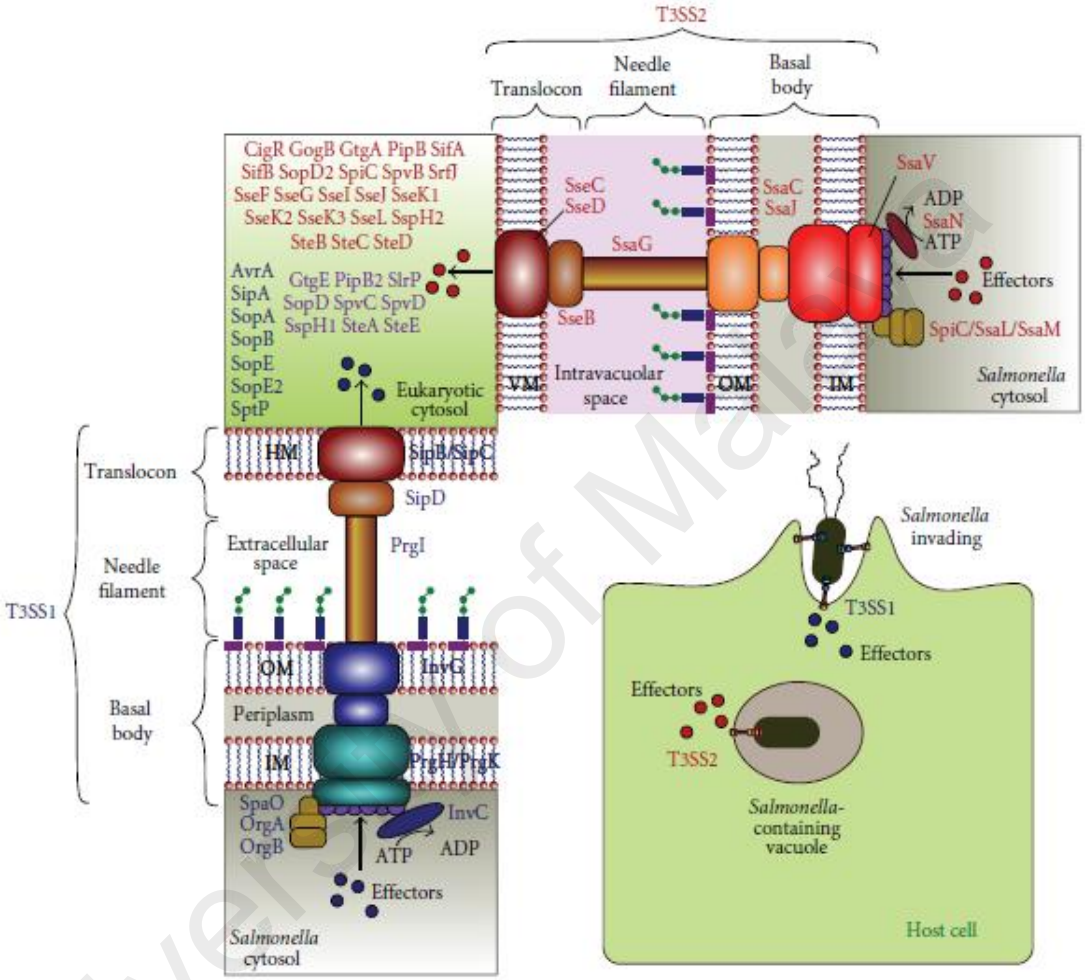


Figure 2.9: Schematic representation of Salmonella T3SS1 and T3SS2 (Hueck, 1998). HM: host plasma membrane; VM: vacuolar membrane; OM: outer membrane; IM: inner membrane. T3SS1 components and effectors are in blue. T3SS2 components and effectors are in red. Effectors translocated through both systems are in purple.

Invasion of *S. typhimurium* into host cells often associates with cell death. There are three processes of T3SS-dependent cell death programmes namely epithelial cell apoptosis, rapid T3SS1-dependent macrophage pyroptosis, and delayed T3SS2-dependent macrophage pyroptosis (Fink & Cookson, 2007). Both pyroptosis and apoptosis are the forms of programmed cell death which depend on caspase activation. On the other hand, one of the T3SS effector playing role in modulating apoptosis in epithelial cells is SlrP which interacts with thioredoxin-1 and ERdj3 (Bernal-Bayard et al., 2010). Considering the limited information on regulation of T3SS, this molecular machine is possibly regulated by quorum sensing system.

2.2.3 Qurorum sensing in *Salmonella typhimurium*

AI-2 has been regarded as a universal quorum-sensing signal that enables interspecies communication (Schauder et al., 2001). The LuxS synthase enzyme, which is responsible for AI-2 biosynthesis, is present in more than 55 Gram-negative and Gram-positive bacteria species including *S. typhimurium* (Surette et al., 1999; Xavier & Bassler, 2003). In *S. typhimurium*, LuxS enzymes use *S*-ribosylhomocysteine (SRH) to synthesize 4,5-dihydroxy-2,3-pentanedione (DPD) which spontaneously cyclizes into AI-2 (Taga et al., 2001). The biological activity of DPD in *S. typhimurium* has been investigated by De Keersmaecker et al. (2005). In that study, formation of *S. typhimurium* biofilm was found to be defective in a luxS mutant and was restored upon addition of DPD under control of lsr (LuxS-regulated) operon. Thus, it could be inferred that LuxS/AI-2 system is responsible for biofilm formation by *S. typhimurium*. In 2008, Soni et al. demonstrated that AI-2-mediated quorum sensing was essential for regulation of expression of outer membrane protein F, Trigger Factor, Transaldolase B, DNA-directed RNA polymerase, phosphoglycerate mutase and PhoP-PhoQ phosphorelay protein in *S. typhimurium*. Although the information on functional linkage

between quorum sensing and T3SS in *S. typhimurium* is still limited, at least a study performed by Sperandio et al. (1999) has demonstrated the modulation of Locus of Enterocyte Effacement (LEE) Pathogenicity Island encoding a type III secretion system by LuxS gene in *E.coli*.

2.2.4 Phosphorelay signalling in *Salmonella typhimurium*

The two component system is a mechanism that controls a highly regulated program of gene expression in response to environmental stimuli. Generally, the two component systems have two phosphorelay steps and consist of a membrane-associated sensor histidine kinase and a cytoplasmic response regulator. In *S. enterica*, one of the phosphorelay system is regulator of capsule synthesis (Rcs) comprising RcsC and RcsD that function as a modified hybrid sensor kinase, and a response regulator, RcsB (Majdalani & Gottesman, 2005). The structure of Rcs phosphorelay system in *S. typhimurium* is shown in Figure 2.10.

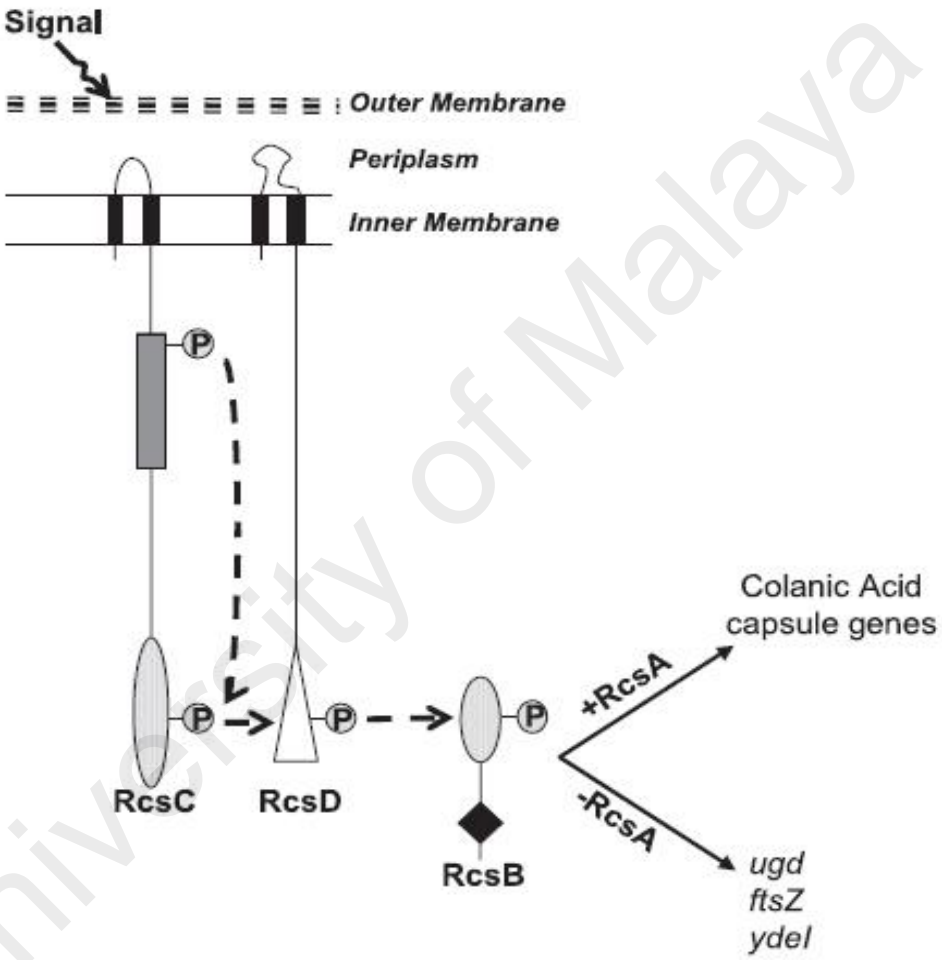


Figure 2.10: Model of the Rcs signalling pathway in *S. typhimurium* (Majdalani & Gottesman, 2005).

During systemic infection, *S. enterica* resides in macrophages and thus there is a need for the bacteria to encode defence mechanisms that enable them to withstand antimicrobial peptide (AMP) insult. AMPs represent a broad spectrum defence mechanisms produced prophylactically by the host cells in the absence of infection and their expression levels are increased in response to cytokines, LPS and bacteria (Erickson & Detweller, 2006). The PhoQ-PhoP phosphorelay system is another two-component system present in *S. enterica*. In this system, PhoQ acts as the sensor kinase whilst PhoP acts as the response regulator or transcriptional regulator. Expression of PhoP is known to be regulated by AI-2-mediated quorum sensing (Soni et al., 2008). Typically, the sensor kinases autophosphorylate at a conserved histidine residue and then transfer the phosphoryl group to a conserved aspartate in the response regulator. The PhoQ-PhoP phosphorelay system mediates AMP resistance by activating genes that lead to reduction of negative charge of lipopolysaccharide (LPS) (Ernst et al., 2001). Modification of LPS causes reduction of affinity of many AMPs for its bacterial membranes (Ernst et al., 2001). Another defence mechanism to resist the AMP is toxin-antitoxin (TA) module.

2.2.5 Toxin-antitoxin (TA) modul of *Salmonella typhimurium*

Toxin-antitoxin (TA) module has received considerable attention in the studies of survival of pathogenic bacteria. In 2013, a newly discovered TA module in *S. typhimurium* namely *shpAB* was discovered (Slattery et al., 2013). It was found to cause a 3- to 4-order-of-magnitude increase in survival of *S. typhimurium* following ampicillin exposure and protected the population against exposure to multiple antibiotics. This TA module is in agreement with the general model for bacterial persistence whereby increased toxin activity within a subpopulation of cells results in inhibition of essential cellular processes leading to dormancy whilst proteolytic activity is required to degrade

those toxins (Lewis, 2007; Lewis & Bates, 2010; Kuroda et al., 2001). In addition to the dormancy in bacterial cells, EPS matrix is also a culprit causing the failure of antibiotic treatment.

2.2.6 EPS matrix of *Salmonella typhimurium*

EPS matrix of bacteria often acts as a physical barrier for survival against antibiotic attack and the immune system of humans. The production of EPS component by Salmonella biofilm has been reported by Prouty et al. (2002). By using ruthenium red stain, a stain specific for polysaccharides, they successfully showed clumps of cells which were either associated biofilm or early microcolony formation (Prouty et al., 2002). Further analysis using SEM with 7000 magnification revealed that the bacterial cells embedded in a putative EPS matrix without the apparent web-like strands (Prouty et al., 2002). Moreover, Rdar morphotype (a multicellular behaviour) of *S. typhimurium* has been shown to produce thin aggregative fimbriae (*agf*) (Romling et al., 2000) and cellulose (Zogaj et al., 2008) as EPS components. Besides EPS matrix, intracellular changes in host is also essential for survival of *S. typhimurium.*

2.2.7 Intracellular changes in *Salmonella typhimurium*-infected hosts

S. typhimurium invades the host cells and manipulate their intracellular components to prevent elimination. Increases in inositol trisphosphate, intracellular Ca^{2+} mobilization, translocation of protein kinase C to the membrane and upregulation of nitric oxide synthase expression have been shown to occur in *S. typhimurium* porin-activated gut macrophages (Gupta et al., 1999). Salmonella invasion of polarized intestinal epithelial cells has also been demonstrated to associate with recruitment focal adhesion proteins such as FAK, p130Cas, paxillin, vinculin, α-actinin and VASP at sites of Salmonella entry (Shi & Casanova, 2006). Since the molecular basis of *S. typhimurium*-host

interaction is a relatively new discovery, most of antibiotics used for controlling salmonellosis appear to target only cellular components of *S. typhimurium.*

2.2.8 Antibiotics for salmonellosis

The use of antibiotics for salmonellosis is limited due to emerging drug resistance over the past two decades. In other words, antibiotics are limited to patients with severe salmonellosis and are not routinely used to treat uncomplicated non-typhoidal *Salmonella* gastroenteritis. Meanwhile, patients with severe diarrhoea are treated only with intravenous fluid for rehydration. Table 2.1 shows common antibiotics used in various countries to control salmonellosis. An alternative strategy to control *S. typhimurium* infection is the application of inhibitor targeting host protein kinase.

Table 2.1: Common antibiotics used to treat salmonellosis.

Antibiotics	Mode of actions	Resistance cases	References
Nalidixic acid	inhibits a subunit of DNA gyrase and topoisomerase IV and induce formation of cleavage complexes.	Yes	Crump et al. (2011)
Ceftriaxone	inhibits bacterial cell wall synthesis by binding to transpeptidases that catalyze the cross-linking of the peptidoglycan polymers forming the bacterial cell wall.	Yes	Crump et al. (2011)
Quinolone	inhibit the topoisomerase II.	Yes	Ercis et al. (2006)
Fluoroquinolones	inhibit type II DNA toposiomerases.	Yes	Hakanen et al. (1999)
Azithromycin	binds to the 50S subunit of the bacterial ribosome which in turn inhibit translation of mRNA.	Unknown	Beeching and Parry (2011)
Gatifloxacin	inhibits the bacterial enzymes DNA gyrase and topoisomerase IV.	Unknown	Beeching and Parry (2011)
Amoxicillin	inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell walls	Unknown	Coughlin et al. (2003)
Cephalosporin	disrupt the synthesis of the peptidoglycan layer forming the bacterial cell wall	Unknown	Coughlin et al. (2003)
Ampicillin	acts as an irreversible inhibitor of the bacterial transpeptidase,	Yes	Poppe et al. (1998)
Chloramphenicol	prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome	Yes	Poppe et al. (1998)

Table 2.1, continued

Antibiotics	Mode of actions	Resistance cases	References
Streptomycin	binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit	Yes	Poppe et al. (1998)
Sulfonamides	act as competitive inhibitors of the enzyme dihydropteroate synthase (DHPS), an enzyme involved in folate synthesis	Yes	Poppe et al. (1998)
Tetracycline	inhibits protein synthesis by blocking the attachment of charged aminoacyl-tRNA to the A site on the ribosome	Yes	Poppe et al. (1998)

2.2.9 Control of *Salmonella typhimurium* infection by inhibition of host protein kinase

Manipulation of host protein kinases by *S. typhimurium* typically involves effector molecules secreted via T3SS1 and T3SS2 (McGhie et al., 2009). Such manipulation is essential for multi stage infection of the host which include invasion of intestinal mucosa, formation of Salmonella-containing vacuole (SCV) and formation of tubulovesicular SCV structures called Salmonella-induced filaments (Sifs) in Golgi apparatus (McGhie et al., 2009). According to Hobbie et al. (1997), the family of mitogen-activated protein (MAP) kinases is a key element in the signaling pathways involved in transducing *S. typhimurium*-initiated signals to cellular responses. This corroborates several lines of studies showing that *S. typhimurium* infection of cultured intestinal epithelial cells leads to activation of MAP kinases ERK-1 and ERK-2, c-Jun NH_2-terminal kinases (JNK), and p38 which in turn causes rearrangement of the host cell cytoskeleton and internalization of the bacteria and IL-8 secretion (Pace et al., 1993; Posenshine 1994; Chen et al., 1996; Mynott et al., 2002). *S. typhimurium* is also known to activate protein kinase AKT1 in the host cell using effector SopB (Coenraad et al., 2007). Application of AKT1 inhibitor, a potent drug for cancer therapy, successfully interrupts the bacterial manipulation of host signalling mechanism thereby hindering the intracellular growth of *S. typhimurium* (Coenraad et al., 2007). It appears that such control strategy for *S. typhimurium* infection also has high potential to minimize the problems associated with antibiotic resistance.

2.3. Protein kinase

Protein kinase is a member of phosphotransferase family and is an enzyme that functions in modifying other proteins by phosphorylation process which could be reversed by the activity of protein phosphatase. A general mechanism of protein

phosphorylation has been explained by Ubersax and Ferrell (2007). Briefly, protein phosphorylation by a protein kinase begins with binding of high-energy phosphate-donating ATP and substrate to the active site of the protein kinase. A phosphoryl group is then transferred from ATP to a histidine, serine, threonine or tyrosine residue of the substrate. Finally, both ADP and phosphorylated substrate are released from the active site of protein kinase. Due to disparities in the charge and hydrophobicity of surface residues, protein kinase bind to and phosphorylate different protein substrates. Protein kinase-mediated cell signaling is a complex communication system which takes place in both extracellular and intracellular spaces to coordinate basic cellular activities (Rhee, 2006).

A number of studies have revealed the presence of protein kinases and phosphoproteins in *S. typhimurium*. Wang and Koshland (1978) demonstrated the phosphoproteins of *S. typhimurium* in the range between 45 kDa and 88 kDa using a combination of *in vitro* phosphate labeling, SDS PAGE and autoradiography. The same approach had been used by Waygood et al. (1984) to analyze the major phosphoenolpyruvate-dependent phosphoproteins in the range between 8 kDa and 65 kDa and ATP-dependent phosphoproteins in the range between 15 kDa and 75kDa. Furthermore, Cho and Ahn (2014) used a combination of two dimensional polyacrylamide gel electrophoresis and MALDI-TOF to study the effect of acid stress on β-lactam antibiotic-resistant *S. typhimurium* and their results showed that ribose-phosphate pyrophosphokinase, phosphoglycerate kinase and adenylate kinase were differentially expressed.

The roles of protein kinases in eukaryotes have been well addressed, however, the roles of bacterial protein kinases remain largely unknown especially in the context of biofilm formation. Recent development in the protein kinase assay system and extensive studies

on the existing protein kinase inhibitors have largely supported the progressive search for the effective antibiofilm agents.

2.3.1 General roles of protein kinases

The role of kinase signaling has frequently been discussed in the context of molecular and cellular processes such as protein expression, metabolism and protein secretion. In 2001, Ping et al. studied how the kinase signaling affects the proteome expression in HeLA S3 cells. In that study, the two dimensional SDS polyacrylamide gel electrophoresis demonstrated significant changes in the number of protein spots, protein abundance, and the posttranslational modifications of proteins in comparison between protein kinase C transgenic mice and non-transgenic mice. The differentially expressed proteins were found to be heat shock protein 70, tropomyosin α chain, troponin T2, αB-crystallin and cardiac isoform of myosin regulatory light chain. In the context of metabolic regulation, Long and Zierath (2006) correlated AMP-activated protein kinase (AMPK) with glucose uptake and lipid oxidation for energy production during nutrient starvation. Serving as an intertissue signal integrator among peripheral tissues, the AMPK responses to diverse hormonal signals and controls whole-body glucose homeostasis. In a more recent work, the kinase signaling has been shown to participate in the protein secretion. A phosphoproteomic study by Yan et al. (2011) revealed a total of 142 phosphorylation sites in the phosphosecretome associated with carcinogenesis, invasion, and metastasis. Many of phosphoproteins were secreted with no previously known function. They also suggested that the phosphorylation may contribute to the conventional protein secretion process. In addition to these, there have been multiple lines of evidences addressing the presence of the protein kinases in bacteria.

2.3.2 Essentiality of protein kinases in biofilm formation

In the context of biofilm formation, protein kinase is often associated with multistep phosphorelay system, a cascade that mediates the coordinated expression of virulence genes in bacteria. This regulatory system which mainly involves histidine kinase has been studied by Mishra et al. (2005) in which the BvgAS phosphorelay system has been shown to regulate biofilm development in Bordotella spp. In parallel with that, Petrova and Sauer (2009) has also demonstrated the important role of histidine kinase-mediated phosphorelay system in initiation of biofilm formation, biofilm maturation and microcolony colonization by *P. aeruginosa*.

Limited data is available on the protein kinases involved in formation of *S. typhimurium* biofilm. Although there have been several works on the protein kinases that potentially associate with biofilm formation in *S. typhimurium* (Table 2.2), there is still a need for more experimental evidences to understand the role of protein kinases in *S. typhimurium* biofilm.

Table 2.2: Protein kinases that are present in *S. typhimurium* and their possibilities to be involved in the biofilm formation based on the previous works.

Protein kinases in *S. typhimurium*	**Potential links to biofilm formation**
PhoQ histidine kinase (Ernst et al., 2001)	Sensor histidine kinase-mediated phosphorelay signaling regulates biofilm formation by Bordetella strains in a biofilm silicone tubing assay (Mishra et al. 2005).
RcsC and RcsD as hybrid sensor histidine kinase (Majdalani & Gottesman, 2005)	Rcs system is required for formation of *E. coli* biofilm (Ferrieres & Clarke, 2003).
Acetate kinase (Chittori et al., 2012)	Acetate kinase is used to phosphorylate acetate to acetyl phosphate which is a global signal that contributes to biofilm development (Wolfe et al. 2003)
Adenylate kinase (White et al., 2010)	Adenylate kinase is secreted by *P. aeruginosa to* contend with alveolar macrophages and mast cells enabling biofilm formation (Markaryan et al. 2001).
Phosphoglycerate kinase (Cho & Ahn, 2014)	Expression level of phosphoglycerate kinase was enhanced during formation of *Streptococcus mutans* biofilm (Welin et al. 2004).
Ribose-phosphate pyrophosphokinase (Cho & Ahn, 2014)	Expression level of ribose-phosphate pyrophosphokinase was upregulated during formation of *Legionella pneumophila* biofilm (Hindre et al. 2008).

2.3.3 Protein kinase assay system

In conjunction with the growing knowledge pertaining to the critical roles of protein kinases in the human diseases, there have been many notable developments of protein kinase assay system. In 2002, Rodems et al. has developed a fluorescence-based kinase assay which is compatible with robotic, ultra-high throughput screening systems. With the coupled-enzyme format, the assay has been shown to perform the rapid and accurate compound screening for the protein kinases. In the meantime, the high-throughput screening of bioactive compounds against protein kinases has also become significant with the development of bioluminescent kinase assay (Koresawa & Okabe, 2004). This assay system has been tested with both serine/threonine and tyrosine kinases, resulting in Z values > 0.8 in either 96-well plate or 384-well plate formats. PepChip kinomic array has been introduced in 2004 (Diks et al., 2004). Basically, the use of this array technology involves printing of consensus peptides for kinases on a solid support, incubation with cell lysates in the presence of radioactive ATP and visualization of radioactive peptides (Diks et al., 2004). This approach absolutely overcomes the problems associated with most of protein analyses such as small number of samples (in-gel kinase assay and Western blot), only one type of phosphorylation is studied per experiment (Western blot and ELISA), time consuming procedure (a combination of 2D electrophoresis and MALDI-TOF) and inability to measure the actual activity of various cellular signalling pathways (a combination of 2D electrophoresis and MALDI-TOF) (Diks et al., 2004). The principle of kinomic array profiling is shown in Figure 2.11.

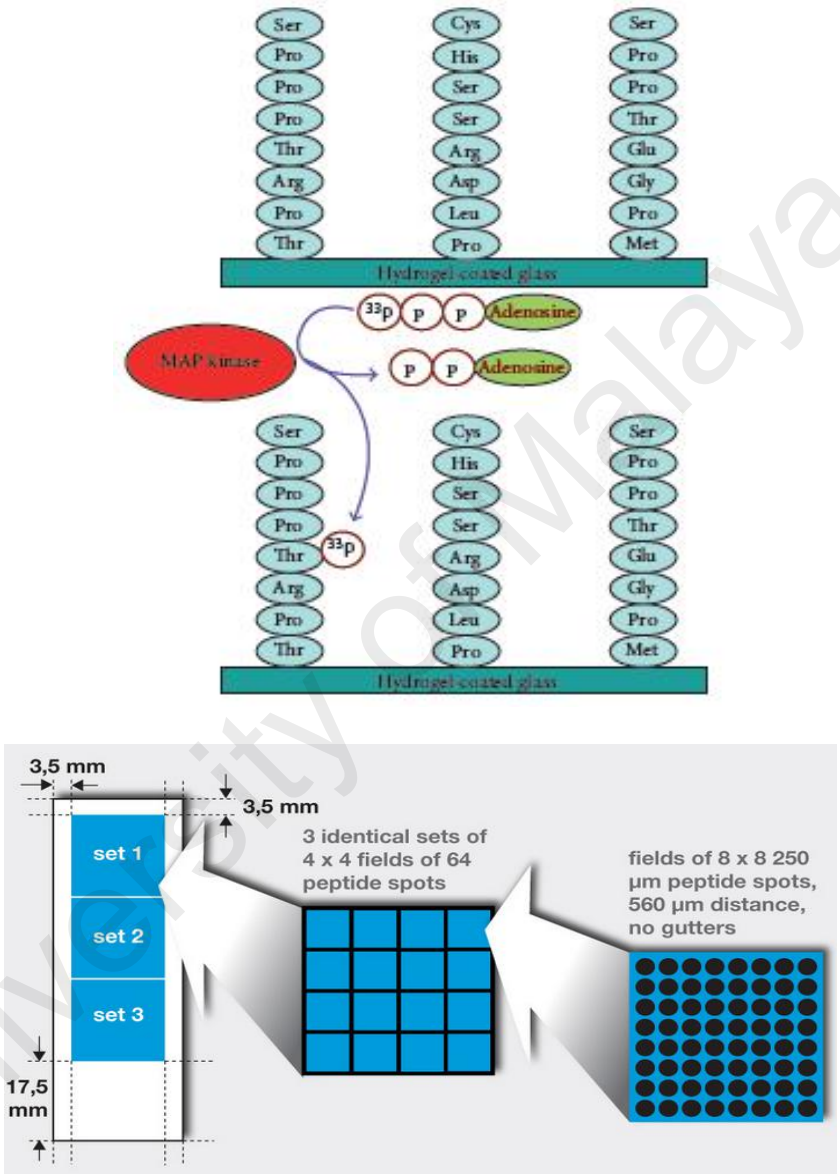


Figure 2.11: Principle of kinome profiling using array technology (Diks et al., 2004) and custom design peptide arrays (www.pepscan.com).

2.3.4 Protein kinase inhibitors

Most protein kinase inhibitors are ATP competitive small molecules that block the binding of ATP to the protein kinase domain thereby reducing its enzymatic activity. The ATP pocket is a deep cleft with a large surface area which enables many polar interactions with the small molecules. This inhibition strategy is challenged by the high conservation of the pocket among the members of the protein kinase family and competition from the high intracellular ATP concentrations which. However, the ATP pocket remains the most druggable site for at least the majority of the kinase targets.

Over the past few decades, a wide range of protein kinase inhibitors has been widely investigated for various biological actions. Although the protein kinase inhibitors are commonly used in the cancer treatment, they have also been introduced to the antimicrobial treatment. This is due to the fact that the progress of both infectious and non-infectious disease is greatly dependent on the kinase signaling (Hale et al., 2006; Force et al., 2004).

In 2006, Ulanowska et al. demonstrated the antibacterial activity of genestein at 0.1 mM against *E. coli, V. harveyi* and *B. subtilis* in a time-dependent manner. Further microscopic analysis revealed the remarkable changes in the bacterial morphologies following the exposure to 0.1 mM genestein. On the other hand, a set of 80 compound protein kinase inhibitors was tested against *Listeria monocytogens* biofilm (Nguyen et al., 2011). Out of 80 kinase inhibitors, 15 were found to inhibit formation of *L. monocytogenes* biofilm including hypericin, staurosporine, tryphostin 9 and sphingosine.

Collectively, these imply that the existing eukaryotic protein kinase inhibitors for cancers are also effective in modulating the bacterial survival although the protein kinases are much less widespread in bacterial metabolism. Whether these protein kinase inhibitors specifically bind to ATP pockets of bacterial protein kinases remain largely unknown. Table 2.3 shows common protein kinase inhibitors with their targets in the cell signaling pathways. Among all these protein kinase inhibitors, DMSO possesses the widest range of pharmacological applications.

Table 2.3: Protein kinase inhibitors commonly used in disease management.

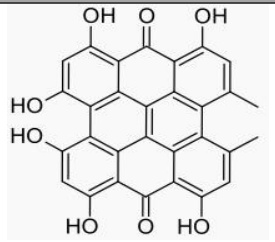
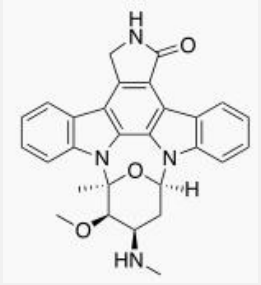
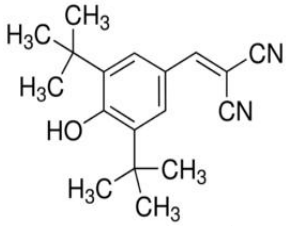
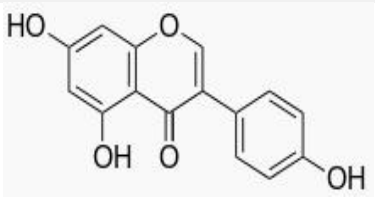
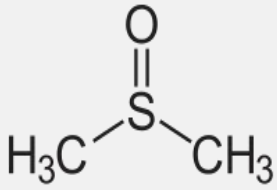
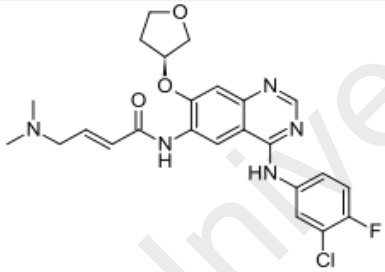
Kinase inhibitor	Structure	Targets	Reference
Hypericin		Protein kinase C in mammalian cells	Takahashi et al.(1989)
Staurosporine		Protein kinase C in cancer cells	Meyer et al. (2006)
Tryphostin 9		Protein tyrosine kinase in herpes simplex virus type 1	Yura et al. (1995)

Table 2.3, continued

Kinase inhibitor	Structure	Targets	Reference
Sphingosine		Protein kinase C in human platelets	Hannun et al. (1986)
Genistein		Protein tyrosine kinase (epidermal growth factor receptor) in cancer cells	Anderson et al. (1998)
Dimethyl sulfoxide		Pyruvate kinase in proliferating rat basophilic leukemia 2H3 (RBL-2H3) cells	Koo and Kim (2009)
Afatinib		EGFR tyrosine kinases in cancer cells	Zhang and Munster (2014).

2.3.4.1 Potential use of dimethyl sulfoxide as antibiofilm agent

Dimethyl sulfoxide (DMSO) specifically inhibits the pyruvate kinase (Koo & Kim, 2009). It is a naturally occurring substance which is also a commercially manufactured dipolar aprotic solvent. It is generally known to be present at low concentrations (<0.05 ppm to 1.8 ppm) as an important flavour and aroma compound in fresh fruits, vegetables and grains. It possesses a wide range of pharmacological applications including drug vehicle, stimulator of cell differentiation, anticoagulant, topical analgesic and free radical scavenger. DMSO at concentrations of 0.5–1.5% has been widely used as a solvent for various pharmacological agents in both cell culture and *in vivo* studies (Jacob & de la Torre, 2009). Table 2.4 below summarizes various scopes of study of DMSO, bacterial protein kinase and biofilm formation in order to highlight a potential linkage between them. DMSO appears to possess a high potential for the control of *S. typhimurium* biofilm.

Table 2.4: Previous works on DMSO, bacterial protein kinase and biofilm formation.

Previous works	Scopes of study	Remarks
Bartsch et al. (1976)	Safety of DMSO	DMSO is a relatively low-toxicity organic solvent with LD_{50} values less than 9.9 ml kg^{-1}
Zuurmond et al. (1996)	Safety of DMSO	50% DMSO cream is safe topical application and is recommend patients suffering from acute reflex sympathetic dystrophy (RSD).
Capriotti and Capriotti (2012)	Safety of DMSO	DMSO concentration up to 50% has been approved by US FDA for dermatological application
Elisia et al. (2016)	Safety of DMSO	Rapid metabolism and excretion of DMSO *in vivo*
Basch and Gadebusch (1968)	Effect of DMSO	DMSO exhibits bactericidal activity against a wide spectrum of bacteria with minimal bactericidal concentrations between 8% and 40%
Koo and Kim (2009)	Effect of DMSO	DMSO specifically inhibits enzymatic activity of pyruvate kinase in proliferating rat basophilic leukemia 2H3 (RBL-2H3) cells and FcepsilonRI-mediated tyrosine phosphorylation of pyruvate kinase in allergenic activated RBL-2H3 cells
Rudy and Wilhelm (1977)	Effect of DMSO	Prolonged exposure (more than five minutes) of enzyme to high concentrations of DMSO results in irreversible inhibition of ATP binding and phosphoprotein formation
Balazovich et al. (1987)	Effect of DMSO	Changes in PKC activity following exposure to varying concentration of DMSO
Thomas et al. (1995)	Effect of DMSO	DMSO inhibits c-Myc expression in different cell types

Table 2.4, continued

Previous works	Scopes of study	Remarks
Antunes et al. (2010)	Effect of DMSO	DMSO inhibits gene expression of OmpR/ ToxR-type transcriptional regulator HilA in *S. typhimurium* and host cell invasion
Zoraghi et al. (2011)	Bacterial protein kinase	cis-3,4-dihyrohyrohamacanthin B and bromodeoxytopsentin effectively inhibit activity of pyruvate kinase in methicillin-resistant *Staphylococcus aureus* (MRSA) with IC_{50} values in the range between 16 and 60 nM
Allan et al. (2014)	Biofilm formation / Bacterial protein kinase	Differential expression of 9 glycolytic proteins including pyruvate kinase and phosphoglycerate kinase during biofilm formation.
Vasu et al. (2015)	Biofilm formation / Bacterial protein kinase	In *Staphylococcus aureus*, elevated synthesis of pyruvate kinase does not increase energy production, but contribute to upregulation of biosynthetic pathways involved in biofilm formation

2.4. Tandem mass spectrometry-based proteomic analysis

Tandem mass spectrometry (MS/MS) is an analytical technique used to measure the molecular mass of small and large samples using multiple analyzers. Its major applications include peptide analysis, drug discovery, neonatal screening, evaluation of food contamination and determination of oil composition. In the context of biochemistry, this analytical technique is often used in amino acid analysis (Quadroni & James, 2000), newborn screening (Carpenter & Wiley, 2002) and determination of macromolecular structure (Hsu & Turk, 2000). Understanding the principles of proteomic technique and tandem mass spectrometry would benefit various experimental designs, however, special evaluation is required when the proteomic analysis involves the heterogeneous biofilm sample.

2.4.1 Overview of proteomics workflow

Briefly, proteomics can be defined as the systematic and high-throughput proteome analysis which includes identification and quantification of proteins. The key technology for the proteomics work is tandem mass spectrometry. The term proteome is abbreviated from "PROTEin complement of the genOME" meaning the complete set of the proteins expressed by the cell (Wilkins et al., 1996). Because proteins are the active cellular components that determine the phenotype of an organism (Reinagel & Speth, 2016), the proteome often varies from one organism to another organism and create different phenotypes. Many human diseases are consequences of proteome abnormalities (Lippolis & Angelis, 2016). To discuss the general proteomic workflow, this section focuses on one dimensional SDS-PAGE coupled with HPLC-tandem mass spectrometry.

2.4.1.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method to separate protein mixture from whole cells, subcellular fractions, column fractions, or immunoprecipitates. A polyacrylamide gel is formed by polymerization of linear polymer-forming acrylamide and the crosslinker bisacrylamide. The pore size of a polyacrylamide gel decreases with increasing total concentration of acrylamide and bisacrylamide. SDS is a negatively charged detergent molecules binds non-covalently to proteins, promotes protein denaturation by disrupting non-covalent bonds, linearizes proteins and imparts a negative charge to linearized proteins. Binding of SDS to the proteins would result in similar charge-to-mass ratios and similar shapes, thereby separating protein molecules according to their molecular mass regardless of charge. Advantages of this analytical method include high loading capacity and great separation of hydrophobic and low-molecular-mass proteins (Jiang et al., 2016). Different dyes can be used to visualize the electrophoretically separated proteins.

2.4.1.2 Gel staining

Lopez et al. (2000) has made a thorough comparison between Coomassie staining, silver staining and SYPRO Ruby staining. Coomassie stain is a disulfonated triphenylmethane textile dye, which binds to the amino groups of proteins in acidic solutions. The benefits of Coomassie staining are its easy application, low cost, and high compatibility with downstream methods such as MS/MS. However, the sensitivity and linear range of Coomassie are still the major limitations of the method. Silver staining is usually performed by using silver nitrate and formaldehyde developer in alkaline carbonate buffer. Silver staining is a relatively sensitive method enabling the detection of protein quantities as low as 0.1 ng, however, its limitations includes low reproducibility due to termination of reaction at an arbitrary time point, narrow linear range and interferences

with the MS/MS analysis by reducing the number of identified peptides and the sequence coverage of the proteins. SYPRO Ruby is a ruthenium metal chelate, which binds to basic amino acid residues. It is as sensitive as silver staining, but it offers a significantly broader linear dynamic range, high reproducibility of the quantitative results and high compatibility with MS/MS. All these features highlight its advantage over Coomassie and silver staining methods (Lopez et al., 2000). Following protein separation and staining, the proteins are typically excised from the gel using a scalpel or a robot to segment the desired area of the gel for in-gel trypsin digestion.

2.4.1.3 Protein digestion

To facilitate protein identification by MS/MS, proteins are usually digested chemically or enzymatically into fragments. This step is essential to reduce the complexity of protein mixtures by generating peptide fragments which are smaller in size and more tractable chemically. Enzymatic digestion of proteins could be performed in solution or gel. Trypsin which is the most commonly used digestive enzyme cuts the peptide bond specifically at the carboxyl end of the basic amino acids arginine and lysine. While conventional in-gel digestion by trypsin is typically carried out overnight at 37^{o}C, the digestion process using trypsin modified by reductive methylation cleavage can be performed at a higher temperature (50^{o}C-55^{o}C) and the digestion time can be significantly reduced from 16 hours to 30 minutes without losing the efficiency of the protein digestion (Shevchenko et al., 1996). A study indicates that a sufficient ratio of enzyme to substrate is 1:20 (Hustoft et al., 2011). C18 ZipTip procedure is often used to purify the digest from the mixture of buffers and salts added during sample preparation, prior to separation by high performance liquid chromatography.

2.4.1.4 High performance liquid chromatography

A high performance liquid chromatography (HPLC) system consists of several components as follows: i) a column packed with nonpolar (hydrophobic) beads, referred to as the stationary phase, ii) a pump that creates pressure that moves the polar mobile phase through the column and iii) a detector that captures the retention time. The sample is diluted in the aqueous solution and added to the mobile phase. As the peptides are pushed through the column, they bind to the beads proportionally to their hydrophobic segments. Thus, hydrophilic peptides would elute faster than hydrophobic peptides. HPLC separation allows only a small subset of peptides to be eluted from the LC column at a particular time into the mass spectrometer. Peptides of similar molecular mass but different hydrophobicity elute from the LC column and enter the mass spectrometer at different times. Reduction in the overlap of the peptides of the same mass dramatically increases the peak resolution. In most cases, strong cation exchange (SCX) chromatography is used as a first separation step while reversed-phase chromatography (RP) is used as a secondary separation step because of its ability to remove salts and its compatibility with MS through electrospray ionization (Lee et al., 2006; Sandra et al., 2009). Both SCX and RP are essential to improve low-level separation of SDS-PAGE (Kwon et al., 2010) and their combination forms the basis of the Multidimensional Protein Identification Technology (MudPIT) approach (Washburn et al., 2001).

2.4.1.5 Protein identification

MS/MS is the major technique for high-throughput protein identification. Typically, protein identification via MS/MS is performed either in the form of top-down proteomics or bottom-up proteomics. Top-down proteomics is the analysis of intact or whole-protein whilst bottom-up proteomic or shotgun proteomics is the analysis of

enzymatically produced peptides. Protein identification within complex mixture usually uses peptide mass fingerprinting (PMF) approach when the genome sequence information is available for the organism under study. If there is no genome sequence information available for the studied organism, proteins can be identified by *de novo* sequencing (Hernandez et al., 2006). In automated MS/MS analyses, the mass spectrometer selects peaks from the MS spectrum for fragmentation and MS/MS analysis thereby creating hundreds of high-resolution spectra for comparison of experimentally collected MS/MS spectra with theoretical MS/MS spectra of peptides derived from protein databases and generation of score values for the matching spectra. PMF is an optimal way to identify the proteins derived from the polyacrylamide gels. PMF analysis may involve various bioinformatics tools such as Mascot, Profound, PeptideSearch and PeptIdent. Many factors are taken into consideration during PMF analysis such as the accuracy of matching peptide peaks, intensities of the peaks, modified amino acids, missed or non-specific cleavages during protein digestion, errors in database sequences, calibration of the instrument, and peaks originating either from other peptides and contaminant molecules, or background noise (Palagi et al., 2006).

2.4.1.6 Protein quantitation

There are two approaches for protein quantitation namely label-based and label free protein quantification. Proteins are labelled with either isobaric tags for relative and absolute quantitation (iTRAQ) or by stable isotope labeling with amino acids in cell culture (SILAC) prior to the separation and comparison are performed by detection of the introduced label during MS analysis. This approach, however, requires expensive isotope labels, specific software, and expertise to analyze data. On the other hand, the label free approaches relies on spectral counting or signal intensity measurement during MS/MS. It is assumed that abundant peptides selected for fragmentation would produce

a higher abundance of MS/MS spectra, corresponding with the protein amount. Nonetheless, spectral counting is more susceptible to variation. The proteomic data is often validated by a variety of experimental approaches.

2.4.1.7 Experimental validation

The common experimental methods for validation of MS/MS data are Western blotting, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry and quantitative real-time polymerase chain reaction (qPCR). These convenient methods are widely accepted for the verification of single protein. However, when multiple MS/MS-identified proteins need to be validated, the costs and analysis time would increase. Additionally, suitable antibodies are not available for all protein targets. Therefore, multiple reaction monitoring (MRM) or selective reaction monitoring (SRM) has been a method of choice for validation of multiple protein targets due to its high-throughput and specificity. MRM requires a triple quadrupole mass spectrometer, where in the first quadrupole (Q1) the known precursor ion is isolated, in Q2 the ion is fragmented and in Q3 the optimum fragment ions are monitored. On the other hand, fluorescence-based ProQ Diamond assay is a common approach to simultaneously validate multiple phosphoproteins identified by MS/MS (Gannon et al., 2008; Sheikh et al., 2016; Lv et al., 2016). It is known to exhibit greater sensitivity than the conventional antibody-based methods in the phosphoproteomic analysis (Stasyk et al., 2005; Wu et al., 2005).

2.4.2 Principles of tandem mass spectrometry

The multiple mass analyses in the tandem mass spectrometry are performed using individual mass spectrometer elements physically separated in space or single mass spectrometer separated in time (Hoffmann, 1996). The MS/MS system consists of three

parts namely ionization source, mass analyzer and detector. Principally, the sample needs to be introduced to ionization source prior to isolation by the first analyzer of the mass spectrometer (Madeira & Florêncio, 2012). The ionized sample molecule (precursor ion, m_p+) is fragmented by collision with an inert gas to produce product ions (m_f+) and neutral fragments (m_n) which are then analyzed by the second mass spectrometer (Paulo et al., 2010). The workflow of tandem mass spectrometry is illustrated in Figure 2.12.

2.4.2.1 Scan modes

In the MS/MS system, there are four main scan modes namely precursor ion scan, product ion scan, neutral loss scan and selected/multiple reaction monitoring (SRM/MRM) (Paulo et al., 2012). Precursor ion scan selects a given product ion and determines the precursor ions. Product ion scan then selects a precursor ion of a given m/z ratio and determines the product ions resulting from fragmentation. Neutral loss scan selects a neutral fragment and detects the fragmentations that lead to such loss. SRM selects a fragmentation reaction and focuses on selected m/z ratios thus increasing sensitivity. Both precursor ion scan and neutral loss scan are not available in time-based mass spectrometers such as ion trap and FTICR.

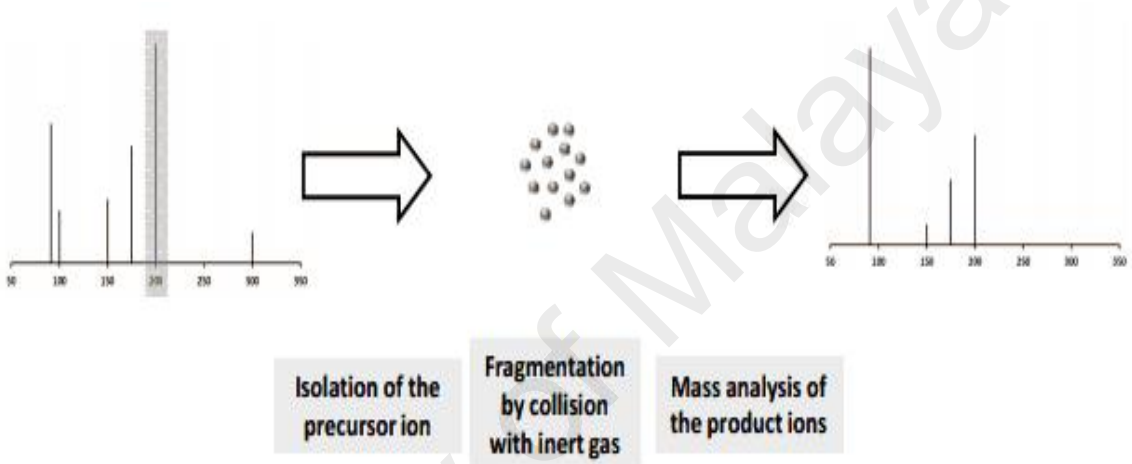


Figure 2.12: Principle of tandem mass spectrometry (Paulo et al., 2012).

2.4.2.2 Ionization methods

Two ionization methods mainly used in the tandem-mass spectrometry-based proteomics are electrospray ionization (ESI) (Fenn et al., 1989) and matrix assisted laser desorption ionization MALDI (Karas & Hillenkamp, 1988). These methods are also referred to as soft ionization methods as they are suitable to generate ions from large molecules without significant fragmentation. Signor and Erba (2013) has made a brief comparison between ESI and MALDI methods as follows: i) ESI ionize molecules directly from liquid phase. ESI-time-of-flight (TOF) coupled to liquid chromatography has been a routine technique for the analysis of intact proteins, because it allows high-accuracy mass determination (≤ 50 ppm). Nonetheless, it is slightly susceptible to the composition of salts and detergents in the sample buffer and to contaminants which are often difficult to eliminate, causing the suppression of the analyte signal; ii) MALDI requires crystallization of molecules with ultraviolet-absorbing organic molecules (matrix molecules) prior to ionization. MALDI-TOF allows intact protein mass determination with sufficient accuracy (≤ 500 ppm) and is less affected by buffer components, detergents and contaminants. Illustration of ESI and MALDI ionization methods are shown in Figure 2.13.

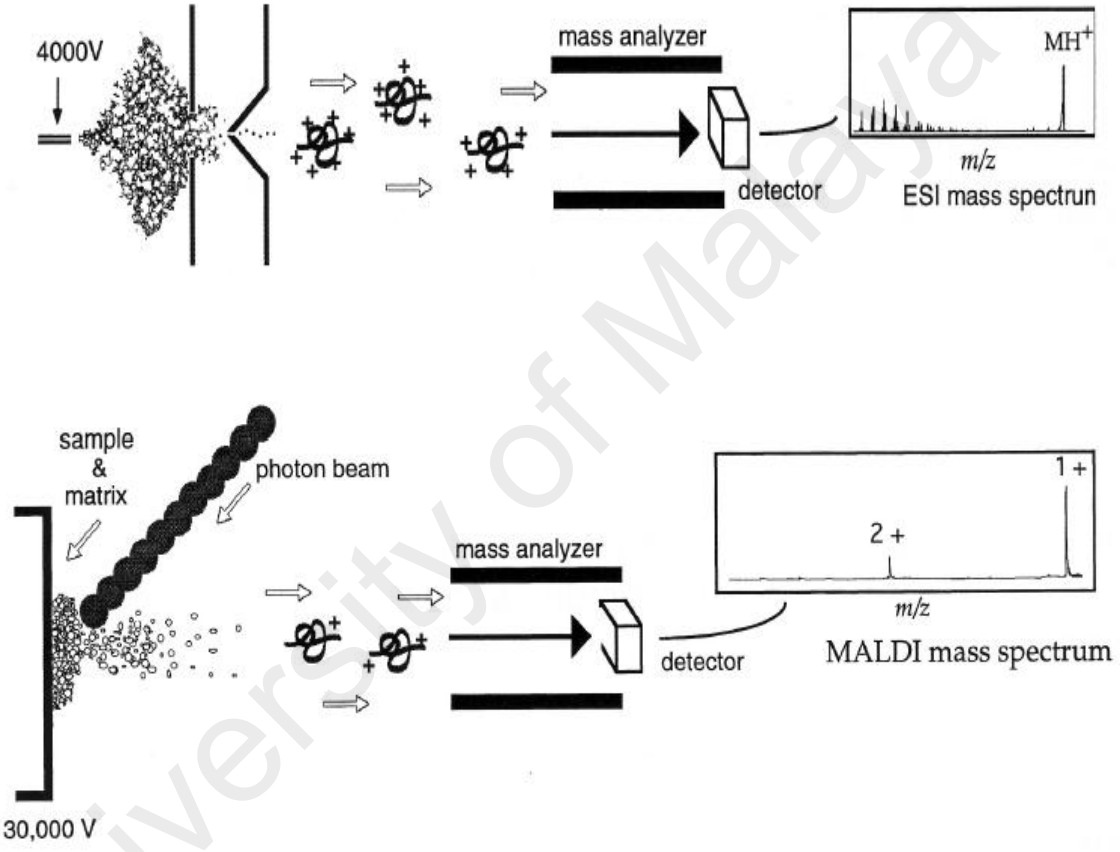


Figure 2.13: Illustrations of ESI (upper panel) and MALDI (lower panel) ionization methods (Hollenbeck et al., 1999).

2.4.2.3 Fragmentation of gas-phase ions

Fragmentation of precursor ions is crucial for tandem mass spectrometry and takes place between first and second mass analyses to activate the precursor ion. This step also ultimately defines the type of product ions in the mass analysis. According to Sleno and Volmer (2004), there are many methods used to fragment the ions and these can result in different types of fragmentation. For examples, collision-induced dissociation (CID) and surface-induced dissociation (SID). In brief, CID involves collision between precursor ions with inert target gas molecules that are accompanied by an increase in internal energy whilst SID requires collision between precursor ions and a solid target surface with or without a self-assembled monolayer causing fragmentation as well as other side reactions. According to Jennings (2000), CID is the most common activation method for the tandem mass spectrometry. A conversion of kinetic energy into internal energy following collisions between the precursor ions and inert gas target (Levsen, 1978) would induce decomposition of precursor ions with higher fragmentation probability (Sleno & Volmer, 2004) and this can be explained by the equation as follows:

$$E_{cm} = [N/(m_p + N)] \times E_{Lab}$$

Where Ecm is internal energy of precursor ion, N is the mass of inert gas target, m_p is the mass of the precursor ion and E_{Lab} is the kinetic energy of precursor ion. Based on the equation above, CID process is highly dependent on the relative masses of the two species namely inert gas target and precursor ion. The larger precursor ions have less internal energy for ion fragmentation through collision processes (Busch et al., 1988).

2.4.2.4 Nomenclature of product ions

A brief discussion on the nomenclature of product ion in this section is based on Mann et al. (2001). Several chemical bonds along the peptide backbone can be broken during CID. The most common ion types are the b and the y ions, which denote fragmentation at the amide bond with charge retention on the N or C terminus, respectively. In proteomics, protein digestion is mostly performed with trypsin enzymes, which have arginyl or lysyl residues as their C-terminal residues. Thus, y ions are the predominant ion series observed in the mass spectra. Nomenclature of product ion is shown in Figure 2.14.

The y ion series of tryptic digests start with masses y1 for the C-terminal amino acid. The next fragmentation peak is y2 ion which differs by the mass of an amino acid residue. Similarly, the b ion series starts with b1 for the N-terminal amino acid and is traced upward in molecular weight. Both b and y ion series confirm the entire peptide sequence. Because not all fragment ions are present at detectable levels and fragments ions can also be produced by double fragmentation of the backbone (internal fragment ions), it is impossible to obtain all of high confidence-peptide sequence.

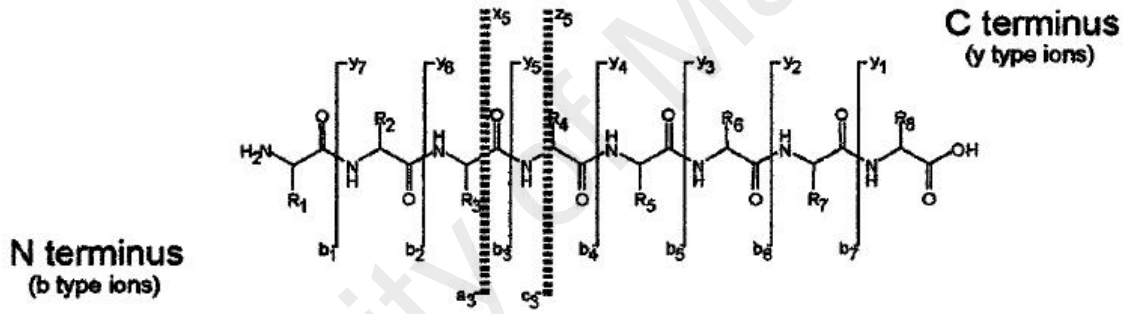


Figure 2.14: Product ion from resulting from CID process.

2.4.3 Types of mass spectrometer

There are four commonly used mass analyzers namely quadrupole, ion trap, time-of-flight (TOF) and Fourier transform ion cyclotron resonance (FTICR). Basically, these mass analyzers differ in accuracy, resolution, mass range, and sensitivity. Accuracy is defined as the ability by which the mass spectrometer can accurately provide m/z information. Resolution is the ability of a mass spectrometer to distinguish between ions of different m/z ratios. Mass range is the m/z range detected by mass spectrometer. Sensitivity is defined as the ability of mass spectrometer to discriminate molecular masses. Combination of two or more mass analyzers produces hybrid mass spectrometers such as triple quadrupole, quadrupole-TOF, quadrupole-ion trap and TOF-TOF (Gygi & Aebersold, 2000). The structure of QTOF mass spectrometer is shown in Figure 2.15. It is similar with a triple quadrupole where the last quadrupole is replaced by a TOF analyser. Principally, ions are produced at atmosphere in the ion source. After passing through gas stream, the ions enter the vacuum system and are focused into the first quadrupole section (q0). The ions are then separated according to their masses in Q1 and dissociated in Q2. The ions enter the orthogonal TOF analyzer through a grid which are then pulsed into the reflector and onto the detector, where they are recorded. In TOF analyser, ions of the same exact mass but slightly different kinetic energies arrive at the detector at exactly the same moment. The mass-to-charge ratio (m/z) is correlated with the time taken by an ion to reach the detector; the lighter ions arrive at the detector first.

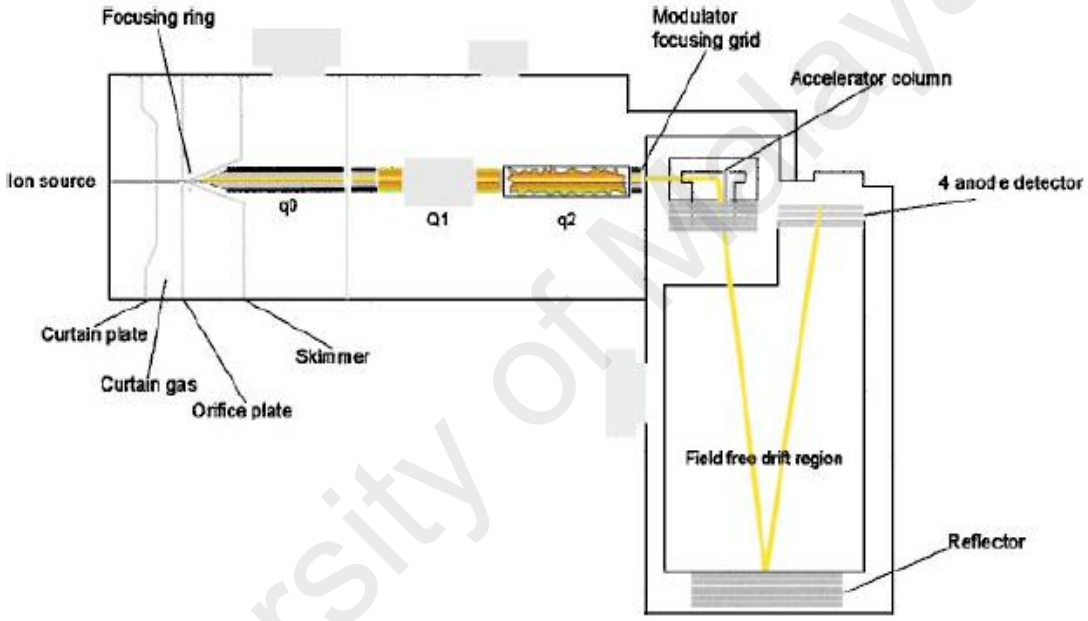


Figure 2.15: Schematic illustration of quadrupole time of flight mass spectrometer (Mann et al., 2001).

Understanding the kinetic energy of precursor ion is essential for general classification of MS/MS (Paulo et al., 2012). High-energy CID produces a broad internal energy distribution. It is very reproducible as changes in the collision conditions such as collision gas, pressure, and temperature do not produce large changes in the product-ion mass spectrum. The high-energy CID which occurs in the kiloeV range is used in TOF-TOF instrument (Paulo et al., 2012). Low-energy CID produces narrower internal energy distributions. Due to that, it less reproducible as changes in the collision conditions may result in a large difference in the product-ion mass spectrum. The low-energy CID which occurs in the 1-100eV range is used in quadrupole instruments (such as triple quadrupole) and trapping instruments (such as ion traps and FTICR) (Paulo et al., 2012).

2.4.4 Tandem mass spectrometry-based proteomic analysis of heterogeneous biofilm

Tandem mass spectrometry has been widely employed in the proteomic analysis of biofilms and EPS matrix. In most studies, the tandem mass spectrometry is combined with liquid chromatography which enables the high-level protein separation prior to protein identification. Table 2.5 highlights the use of tandem mass spectrometry in the studies of microbial biofilms.

Table 2.5: Proteomic analyses which employ tandem mass spectrometry to identify biofilm proteins.

Microorganisms	Instruments	Findings
Campylobacter jejuni	two dimensional gel electrophoresis and capillary liquid chromatography coupled to quadrupole time-of-flight (QTOF) mass spectrometer	Identification of flagellar motility complex proteins associated with pellicle formation at air-liquid interface and cell attachment to solid surface (Kalmokoff et al., 2006)
Pseudomonas aeruginosa	liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometer	Identification of 21 phosphoproteins in 24-hour biofilm (Petrova & Sauer, 2009)
Salmonella typhimurium	a combination of two dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer	Identification of at least 175 unique proteins which were present in biofilm fraction and absent in planktonic fraction (Hamilton et al., 2009)
Vibrio cholerae	A combination of high performance liquid chromatography and LTQ linear ion-trap mass spectrometer	Identification of Bap1 and RbmA proteins which were associated with extracellular matrix of biofilm (Absalon et al., 2011)
Streptococcus pneumoniae	two dimensional ultra performance liquid chromatography coupled to quadrupole time-of-flight	Identification of nine differentially expressed glycolytic enzymes (such as gapA, pgk, and pyk) during biofilm formation (Allan et al., 2014)

Table 2.5, continued

Microorganisms	Instruments	Findings
Salmonella typhimurium	a combination of two dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer	Identification of differentially expressed proteins (such as ribose-phosphate pyrophosphokinase, phosphoglycerate kinase and adenylate kinase) following exposure to acid stress (Cho & Ahn, 2014)

Technical and biological variations are known to cause differences in the protein abundance detected in the polyacrylamide gel images. While various technical variations such as pipetting errors, sample loss during electrophoresis, inconsistent staining time and inconsistent image acquisition (Challapalli et al., 2004) are typically easy to overcome, biological variation which is due to sample heterogeneity is a more complicated issue in the proteomic analysis. Bacteria cells growing in biofilm growth mode are physiologically heterogeneous depending on their spatial location and chemical gradient of nutrients (Xu et al., 1998; Stewart & Franklin, 2008), making the quantitation of protein expression changes inconsistent and challenging (Williamson et al., 2012).

Dynamic range of molecular expression in the heterogeneous biofilm have been addressed in the past few years. In thick biofilm, the bacterial cells at the top of the biofilms exhibits distinct pattern of gene expression, growth and antibiotic tolerance as compared to those of bacterial cells at the bottom of biofilm (Williamson et al., 2012). The high heterogeneity within the biofilm also contributes to different levels of metabolism and subproteome expression (Couto et al., 2015). Moreover, consistent proteomic identification of surface proteins of uropathogenic *E. coli* (UPEC) is hampered by its extensive genotypic and phenotypic heterogeneity (Wurpel et al., 2016). Across many published works on proteomic analysis of biofilms, the issue of biofilm heterogeneity is often neglected and is probably the important cause of dynamic range of protein expression. This could be seen in many cases in which the proteomic analyses are often necessary to harvest the entire biofilm community for sufficient protein samples and provide information as an average of that for the entire biofilm (Williamson et al., 2012). According to An and Parsek (2007), the average molecular expression across the entire biofilm population do not account for physical and chemical

heterogeneities in the biofilms. The key factors to explain the inconsistent protein expression are as follows: i) the mixture between monolayer and multilayer biofilms, ii) biofilm thickness, iii) spatial location of biofilm cells and iv) chemical gradient of nutrients (Entcheva-Dimitrov & Spormann, 2004; Xu et al., 1998; Stewart & Franklin, 2008; Karatan & Watnick, 2009). To resolve this problem, researchers may consider the following strategies to obtain the homogeneous biofilm fraction for quantitative proteomic analysis.

2.4.4.1 Isolation of monolayer biofilm

A quantitative proteomic analysis of biofilm should be performed at the time points which are not associated with the maturity and heterogeneity of biofilm. Based on the model of biofilm development established by Monroe (2007) and Karatan and Watnick (2009), the stages where irreversible attachment and early phase of biofilm architecture occur are defined as biofilm monolayer and are less heterogeneous. Considering, their similar molecular expression pattern (Williamson et al., 2012) and metabolic activity (Couto et al., 2015), these stages are suitable for quantitative proteomic analysis. According to Moorthy and Watnick (2004), monolayer biofilm could be produced by inhibition of expression of genes associated with carbohydrate namely mannose-sensitive haemagglutinin type IV pilus (MSHA) or cultivation of biofilm using minimal medium lacking monosaccharides. Over a 24 h-period, the monolayer biofilm is unable to progress to a multilayer biofilm in the absence of monosaccharides. Their findings have concluded that a surface is not absolutely necessary for creation of the biofilm state. Producing the monolayer biofilm using monosaccharide-deficient medium is feasible, however, such minimal medium lacking monosaccharides may not be widely commercialized. Additionally, the total proteins from the monolayer biofilm may be insufficient for large-scale proteomic analysis.

2.4.4.2 Laser capture microdissection

Laser capture microdissection (LCM) technique is a technique for isolation of specific subpopulation of cells of interest from microscopic area. Williamson et al. (2012) reported the advantage of this technique in the transcriptomic and metabolic studies of vertical biofilm strata. They successfully captured biofilm subpopulations from the top 30 μm and bottom 30 μm of *P. aeruginosa* PAO1 biofilms (average thickness of ~ 350 μm) and revealed the distinct pattern of gene expression, antibiotic susceptibility and green fluorescent protein (GFP)-based metabolic activity between different biofilm subpopulations. Although the technique has been shown to be readily compatible with subsequent analysis of gene expression, limited data is available on its application in the proteomic analysis of isolated biofilm subpopulations. Also, application of LCM in the proteomic study of heterogeneous biofilm is subjected to equipment availability.

2.4.4.3 Subtractive proteomic analysis

Proteins expressed in biofilms are typically categorized into upregulated, downregulated and unique expression (Oosthuizen et al., 2002). On assumption that the biofilm sample is homogenous, most researchers often ignore the inconsistent pattern of protein expression. In fact, the analysis of upregulated and downregulated biofilm proteins deserve further consideration especially when the biofilms have reached the mature and heterogeneous stages that typically reflect in the large standard deviation of protein expression level. Therefore, the unique proteins which are uniquely expressed in one state but not the other state are likely to be not influenced by biofilm heterogeneity. This assumption is based on the concept of subtractive proteome analysis which identifies the proteins exclusively expressed in one state but not the other. This approach has been evidenced to resolve the problem of heterogeneous proteome sample and dynamic range of protein expression (Schirmer et al., 2003; Oh et al., 2004). The same approach has

been used by Hamilton et al. (2009) to identify 175 unique proteins which were specifically present in biofilm fraction and absent in planktonic fraction. Recently, Husi et al. (2016) used the subtractive proteomic approach to identify diagnostic cancer biomarkers from urine samples. They observed that such analytical approach was useful to reduce the complexity of urinary proteome and remove unrelated profiling data. On the other hand, the subtractive comparison of bacterial genome and proteome with those of human using bioinformatics tools have also been used to identify the potential drug and vaccine targets which are essential to the survival of pathogens and non-homologous to human (Sakharkar et al., 2004; Sharma et al., 2008; Shobana & Thiagarajan, 2011; Shinde et al, 2013). Collectively, it could be inferred that the subtractive comparison of protein profile for identification of the unique proteins is a feasible, straightforward and cost-effective strategy to study the heterogeneous biofilms.

CHAPTER 3: MATERIALS AND METHODS

3.1 General chemicals

Glycine (CALBIOCHEM), sodium acetate (CALBIOCHEM), acetic acid (MERCK), methanol (MERCK), ethanol (MERCK), nutrient broth (Difco Laboratories, USA), crystal violet (Sigma, USA), resazurin (Sigma, USA), sodium dodecyl sulfate (Sigma, USA), phenylmethane sulfonyl fluoride (Sigma, USA), coomassie brilliant blue R-250 (Fluka), Laemmli sample buffer (Biorad), Tris base (CALBIOCHEM), acrylamide (Sigma, USA), N,N'-methylenebisacrylamide, (Sigma, USA), sodium chloride (Sigma, USA), 2-mercaptoethanol (Sigma, USA), ammonium bicarbonate (Sigma, USA), acetonitrile (Sigma, USA), trifluoroacetic acid (Sigma, USA).

3.2 Kits

PeppermintStick Phosphoprotein Molecular Weight Standards (Invitrogen, USA), TrypsinGold (Promega). Bradford dye reagent (BioRad, USA), BLUeye Prestained Protein Standard (Sigma, USA), Pro-Q® Diamond Phosphoprotein Gel Stain (Invitrogen, USA).

3.3 Analytical instruments

NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, USA), Synergy H1 Hybrid microplate reader (BioTek Instruments Inc., USA), Spectronic 20 Genesys spectrophotometer (Spectronic Instruments, NY), Nicolet 6700 ATR-FTIR spectrometer (Thermo Fisher Scientific Inc., USA), Infinite Focus Microscope G4 (Alicona, Austria), Mini-PROTEAN Tetra Cell (BioRad, USA), Prominence nano HPLC system (Shimadzu, Japan) coupled to a 5600 TripleTOF mass spectrometer (AB Sciex, USA).

3.4 Software and database

Transmembrane Helix Hidden Markov Model (http://www.cbs.dtu.dk/services/TMHMM/), Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/), Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/), Prosite (http://prosite.expasy.org/), Search Tool for the Retrieval of Interacting Genes/Proteins (http://string-db.org/), Database of Essential Genes (http://www.essentialgene.org/), Type III Secretion System (http://gecco.org.chemie.uni-frankfurt.de/T3SS_prediction), SubLoc (www.bioinfo.tsinghua.edu.cn/SubLoc/), Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ImageJ (www.imagej.nih.gov/ij/), ScanProsite (http://prosite.expasy.org/scanprosite/),

3.5 Test microorganisms

Salmonella typhimurium ATCC 14028, *Escherichia coli* ATCC 1299 *and Pseudomonas aeruginosa* ATCC 10145 were maintained on nutrient agar media (Difco Laboratories, USA). Batch cultures of test microorganisms were grown at 37°C in nutrient broth (Difco Laboratories, USA). Culture purity was assessed regularly by Gram-staining and colony morphology. For all biofilm assays, turbidity of bacterial inoculum was adjusted to 9×10^8 CFU/mL.

3.6 Test compounds

Protein kinase inhibitors used in this study were dimethyl sulfoxide (Merck, Germany) and afatinib (Abcam, UK). DMSO and afatinib were tested alone and in combination. Afatinib was prepared in the concentration range between 0.0032 and 3.2 µg/mL in which the final concentration of DMSO was less than 1.0%. This range of concentration was known to be effective against cancerous cells and malarial parasites (0.00024-243

μg/mL) (Ioannou et al., 2011; Lacouture et al., 2013; Sun et al., 2014). DMSO was tested in the range between 1% and 32% which is safe for topical and oral applications (Zuurmond et al., 1996; Morton, 1993). On the other hand, the preparation of DDA involved dissolution and dilution of afatinib in absolute DMSO to obtain various test concentrations. All test solutions were prepared fresh prior to antibiofilm assay.

3.7 Experimental design

Antibiofilm study began with general screening of antibiofilm effects of protein kinase inhibitors against biomass, viability and EPS matrix. The effective compound was then selected and analysed for differential protein expression in *S. typhimurium* biofilm using SDS-PAGE. The proteins of interest in the polyacrylamide gels were digested with trypsin and identified by MS/MS analysis. Several of the MS/MS-identified proteins were validated using a highly sensitive fluorescence-based assay. To gain deeper insights into the MS/MS-identified proteins, a series of *in silico* analyses were performed to mainly evaluate their functional linkages and potential as therapeutic targets. The workflow of antibiofilm study is shown in Figure 3.1.

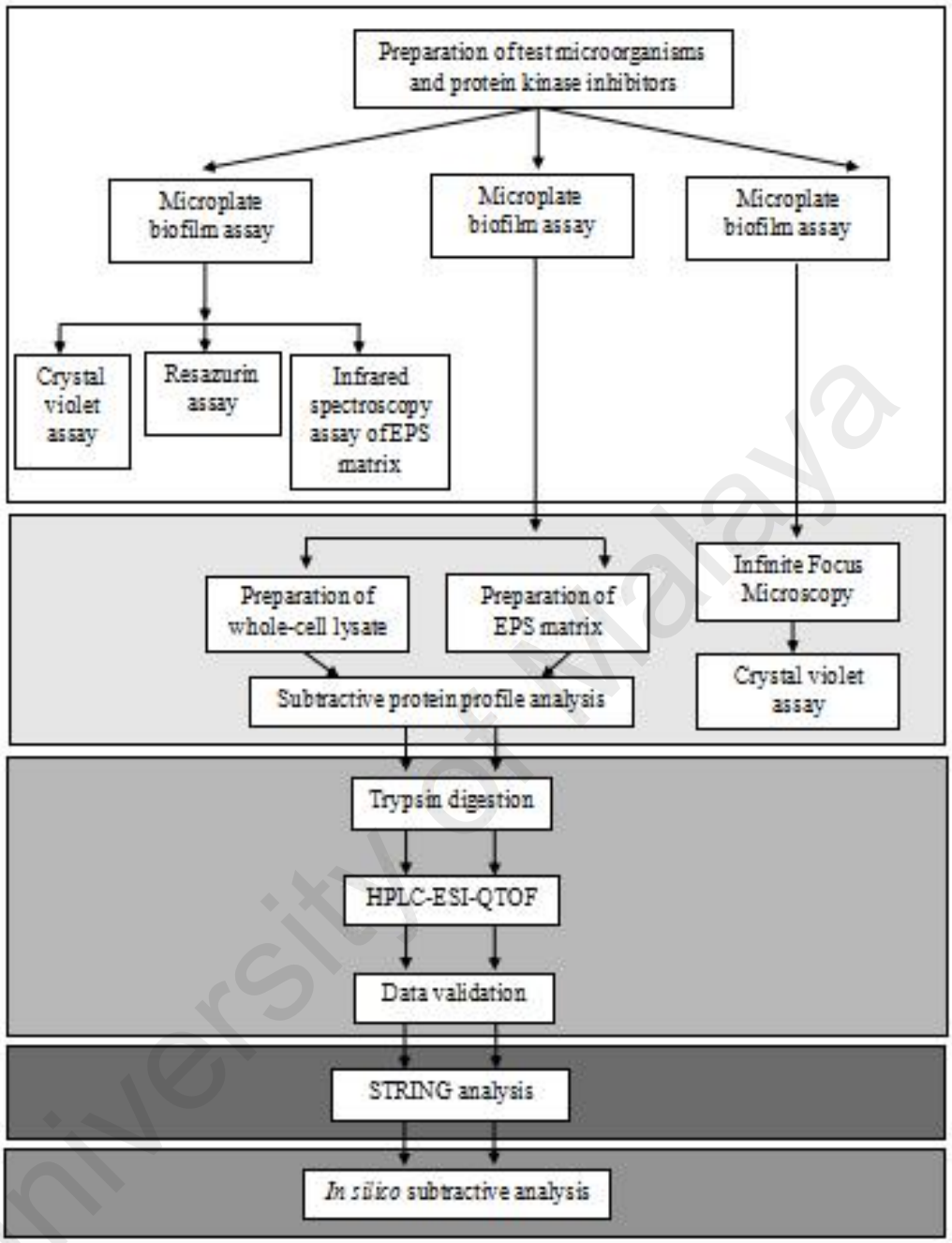


Figure 3.1: Workflow of antibiofilm study. Color scale indicates the depth of study.

3.8 Antibiofilm screening

Antibiofilm screening involved biofilm assay in 96-well microplate and microcentrifuge tube. Biofilm fractions from the 96-well microplates were analysed using crystal violet assay and resazurin assay. Biofilm fractions from the microcentrifuge tubes were analysed using attenuated total reflectance fourier transform infrared (ATR-FTIR) spectroscopy. The effective antibiofilm compound was further studied for protein profiling of whole-cell and EPS proteins to determine its molecular mechanism

3.8.1 Biofilm formation in 96-well microplate

Overnight inoculums (100 µL) of test microorganisms were loaded into microplate wells. Fresh nutrient broth (70 µL) (Difco Laboratories, USA) and inhibitor (80 µL) solution were then added. The mixture was incubated at 37ºC for 24 hours. Wells containing 100 µL of overnight inoculum and 150 µL of fresh nutrient broth were used as controls.

3.8.2 Resazurin assay

Following 24 hour incubation, nutrient medium containing stationary-phase planktonic cells was aspirated from the 96-well microplates and loaded into another 96-well microplate whilst biofilm cells were rinsed with distilled water twice to remove non-adherent cells. Both the planktonic and biofilm fractions (in separate 96-well microplates) were then incubated with 0.02% resazurin (Sigma, USA). Determination of cell viability was based on the irreversible reduction of the blue colored resazurin to pink colored resorufin after at least two hours of incubation at 37ºC.

3.8.3 Crystal violet assay

After 24 hour incubation, nutrient medium containing stationary-phase planktonic cells was discarded from 96-well microplates whilst biofilm cells were rinsed with distilled water twice to remove non-adherent cells. The biofilm fraction was then heat-fixed for 30 minutes, stained with 0.5% crystal violet (Sigma, USA) at room temperature for five minutes and destained with distilled water gently. Stained biofilm biomass was then dissolved in absolute ethanol (Merck, Germany) and measured spectrophotometrically at 600 nm using a Synergy H1 Hybrid microplate reader (BioTek Instruments Inc., USA).

3.8.4 Biofilm formation in microcentrifuge tube

Overnight inoculums (400 µL) of test microorganisms were loaded into 1.5 mL microcentrifuge tubes. Fresh nutrient broth (280 µL) (Difco Laboratories, USA) and inhibitor solution (320 µL) were then added. The mixture was incubated at 37^{o}C for 24 hours. Tubes containing 400 µL of overnight inoculum and 600 µL of fresh nutrient broth were used as controls.

3.8.5 Extraction of EPS matrix

Following 24 hour incubation, nutrient medium containing stationary-phase planktonic cells was discarded from 1.5 mL microcentrifuge tubes whilst biofilm cells were rinsed with distilled water twice to remove non-adherent cells. Extraction of EPS matrix was performed as previously described by Lewandowski and Beyenal (2013) with slight modification. In brief, the biofilm fraction was suspended in 300 µL of 0.9% NaCl (Sigma, USA) and vigorously vortexed for five minutes. A minor modification was made in this step whereby the 0.9% NaCl solution was supplemented with 1mM PMSF (Sigma, USA) and 1% SDS (Sigma, USA). The suspension was centrifuged at 4000g

for 15 minutes at 4°C and the resulting supernatant was subjected to incubation with 1200µL of absolute ethanol (Sigma, USA) overnight at -20°C. The mixture was then centrifuged at 4000g for 15 minutes at 4°C and pellet containing EPS matrix was suspended in 400 µL of deionized water.

3.8.6 ATR-FTIR spectroscopy

Analysis of EPS matrix was performed using a Nicolet 6700 ATR-FTIR spectrometer (Thermo Fisher Scientific Inc., USA). Suspension of EPS matrix (100 µL) was positioned in direct contact with a diamond crystal and scanned in the range between 4000 and 400 cm^{-1} at a resolution of 2 cm^{-1}. A total of 16 IR spectra were acquired for each sample. Data collection, visualization and processing was performed using OMNIC software spectrometer (Thermo Fisher Scientific Inc., USA). IR spectra were automatically baseline corrected and normalized to produce a spectrum of EPS matrix. To validate the data obtained from IR spectroscopy assay, the suspension of EPS matrix was then analysed for total EPS proteins using standard Bradford protein assay.

3.8.7 Bradford protein assay

Bradford protein assay was performed in 1.5 mL microcentrifuge tubes. Standards (bovine serum albumin, BSA) were prepared in the range between 2 µg and 200 µg in a final volume of 800 µL. Samples were diluted with distilled water in a final volume of 800 µL. Bradford reagent (200 µL) was added to each tube, vortexed and incubated for five minutes at room temperature. Absorbance was measured at 595 nm using Spectronic 20 Genesys spectrophotometer (Spectronic Instruments, NY). Standard curve was produced using scatter plot.

3.8.8 Statistical analysis

All data from biofilm biomass microplate assay and EPS protein assay were expressed as mean ± standard deviation with n = 3. Independent T-test was performed to determine the degree of significant difference between control and test groups. A value of $p<0.05$ was considered significant. The Pearson correlation coefficient test was performed to determine the strength of association between biofilm biomass and EPS proteins.

3.9 Infinite Focus Microscopy

Infinite Focus Microscope (IFM) analysis was conducted to study heterogeneity of *S. typhimurium* biofilm. Briefly, this analysis involved preparation of mild steel coupons, biofilm formation in 6-well microplate and biofilm imaging. To verify the finding from IFM analysis, a biofilm assay in sterile test tube and crystal violet assay were performed.

3.9.1 Preparation of mild steel coupons

Mild steel ASTM A283-D coupons with dimensions of 15 mm x 15 mm x 1 mm were used for IFM analysis. The coupons were ground with SiC paper to a smooth surface and subsequently polished to a mirror-finish surface using alumina paper. The polished coupons were rinsed with deionized water thrice, followed by degreasing with acetone, sterilized by immersing into ethanol solution (70%) for 4 hours, and air dried. All the coupons were stored in a desiccator prior to the medium exposure.

3.9.2 Exposure of mild steel coupons to biofilm assay

The mild steel coupons were introduced into the biofilm assay in 6-well microplate with the surface of interest facing upward. Overnight inoculum (2 mL) of test microorganism

was loaded into microplate wells. Fresh nutrient broth (1.4 mL) (Difco Laboratories, USA) and inhibitor solution (3.6 mL) were then added. The mixture was incubated at 37^{o}C for 24 hours. Wells containing 2 mL of overnight inoculum and 3 mL of fresh nutrient broth were used as controls. The mild steel coupons were also exposed to fresh nutrient broth without the bacterial inoculum.

3.9.3 Biofilm imaging

After 24 hour incubation, the mild steel coupons were removed from the 6-well microplate for characterization. Imaging of the biofilm was performed using Infinite Focus Microscope G4 (Alicona, Austria). Magnification of 20× with depolarized light was used. The light source was set to 1.00 with the brightness of the light source set at 107μS. Vertical and lateral resolution were established at 100 nm and 8.80 μm, respectively. Two dimensional images of the biofilms were captured in pseudo color mode.

3.10 Subtractive protein profile analysis

Investigation of antibiofilm mechanism of selected protein kinase inhibitor (Section 3.8) involved biofilm formation in 6-well microplate. Biofilm and EPS fractions from the 6-well microplates were enriched by pooling them from three independent assays (n=3) into a centrifuge tube. In turn, analysis of whole-cell and EPS protein profiles was performed using SDS-PAGE and Coomassie brilliant blue staining. Because the homogeneous sample is critical in proteomic research to generate accurate and informative data, analysis of biofilm heterogeneity by Infinite Focus Microscopy (Section 3.9) was performed to assist the selection of protein bands for high-confidence protein identification which was based on the subtractive comparison of protein profiles between control and test biofilm fractions.

3.10.1 Biofilm formation in 6-well microplate

Two mL of overnight inoculum of test microorganism was loaded into microplate wells. Fresh nutrient broth (1.4 mL) (Difco Laboratories, USA) and inhibitor solution (3.6 mL) were then added. The mixture was incubated at 37°C for 24 hours. Wells containing 2 mL of overnight inoculum and 3 mL of fresh nutrient broth were used as controls.

3.10.2 Whole-cell protein extraction and protein determination

Following 24 hour incubation, nutrient medium containing stationary-phase planktonic cells was discarded from the 6-well microplates while biofilm fractions were suspended in 0.9% NaCl solution supplemented with 1 mM PMSF (Sigma, USA) and 1% SDS (Sigma, USA) and pelleted at 10,000 rpm for 10 minutes. Pellets and supernatants were used for whole-cell protein extraction and EPS protein extraction respectively. Whole-cell proteins were extracted from the resulting pellet using detergent lysis protocol whereby the biofilm fractions were incubated in 25 mM Tris, 150 mM NaCl, 0.5% SDS at 95°C for 15 minutes. The whole-cell protein fractions were then pelleted at 10,000 rpm for 10 minutes. Protein determination was performed using standard Bradford assay (Section 3.8.7).

3.10.3 Extraction of EPS proteins and protein determination

EPS proteins were extracted from the resulting supernatants by incubation with absolute ethanol (Sigma, USA) at -20°C overnight at the ratio of 1:4. The mixtures were then centrifuged at 4000g for 15 minutes at 4°C and the pellets containing EPS proteins were suspended in 25 mM Tris, 150 mM NaCl, 0.5% SDS. Protein determination was performed using standard Bradford assay (Section 3.8.7).

3.10.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Small format gels (7 cm x 8 cm) with 1.5 mm thickness and 5 wells were used for protein analysis. Whole-cell and EPS proteins were dissolved in 1× SDS sample buffer at the ratio of 1:1 and heated at 95°C for five minutes. Biofilm proteins were then separated on the polyacrylamide gels consisting of 12% separating gel and a 4% stacking gel using Mini-PROTEAN Tetra Cell (BioRad, USA). Three replicates of polyacrylamide gel were run in 25 mM Tris, 192 mM glycine, 0.1% SDS for 90 minutes at 100V until the blue dye front reached the bottom of the polyacrylamide gel. The protein samples were run alongside BLUeye Prestained Protein Standard (10– 245 kDa).

3.10.5 Coomassie Brilliant Blue R250 staining and gel imaging

The polyacrylamide gels were stained in 0.25% Coomassie Brilliant Blue R250, 45% methanol, 10% glacial acetic acid overnight with agitation at 60 rpm. The polyacrylamide gels were then destained in 30% methanol, 10% glacial acetic acid for one hour with agitation at 60 rpm. Gel image acquisition was performed using ImageScanner III (GE Healthcare, USA) whilst gel image analysis was performed using publicly available ImageJ software (Natale et al., 2011). Subtractive comparison of protein profiles was performed to identify the unique protein bands which were either expressed only in control biofilm fraction or test biofilm fraction respectively. The unique protein bands were then manually excised using a clean scalpel blade and subjected to protein identification.

3.11 Tandem mass spectrometry

Tandem mass spectrometric analysis involved a combination of trypsin digestion, chromatographic separation, peptide mass fingerprinting and database search.

3.11.1 Trypsin digestion

The unique protein bands (Section 3.10.5) were subjected to trypsin digestion according to standard techniques (Bringans et al., 2008). Excised protein bands were destained with 25 mM ammonium bicarbonate in 50 : 50 acetonitrile : dH_2O for 45 minutes. The destaining process was repeated three times. The destained and washed gel slices were then vacuum-dried and stored at -20°C until further use. Ten µL of trypsin digest solution (12.5 µg/mL trypsin, 25 mM ammonium bicarbonate) was added to each gel slice and incubated overnight at 37°C. The digested peptides were then extracted by incubation with 10–20 mL acetonitrile containing 1% trifluoroacetic acid for 20 minutes, depending on the size of the gel slice. The pooled extracts were dried by Speed-Vac concentrator at room temperature and desalted using C18 ZipTip (Millipore). Concentration of eluted peptides from the C18 ZipTip were then measured using NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, USA) for subsequent tandem mass spectrometry analysis.

3.11.2 Nano HPLC-ESI-QTOF

Tryptic peptides were analysed by Prominence nano HPLC system (Shimadzu, Japan) coupled to a 5600 TripleTOF mass spectrometer (AB Sciex, USA). Tryptic peptides were loaded onto an Agilent Zorbax 300SB-C18 (300 Å, 150 mm, 3.5 µm) (Agilent Technologies) and separated with a 2-40% linear gradient of 98% acetonitrile / 0.1% formic acid at a flow rate of 40 µL/min. For TripleTOF 5600 mass spectrometer, the parameter settings used were as follows: ion spray voltage floating (ISVF) 2300 V, curtain gas (CUR) 20, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 20, declustering potential (DP) 70 V. For information-dependent acquisition (IDA), a MS survey scan in the mass range between 350 and 1250 was followed by MS/MS scan in the mass range between 100 and 1800. For switching criteria, ions in the mass range

between 400 and 1250, charge state between 2 and 5 and ion exclusion for 10 seconds were used.

3.11.3 Database search

QTOF mass spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) and SwissProt database. The search parameters used were as follows: mass values: monoisotopic; peptide mass tolerance: ±0.2 Da; miss cleavage: 1; variable modifications: phospho (ST), phospho (Y). For high-confidence protein identification, protein identified by two or more peptides were selected (Kwon et al. 2010).

3.12 Phosphoprotein analysis

Electrophoretic separation of biofilm proteins was performed as previously described (Section 3.10.4). The resulting polyacrylamide gels were then subjected to ProQ diamond staining (Eugene, USA) based on manufacturer's recommendation with minor modifications. The polyacrylamide gels were fixed in 50% (v/v) methanol, 10% (v/v) trichloroacetic acid for 30 minutes with agitation at 60 rpm. Following wash step with ultrapure water for 10 minutes, the polyacrylamide gels were incubated in the fluorescent dye in the dark for 60 minutes with agitation at 60 rpm. Destaining was achieved with wash using 20% (w/v) acetonitrile, 50 mM sodium acetate, pH 4.0 in the dark for 30 minutes with agitation at 60 rpm. Fluorescently labeled phosphoproteins were visualized and photographed using an Alpha Imager Gel Documentation System (Alpha Innotech, CA). Detection of phosphoproteins at the positions similar to those of unique protein bands (Section 3.10.5) would validate the QTOF data (Section 3.11.3).

3.13 Bionformatic analysis

Bioinformatic analysis was performed to understand the molecular mechanism underlying the antibiofilm effect of 32% DMSO against *S. typhimurium*. All QTOF-identified proteins (Section 3.11.3) were extensively analysed for functional categories, subcellular locations, transmembrane helices, T3SS signal peptides, functional linkages and protein sequence similarity.

3.13.1 General characterization

Classification of QTOF-identified proteins according to biological process was performed based on SwissProt/TrEMBL database. Prediction of subcellular localization was carried out using SubLoc v1.0. Transmembrane helices were predicted using TMHMM Server v2.0. T3SS signal peptides were predicted using T3SS_Prediction with default parameters (prediction method: artificial neural network; threshold value: 0.4). Additional analysis of hypothetical proteins involved domain search against Conserved Domain Database (CDD), motif search using ScanProsite and BLASTp search against non-redundant database with e-value cut-off score of $1e^{-06}$. In the case of EPS proteins, they should fulfil at least one of the following criteria: i) contained signal peptide according to SignalP 4.0, ii) secreted via non-classical secretion pathway according to SecretomeP 2.0, iii) contained eukaryotic-like domains occurring in secreted proteins and iv) contained transmembrane domain according to TMHMM Server v2.0.

3.13.2 Analysis of functional linkages

Prediction of protein interaction network was performed using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database v9.0. This analysis was set at high stringency (STRING score: 0.7) and was based on seven criteria namely

neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining. The protein interaction network was presented under confidence view. The strength of functional linkages was indicated by thickness of solid lines while proteins interactors were represented as nodes. Gene ontology (GO) enrichment analyses of biological process and pathway were performed using false discovery rate (FDR) correction to identify significant ($p<0.05$) biological processes and pathways associated with the protein network. Clustering algorithm of k-means was used to form non-overlapping protein clusters. The resulting protein clusters were then separated manually for better visual representation and understanding.

3.13.3 *In silico* subtractive analysis

Subtraction of protein dataset between pathogen and host would provide information for a set of proteins that are likely to be essential to the pathogen but absent in the host. BLASTp search against DEG database (Zhang et al., 2004) with e-value cut-off score of $1e^{-06}$ was performed to identify the QTOF-identified proteins essential for the survival of *S. typhimurium* pathogen. BLASTp (Altschul et al., 1990) search against the non-redundant database (restricted to *Homo sapiens*) with e-value cut-off score of $1e^{-06}$ was conducted to identify the QTOF-identified proteins which are non-homologous to human proteome.

CHAPTER 4: RESULTS

4.1 Overview of main results

Table 4.1 shows summary of the main results.

4.2 Antibiofilm activities

Each protein kinase inhibitor was subjected to resazurin assay (Section 3.8.2), crystal violet assay (Section 3.8.3), ATR-FTIR spectroscopy (Section 3.8.6) and Bradford protein assay (Section 3.8.7) for analyses of biofilm viability, biofilm biomass, chemical composition of EPS matrix and total EPS proteins respectively. Considering the high diversification in biofilm, a combination of protein kinase inhibitors was also investigated. Their antibiofilm effects on *S. typhimurium* biofilm were compared with those on *E. coli* and *P. aeruginosa.* Protein kinase inhibitor demonstrating the effectiveness against viability, biomass and EPS matrix of *S. typhimurium* biofilm was selected for whole-cell and EPS protein analyses.

4.2.1 Killing effects

The killing effects of DMSO and afatinib against biofilm and planktonic fractions are shown in Table 4.2 whilst a representative viability profile of *S. typhimurium* biofilm is shown in Figure 4.1. Biofilm fractions were killed only by 32% DMSO whilst planktonic fractions were affected differentially by DMSO (Table 4.2a). Biofilm fractions of *E. coli* and *P. aeruginosa*, but not *S. typhimurium*, were observed to be killed by 3.2 μg ml^{-1} afatinib whereas none of the planktonic fractions were killed by 3.2 μg ml^{-1} afatinib (Table 4.2b). 32% DMSO-dissolved afatinib (DDA) was effective against all biofilm and planktonic fractions (Table 4.2c). Because 32% DMSO and DDA were found to be effective against all biofilm and planktonic fractions, they were selected for further analyses.

Table 4.1: Overview of the main results

Section	Main results
4.2	The antibiofilm effects of protein kinase inhibitors: a. The antibiofilm effects on *S. typhimurium* were compared with those on *E. coli* and *P. aeruginosa*. b. 32% DMSO displayed inhibitory effects on viability, biomass and EPS matrix of *S. typhimurium, E. coli* and *P. aeruginosa* biofilms. c. Afatinib alone displayed no antibiofilm activity. d. The combination of protein kinase inhibitors (32% DMSO and 3.2 µg ml^{-1} afatinib) displayed greater inhibitory effects on viability, biomass and EPS matrix of *S. typhimurium, E. coli* and *P. aeruginosa* biofilms. e. Of the two protein kinase inhibitors, DMSO was shown to be effective on biofilms and was therefore selected for further investigation to understand the possible antibiofilm mechanism against *S. typhimurium*.
4.3	Biofilm heterogeneity: a. The changes in two dimensional topographic profile of biofilm surface were observed in IFM analysis indicating biofilm heterogeneity and highlighting the relevant use of subtractive protein profile analysis.
4.4	Subtractive protein profile analysis of whole-cell biofilm: a. The subtractive approach was used due to biofilm heterogeneity and inconsistent expression level of major protein bands in *S. typhimurium* at 24 h across seven independent experiments. b. Treatment with 32% DMSO triggered changes in whole-cell protein expression in *S. typhimurium* biofilm and two unique protein bands, 25.4 kDa and 51.2 kDa were recognized. c. The unique proteins were further analyzed by tandem mass spectrometry. QTOF-based identification of 25.4 kDa and 51.2 kDa protein bands from whole-cell biofilm: a. 29 proteins were identified from 25.4 kDa protein band while 46 proteins were identified from 51.2 kDa protein band. b. Five phosphoproteins were identified based on neutral loss of 98 Da. Phosphoproteins in *S. typhimurium* biofilm: a. In-gel detection of phosphoproteins was performed using ProQ Diamond staining. b. The protein bands of 25.4 kDa and 51.2 kDa were found to contain phosphoproteins, validating the QTOF data and allowing in-depth bioinformatics analyses. c. The phosphorylation level of 25.4 kDa and 51.2 kDa protein bands correlated with the time course of biofilm formation. d. The inhibitory effect of DMSO on phosphorylation of 51.2 kDa protein band was observed. Bioinformatic analyses of QTOF-identified whole-cell proteins: a. Fifty seven out of 75 QTOF-identified whole-cell proteins were predicted to be localized in cytoplasm. b. Four out of 75 QTOF-identified whole-cell proteins were predicted to contain transmembrane domains.

Table 4.1, continued

Section	Main results
	c. Seven out of 75 QTOF-identified whole-cell proteins were predicted to be TTSS effector proteins. d. Sixty six out of 75 QTOF-identified whole-cell proteins were predicted to be functionally linked by at least one STRING criterion. e. Nine biological processes and nine pathways were determined to be significant ($p<0.05$) in the protein interaction network. f. Forty one out of 75 QTOF-identified whole-cell proteins were found to be essential and non-homologous to human, making them ideal therapeutic targets for biofilm control.
4.5	Subtractive protein profile analysis of EPS matrix: a. The subtractive approach was used due to biofilm heterogeneity and inconsistent secretion level of EPS protein at 24 h across seven independent experiments. b. Treatment with 32% DMSO triggered changes in EPS protein secretion and one unique protein band, 51.2 kDa was recognized. c. The unique proteins were further analyzed by tandem mass spectrometry. QTOF-based identification of 51.2 kDa protein band from EPS matrix: d. Three proteins were identified from 51.2 kDa protein band. Bioinformatic analysis of QTOF-identified EPS proteins: e. All the QTOF-identified EPS proteins fulfilled the criteria of secreted proteins. f. All the QTOF-identified EPS proteins were predicted to be functionally linked by several STRING criteria. g. No QTOF-identified EPS proteins were found to be essential to the survival of *S. typhimurium* and were non-homologous to human proteome.

Table 4.2: Killing effects of DMSO (a), afatinib (b) and DDA (c) against test microorganisms. Data are expressed as + and – which indicate killing effect and no killing effect respectively. Effective test concentration to be analysed against biofilm biomass and EPS matrix are shown by *.

a) DMSO

Test microorganisms	Control	Test concentrations (%)					
		32 *	20	10	5	2	1
Biofilm fractions							
E. coli ATCC1299	-	+	-	-	-	-	-
P. aeruginosa ATCC10145	-	+	-	-	-	-	-
S. typhimurium ATCC14028	-	+	-	-	-	-	-
Planktonic fractions							
E. coli ATCC1299	-	+	+	+	+	+	+
P. aeruginosa ATCC10145	-	+	+	+	+	-	-
S. typhimurium ATCC14028	-	+	+	+	+	+	-

b) Afatinib (DMSO <1%)

Test microorganisms	Control	Test concentrations (µg/mL)			
		3.2	0.32	0.032	0.0032
Biofilm fractions					
E. coli ATCC1299	-	+	-	-	-
P. aeruginosa ATCC10145	-	+	-	-	-
S. typhimurium ATCC14028	-	-	-	-	-
Planktonic fractions					
E. coli ATCC1299	-	-	-	-	-
P. aeruginosa ATCC10145	-	-	-	-	-
S. typhimurium ATCC14028	-	-	-	-	-

c) DDA (32% DMSO in combination with 3.2 µg/mL afatinib) *

Test microorganisms	Control	Type of bacterial fractions	
		Biofilm fraction	Planktonic fraction
E. coli ATCC1299	-	+	+
P. aeruginosa ATCC10145	-	+	+
S. typhimurium ATCC14028	-	+	+

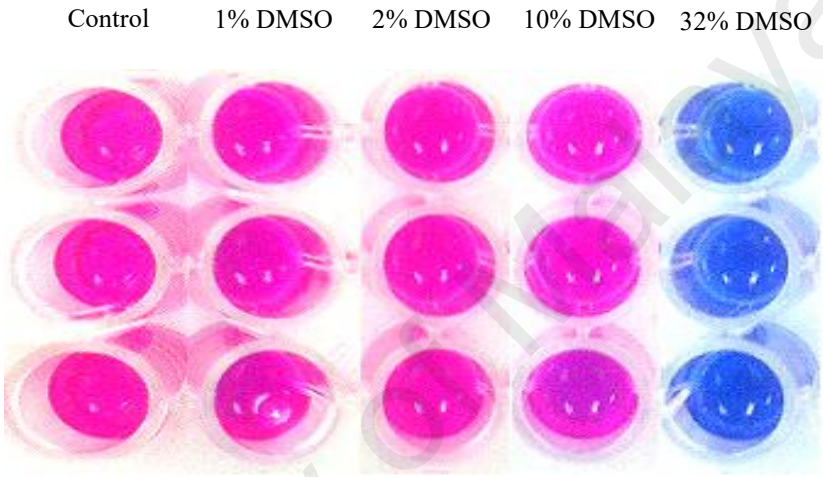


Figure 4.1: Representative viability profile of *S. typhimurium* biofilm. Live biofilm cells would metabolize blue colored resazurin to pink colored resorufin. Pink and blue colors indicate live and dead cells respectively.

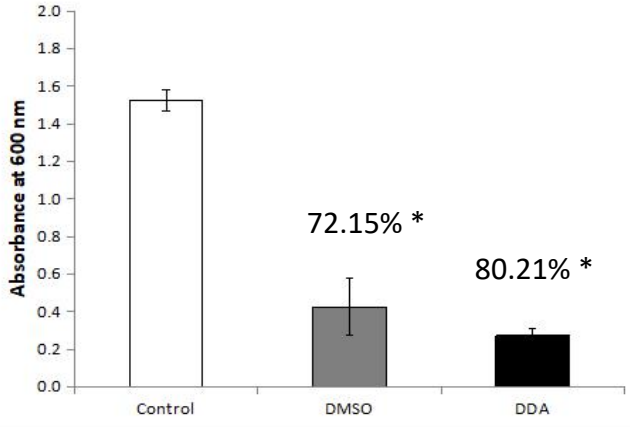
4.2.2 Reduction in biofilm biomass

Biofilm biomass of test microorganisms is depicted in Figure 4.2. Treatment with 32% DMSO and DDA was found to significantly ($p<0.05$) reduce biofilm biomass of all test microorganisms. In all cases, treatment with DDA was found to cause greater reduction in biofilm biomass than 32% DMSO. Biomass of *S. typhimurium* biofilm was the most susceptible to DMSO and DDA treatments whilst biomass of *E. coli* biofilm was the least susceptible.

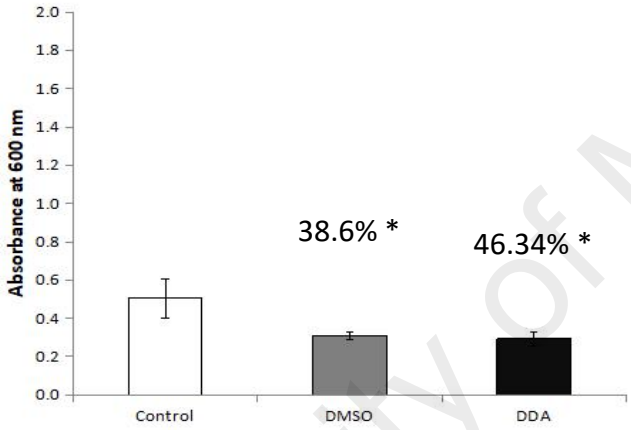
4.2.3 Chemical modification of EPS matrix

Figure 4.3 shows ATR-FTIR spectra of EPS matrix. Noticeably, adequate IR absorbance signal was successfully obtained for a variety of functional groups making the EPS matrix highly charged. As a result of treatment with 32% DMSO and DDA, spectral variations of EPS matrix in *E. coli*, *P. aerugionsa* and *S. typhimurium* biofilms were observed in the range between 1700 and 900 cm^{-1}. Functional groups in the EPS matrix affected were C=O stretch (amide I), N-H stretch (amide II), C=C stretch, N-O asymmetric stretch, C=C stretch, P=O symmetric stretch, C-O-C symmetric stretch and C-O stretch at the frequencies of 1652 cm^{-1}, 1558 cm^{-1}, 1538 cm^{-1}, 1506 cm^{-1}, 1456 cm^{-1}, 1080 cm^{-1}, 1044 cm^{-1} and 1018 cm^{-1}respectively. The decrease in intensity of IR absorbance peaks at all frequencies appeared to be more profound in DDA-treated biofilm.

a) *S. typhimurium*



b) *E. coli*



c) *P. aeruginosa*

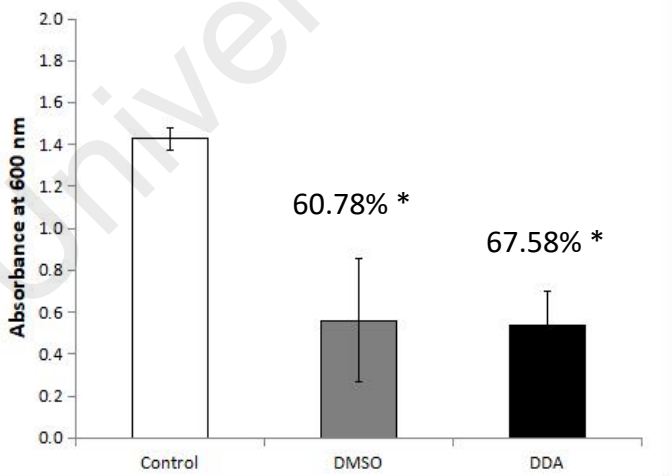
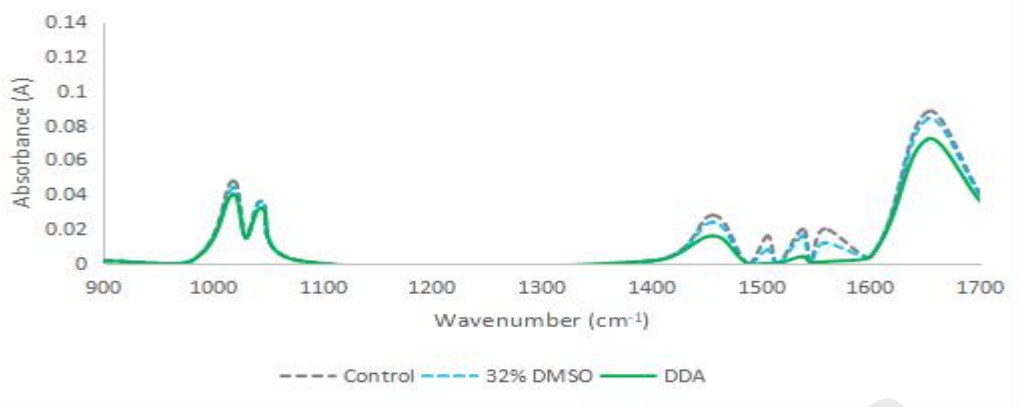
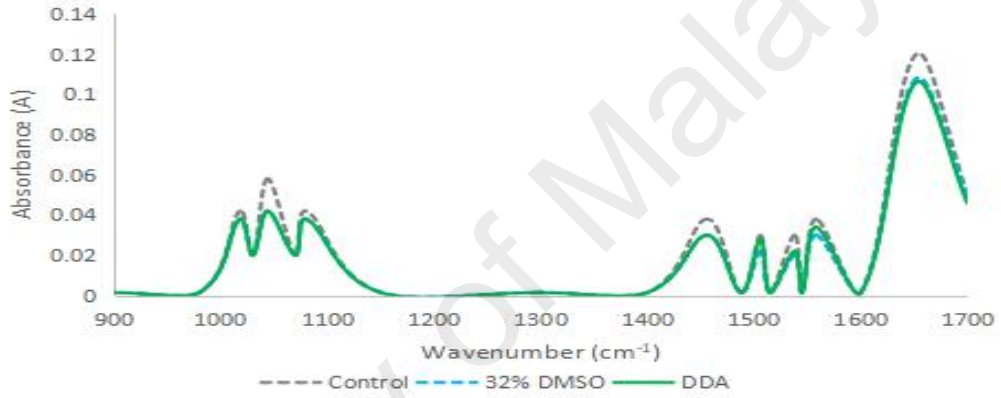


Figure 4.2: Biofilm biomass of *S. typhimurium* (a), *E. coli* (b) and *P. aeruginosa* (c). DMSO: dimethyl sulfoxide; DDA: dimethyl sulfoxide-diluted afatinib. Each column represents the mean ± standard deviation with n = 3. Percentage values of biofilm inhibition are also indicated. Significant differences ($p<0.05$) compared to control are shown by *.

a) *S. typhimurium* ATCC14028



b) *E. coli* ATCC1299



c) *P. aeruginosa* ATCC10145

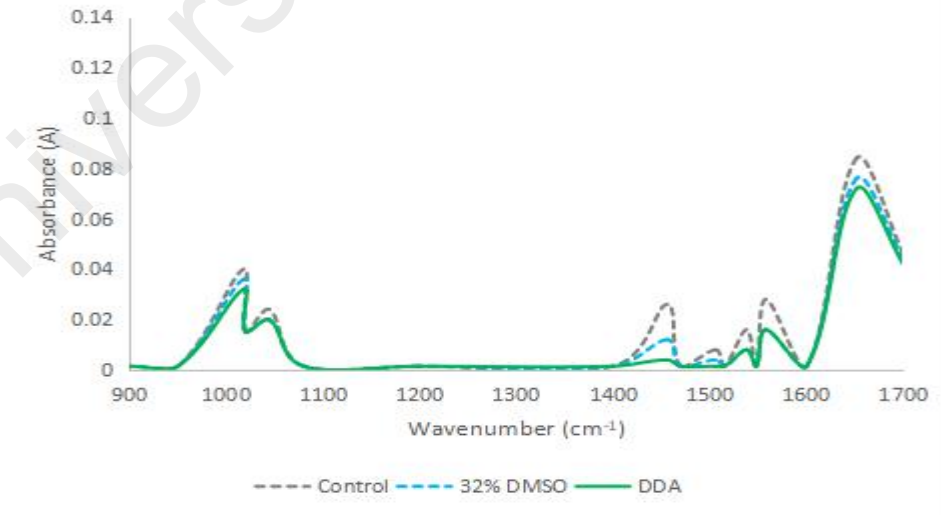


Figure 4.3: ATR-FTIR spectra of EPS matrix of *S. typhimurium* (a), *E. coli* (b) and *P. aeruginosa* (c).

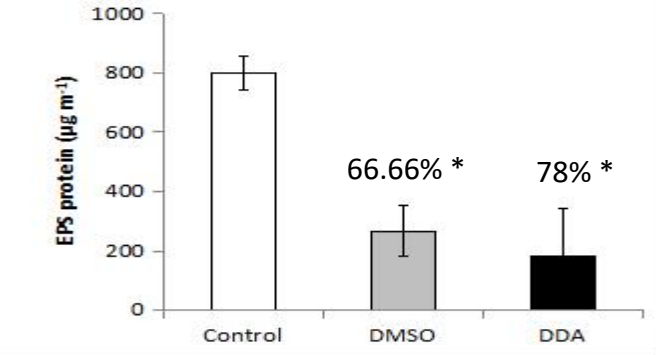
4.2.4 Reduction in EPS proteins

Total EPS protein is presented in Figure 4.4. Treatment with 32% DMSO and DDA caused significant reduction ($p<0.05$) in EPS proteins in all test microorganisms. In all cases, treatment with DDA was found to cause greater reduction in EPS proteins than 32% DMSO. The EPS protein fraction of *S. typhimurium* biofilm was the most susceptible to 32% DMSO while EPS protein fraction of *P. aeruginosa* biofilm was the least. EPS protein fraction of *E.coli* biofilm was the most susceptible to DDA whilst EPS protein fraction of *P. aeruginosa* biofilm was the least. Notably, reduction in total EPS protein verified the chemical modifications of EPS matrix as shown in Figure 4.3.

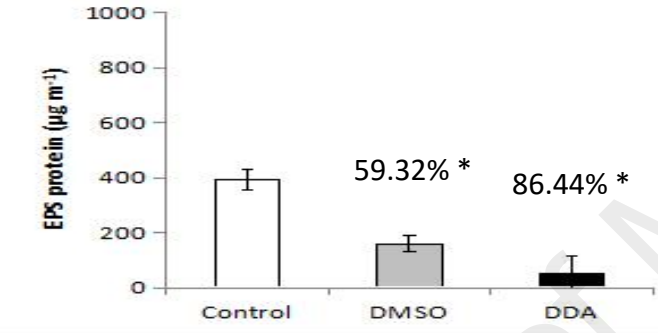
4.2.5 Correlation between biomass and EPS proteins

Clear reducing trends observed in biofilm biomass (Figure 4.2) were also observed in EPS proteins (Figure 4.4), suggesting statistical relationships between them and thus, Pearson correlation coefficient test was performed. Table 4.3 shows the relationship between biomass and EPS protein. R^2 values for *E. coli, P. aeruginosa* and *S. typhimurium* were found to be 0.717, 0.649 and 0.902 respectively. All the observed correlations were strong and significant ($p<0.05$).

a) *S. typhimurium*



b) *E. coli*



c) *P. aeruginosa*

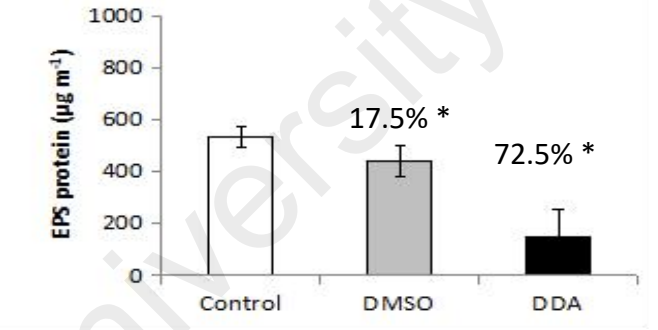


Figure 4.4: EPS proteins of *S. typhimurium* (a), *E. coli* (b) and *P. aeruginosa* (c). DMSO: dimethyl sulfoxide; DDA: dimethyl sulfoxide-diluted afatinib. Each column represents the mean ± standard deviation with n = 3. Percentage values of protein reduction are indicated. Significant differences ($p<0.05$) compared to control are shown by *

Table 4.3: Relationship between biofilm biomass and EPS proteins in the presence of 32% DMSO and DDA.

Test microorganisms	R^2 value	Correlational strength	P value	Significance
E. coli ATCC1299	0.717	Strong	< 0.05	Yes
P. aeruginosa ATCC10145	0.649	Strong	< 0.05	Yes
S. typhimurium ATCC14028	0.902	Strong	< 0.05	Yes

4.3 Heterogeneous biofilm

IFM analysis (Section 3.9.3) was performed to study biofilm heterogeneity. Two dimensional topographic profile highlighted the biofilm heterogeneity and relevant use of subtractive protein profile analysis.

A representative two dimensional topographic profile of biofilm surface is shown in Figure 4.5. The thick biofilm was indicated by yellow color in the pseudocolor mode. It should be noted that the color/height scale accompanying each individual image could not be generalized to other images as each sample was unique in terms of their surface topography and reflectance. It was clear that the biofilms were heterogeneous in thickness and surface coverage. Scanning by IFM also revealed biofilm formation in the presence of 32% DMSO.

Figure 4.6 depicts heterogeneous distribution of crystal violet-stained biofilms in test tubes. Direct observation showed that the biofilms were unevenly distributed. They were mainly formed at air-liquid interfaces and at the bottom of test tubes. The intensity of biofilm detected across different sections of test tubes were also not consistent. This staining profile validated biofilm heterogeneity as shown by IFM analysis. In addition, treatment with 32% DMSO was found to inhibit formation of biofilm at the air-liquid interface.

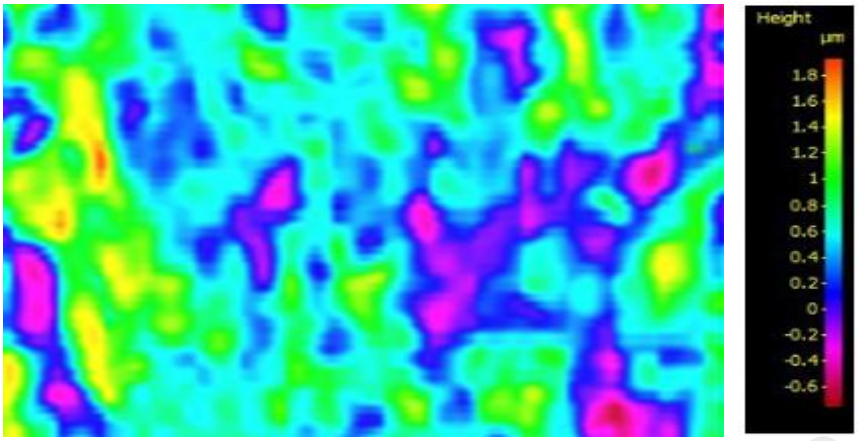


Figure 4.5: Representative two dimensional pseudocolor image of biofilm formed on mild steel surface after 24 hours. The color/height scale correspond to the varying topography, reflectance and thickness of biofilm.

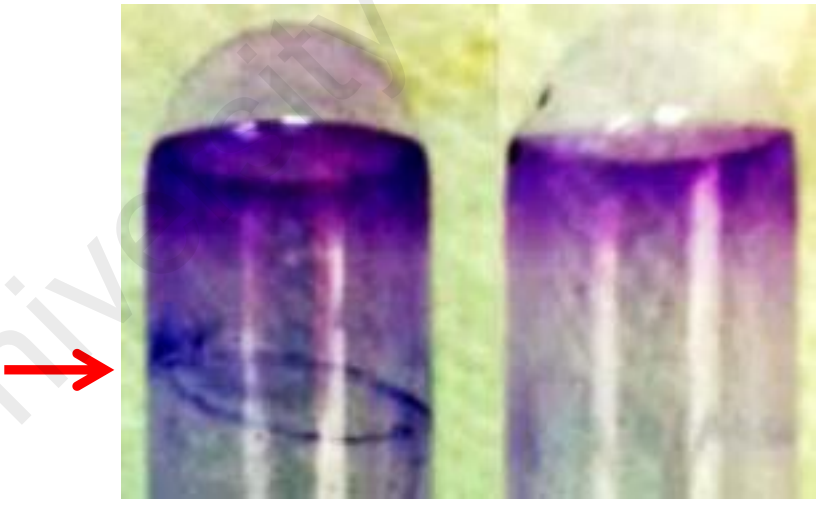


Figure 4.6: Crystal violet-stained biofilms in test tubes. Arrow indicates pellicle formation at air-liquid interface. Left panel: control biofilm; right panel: 32% DMSO-treated biofilm.

4.4 Differential whole-cell protein profiles

Treatment with 32% DMSO effectively inhibited *S. typhimurium* biofilm (Section 4.2.1-4.2.2) and was therefore, selected for whole-cell protein expression analysis. Despite its notable antibiofilm effects, 32% DMSO-dissolved afatinib (DDA) was not subjected to whole-cell protein expression analysis because there was insignificant difference ($p>0.05$) in biofilm biomass of *S. typhimurium* observed between 32% DMSO and DDA treatments (Section 4.2.2), suggesting that 32% DMSO and DDA share a similar molecular basis. Whole-cell proteins of *S. typhimurium* biofilm were fractionated using one dimensional SDS-PAGE (Section 3.10.4), visualized using Coomassie brilliant blue staining (Section 3.10.5) and analysed using ImageJ software (Section 3.10.5).

4.4.1 SDS-PAGE gel images

To optimize the whole-cell protein extraction, detergent lysis of 24 h biofilm fraction was performed at four different temperatures and incubation times as follows: protocol 1: 4^{o}C for 10 minutes; protocol 2: 27^{o}C for 30 minutes; protocol 3: 95^{o}C for one minute and protocol 4: 95^{o}C for five minutes. Only protocol 4 produced approximately 18 protein bands in the range between 17 kDa and 78 kDa. Several major protein bands (78.1 kDa, 51.2 kDa, 41.5 kDa, 37.3 kDa, 35.1 kDa, 27.6 kDa, and 25.4 kDa) were found to be highly reproducible across multiple electrophoretic separations. Thus, protocol 4 was used for the subsequent protein analyses.

To study the consistency of whole-cell protein expression, the experiments were repeated seven times in the absence of DMSO. The one dimensional PAGE protein banding pattern of *S. typhimurium* biofilm at 24 h was highly reproducible, however, the intensity of all protein bands was inconsistent although the protein amount loaded into

the polyacrylamide gels (4.5 μg for each lane) and other technical procedures (such as pipetting action, gel staining and gel image acquisition) were standardized across all independent experiments. Figure 4.7 depicts intensity of a representative protein band from 24-h-old biofilm across seven independent experiments. Coefficient of variation for 27.6 kDa protein band was found to be 21.47%.

Whole-cell protein expression profiles in the absence and presence of 32% DMSO are presented in Figure 4.8. Treatment with 32% DMSO triggered changes in whole-cell protein expression in *S. typhimurium* biofilm at 24 h. Intensity of all the major protein bands in *S. typhimurium* biofilm decreased following treatment with 32% DMSO. Subtractive comparison of protein profiles identified two unique protein bands, 25.4 kDa and 51.2 kDa that were expressed only in control fraction and not in 32% DMSO-treated fraction. The absence of 25.4 kDa and 51.2 kDa protein bands in 32% DMSO-treated biofilm was confirmed by densitometric analysis. Meanwhile, treatment with 32% DMSO produced no changes in whole-cell protein expression in *S. typhimurium* biofilm at 2 h.

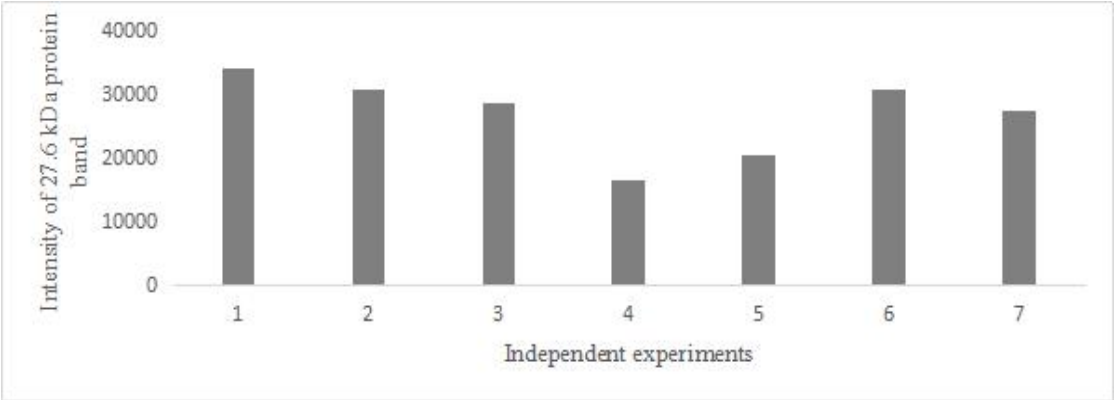


Figure 4.7: Expression level of a representative whole-cell protein band from 24-h-old biofilms across seven independent experiments. Coefficient of variation is the ratio of the standard deviation to the mean which is expressed as a percentage. Densitometric analyses of protein band was performed using publicly available ImageJ software.

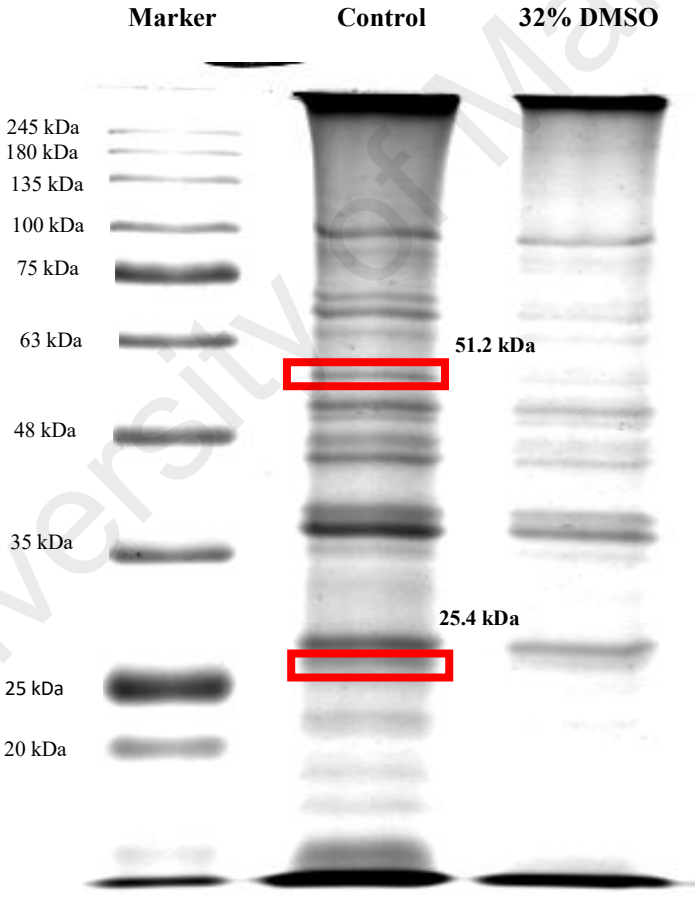


Figure 4.8: Whole-cell protein expression in *S. typhimurium* biofilm following treatment with 32% DMSO at 24 h. Red boxes highlight the unique protein bands identified by subtractive profile analysis. Estimated protein amount for each lane was 4.5 µg.

4.4.2 QTOF-identified whole-cell proteins

Nano-HPLC-ESI-QTOF (Section 3.11.2) was performed to identify the unique proteins (Section 4.4.1). In-gel phosphoprotein detection (Section 3.12) was performed to validate QTOF-identified phosphoproteins and determine the effects of DMSO on protein phosphorylation in *S. typhimurium* biofilm. A series of bioinformatics analyses (Section 3.13) were carried out to predict the functional characteristics, functional linkages and therapeutic potential of QTOF-identified whole-cell proteins.

Figure 4.9 shows the distribution of QTOF-identified whole-cell proteins. Approximately 55% of QTOF-identified whole-cell proteins showed the Mascot score in the range between 50 and 149. A list of QTOF-identified whole-cell proteins is presented in Table 4.4. Twenty nine proteins and 46 proteins were identified from the protein bands of 25.4 kDa and 51.2 kDa respectively. It should be noted that these whole-cell proteins were expressed only in control biofilm and not in 32% DMSO-treated biofilm. Figure 4.10 shows the relative abundance of QTOF-identified whole-cell proteins which was calculated using exponentially modified protein abundance index (emPAI). Approximately 90% of QTOF-identified whole-cell proteins were classified as low abundance proteins (emPAI value < 1). The most abundant protein was Trigger factor while the least abundant protein was ParB-like partition protein

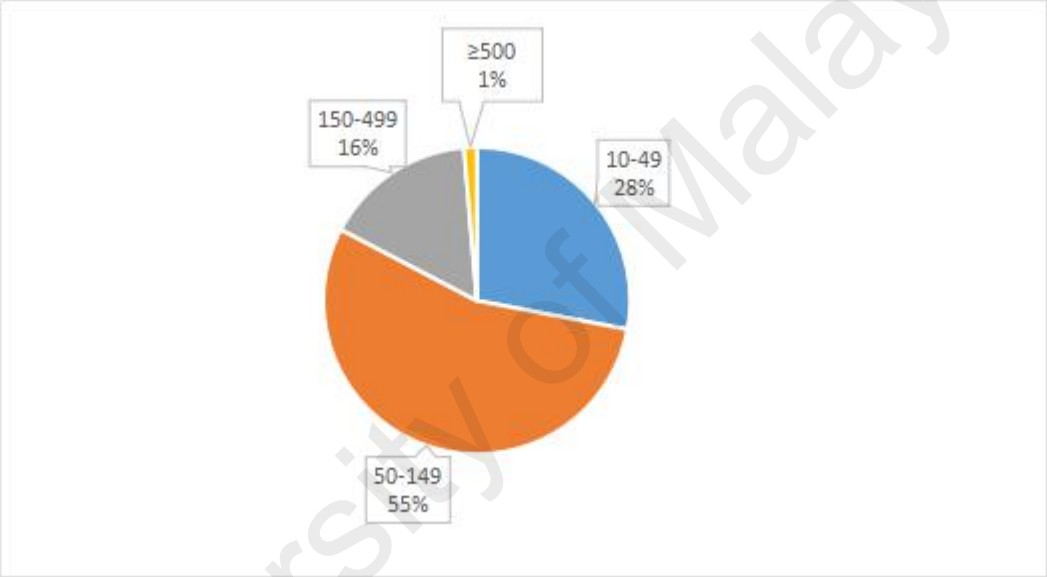


Figure 4.9: Distribution of QTOF-identified whole-cell proteins according to Mascot score.

Table 4.4: Whole-cell proteins from *S. typhimurium* biofilm whose expression is inhibited by treatment with 32% DMSO.

Accession (SwissProt)	Mascot score	Peptides matched	Sequence coverage (%)	Protein identity	Functional categories
25.4 kDa protein band					
EKT05435	25	18	8	Hypothetical protein	Unknown
PHOP_SALTY	210	6	33	Virulence transcriptional regulatory protein PhoP	Cell signaling
CAA68205	74	7	13	Pyruvate kinase like protein	Metabolism
OMPA_SALTY	306	18	44	Outer membrane protein A	Transport
CQE27718	33	4	7	Outer membrane protein TolC	Transport
METQ_SALTY	105	2	39	D-methionine-binding lipoprotein MetQ	Transport
NFSB_SALTY	51	2	11	Oxygen-insensitive NAD(P)H nitroreductase	Metabolism
DKGA_SALTY	49	2	9	2,5-diketo-D-gluconic acid reductase A	Metabolism
RIDA_SALTY	74	2	17	2-iminobutanoate/2-iminopropanoate deaminase	Metabolism
SDHB_SALTY	41	2	4	Succinate dehydrogenase iron-sulfur subunit	Metabolism
TRAT_SALTM	115	8	23	TraT complement resistance protein	Conjugation
CRF14171	55	3	12	CDP-diacylglycerol pyrophosphatase	Metabolism
CQM44525	91	3	29	Conjugal transfer surface exclusion protein TraT	Conjugation
KNB28456	50	3	8	Elongation factor Tu	Transcr. / Translt.
CQL59035	124	3	15	Heat shock protein GrpE	Defense response
KNB30613	50	5	3	uridine phosphorylase	Metabolism
ABU94666	97	9	6	Malate dehydrogenase	Metabolism
AIE04707	73	2	6	Phosphoglycerate mutase	Metabolism
CQB98037	94	3	9	Deoxyribose-phosphate aldolase	Metabolism
KLT32369	68	4	10	ABC transporter permease	Transport
KPF20588	67	3	12	Cystine transporter subunit	Transport
CQC73656	28	2	7	Succinyl-CoA synthetase subunit beta	Metabolism
KNB37322	74	3	13	Recombinase A	DNA repair
CBW17697	15	3	8	Hypothetical protein	Unknown function
AIE04154	54	2	6	Translation elongation factor Ts	Transcr. / Translt.
CCF76845	14	2	3	ParB-like partition protein	Cell proliferation
CQO45143	50	2	6	Acetylglutamate kinase	Metabolism
KMM51232	39	4	15	Biopolymer transporter ExbB	Transport
AFD58573	59	3	6	Universal stress protein	Defense response
51.2 kDa protein band					
KPE56665	100	10	27	Periplasmic trehalase	Metabolism
CBW16311	20	2	3	Deoxyguanosinetriphosphate triphosphohydrolase	Metabolism
CQB00605	64	8	19	Phosphoenolpyruvate carboxykinase	Metabolism
CQG33171	108	9	24	Fructose-bisphosphate aldolase class 1	Metabolism
KNB35622	51	2	4	Ribose-phosphate pyrophosphokinase	Metabolism
KNB35421	49	7	19	Enolase	Metabolism
AAZ73301	42	8	16	ATP synthase subunit beta	Transport

Table 4.4, continued

Accession (SwissProt)	Mascot score	Peptides matched	Sequence coverage (%)	Protein identity	Functional categories
KNB34569	40	3	8	Manganase containing catalase	Metabolism
OTSA_SALTS	56	4	8	Alpha,alpha-trehalose-phosphate synthase [UDP-forming]	Metabolism
SYR_SALTS	45	4	3	Arginine-tRNA ligase	Metabolism
FHLA_SALTS	17	3	2	Formate hydrogenlyase transcriptional activator	Transcr. / Translt.
CCF76853	15	2	6	Putative transposase	Cell proliferation
ARGT_SALTY	82	4	27	Lysine/arginine/ornithine-binding periplasmic protein	Transport
AHPF_SALTY	169	10	22	Alkyl hydroperoxide reductase subunit F	Metabolism
FLJB_SALTY	126	6	25	Phase 2 flagellin	Cell motility
AAZ79774	155	6	29	Phase 1 flagellin	Cell motility
CUB77306	1176	74	54	Trigger factor	Transcr. / Translt.
CUC07050	169	5	42	30S ribosomal protein	Transcr. / Translt.
KMJ21897	142	3	23	50 ribosomal protein L1	Transcr. / Translt.
ESE73385	208	13	39	ATP synthase subunit alpha	Transport
AFD30527	171	21	32	Heat shock protein GroEL	Defense response
AAK95374	428	25	41	Heat shock protein 60 kDa	Defense response
AFD60284	58	2	14	DNA directed RNA polymerase	Transcr. / Translt.
WP_050961606	30	3	20	Nucleoside diphosphate kinase	Metabolism
KPE94525	236	11	23	Membrane protein insertase	Transport
BAA00904	204	7	19	Rho protein	Transcr. / Translt.
CBG24394	167	11	24	Pyruvate kinase	Metabolism
WP_001220237	254	11	32	Adenylate kinase	metabolism
KNB36654	358	25	38	Dihydrolipoamide dehydrogenase	Metabolism
KPF04252	79	7	5	Alkyl hydroperoxide reductase	Metabolism
CQF88416	80	9	21	Negative regulator of mulitple antibiotic resistance	Transcr. / Translt.
KNB38983	98	3	6	Succinate-semialdehyde dehydrogenase	Metabolism
CUB84645	74	6	12	Serine hydroxymethyltransferase	Metabolism
KNB34251	43	2	8	Triosephosphate isomerase	Metabolism
ADX15753	74	7	26	Transaldolase B	Metabolism
CQE26958	30	3	19	Oxidoreductase	Metabolism
CQK37686	119	4	7	Flagellar biosynthesis protein FliC	Cell motility
AAA27114	96	11	37	Glyceraldehyde-3-phosphate dehydrogenase	Metabolism
CCW73166	106	14	23	Protein-export membrane protein	Transport
KNB39376	61	2	6	Phosphoglycerate kinase	Metabolism
KPF30874	57	2	5	Ornithine carbamoyltransferase	Metabolism
CUB97222	130	8	12	Glycerol kinase	Metabolism
KNU68004	404	19	46	Elongation factor Tu	Transcr. / Translt.
AAA56997	40	2	9	Iron superoxide dismutase	Defense response
AIE08185	42	2	10	Glucose-6-phosphate isomerase	Metabolism
CQF47592	45	3	9	Alcohol dehydrogenase	Metabolism

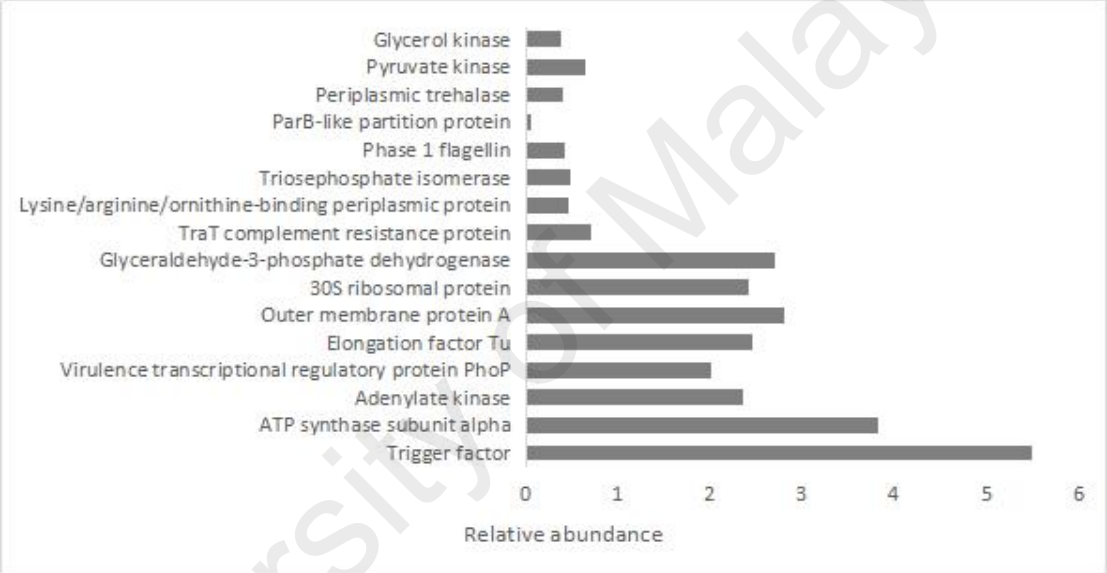


Figure 4.10: Representative low and high-abundance QTOF-identified whole-cell proteins.

Table 4.5 depicts a list of representative peptides with missed trypsin cleavage for the whole-cell proteins. Missed trypsin cleavage was detected in approximately 13% of QTOF-identified whole-cell proteins. QTOF-identified phosphopeptides are portrayed in Table 4.6. The phosphorylated serine and threonine residues were detected in Virulence transcriptional regulatory protein (PHOP_SALTY), Arginine--tRNA ligase (SYR_SALTS), Formate hydrogenlyase transcriptional activator (FHLA_SALTS), hypothetical protein (CBW17697) and ParB-like partition protein (CCF76845).

4.4.2.1 Validation of QTOF data

The QTOF analysis identified two and three phosphoproteins from the protein bands of 25.4 kDa and 51.2 kDa respectively whose expression was inhibited by treatment with 32% DMSO. Successful validation of QTOF data by ProQ Diamond assay allowed in-depth bioinformatics analyses for important insights into antibiofilm mechanism. On the other hand, protein kinase activity in *S. typhimurium* in the absence and presence of DMSO was also determined using ProQ Diamond assay.

Phosphoprotein bands of 21.4 kDa and 51.2 kDa are shown in Figure 4.11. Fluorescence ProQ diamond assay successfully detected the presence of phosphoprotein bands of 25.4 kDa and 51.2 kDa thereby validating the phosphoproteins identified by QTOF. The expression pattern of phosphoprotein bands observed in this assay was similar to that of total protein bands observed in Coomassie-stained polyacrylamide gels (Section 4.4.1).

Table 4.5: Representative peptides with missed trypsin cleavage. Bold and underlined letters indicate incomplete cleavage sites.

m/z	Missed cleavage	Score	Peptide / QTOF-identified proteins
557.8151	1	17	LIAATN**R**DLK / Formate hydrogenlyase transcriptional activator
574.3206	1	15	**R**MAALTLLMK / Putative transposase
537.3065	1	31	GTVVTG**R**VER / Elongation factor Tu
791.4475	1	92	VTITIAADSIETAV**K**SELVNVAK / Trigger factor
538.9462	1	60	INSA**K**DDAAGQAIANR / Phase 1 flagellin
489.9204	1	57	**K**SSEVYGQTNIGGK / Glycerol kinase

Table 4.6: Phosphopeptides identified by QTOF analysis. Bold and underlined letters indicate phosphorylated residues.

m/z	Phospho residues	Score	Phosphopeptides / Identified proteins
463.2967	Ser-171	4	NNGKVV**S**K / Virulence transcriptional regulatory protein PhoP
701.3521	Thr-145	4	**T**LEFLGHHVIR / Arginine--tRNA ligase
515.3202	Ser-679	1	TTLL**S**RMK / Formate hydrogenlyase transcriptional activator
557.8151	Thr-521	17	LIAA**T**NRDLK / Formate hydrogenlyase transcriptional activator
691.3335	Ser-168	3	NI**S**EYNFLSSK / Hypothetical protein
494.2693	Thr-271	15	AVLN**T**FSR / Hypothetical protein
784.3000	Ser-168	8	TPAQIGDLLGY**S**PR / ParB-like partition protein

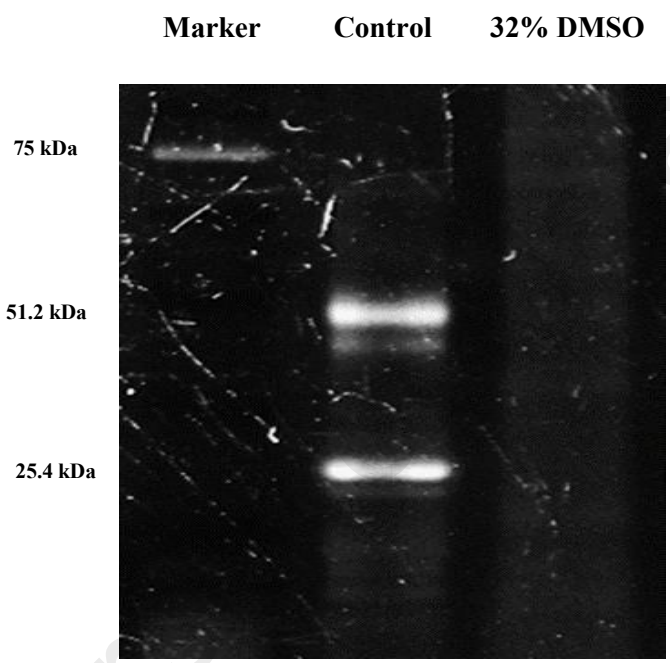


Figure 4.11: Phosphoprotein bands detected by ProQ diamond assay. 51.2 kDa: FHLA_SALTS, CBW17697, CCF76845; 25.4 kDa: PHOP_SALTY, SYR_SALTS.

4.4.2.2 General characteristics of QTOF-identified whole-cell proteins

For general characterization, all the QTOF-identified whole-cell proteins were analyzed for subcellular localization, transmembrane domain and Type III secretion signal. In the case of hypothetical proteins, identification of protein domains and sequence similarity search were also performed.

Functional classification of QTOF-identified whole-cell proteins based on Swiss-Prot/TrEMBL database is shown in Figure 4.12. The QTOF-identified whole-cell proteins involved in metabolism (51%), transport (16%) and transcription/translation (12%) were found to be mainly suppressed by treatment with 32% DMSO. Figure 4.13 shows subcellular localization of QTOF-identified whole-cell proteins. Approximately 76% of QTOF-identified whole-cell proteins were predicted to be localized in the cytoplasm. Percentage of membrane protein is depicted in Figure 4.14. Approximately 5% of QTOF-identified whole-cell proteins were predicted to contain transmembrane domains. Figure 4.15 presents percentage of T3SS effector proteins. Approximately 9% of QTOF-identified whole-cell proteins were predicted to be T3SS effector proteins.

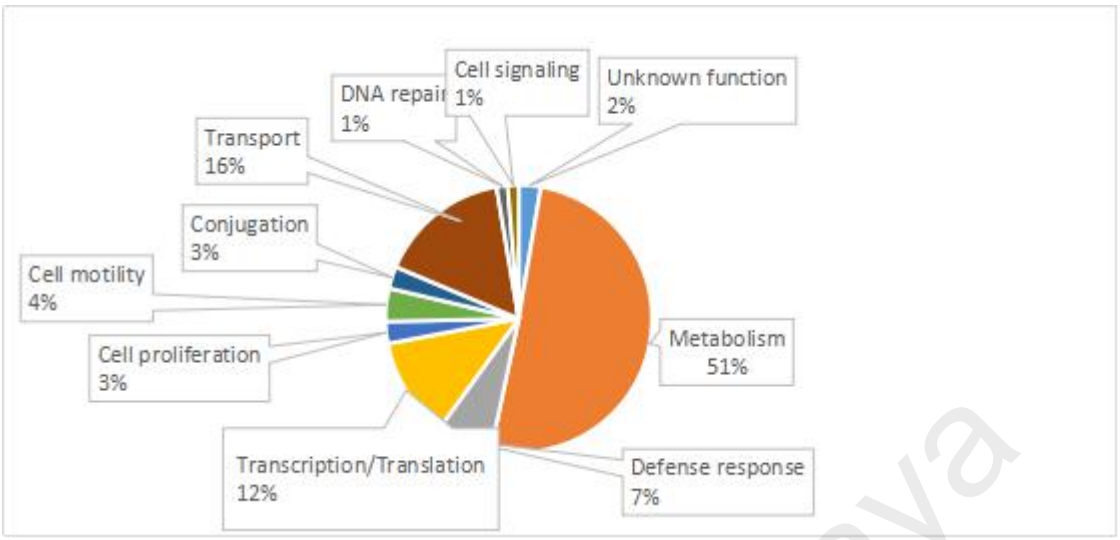


Figure 4.12: Classification of QTOF-identified whole-cell proteins based on functional categories

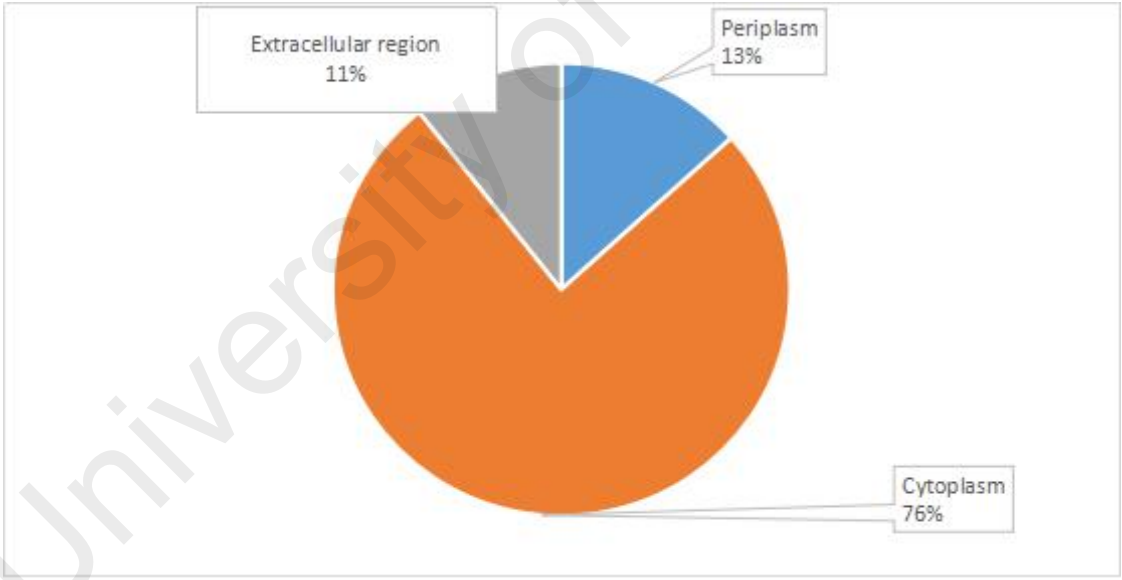


Figure 4.13: Subcellular localization of QTOF-identified whole-cell proteins.

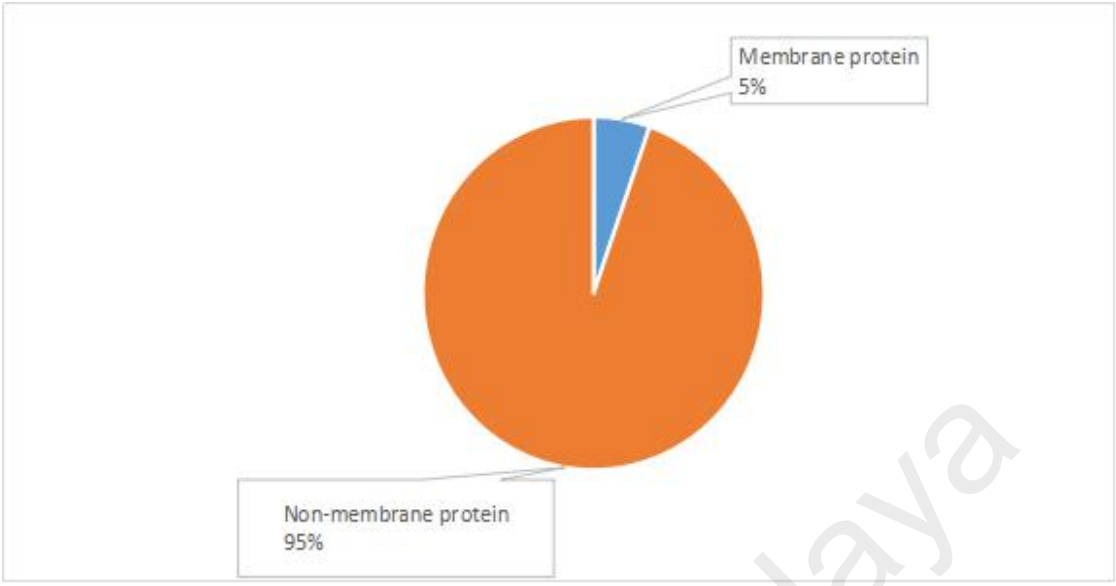


Figure 4.14: Percentage of QTOF-identified whole-cell proteins classified as membrane proteins

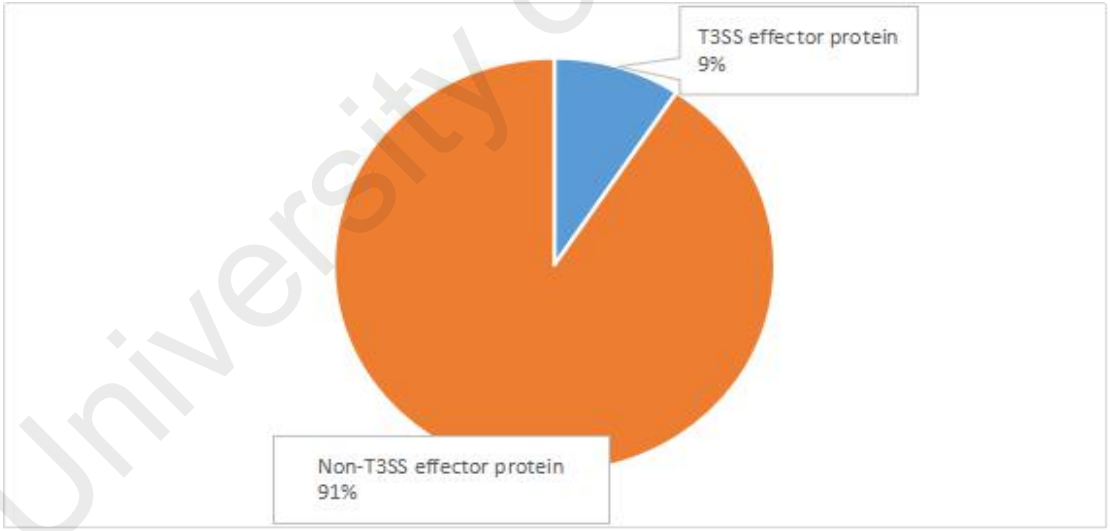


Figure 4.15: Percentage of QTOF-identified whole-cell proteins classified as T3SS effector proteins.

Table 4.7 shows characteristics of hypothetical proteins identified by the QTOF analysis. BLASTp search showed 82% sequence similarity between EKT05435 and putative GTP-binding protein from *Citrobacter freundii.* EKT05435 was also predicted to be localized in periplasm and contain transmembrane domain. Therefore, it was assigned as a membrane GTP-binding protein involved in signal transduction mechanism. On the other hand, CBW17697 was predicted to contain cell attachment sequence. It was also predicted to be localized in cytoplasmic compartment and contain Type III secretion system signal peptide. Therefore, it was assigned as a secreted protein involved in direct injection of bacterial toxin from the bacterial cytoplasm into the cytoplasm of host's cells.

4.4.2.3 Functional associations of QTOF-identified whole-cell proteins

The protein interaction network was analysed using STRING database (Section 3.13.2) by which the prediction of functional associations was based on neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining. This database is an extensive collection of pre-computed protein interaction data and protein interaction score which makes use of probabilistic scoring for high-confidence protein interactions. GO enrichment analysis successfully determined the biological processes and pathways inhibited by treatment with 32% DMSO while the use of k-means clustering algorithm efficiently produced non-overlapping protein clusters.

Table 4.7: Characteristics of hypothetical proteins identified by QTOF analysis.

Accession	EKT05435	CBW17697
Length	156 aa	370 aa
Phosphoprotein	No	Yes
Subcellular location	Periplasmic	Cytoplasmic
Transmembrane domain	Yes	No
T3SS signal	No	Yes
Conserved domain or motif	Accession: cl19471 DUF945 superfamily This family consists of several hypothetical bacterial proteins of unknown function.	Accession: PS00016 Cell attachment sequence The sequence Arg-Gly-Asp is crucial for interaction with cell surface receptor and cell adhesion.
Homologue	KFB96996 Putative GTP-binding protein *Citrobacter freundii* ATCC 8090 E-value: $5e^{-10}$ Identity: 82%	No

Functional associations among QTOF-identified whole-cell proteins are depicted in Figure 4.16. A total of 66 QTOF-identified whole-cell proteins were predicted to be functionally linked by at least one STRING criterion while 268 interactions were successfully produced from this protein dataset. Most of the interacting proteins were positioned at the center of protein interaction network while the rest were positioned at the periphery.

Table 4.8 and Table 4.9 show significant ($p<0.05$) biological processes and pathways respectively. Nine biological processes and nine pathways showed significant probability scores ($p<0.05$). The majority of QTOF-identified whole-cell proteins were found to constitute glycolytic pathway. Figure 4.17 shows KEGG pathway of glycolysis in *S. typhimurium*.

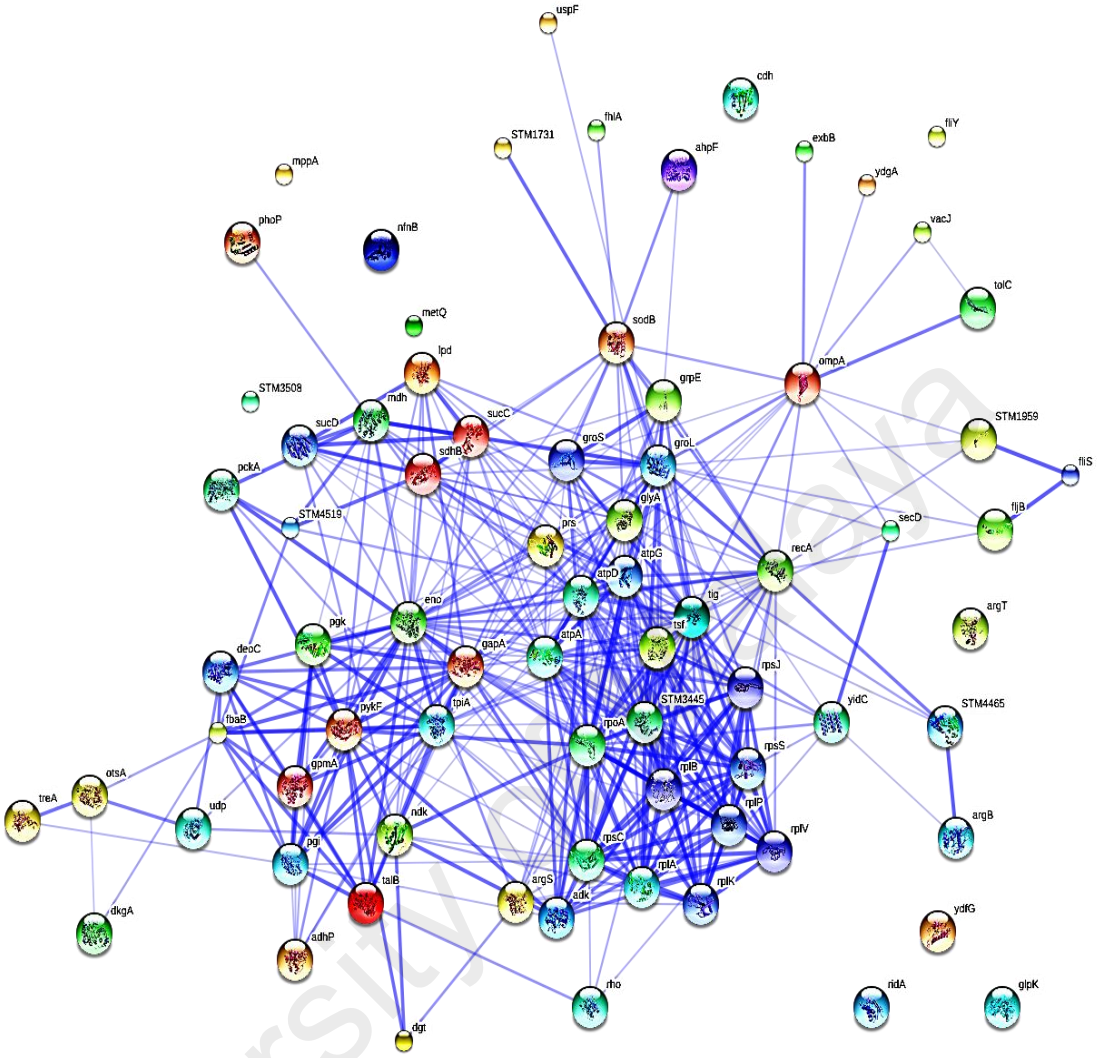


Figure 4.16: Protein interaction network showing functional associations between QTOF-identified whole-cell proteins. The interaction map is presented under confidence view, whereby stronger associations are represented by thicker lines or edges and vice versa, whereas proteins are represented as nodes. Default settings (high confidence: 0.7; criteria for linkage: neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining) were used to generate the interaction map.

Table 4.8: GO enrichment analysis of the biological processes involved in the STRING protein interaction network.

GO BIOLOGICAL PROCESS	P-VALUE
Purine nucleotide metabolic process	$2.429e^{-10}$
ATP generation	$4.349e^{-8}$
Glycolytic process	$4.349e^{-8}$
Phosphorelay signal transduction system	$8.219e^{-1}$
Ion transport	$3.360e^{-1}$
Protein folding	$6.919e^{-3}$
Gluconeogenesis	$1.060e^{-5}$
Tricarboxylic acid metabolic process	$2.599e^{-2}$
Cell motility	$7.079e^{-1}$

Table 4.9: GO enrichment analysis of the pathway involved in the STRING protein interaction network.

GO PATHWAY	P-VALUE
Glycolysis	$1.610e^{-9}$
TCA cycle	$2.379e^{-5}$
Biosynthesis of secondary metabolites	$1.509e^{-7}$
Pentose phosphate pathway	$7.680e^{-3}$
Purine metabolism	$1.909e^{-1}$
Oxidative phosphorylation	$5.199e^{-2}$
Pyruvate metabolism	$1.130e^{-1}$
Flagellar assembly	$1.570e^{-1}$
Beta-Lactam resistance	$2.439e^{-1}$

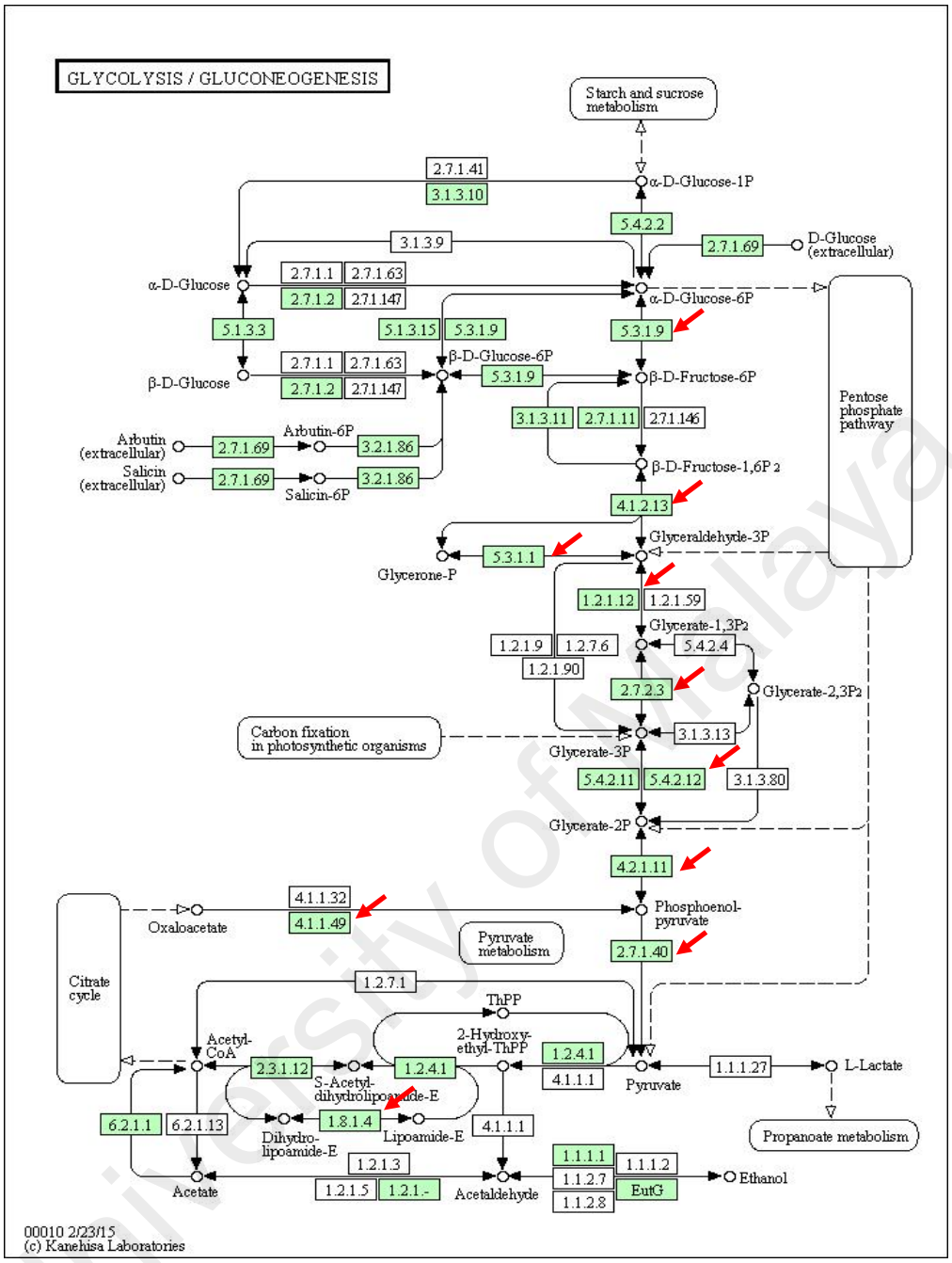


Figure 4.17: Glycolysis/Gluconeogenesis pathway in *S. typhimurium* obtained from KEGG database. Red arrows indicate unique proteins that are present in control biofilm and absent in 32% DMSO-treated biofilm. 5.3.1.9: Glucose-6-phosphate isomerase; 4.1.2.13: Fructose-bisphosphate aldolase class 1; 5.3.1.1: Triosephosphate isomerase; 1.2.1.12: Glyceraldehyde-3-phosphate dehydrogenase; 2.7.2.3: Phosphoglycerate kinase; 5.4.2.11: Phosphoglycerate mutase; 4.2.1.11: Enolase; 4.1.1.49: Phosphoenol pyruvate; 2.7.1.40: Pyruvate kinase; 1.8.1.4: Dihydrolipoamide dehydrogenase.

Non-overlapping protein clusters are shown in Figure 4.18. All the clusters, except cluster 1, were found to compose of densely connected nodes and display strong intracluster connections. Many of the interacting proteins were also linked to those in other clusters. Cluster 1 composed of 23 proteins which were poorly connected to each other and thus no major biological process could be assigned. Cluster 2 consisted of 15 proteins which were mainly involved in ATP and carbohydrate metabolism. Cluster 3 comprised of eight proteins which were mostly involved in transcription and translation. Cluster 4 composed of 20 proteins which were predominantly associated with cell membrane and cellular protection mechanism.

4.4.2.4 QTOF-identified whole-cell proteins as ideal therapeutic targets

In silico subtractive analysis (Section 3.13.3) was executed to evaluate the ideality of QTOF-identified whole-cell proteins as therapeutic targets for biofilm control. An ideal target for antibacterial drug discovery should fulfill three following criteria: i) it plays an important role in disease mechanism, ii) it is non-homologous to the host proteome and iii) it plays an essential role in the survival of pathogen. In this analysis, the QTOF-identified whole-cell proteins were compared with the proteome of human host and analysed against DEG database. The QTOF-identified whole-cell proteins with score greater than threshold of $1e^{-06}$ and identity less than 30% were considered non-homologous. Table 4.10 and 4.11 portray summary of *in silico* subtractive analysis and a list of non-homologous whole-cell proteins respectively. Approximately 54.67% of QTOF-identified whole-cell proteins were found to be essential to the survival of *S. typhimurium* and were non-homologous to human proteome, making them ideal therapeutic targets for biofilm control.

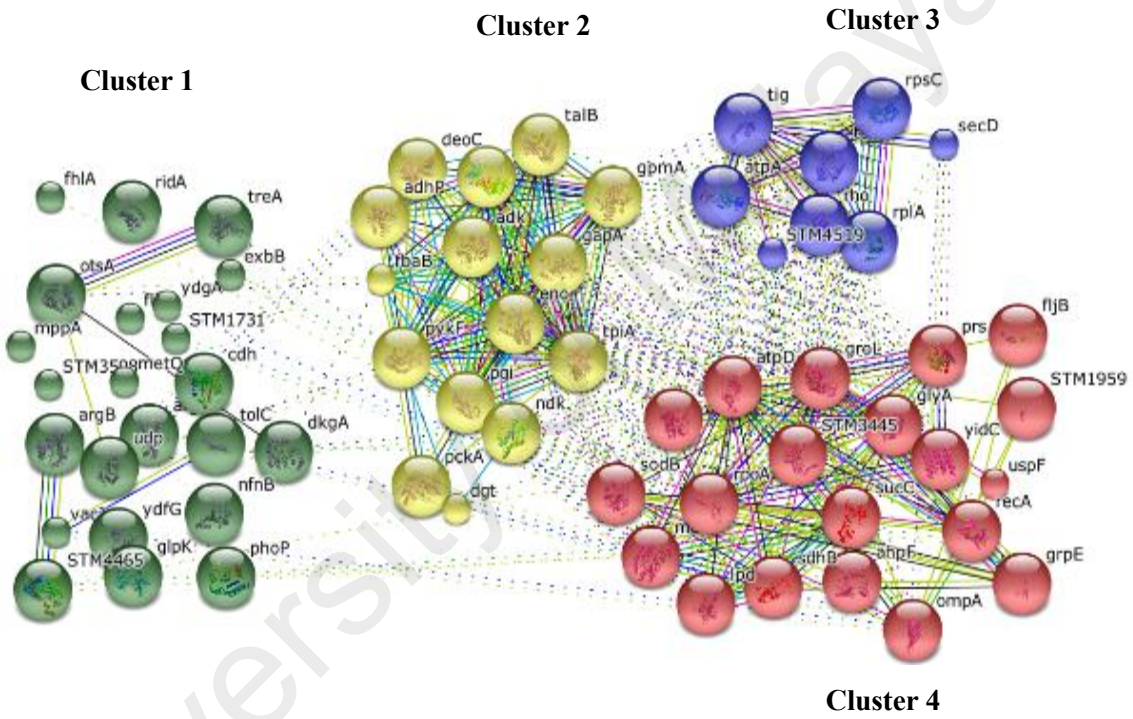


Figure 4.18: Non-overlapping protein clusters produced by k-means clustering algorithm.

Table 4.10: Summary of *in silico* subtractive analysis of QTOF-identified whole-cell proteins.

Proteins	Number	%
QTOF-identified whole-cell proteins	75	100
QTOF-identified whole-cell proteins without matches in human host	47	62.67
QTOF-identified whole-cell proteins with matches in DEG database	41	54.67
QTOF-identified whole-cell proteins considered as ideal therapeutic targets	41	54.67

Table 4.11: QTOF-identified whole-cell proteins which are essential to the survival of *S. typhimurium* and non-homologous to hosts.

Accession	Protein name	Gene name	Functional categories
EKT05435	Hypothetical protein	Unknown	Unknown
OTSA_SALTS	Alpha,alpha-trehalose-phosphate synthase [UDP-forming]	otsA	Carbohydrate metabolism
SYR_SALTS	Arginine-tRNA ligase	argS	Proline metabolism
FHLA_SALTS	Formate hydrogenlyase transcriptional activator	fhlA	Transcription/Translation
PHOP_SALTY	Virulence transcriptional regulatory protein	phoP	Cell signaling
CAA68205	pyruvate kinase like protein	pykF	Carbohydrate metabolism
CCF76853	Putative transposase	ydgA	Cell proliferation
OMPA_SALTY	Outer membrane protein A	ompA	Transport
CQE27718	Outer membrane protein TolC	tolC_2	Transport
METQ_SALTY	D-methionine-binding lipoprotein MetQ	metQ	Transport
ARGT_SALTY	Lysine/arginine/ornithine-binding periplasmic protein	argT	Transport
NFSB_SALTY	Oxygen-insensitive NAD(P)H nitroreductase	nfsB	Cofactor metabolism
DKGA_SALTY	2,5-diketo-D-gluconic acid reductase A	dkgA	Vitamin metabolism
AHPF_SALTY	Alkyl hydroperoxide reductase subunit F	ahpF	Cofactor metabolism
RIDA_SALTY	2-iminobutanoate/2-iminopropanoate deaminase	ridA	Amino acid metabolism
SDHB_SALTY	Succinate dehydrogenase iron-sulfur subunit	sdhB	Carbohydrate metabolism
FLJB_SALTY	Phase 2 flagellin	fljB	Cell motility
AAZ79774	Phase 1 flagellin	fliC	Cell motility
CUB77306	Trigger factor	tig	Transcription/Translation
CUC07050	30S ribosomal protein	rpsC	Transcription/Translation
KMJ21897	50S ribosomal protein L1	rplA	Transcription/Translation
ESE73385	ATP synthase subunit alpha	atpA	Transport
AFD30527	Heat shock protein GroEL	groL	Defense response
AAK95374	Heat shock protein 60 kDa	hsp60	Defense response
KNB28456	Elongation factor Tu	tuf	Transcription/Translation
AFD60284	DNA directed RNA polymerase	pez	Transcription/Translation
CQL59035	Heat shock protein GrpE	grpE_2	Defense response
KNB30613	Uridine phosphorylase	udp	Nucleotide metabolism
AIE04707	Phosphoglycerate mutase	gpmA	Carbohydrate metabolism
BAA00904	Rho protein	rho	Transcription/Translation
KPF04252	Alkyl hydroperoxide reductase	ahp	Cofactor metabolism
CQF88416	Negative regulator of mulitple antibiotic resistance	mppA_2	Transcription/Translation
CQK37686	Flagellar biosynthesis protein FliC	fliC_1	Cell motility
CCW73166	Protein-export membrane protein	SecD	Transport
KNB37322	Recombinase A	recA	DNA repair
CQO45143	Acetylglutamate kinase	argB	Amino acid metabolism
KMM51232	Biopolymer transporter ExbB	exbB	Transport
CQF47592	Alcohol dehydrogenase	adhP	Cofactor metabolism
KPE94525	Membrane protein insertase	yidC	Transport
KLT32369	ABC transporter permease	abc	Transport
KPF20588	Cystine transporter subunit	fliY	Transport

4.5 Differential EPS protein profiles

Treatment with 32% DMSO effectively inhibited EPS matrix of *S. typhimurium* biofilm (Section 4.2.3-4.2.4). Despite its remarkable antibiofilm effects, DDA was not subjected to SDS-PAGE analysis of EPS protein because there was no significant difference ($p>0.05$) in EPS proteins observed between 32% DMSO and DDA treatments (Section 4.2.4), suggesting that 32% DMSO and DDA may share a similar molecular basis. EPS proteins of *S. typhimurium* biofilm were fractionated using one dimensional SDS-PAGE (Section 3.10.4), visualized using Coomassie brilliant blue staining (Section 3.10.5) and analyzed using ImageJ software (Section 3.10.5).

4.5.1 SDS-PAGE gel images

To optimize the EPS protein extraction, ethanol precipitation of EPS fraction (24 hour) was carried out at four different temperatures and incubation times as follows: protocol 1: 4^{o}C for two hours; protocol 2: 4^{o}C overnight; protocol 3: -20^{o}C for two hours and protocol 4: -2^{o}C overnight. Only protocol 4 produced at least nine visible protein bands in the range between 17 kDa and 78 kDa. Three protein bands (27 kDa, 35 kDa and 49 kDa) were found to be highly reproducible across multiple electrophoretic separations. Protocol 4 was used for the subsequent protein analyses.

To study the consistency of EPS protein secretion, the experiment was repeated seven times in the absence of DMSO. The one dimensional PAGE protein banding pattern of EPS protein at 24 hour was highly reproducible, however, the intensity of all EPS protein bands was inconsistent although protein amount loaded into the polyacrylamide gels (2 µg for each lane) and other technical procedures (such as pipetting action, gel staining and gel image acquisition) were standardized across all independent experiments.

Figure 4.19 shows protein profiles of whole-cell lysate and EPS matrix. The one dimensional PAGE protein banding pattern of control EPS matrix was 78% similar to that of control whole-cell biofilm while 32% DMSO-treated EPS matrix was 75% similar to that of 32% DMSO-treated whole-cell biofilm. Treatment with 32% DMSO caused changes in the EPS protein secretion at 24 hour. Subtractive comparison of protein profiles recognized one unique protein band, 51.2 kDa, that was secreted only in 32% DMSO-treated EPS matrix and not in control EPS matrix. The absence of 51.2 kDa protein bands in control EPS matrix was confirmed by densitometric analysis.

4.5.2 QTOF-identified EPS proteins

From the subtractive protein profile analysis, the protein band of 51.2 kDa was found to be secreted only in 32% DMSO-treated EPS matrix and not in control EPS matrix. Identification of EPS proteins involved nano HPLC-ESI-QTOF. To be classified as secreted proteins, all QTOF-identified EPS proteins should fulfill or are related to at least one of the following criteria: signal peptide cleavage sites, non-classical secretion pathway, eukaryotic-like domain and transmembrane domain.

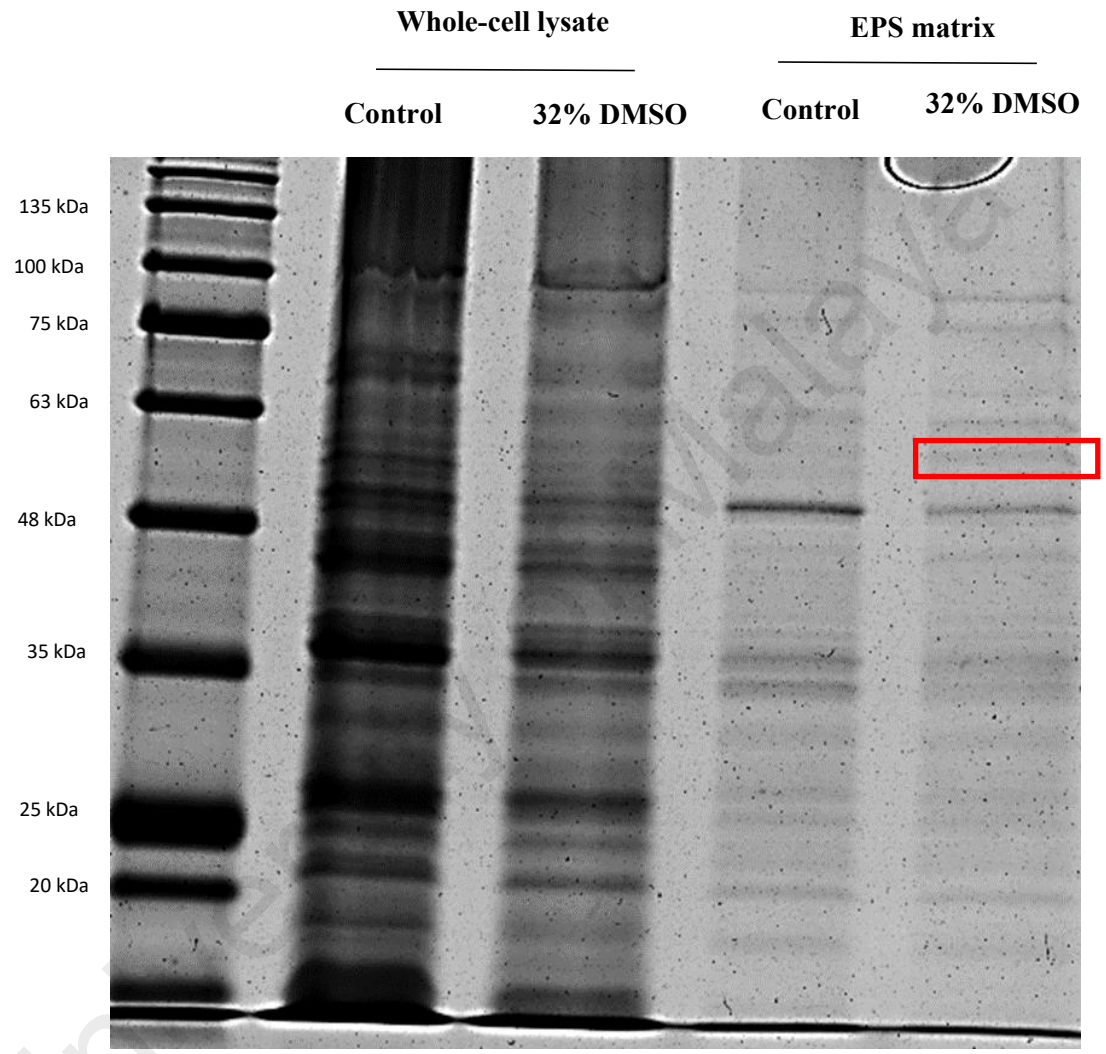


Figure 4.19: Protein profiles of whole-cell lysate and EPS matrix of *S. typhimurium* biofilm (24 hour) in the absence and presence of 32% DMSO. Estimated protein amounts for whole-cell lysate and EPS matrix were 4.5 µg and 2 µg respectively. Red boxes highlight the unique EPS protein band (51.2 kDa) recognized by subtractive analysis.

A list of EPS proteins identified by QTOF analysis are portrayed in Table 4.12. Three EPS proteins from 51.2 kDa protein band were identified as elongation factor Tu (KNU68004), enolase (KNB35421) and phosphoglycerate kinase (KNB39376). It should be noted that these EPS proteins were secreted only in 32% DMSO-treated EPS matrix and not in control EPS matrix. Also, these proteins were present only control biofilm and not in 32% DMSO-treated biofilm (Table 4.3). Table 4.13 presents the results of *in silico* analysis of secreted proteins. All the QTOF-identified EPS proteins were categorized as secreted proteins. No missed trypsin cleavage and phosphopeptides were detected in the QTOF-identified EPS proteins. From ProQ Diamond assay, no EPS phosphoproteins were detected over the course of biofilm formation.

4.5.2.1 Functional association of QTOF-identified EPS proteins

Functional interactions among QTOF-identified EPS proteins are depicted in Figure 4.20. Enolase was predicted to be functionally linked to phosphoglycerate kinase (by five STRING criteria) and elongation factor Tu (by two STRING criteria) while no functional linkage between phosphoglycerate kinase and elongation factor Tu was predicted. GO enrichment analyses of biological process and pathway were executed using false discovery rate (FDR) correction to identify significant ($p<0.05$) biological processes and pathways associated with the protein association. Glycolysis was determined to be significant for this protein association.

4.5.2.2 QTOF-identified EPS protein as ideal therapeutic target

No QTOF-identified EPS proteins were found to be essential to the survival of *S. typhimurium* and non-homologous to human proteome.

Table 4.12: EPS proteins from *S. typhimurium* biofilm whose secretion is inhibited by treatment with 32% DMSO.

Accession	Mascot score	Peptide matches	Sequence coverage (%)	Description	Functional categories
KNB39376	211	3	14	Phosphoglycerate kinase	Carbohydrate metabolism
KNU68004	131	2	2	Elongation factor Tu	Translation
KNB35421	232	6	14	Enolase	Carbohydrate metabolism

Table 4.13: EPS proteins which are classified as secreted proteins. Data are expressed as + and – which indicate calculated probability above threshold level and calculated probability below threshold level respectively.

Accession	Protein name	Prediction software				Secreted proteins
		SignalP 4.0	SecretomeP 2.0	EffectiveELD	TMHMM	
KNB39376	Phosphoglycerate kinase	-	-	+	-	**Yes**
KNU68004	Elongation factor Tu	-	-	+	-	**Yes**
KNB35421	Enolase	-	-	+	-	**Yes**

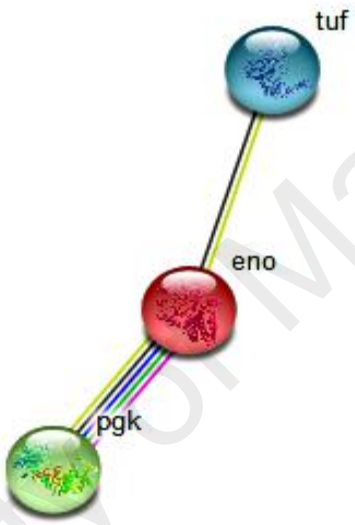


Figure 4.20: Protein interaction network showing functional associations between QTOF-identified EPS proteins. The interaction map is presented under confidence view, whereby stronger associations are represented by thicker lines or edges and vice versa, whereas proteins are represented as nodes. Default settings (high confidence: 0.7; criteria for linkage: neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining) were used to generate the interaction map.

CHAPTER 5: DISCUSSION

5.1 General discussion

Preliminary antibiofilm screening often involves determination of viability, total biomass and EPS matrix of biofilms. These parameters have been commonly evaluated in the antibiofilm studies due to the fact that the persistence and establishment of the physical architecture of biofilms are dependent on the EPS matrix (Suci et al., 1994; Kondoh & Hashiba, 1998; Baillie & Douglas, 2000; Zhao et al., 2016). Skogman et al. (2012) has also addressed that the effective antibiofilm agent should fulfill the following criteria: a) reduction in biofilm viability, b) reduction in biofilm biomass and c) chemical modification of EPS matrix. In the present study, microplate assay system was employed to analyze the biofilms of *S. typhimurium*, *E. coli* and *P. aeruginosa* in the absence and presence of DMSO and its combination with afitinib. This straightforward, high throughput and user-friendly assay system has previously been employed in numerous antibiofilm studies (Stepanovic et al., 2000; Hamilton et al., 2009; Sousa et al., 2009; Absalon et al., 2011; Yahya et al., 2014; Gomes et al., 2014). EPS matrix is known to influence the functional and structural integrity of biofilms on surfaces (Flemming et al., 2000). It contains many functional groups such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups which contribute to its high binding capacity (Ha et al., 2010). In the present study, ATR-FTIR spectroscopy was employed to investigate chemical composition of EPS matrix of control and test biofilm fractions. This direct and non-destructive method has previously been used in other biofilm studies (Suci et al., 1994; Kondoh & Hashiba, 1998; Baillie & Douglas, 2000; Nivens et al., 2001; Omoike & Chorover, 2004; Al-Qadiri et al., 2008; Jiao et al., 2010).

After preliminary antibiofilm screening, *S.typhimurium* biofilm was analyzed for heterogeneity. It has been established that the biofilms formed on the surface are highly

heterogeneous (Xu et al., 1998; Entcheva-Dimitrov & Spormann, 2004; Merode et al., 2006; Stewart & Franklin 2008; Karatan & Watnick 2009; Gomes et al., 2014; Sherry et al., 2014). The mechanisms that contribute to the genetic and physiological heterogeneity of biofilms include microscale chemical gradients, adaptation to local environmental conditions, stochastic gene expression and the genotypic variation (Stewart & Franklin, 2008). Conventionally, heterogeneity of biofilm is investigated using crystal violet assay. According to Gomes et al. (2014) and Sherry et al. (2014), crystal violet assay could provide information on diverse spatial location and biomass of heterogeneous biofilms. In the present study, the heterogeneity of *S. typhimurium* biofilm was mainly investigated by IFM. This high-resolution topographical analysis is rarely used for biological samples such as biofilm, however, Mahat et al. (2012) has investigated the heterogeneous nature of *P. aeruginosa* biofilm on mild steel coupons using IFM.

Following analysis of biofilm heterogeneity, the molecular mechanism underlying the antibiofilm effect was further investigated. In the present study, one dimensional SDS PAGE was performed on whole-cell protein and EPS protein fractions to understand the molecular basis of antibiofilm effects based on differential protein expression. This analytical method was used because: i) it is known to effectively fractionate all types of proteins including hydrophobic and low-molecular mass proteins (Jiang et al., 2016), ii) one dimensional SDS PAGE, unlike two dimensional SDS PAGE, does not limit the electrical properties and solubility of proteins (Kwon et al,. 2010) and one dimensional SDS PAGE protein banding pattern of Salmonella serovars has been established for comparative molecular analysis (Aksakal, 2010). In addition, one dimensional SDS PAGE was also used as an analytical step to evaluate differential protein expression following treatment with DMSO, not a preparative step prior to protein identification.

The similar approach has been performed by Andersen et al. (2002) to study the expression of human nucleolar proteins in the absence and presence of actinomycin D. Instead of direct analysis of up-regulated and down-regulated proteins, subtractive comparison of protein profile was performed herein as the biofilm formed by *S. typhimurium* was found to be heterogeneous. This is because the global proteomic approach which often analyzes the entire biofilm community is not adequate for discovering differential protein expression in the heterogeneous biofilm and there is a variation in the abundances of mRNA transcripts by several order of magnitudes even for cells in close proximity to each other (Williamson et al., 2012). Moreover, the analysis of quantitative proteomic data also becomes inconsistent and challenging (Williamson et al., 2012) unless the homogeneous biofilm fraction is isolated using suitable methods such as laser capture microdissection (LCM) (Moorthy & Watnick, 2004; Lenz et al., 2008; Pérez-Osorio et al., 2010; Williamson et al., 2012). The protein profiling approach taken in the present study is in line with previous studies demonstrating the subtractive protein profiling of heterogeneous proteome samples (Schirmer et al., 2003; Oh et al., 2004; Husi et al., 2016).

The selected protein bands from the subtractive protein profiling were then analyzed using LC-MS/MS. The standard shotgun proteomic strategy involves a combination of trypsin digestion and LC-MS/MS (Aebersold & Mann, 2003). Typically, protein fractionation is required as a preparative step to assist LC-MS/MS in achieving deep coverage of the very complex proteome such as those represented in whole-cell lysates (Thakur et al., 2011). This would result in slight increases in the required starting material and measurement time. On the other hand, an increase in the column length would increase the chromatographic resolution (Thakur et al., 2011). For example, Yates et al. (2006) showed that application of LC system with extended column length

of 50 cm produced 30% increase in protein identification. In the present study, nano-HPLC-ESI-QTOF was used to perform peptide mass fingerprinting as it is highly sensitive in identification of low-abundance proteins and phosphoproteins. Both low-abundance (Jiao et al., 2011) and phosphoproteins (Petrova & Sauer, 2009) are known to play crucial roles in biofilm formation. Fluorescence-based ProQ Diamond assay was subsequently performed to validate QTOF-identified phosphoproteins because it has greater sensitivity than the conventional antibody-based methods in the phosphoprotein detection (Stasyk et al., 2005; Wu et al., 2005). In addition, the assay format of ProQ Diamond assay is similar to the assay format of whole-cell protein analysis, making the validation process more precise, sensitive and reliable.

It has been established that the cellular proteins often work in the specialized group of varying size to modify one another and transport other proteins in both normal and diseased cells. Therefore, functional association was expected to be present among the QTOF-identified proteins whose expression was inhibited by treatment with 32% DMSO. In the present study, the protein interaction network was analysed using STRING database by which the prediction of functional associations between QTOF-identified whole-cell proteins and EPS proteins was based on neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining. This bioinformatics tool was used as it provided an extensive collection of pre-computed protein interaction data and protein interaction score which makes use of probabilistic scoring for high-confidence protein interactions. Application of STRING database would provide extra dimension to the proteome data (Larance & Lamond, 2015). This has been shown by the current trend in the proteomic studies which incorporate STRING analysis (Thio et al., 2013; Pérez-Vázquez et al., 2014; Thio et al., 2015; Cohn et al., 2016).

A progress in antimicrobial strategy has highlighted that therapeutic targets should fulfill the following three criteria: a) play major roles in disease mechanisms b) essential to survival of pathogens and c) non-homologous to host species (Dutta et al., 2006; Munikumar et al., 2012). To confirm this, *in silico* subtractive analysis has been a method of choice due to its rapidity and public availability of genome information. Over the last thirteen years, *in silico* subtractive analysis has received increasing attention for identifying the potential drug and vaccine targets (Dutta et al., 2006; Munikumar et al., 2012). In the present study, the QTOF-identified whole-cell proteins and EPS proteins were evaluated for their ideality as antibiofilm targets using an *in silico* subtractive approach. To our knowledge, this is the first study that combines the standard shotgun proteomic workflow with *in silico* subtractive analysis.

5.2 Biofilm inhibition

DMSO is a polar aprotic solvent commonly used to treat a wide range of diseases such as inflammation and musculoskeletal disorders (Lockie & Norcross, 1967). The systemic toxicity of DMSO is low while the therapeutic properties of DMSO are attributed to its own rapid penetration and ability to enhance the penetration of other substances (Brayton, 1986). To date, there is no published report on antibiofilm potential of DMSO. In the present study, DMSO exhibited poorer killing effects on the biofilm fractions than the planktonic fractions (Section 4.2.1, Table 4.2). This result is in agreement with several studies on the effects of antimicrobial agents on biofilm fractions (Costerton et al., 1999; Sauer, 2003). They showed that the biofilm fraction exhibited greater protection against antibiotics and the human immune system than planktonic fractions as a result of the changes in gene and protein expression patterns. EPS matrix is also known to protect the biofilms by limiting antimicrobial penetration (Suci et al., 1994; Yang et al., 2011). In the present study, only 32% DMSO was

effective in killing biofilm fractions (Section 4.2.1, Table 4.2). This effective concentration is relatively high for application to antibiofilm therapy. According to an *in vivo* study, DMSO is a relatively low-toxicity organic solvent with LD50 values of 6.2 ml/kg and 9.9 ml/kg in mice and rats respectively (Bartsch et al., 1976). The safe application of DMSO for topical and oral treatments of human diseases typically involves the concentration up to 40% (Morton, 1993; Zuurmond et al., 1996) whilst the DMSO concentration up to 50% DMSO solution has been approved by US FDA for dermatological applications (Capriotti & Capriotti, 2012). The use of high dosage of DMSO *in vivo* is permitted because DMSO is rapidly metabolized to $DMSO_2$ and DMS and excreted, primarily through urinary excretion or in expired air, respectively (Elisia et al., 2016). It is expected that application of 32% DMSO to antibiofilm therapy may produce minimal or no adverse effects on humans. This suggestion is in line with Gaylord Chemical Company (2007) reported that the ocular effects resulting from DMSO treatments of dogs, rabbits, guinea pigs and swine were species-specific and not reproducible in primates, including humans.

Afatinib is a protein tyrosine kinase inhibitor commonly used to control cancerous cells (Zhang & Munster, 2014). Other pharmacological applications of afatinib remain largely unknown, however, a study has suggested its application for malarial control (Sun et al., 2014). In the present study, the biofilm fractions were more susceptible to afatinib alone than planktonic fractions (Section 4.2.1, Table 4.2). This finding contradicts the facts that biofilm fractions are less susceptible to antimicrobials (Suci et al., 1994; Costerton et al., 1999; Sauer, 2003; Yang et al., 2011). However, our result is in agreement with Spoering and Lewis (2001). They showed that stationary planktonic fraction produced more persisters and was relatively more resistant towards ofloxacin

and tobramycin than biofilm fraction. It is suggested that afatinib alone possesses poor antimicrobial effectiveness and is therefore not evaluated for other biofilm parameters.

The decreased susceptibility of biofilms to antimicrobial agents is due to a process of phenotypic diversification in the adherent populations which would result in formation of multiple cell types within single-species biofilms (Lewis, 2007). The phenotypic diversification effectively protects the adherent populations from the attack by any single antibiotic. This has led to a general assumption that the combination therapy with chemically distinct compounds would be more effective against biofilm than the single therapy. In the present study, 32% DMSO in combination with 3.2 μg/mL afatinib (DDA) was found to be effective in killing all microbial fractions (Section 4.2.1, Table 4.2). The similar strategy has previously been used by Harrison et al. (2008) in the antibiofilm study of metals and biocides.

According to Skogman et al. (2012), an effective antibiofilm agent should fulfill three following criteria: a) killing of biofilm, b) reduction in biofilm biomass and c) chemical modification of EPS matrix. This is due to an emerging understanding that a combination of these criteria does not only limit the bacterial colonization on the surface but also interrupts the metabolism of high density biofilms. In the present study, we proceeded to investigate only 32% DMSO and DDA for biofilm biomass and EPS matrix as they effectively killed all biofilm fractions (Section 4.2.1, Table 4.2). Other test concentrations (1-10% DMSO and 0.0032-0.32 μg/mL afatinib) were excluded from the subsequent analyses as they failed to kill the biofilm fractions and were therefore considered ineffective against the biofilm fractions.

In the present study, the biofilm-biomass reducing effect of DDA was found to be greater compared to 32% DMSO (Section 4.2.2, Figure 4.2). However, it may not reflect the synergistic action between 32% DMSO and afatinib as there was insignificant difference ($p>0.05$) in biofilm biomass observed between 32% DMSO and DDA treated groups. The reduction in biofilm biomass following treatment with chemicals has been reported (Nguyen et al., 2012; Halstead et al., 2015; Sharma et al., 2015). It is expected that the molecular basis underlying the antibiofilm effects of DDA is not apparently distinct from 32% DMSO.

ATR-FTIR is often used to monitor bacterial adherence, biofilm formation and bacterial response towards chemicals. In this analysis, samples are measured on a single crystal which result in the high-sensitivity IR spectra and long-term measurements could be performed without water vapour interference and instrument instability. In the present study, 32% DMSO and DDA treatments were found to produce changes in the IR spectra of proteins (1700 - 1500 cm^{-1}), nucleic acids and polysaccharides (1300 - 900 cm^{-1}) in EPS matrix (Section 4.2.3, Figure 4.3). This finding is consistent with a previous study on the antibiotic penetration in *P. aeruginosa* biofilm (Suci et al., 1994). They demonstrated significant changes in the IR bands associated with proteins, nucleic acids and carbohydrates following exposure to ciprofloxacin, reflecting chemical modification of biofilm components. It was also clear that DMSO was not incorporated into EPS matrix as there were no strong IR absorbance bands between 1100 cm^{-1} and 900 cm^{-1} corresponding S-O and CH_3 vibrations (Kelava & Culo, 2011). It is probable that the biofilm inhibition by 32% DMSO and DDA is mediated by chemical modification EPS matrix.

The results from the present study demonstrated significant reduction in biofilm biomass (Section 4.2.2, Figure 4.2) and EPS proteins (Section 4.2.4, Figure 4.4) following treatment with 32% DMSO and DDA. DMSO typically induces the electrostatic repulsion between proteins and alters their interactions and solubility (Hansen et al., 2016). Therefore, treatment with 32% DMSO may form the electrostatic repulsion that facilitates solubilisation of EPS proteins which in turn weaken the adhesion force between proteinaceous biofilm and the surface. This suggestion is generally compatible with previous studies on the mode of action of antibiofilm agents (Suci et al., 1994; Kondoh & Hashiba, 1998; Baillie & Douglas, 2000; Xavier et al., 2005)

Correlation between biofilm biomass and EPS proteins has received increasing attention, in order to understand the overall process of biofilm formation. The relation between EPS protein and structural integrity of biofilm biomass has been addressed by previous studies (Burdman et al., 2000; Decho, 2000; Flemming et al., 2000; Ha et al., 2010). In the present study, correlation between EPS proteins and biofilm biomass was found to be consistent across all test microorganisms (Section 4.2.5, Table 4.2). A similar observation has previously been reported by Sousa et al. (2009). They investigated the correlation between EPS proteins and biofilm biomass in four clinical isolates of *Staphylococcus epidermidis* (9142, IE214, IE186 and 1457) and found that EPS proteins correlated with total biofilm biomass and cellular metabolic activity. Meanwhile, in the cases where biofilm fractions are challenged with antibacterial agents, there are strong correlations between EPS proteins and biofilm biomass (Suci et al., 1994; Kondoh & Hashiba, 1998; Baillie & Douglas, 2000).

Inhibition of *S. typhimurium* biofilm by various chemicals has previously been reported. Tabak et al. (2007) studied the effect of triclosan (5-chloro-2-(2, 4-dichlorophenoxy) phenol) on *S. typhimurium* biofilm. They demonstrated that treatment with triclosan reduced the number of viable biofilm cells and caused upregulation of genes encoding for efflux pump and cellulose. In 2010, Vestby et al. investigated the influence of synthetic brominated furanone on the antibofilm activity of disinfectants against *S. typhimurium*. They showed that pretreatment with furanone significantly potentiated the inhibitory effects of hypochlorite and benzalkonium chloride on biofilm biomass and CFU count.

5.3 Heterogeneity of *S. typhimurium* biofilm

Any given microbial cell within the biofilm often experiences a slightly different environment compared to other microbial cells within the same biofilm due to the gradients of nutrients, oxygen, waste products and signalling factors (Stewart & Franklin, 2008). These phenomena contribute to physical and physiological heterogeneities in biofilm, and differential susceptibility to antimicrobial treatment. In order to obtain a clue to heterogeneous molecular expression in *S. typhimurium* biofilm, IFM analysis was performed because it could provide high-resolution topographical images which are useful to analyse the fluctuating thickness and spatial distribution of biofilm. For IFM analysis, the mild steel coupon was used for biofilm adherence because the mild steel is a common food contact surface associated with Salmonella contamination (Zuraw, 2014). In the present study, *S. typhimurium* biofilm was found to be heterogeneous in thickness and surface coverage (Section 4.3, Figure 4.5). This finding is compatible with an earlier study performed by Korber et al. (1997). They showed that heterogeneity of *Salmonella enteritidis* biofilm in flow cells (0.3 cm s^{-1} flow velocity) increased marginally over the time whereby the mean thickness of *S.*

enteritidis biofilm increased from 4.5 ± 1.3 µM (48 h) to 7.7 ± 1.6 µM (72 h). Furthermore, Sha et al. (2013) reported that Salmonella isolates were repeatedly detected by quantitative qPCR technique in heterogeneous aquatic biofilms and remained viable in such biofilms in high number for some time. It is suggested that the study of heterogeneous *S. typhimurium* biofilm requires protein profiling approach which is different from that for homogeneous bacterial cell population.

5.4 Subtractive one dimensional protein profiling

One dimensional SDS-PAGE analysis offers a wide range of applications including determination of molecular mechanism (Mundy & Chua, 1988), optimization of protein extraction (Paulo et al., 2010), evaluation of protein stability (Paulo et al., 2010), bacterial typing (Aksakal, 2010). In the context of antibiofilm study, protein band intensities from SDS-PAGE could reveal trends, consistencies and differences between control and test biofilm fractions. In the present study, treatment with 32% DMSO triggered changes in the protein profiles of whole biofilm cells (Section 4.4.1, Figure 4.9) and EPS matrix (Section 4.5.1, Figure 4.27). These findings are consistent with differential protein banding pattern resulting from the exposure of microbial cells to antibacterial agents and environmental stresses. Decreases in protein band intensities in one dimensional SDS-PAGE has been shown to result from treatment with abscisic acid (Lin & Ho, 1986), tetracyclin antibiotic (Bangen et al., 2004), clindamycin and linezolid antibiotics (Stevens et al., 2007), *Psidium cattleianum* leaf extract (Brighenti et al., 2008), salts (Ganjian et al., 2012) and berberine alkaloid (Peng et al., 2015). It is pertinent to infer that one dimensional SDS-PAGE is sufficient to provide a preliminary insight into the molecular basis of antibiofilm effects of 32% DMSO. Meanwhile, the subtractive analysis recognized two unique protein bands which were expressed only in control biofilm and not in 32% DMSO-treated biofilm namely 25.4 kDa and 51.2 kDa

(Section 4.4.1, Figure 4.9). The 51.2 kDa protein band was also found to be secreted in 32% DMSO-treated EPS matrix and not secreted in control EPS matrix (Section 4.5.1, Figure 4.27). Subtractive protein profiling of heterogenous samples has previously been reported (Schirmer et al., 2003; Oh et al., 2004; Husi et al., 2016) whilst 25.4 kDa and 51.2 kDa protein bands have been shown to be consistently expressed in 34 Salmonella serovars from various host species (Aksakal, 2010).

5.5 High-confidence protein identification

Sensitive and selective LC-MS/MS analysis is essential for high-confidence protein identification. Production of an MS/MS mass spectrum involves the fragmentation of a precursor ion by various methods such as information-dependent acquisition (IDA), SWATH (sequential window acquisition of all theoretical fragment-ion spectra), and MS (All) methods (Zhu & Snyder, 2014). In the present study, nano HPLC-ESI-QTOF with IDA method successfully identified 29 proteins and 46 proteins from the protein bands of 25.4 kDa and 51.2 kDa respectively (Section 4.4.2, Table 4.3). It should be emphasized that expression of these whole-cell proteins in *S. typhimurium* biofilm was inhibited by treatment with 32% DMSO. A considerably high number of biofilm proteins identified for each whole-cell protein band corroborates the fact that information-dependent acquisition (IDA) enhances the speed, sensitivity and quantification capabilities of a TOF MS (Andrews et al., 2012). Missed trypsin cleavage which results from incomplete proteolysis may cause signal loss and underestimated quantification in QTOF analysis (Lawless & Hubbard, 2012). Only 13% missed trypsin cleavage was observed in protein identification indicating the high-reliability QTOF data. The majority of QTOF-identified whole-cell proteins were associated with the general metabolism and were predicted to be localized in cytoplasm. This result is in line with the data from resazurin assay (Section 4.2.1, Figure 4.1) and a large body of

evidence that metabolic reactions are essential for biofilm development (Paolino & Kahket, 1985; Percival et al., 2006; Hamilton et al., 2009; Morales et al., 2013). Inhibition of expression of various metabolic proteins by treatment with 32% DMSO may weaken the viability of *S. typhimurium* biofilm following treatment with 32% DMSO.

At least 20 *S. typhimurium* whole-cell proteins identified herein have previously been shown to be responsive to the phenotypic changes and various treatments including signalling molecule, biocides, acid stress and antibiotics.

Trigger factor

Trigger factor is involved in protein folding and protein export. It acts as a chaperone by maintaining the newly synthesized secretory and non-secretory proteins in an open conformation. Expression of trigger factor in *S. typhimurium* has previously been reported (Soni et al., 2008). They demonstrated that expression level of Trigger factor in wild type and luxS mutant strains was upregulated 2.69 fold and 4.69 fold respectively, in the presence of autoinducer AI-2.

Virulence transcriptional regulatory protein PhoP

Virulence transcription regulatory protein PhoP is a family member of the two-component regulatory system PhoP/PhoQ which regulates the expression of genes involved in virulence, adaptation to acidic and low Mg2+ environments and resistance to host defense antimicrobial peptides. It is required to attenuate bacterial growth within fibroblast cells and to enhance bacterial resistance to bile in intestinal cells. Expression of virulence transcription regulatory protein PhoP in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They showed that expression level of virulence

transcription regulatory protein PhoP was downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Heat shock protein

Heat shock protein is a family member of proteins that are produced by cells in response to exposure to stressful conditions. It is involved in the degradation of certain denaturated proteins, including DnaA, during heat shock stress. Expression of heat shock protein in *S. typhimurium* has previously been reported (Soni et al., 2008). They revealed that expression level of heat shock protein in luxS mutant *S. typhimurium* strain was downregulated 2.17 fold in the presence of autoinducer AI-2.

Transaldolase B

Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway. It links the pentose phosphate pathway to glycolysis. Expression of transaldolase B in *S. typhimurium* has previously been reported (Soni et al., 2008). They demonstrated that expression level of Transaldolase B in luxS mutant *S. typhimurium* strain was upregulated 2.26 fold in the presence of autoinducer AI-2.

Phase 1 Flagellin

Flagellin is the subunit protein which polymerizes to form the filaments of bacterial flagella. It is a virulence factor that is recognized by the innate immune system in a wide range of organisms. Expression of Phase 1 Flagellin in *S. typhimurium* has previously been reported by (Cho & Ahn, 2013). They showed that expression level of Phase 1 Flagellin was upregulated following exposure to acid stress

Adenylate kinase

Adenylate kinase is an important component in cellular energy homeostasis and in adenine nucleotide metabolism. It catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Expression of adenylate kinase in *S. typhimurium* has previously been reported (Cho & Ahn, 2013). They revealed that expression level of adenylate kinase was downregulated following exposure to acid stress.

Alcohol dehydrogenase

Alcohol dehydrogenases is an enzyme that facilitates the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide from NAD+ to NADH. Expression of alcohol dehydrogenase in *S. typhimurium* has previously been reported (Cho & Ahn, 2013). They demonstrated that expression level of adenylate kinase was upregulated following exposure to acid stress.

Phosphoglycerate kinase

Phosphoglycerate kinase is a major enzyme in glycolysis. It is involved in the second step of the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate. Expression of phosphoglycerate kinase in *S. typhimurium* has previously been reported (Cho & Ahn, 2013). They showed that expression level of phosphoglycerate kinase was downregulated following exposure to acid stress.

Periplasmic trehalase

Trehalase is a glycoside hydrolase enzyme. It catalyzes the conversion of trehalose to glucose which can subsequently be taken up by the phosphotransferase-mediated uptake system. Expression of periplasmic trehalase in *S. typhimurium* has previously been

reported (Hamilton et al., 2009). They discovered that periplasmic trehalase was expressed in biofilm fraction and not expressed in planktonic fraction.

Lysine/arginine/ornithine-binding periplasmic protein

Lysine/arginine/ornithine-binding periplasmic protein constitutes ABC transporter which is involved in lysine, arginine and ornithine transport. It stimulates ATPase activity of ATP-binding protein. Expression of lysine/arginine/ornithine-binding periplasmic protein in *S. typhimurium* has previously been reported (Hamilton et al., 2009). They found that the expression level of lysine/arginine/ornithine-binding periplasmic protein was upregulated 4.9 fold in biofilm fraction compared to planktonic fraction.

Enolase

Enolase is an enzyme essential for the degradation of carbohydrates via glycolysis. It catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. Expression of enolase in *S. typhimurium* has previously been reported (Coldham et al., 2006). They demonstrated that expression level of enolase was significantly upregulated following the exposure to ciprofloxacin antibiotic.

Superoxide dismutase

Superoxide dismutase is an enzyme that destroy superoxide free radical by dismutation (or partitioning) of the superoxide (O2−) radical into oxygen and hydrogen peroxide. It is known to participate in a wide range of biological process including cell aging and response to antibiotic. Expression of superoxide dismutase in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They showed that expression level of superoxide dismutase was significantly downregulated following the exposure to a

mixture of oxidizing compounds containing inorganic peroxygen compounds, inorganic salts, organic acid, anionic detergent, fragrance, and dye (OXC)

ATP synthase subunit beta

ATP synthase subunit beta is an enzyme that produces energy storage molecule ATP from ADP in the presence of a proton gradient across the membrane. Rotation of the gamma subunit produces conformational changes which are essential for ATP synthesis. Expression of ATP synthase subunit beta in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They revealed that expression level of ATP synthase subunit beta was significantly downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Outer membrane protein TolC

Outer membrane protein TolC is an enzyme required for the function of several efflux systems. It forms trimeric channels that allow export of a variety of substrates in Gram negative bacteria. Expression of outer membrane protein TolC in *S. typhimurium* has previously been reported (Coldham et al., 2006). They demonstrated that expression level of outer membrane protein TolC was significantly upregulated following the exposure to ciprofloxacin antibiotic

Alkyl hydroperoxide reductase

Alkyl hydroperoxide reductase is an antioxidant enzyme that controls the level of peroxide free radical. Its catalytic mechanism involves cysteine-sulfenic acid active site. Expression of alkyl hydroperoxide reductase in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They found that expression level of alkyl hydroperoxide reductase was significantly downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Oxygen-insensitive NAD(P)H nitroreductase

Oxygen-insensitive NAD(P)H nitroreductase is an enzyme that reduces a variety of nitroaromatic compounds using NADH/NADPH as source of reducing equivalents. It is able to reduce nitrofurazone drug typically used as antibiotics or antimicrobials. Expression of oxygen-insensitive NAD(P)H nitroreductase in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They discovered that expression level of oxygen-insensitive NAD(P)H nitroreductase was significantly downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Malate dehydrogenase

Malate dehydrogenase is an enzyme that catalyzes the reversible oxidation of malate to oxaloacetate. It is involved in tricarboxylic acid cycle and gluconeogenesis. Expression of malate dehydrogenase in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They demonstrated that expression level of malate dehydrogenase was downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Flagellar biosynthesis protein FliC

Flagellar biosynthesis protein FliC is a structural protein that forms filament, a component of typical bacterial flagellum. Its expression in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They demonstrated that expression level of Flagellar biosynthesis protein FliC was significantly downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Outer membrane protein A

Outer membrane protein A is a protein that acts as a porin with low permeability allowing slow penetration of small solutes. It is located in the membrane of gram-negative bacteria and plays a multifunctional role in bacterial physiology and pathogenesis. Expression of outer membrane protein A in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They showed that expression level of outer membrane protein A was significantly downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Glucose phosphate isomerase

Glucose phosphate isomerase is an enzyme that synthesizes D-glyceraldehyde 3-phosphate and glycerone phosphate from D-glucose. It is involved in glycolysis and gluconeogenesis. Expression of glucose phosphate isomerase in *S. typhimurium* has previously been reported (Karatzas et al., 2008). They revealed that expression level of glucose phosphate isomerase was downregulated following the exposure to a biocide composed of tar acids, organic acids, and surfactants (TOP).

Many bacterial proteins have been shown to be involved in the biofilm formation including pyruvate kinase (Moorthy & Watnick, 2004), adenylate kinase (Yadav et al., (2012), virulence transcriptional regulatory protein PhoP (Mishra et al., 2005), flagellin (Kalmokoff et al., 2006), outer membrane protein A (Lower et al., 2005), putative transposase (Villa & Carattoli, 2005), trigger factor (Wen et al., 2005), enolase (Welin et al., 2004), pyruvate kinase (Welin et al., 2004), phosphoglycerate kinase (Welin et al., 2004), phosphoglycerate mutase (Welin et al., 2004), glyceraldehyde-3-phosphate dehydrogenase (Welin et al., 2004), DNA-directed RNA polymerase (Welin et al., 2004), elongation factor Ts (Welin et al., 2004) and trigger factor (Inagaki et al., 2009).

EPS matrix is critical for the spatial organization and structural stability during biofilm development. One of the major component of EPS matrix is protein (Jiao et al., 2011). Numerous proteomic studies have revealed that many of EPS proteins are involved in cell motility and secretion, amino acid metabolism, carboydrate metabolism, cell wall biogenesis, transcription, defense mechanism and replication (Gallaher et al., 2006; Eboigbodin & Biggs, 2008; Jiao et al., 2011). In the present study, nano HPLC-ESI-QTOF with IDA method successfully identified three EPS proteins (phosphoglycerate kinase, elongation factor Tu and enolase) from the protein bands of 51.2 kDa respectively (Section 4.5.2, Table 4.11). Secretion of these EPS proteins in *S. typhimurium* biofilm was induced by treatment with 32% DMSO. The result from the present study is in accordance with Berrier et al. (2000). They showed that *E. coli* secreted elongation factor Tu upon the treatment with Tris-EDTA. The result from the present study also contradicts a study performed by Yang et al. (2011). They demonstrated that enolase of *Bacillus subtilis* was regularly secreted into extracellular environment, however, treatment with chloramphenicol antibiotic did not affect the secretion of enolase. Although phosphoglycerate kinase is known to be secreted by tumour cells (Lay et al., 2000), secretion of this enyzme by bacterial cells remains largely unknown. On the other hand, glycolytic enzymes (enolase and phosphoglycerate kinase) were secreted only in 32% DMSO treated EPS matrix and not in control EPS matrix. This result is in accordance with Chiba et al. (2015). They believed that a combination glycolytic enzymes with proteases and deoxyribonucleases in extracellular matrices is effective in inhibiting biofilm formation and promoting biofilm dispersal. This implies that inhibition of *S. typhimurium* biofilm by 32% DMSO may be mediated by secretion of the glycolytic enzymes. The majority of secretome analysis of *S. typhimurium* have focused on the roles of Salmonella effector proteins in bacterial colonization of the host (Gerlach et al., 2007; Auweter et al., 2011; Niemann et al.,

2011). Nevertheless, the study on the effect of antimicrobial treatment on EPS matrix of *S. typhimurium* biofilm is still not established. Thus, the present study provides the first evidence of the changes in EPS protein profile of *S. typhimurium* biofilm following treatment with 32% DMSO.

5.6 Coordinated control of multiple pathways essential for biofilm formation

Protein-protein interactions mediate metabolic pathways, signaling pathways, cellular processes and mechanism leading to normal and diseased states in organisms (Gonzalez & Kann, 2012). Therefore, elucidation of protein interaction networks can provide information on the molecular basis of disease which in turn can develop methods for prevention, diagnosis and treatment (Gonzalez & Kann, 2012). According to De Las Rivas and De Luis (2004), protein-protein interactions may refer to physical binding, metabolic or genetic correlation, co-localization and co-expression. Since many decades ago, a wide range of approaches have been identified for the study of protein-protein interaction including x-ray crystallography, NMR spectrocopy, yeast two-hybrid and computational predictions (Gonzalez & Kann, 2012). In the present study, STRING analysis successfully identified 268 functional interactions from the dataset of QTOF-identified whole-cell proteins (Section 4.4.2.3, Figure 4.23). GO enrichment analysis identified several biological processes in *S. typhimurium* biofilm to be significantly ($p<0.05$) affected by treatment with 32% DMSO including glycolysis, ion transport, phosphorelay signaling and flagellar biosynthesis (Section 4.4.2.3, Table 4.7).

Several functional linkages between the QTOF-identified whole-cell proteins have previously been reported.

Functional linkages between glyceraldehyde-3-phosphate dehydrogenase, recombinase A, virulence transcription regulatory protein PhoP and malate dehydrogenase

Functional linkages between glyceraldehyde-3-phosphate dehydrogenase, recombinase A, virulence transcription regulatory protein PhoP and malate dehydrogenase shown in the present study corroborate the fact that these proteins are housekeeping gene products in Salmonella species (McQuiston et al., 2012). The use of these houskeeping proteins has been shown to successfully produce a robust phylogeny of the Salmonella species and subspecies that clearly defines the lineages comprising diphasic and monophasic subspecies (McQuiston et al., 2012). Inhibition of expression of these housekeeping proteins by treatment with 32% DMSO may cause severe interruption of basic cellular maintenance of *S. typhimurium* biofilm.

Functional linkages between flagellin, ATP synthase subunit alpha, ATP synthase subunit beta, enolase, heat shock protein 60 kDa, outer membrane protein A and outer membrane TolC

Functional linkages between flagellin, ATP synthase subunit alpha, ATP synthase subunit beta, enolase, heat shock protein 60 kDa, outer membrane protein A and outer membrane TolC shown in the present study are in accordance with Coldham et al. (2006). They demonstrated that all of these proteins were co-expressed in *S. typhimurium*. These functional linkages also confirm the fact that ATP synthase supports the function of flagellin proteins. Inhibition of expression of these proteins by treatment with 32% DMSO may affect the biofilm dispersion.

Functional linkages between 10 glycolytic enzymes

Functional linkages between 10 glycolytic enzymes shown in the present study are compatible with Fraenkel (1996) regarding the metabolic connections between them with phosphofructokinase as the key component. According to Bowden et al. (2009), glycolysis is essential for intracellular replication and survival of *S. typhimurium* in macrophages. Inhibition of expression of glycolytic enzymes by treatment with 32% DMSO may weaken the pathogenicity of *S. typhimurium* biofilm.

The biological pathways identified in the present study namely glycolysis (Moorthy & Watnick, 2004), purine biosynthesis (Yadav et al., 2012), phosphorelay signaling (Mishra et al., 2005), flagellar biosynthesis (Kalmokoff et al., 2006), ion transport (Lower et al., 2005), transposition (Villa & Carattoli, 2005), protein synthesis (Wen et al., 2005) and DNA repair (Inagaki et al., 2009), have previously been shown to be involved in the biofilm formation. Therefore, inhibition of *S. typhimurium* biofilm by treatment with 32% DMSO may be mediated by interruption of the coordinated control of multiple biological pathways. This has led to a possible molecular basis as illustrated in Figure 5.1. In brief, this biofilm inhibition model involves five sequential stages namely planktonic stage, cell attachment, microcolony, mature biofilm and biofilm dispersal. Inhibition of expression of proteins associated with all the identified biological pathways would result in functional interference with the normal biofilm life cycle.

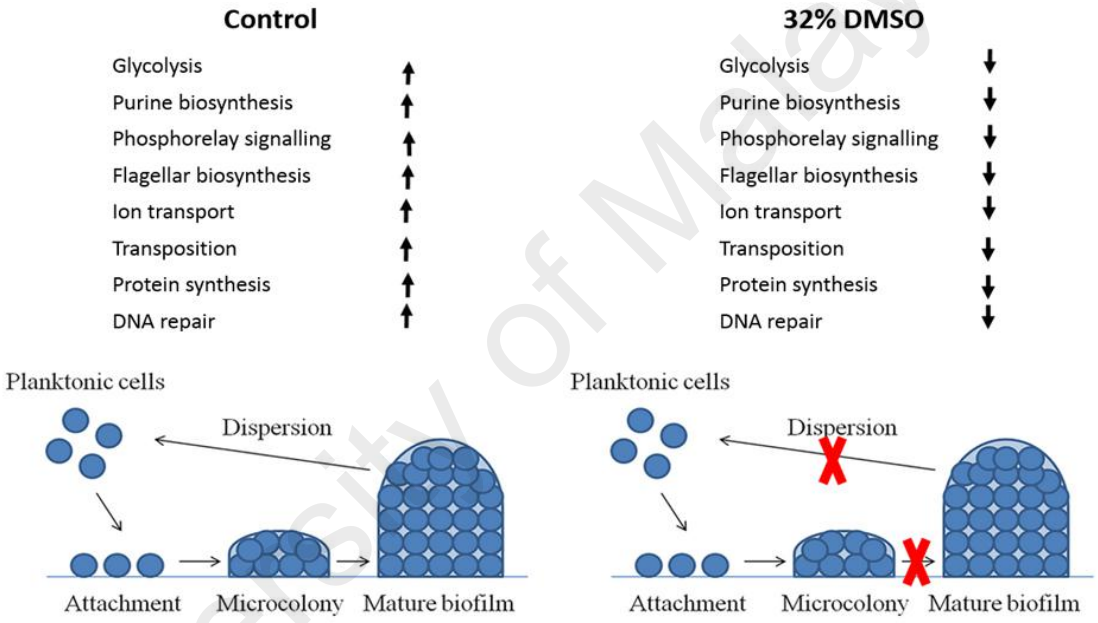


Figure 5.1: A proposed molecular basis for the antibiofilm effect of 32% DMSO. (↑) and (↓) indicate protein expression and inhibition respectively.

In the functional interaction networks, hubs are nodes with high connectivity or molecules that have the high number of functional interactions. The hub molecules are expected to play crucial roles in the cellular processes and hence possess biological significance (Jeong et al., 2001). Across many studies, hub proteins are known to associate with cancer, leukemia and asthma (Jonsson & Bates, 2006; Lu et al., 2007; Haferlach et al,. 2010; Jamil et al., 2012). In the present study, four QTOF-identified whole-cell proteins were identified as the hub proteins namely elongation factor Tu, glyceraldehyde-3-phosphate dehydrogenase and ATP synthase subunit alpha and trigger factor (Section 4.4.2.3, Figure 4.23). They were found to have more than 10 interaction partners. This finding is in accordance with a large-scale study on protein-protein interaction in *E. coli.* (Butland et al., 2005). They demonstrated the presence of highly connected nodes in the protein interaction network and their removal markedly affected the robust protein interaction network. They also revealed that the protein connectivity was proportional to the number of genomes a homologue was detected in. It is postulated that treatment with 32% DMSO may interfere with the protein connectivity in *S. typhimurium* biofilm.

5.7 Inhibitory effects of DMSO on protein phosphorylation

Protein kinase inhibitors represent an important and still emerging class of targeted therapeutic agents. While numerous studies largely determine the effects of protein kinase inhibitors on enzymatic activities, the current studies of protein kinase inhibitors tend to explicitly address the impact of protein kinase inhibitors on the gene and protein expression (Kumahara et al., 1999; Antunes et al., 2010; Klopfleisch et al., 2012; Gharechahi et al., 2014; Bullard et al., 2015). This progressive shift in the analytical approach for studying protein kinase inhibitors may be due to the following reasons: i) effects of compounds on protein kinase activity have been well investigated, ii)

information on modulation of multiple cellular pathways by the protein kinase inhibitors is still limited and iii) advancement of key technologies for gene and protein identification. DMSO is a potent protein kinase inhibitor (Balazovich et al., 1987; Koo & Kim, 2009). This compound was initially hypothesized to inhibit protein phosphorylation in *S. typhimurium* biofilm.

In the present study, 25.4 kDa and 51.2 kDa protein bands were found to contain phosphoproteins based on ESI-QTOF analysis. This finding is in agreement with the fact that both Q-TOF and triple quadrupole analyzers are effective in detection of phosphopeptides (Steen et al., 2001). This is because, in ESI-QTOF analysis which uses positive ionization mode, phosphoserine- and phosphothreonine-containing peptides tend to undergo β-elimination reaction causing a neutral loss of 98 Da (H_3PO_4) or 80 Da (HPO_3) following collision-induced dissociation (CID) in Q2. Such phenomenon is useful for selective detection of phosphopeptides by tandem mass spectrometry (Schlosser et al., 2001). The expression of QTOF-identified phosphoproteins was validated by ProQ Diamond assay which is a common approach to simultaneously detect multiple phosphoproteins identified by MS/MS (Gannon et al., 2008; Sheikh et al., 2016; Lv et al., 2016). ProQ Diamond assay is also known to exhibit greater sensitivity than the conventional antibody-based methods in the phosphoproteomic analysis (Stasyk et al., 2005; Wu et al., 2005). The result from ProQ Diamond assay corroborates several previous works on the phosphoproteins in *S. typhimurium* lysates (Wang & Koshland, 1982; Waygood et al., 1984; Peri et al., 1984). These studies detected the phosphoproteins in the range between 8 kDa and 65 kDa. On the other hand, proteomic detection of phosphoroteins in biofilm has previously been reported by Petrova and Sauer (2009). Interestingly, the present study provides the first evidence of the occurrence of phosphoproteins in *S. typhimurium* biofilm.

Phosphorylation of Virulence transcription regulatory protein PhoP and ParB-like partition protein has previously been reported.

Phosphorylated PhoP protein

PhoP-PhoQ system controls the virulence of *S. typhimurium* at the transcriptional level (Fields et al., 1989). Its phosphorylation mechanism has previously been reported (Soncini et al., 1995). They demonstrated that environmental signals stimulated PhoQ protein to phosphorylate DNA-binding PhoP protein which in turn increased the amounts of PhoQ and PhoP proteins. As a result, expression level of PhoP-activated genes increased substantially. Recently, selected reaction monitoring (SRM)-based proteomics has been applied to study Salmonella PhoP/PhoQ regulation (Hu et al., 2016). Their results demonstrated the PhoP phosphorylation, dynamic activation of PhoQ/PhoP-system and repression of iron homeostasis by PhoP-PhoQ system.

Phosphorylated Par-B like partition protein

ParB-like partition protein is a centromere binding protein required for assuring stable transmission of bacterial plasmids during cell division. Its phosphorylation mechanism has previously been studied by Motallebi-Veshareh (1990). They suggested that ATP hydrolysis by the ParA protein might result in phosphorylation of the ParB protein which then influenced the separation of paired plasmid molecules at the cell division plane.

The primary source of phosphoproteins in *S. typhimurium* is phosphoenolpyruvate (PEP) whilst the molecular mass of PEP-dependent phosphoproteins ranges between 8 kDa and 65 kDa (Waygood et al., 1984). In this case, the present study did not reveal any relation between phosphoproteins and PEP metabolism, however, it could be assumed

that the inhibition of expression of phosphoenolpyruvate carboxykinase (Section 4.4.2, Table 4.3) which catalyse formation of PEP from the decarboxylation of oxaloacetate and hydrolysis of guanosine triphosphate molecule may cause an interruption of PEP source for phosphoprotein production thereby decreasing the phosphorylation level of 51.2 kDa protein band. The findings from the present study suggest the kinase inhibitory effect of DMSO on *S. typhimurium* bioflm.

5.8 Ideal therapeutic targets for biofilm control

Numerous biofilm control strategies have been researched to overcome environmental, industrial and medical implications of biofilms. These include super high magnetic field (Okuno et al., 1993), high pulsed electrical field (Liu et al., 1997), passive and active polymer coatings (Hetrik & Scoenfisch, 2006), slippery surfaces with physico-chemical properties (Callow & Callow, 2011), ultrasound (Guo et al., 2011) and electrochemical coatings (Fraunhofer-Gesellschaft, 2012). However, many of them require further improvement to eradicate the highly dynamic and complex biofilm communities. Recent antimicrobial strategies emphasize the functionality, essentiality and non-homology of theraputic targets (Dutta et al., 2006; Munikumar et al., 2012). To improve the biofilm control strategy, identification of functional, essential and non-homologous proteins from *S. typhimurium* deserves further attention. In the present study, approximately 54.67% of QTOF-identified whole-cell proteins were found to be essential to the survival of *S. typhimurium* and were non-homologous to human proteome (Table 4.10). Many of them were found to associate with carbohydrate metabolism, amino acid metabolism and cofactor metabolism. The finding from the present study is in line with a recent bioinformatic work on *Salmonella enterica* (Verma et al., 2016). In that study, they showed that a total of 143 essential and non-homologous genes from *S. enterica* Gram negative pathogen were involved in

various metabolic pathways including glycan metabolism, terpenoid metabolism, carbohydrate metabolism, amino acid metabolism and cofactor metabolism. The finding from the present study also supports the fact that *in silico* subtractive analysis is efficient in speeding up drug discovery process by identifying essential and non-homologous drug targets which can avoid the potential cross-reactivity with host encountered in the classical approaches (Dutta et al., 2006; Munikumar et al., 2012). The essential and non-homologous proteins of *S. typhimurium* biofilm identified in the present study may represent the key proteins that offer new diagnostic and therapeutic approaches. On the other hand, treatment with 32% DMSO is believed to be an effective strategy to control *S. typhimurium* biofilm because it inhibits expression of many essential and non-homologous proteins.

5.9 Potential applications of DMSO as an antibiofilm agent

The present study successfully demonstrates the antibiofilm activities (Section 4.2) and molecular basis of DMSO (Section 4.4 – 4.5) against *S. typhimurium* biofilm. Considering its low systemic toxicity (Morton, 1993; Zuurmond et al., 1996; Capriotti & Capriotti, 2012) and metabolic removal from human body system (Elisia et al., 2016), DMSO could be used in a wide range of medical and industrial applications. First, DMSO could be formulated as a catheter maintenance solution. The catheter is a thin tube inserted into body cavity during a surgical procedure which is often associated with the biofilm infection. Application of antibiofilm agent in the treatment of intravascular catheter has been reported by Aslam et al. (2008). They used N-acetylcysteine (NAC) to control bacterial infection on hemodialysis catheter. Second, DMSO could also be formulated as a skin antiseptic. Application of low concentration of DMSO in the skin antiseptic has been reported by Tarrand et al. (2012). They showed that 4%-5% DMSO

increased the efficiency of isopropanol based antiseptics against a variety of bacteria and no skin irritation and redness were observed in the clinical studies. Nonetheless, Tarrand et al. (2012) did not investigate the antibiofilm potential of DMSO.

5.10 Limitation of study

Although all the technical procedures such as preparation of bacterial inoculum density and assay incubation were standardized, the biofilm formed on the surface after 24 hours remained heterogeneous which is not suitable for quantitative proteomics. The diverse spatial location of biofilm cells and varying biofilm thickness caused highly inconsistent protein expression across the replicates and independent experiments making the analysis of up-regulated and down-regulated proteins statistically inaccurate which was reflected in the large standard deviation of protein density values. This unavoidable limitation led to the use of subtractive polyacrylamide gel analysis approach highlighting only the unique protein bands which were either expressed only in control biofilm fraction or test biofilm fraction respectively. Although this approach seemed to limit the useful information regarding the molecular mechanism of protein kinase inhibitor, it should be noted that the homogeneity of sample is very crucial to obtain consistent protein expression for the large-scale and high-confidence quantitative proteomics. To solve the experimental challenge of biofilm heterogeneity, a technique called laser capture microdissection (LCM) may be effective to isolate the specific subpopulation of biofilm cells of interest under direct microscopic observation. Thus, the combination of LCM and quantitative proteomics would be an ideal approach for the high-confidence protein identification and quantitation in the study of heterogeneous biofilms.

CHAPTER 6: CONCLUSION

6.1 Present investigation

In this PhD thesis, two protein kinase inhibitors namely dimethyl sulfoxide (DMSO) and afatinib were evaluated against *S. typhimurium* biofilm and results demonstrated that both 32% DMSO and its combination with 3.2 µg/mL afatinib which was termed DMSO-diluted afatinib (DDA) were effective in killing biofilm cells, reducing biofilm biomass and chemically modifying EPS matrix. In all cases, DDA exhibited greater antibiofilm effects compared to 32% DMSO. These antibiofilm effects were also observed in other biofilms. However, only 32% DMSO was regarded as an effective candidate against *S. typhimurium* biofilm due to the evidence that afatinib alone was not effective against biofilm and no significant differences in biomass and EPS proteins between 32% DMSO-treated biofilm and DDA-treated biofilm. The latter suggests the similar molecular basis for both 32% DMSO and DDA.

S. typhimurium biofilm was observed to be heterogeneous in thickness and surface coverage during IFM analysis. Therefore, subtractive protein profiling was the ideal approach to understand the molecular basis underlying the antibiofilm effects of 32% DMSO on *S. typhimurium* biofilm. Subtractive protein profile analysis recognized two unique protein bands (25.4 kDa and 51.2 kDa) of whole biofilm cells which were expressed only in control biofilm and not in 32% DMSO-treated biofilm. In turn, nano high performance liquid chromatography-electrospray ionization-quadrupole time-of-flight (Nano-HPLC-ESI-QTOF) mass spectrometry successfully identified 29 and 46 proteins from protein bands of 25.4 kDa and 51.2 kDa respectively. Five of the QTOF-identified whole-cell proteins showing neutral loss of H_3PO_4 in their spectra were assigned as phosphoproteins whose expression was validated by fluorescence-based ProQ Diamond assay. On the other hand, the protein band of 51.2

kDa was also observed to be uniquely secreted into 32% DMSO-treated EPS matrix. The nano-HPLC-ESI-QTOF successfully identified three proteins from this protein band, however, none of these proteins showed neutral loss of H_3PO_4 in their spectra. Subsequently, STRING protein interaction network analysis identified several biological processes to be significantly ($p<0.05$) inhibited by treatment with 32% DMSO including glycolysis, PhoP-PhoQ phosphorelay signalling and flagellar biosynthesis. Meanwhile, *in silico* subtractive analysis revealed that 41 out of 75 QTOF-identified whole-cell proteins were essential for survival of *S. typhimurium* and non-homologous to human proteome, making them ideal therapeutic targets for biofilm control.

The kinase inhibitory effect of DMSO on *S. typhimurium* biofilm was demonstrated using fluorescence-based ProQ Diamond assay. DMSO was found to inhibit biofilm formation and phosphorylation of 51.2 kDa protein band in a concentration-dependent manner.

Altogether, the present study demonstrates an original work on the antibiofilm effects of DMSO against *S. typhimurium* biofilm and its mechanism of action targeting multiple biological pathways essential for biofilm formation. The ideality of 32% DMSO treatment is established by the evidence that the majority of *S. typhimurium* bioflm proteins whose expression was inhibited by 32% DMSO were essential and non-homologous. In addition, the finding from the present study also suggests that the phosphoproteins may play essential roles in the formation of *S. typhimurium* biofilm. Finally, the work presented in this PhD thesis develops a new understanding on how to combat highly resistant *S. typhimurium* which may be helpful in improving the treatment of human gastroenteritis.

6.2 Future directions

There are several potential future research works related to proteome of *S. typhimurium* biofilm as follows:

1. Development of proteome map of *S. typhimurium* biofilm using a combination of laser capture microdissection and liquid chromatography mass spectrometry. This study will lead to a more accurate comparative analysis of heterogeneous proteome expression in *S. typhimurium* biofilm.

2. A combination of laser capture microdissection and liquid chromatography mass spectrometry to study formation of *S. typhimurium* biofilm in the presence of DMSO. This study will provide a new insight into how a homogeneous fraction of *S. typhimurium* biofilm respond towards treatment with DMSO.

3. Subtractive protein profiling of DMSO-treated *S. typhimurium* biofilm using gel-based and gel free approaches. This study will validate the impact of DMSO on *S. typhimurium* proteome expression.

4. Subtractive protein profiling to evaluate the effects of DMSO on *S. typhimurium* biofilm under aerobic and anaerobic conditions. This study will reveal the role of oxygen in the antibiofilm action of DMSO against *S. typhimurium* biofilm.

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