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Mass Spectrometry-Based Proteomic Investigation of Heterogeneous Biofilms: A Review

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ABSTRACT

Biofilm represents a major public health concern. It is a highly structured and heterogeneous microbial population that is well protected by a hydrated extracellular matrix. In most cases, the difficulties in combating a wide spectrum of biofilm-associated diseases are due to the presence of dormant cells and differential molecular expression. Proteomics is the large-scale and systematic study of cellular proteome expression at any given time by mass spectrometry. It allows high-sensitivity and high-specificity identification of differentially expressed proteins in the biofilms. Over the past few decades, multiple lines of proteomic works have successfully elucidated various aspects of the biofilm including developmental stages, antimicrobial resistance, and survival mechanisms. However, the heterogeneity of biofilms may contribute to inconsistent proteome expression throughout a proteomic experiment. This is due to the fact that the mature biofilm is often associated with the mixture between monolayer and multilayer biofilms, thick microbial population, and chemical gradient of nutrients. This review highlights the biofilm heterogeneities, the principle of mass spectrometry in proteomics, and the possible strategies for quantitative proteomic analysis of heterogeneous biofilms. It is suggested that isolation of monolayer biofilm, laser capture microdissection, flow cytometry, and subtractive proteome profiling may be considered for an accurate and reliable quantitative proteomics experiment.

Keywords: biofilm, proteomics, mass spectrometry, heterogeneity

INTRODUCTION

Biofilm is a sessile and densely packed microbial community representing the primary mode of microbial existence. The biofilm which is encapsulated in the extracellular matrix can form on biotic surfaces which include plants, animals, and other microbes. It can also grow on abiotic surfaces such as minerals, plastics, steel, the chitinous covering of dead organisms, and air-liquid interfaces. It has been established that biofilm formation is a biological process that occurs in four main stages namely (1) microbial attachment to a surface, (2) formation of microcolony and production of extracellular matrix, (3) biofilm maturation, and (4) biofilm dispersal [1]. Numerous works have shown that the biofilm fraction differs from the free-floating fraction in the gene and protein expression patterns conferring greater defense against antibiotics and the human immune system [2-3]. Since the last few decades, proteomics has become an important approach to understand various aspects of the biofilm including developmental stages, antimicrobial resistance, and survival mechanisms.

Proteomics is the systematic and high-throughput proteome analysis that includes the identification and quantification of proteins. The common workflow in a proteomics experiment begins with protein extraction, followed by protein assay, protein separation, enzymatic digestion using trypsin enzyme, ionized peptide separation, and mass analysis [4]. Alternatively, the ionized peptides are further fractionated by high-performance liquid chromatography (HPLC) followed by electrospray ionization-quadrupole time of flight (ESI-QTOF) mass spectrometry. The combination of HPLC and ESI-QTOF provides not only the protein identity but also quantitative information and post-translational modification for that particular protein [4]. The high resolution, sensitivity, and mass accuracy offered by the mass spectrometry-based proteomics have advanced various areas of antibiofilm drug discovery and development. Nonetheless, the biofilm heterogeneity that underlies inconsistent proteome expression has received poor attention. Therefore, the objective of this review was to highlight the biofilm heterogeneities, the principle of mass spectrometry in proteomics, and the strategies for quantitative proteomic analysis of heterogeneous biofilms.

BIOFILM HETEROGENEITIES

Attachment of microbial cells to a surface would lead to the 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 a transition to the permanent attachment upon changes in the membrane potential [5]. The production of an adhesive extracellular matrix facilitates intercellular adhesion leading to the formation of a multilayer of biofilm. The structural differences between the monolayer and multilayer biofilms have been studied by [6] using confocal scanning laser microscopy as shown in Figure 1. It is apparent that monolayer and multilayer biofilms are different in the degree of heterogeneity.

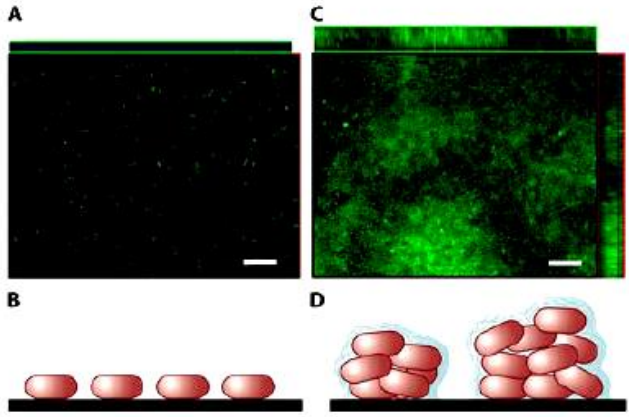

Figure 1: Distribution of Monolayer (A and B) and Multilayer (C and D) Biofilms (Source by Karatan and Watnick, 2009)

The biofilm cells are physiologically heterogeneous because of spatial location, and chemical gradient of oxygen, nutrients, waste products and signalling molecules [7]. A major factor known to contribute to these phenomena is the formation of a biphasic biofilm consisting a homogeneous monolayer biofilm intermixed with heterogeneous multilayer biofilm which are different in microbial load, distribution and thickness [6]. According to [8], the monolayer biofilm is a transient stage in biofilm development, making it difficult to differentiate from the mature biofilm.

The heterogeneous biofilms show greater adhesion and higher biomass than homogeneous biofilms due to lower zeta potential and electrostatic repulsion [9]. Basically, zeta potential is the cell surface charges whilst electrostatic repulsion is the force between bacterial cells and the surface repelling each other as both of them are negatively charged. Furthermore, the biofilm heterogeneity increases with incubation time as shown by the large standard deviation of the biomass and average thickness values [10]. These findings are in agreement with [11] 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 such as types of surface, nutritional levels, temperature, oxygen level, salinity, pH, and antibiotics.

PROTEOME EXPRESSION OF BIOFILM

The proteome is the complete set of proteins that can be expressed by a cell under a certain condition. Differential proteome expression in the biofilms is often observed during the transition from planktonic state to biofilm state, antimicrobial treatment, progressive developmental stages, and exposure to stressful environments. In most biofilm studies, mass spectrometry is combined with liquid chromatography which enables the high-level protein separation prior to the identification of differentially expressed proteins. Table 1 highlights the use of mass spectrometry in several studies of microbial biofilms.

Table 1: Proteomic Analyses which Employ 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 the air-liquid interface and cell attachment to the solid surface [12].

Pseudomonas aeruginosa	Liquid chromatography coupled to hybrid quadrupole time-of-flight (QTOF) mass spectrometer	Identification of 21 phosphoproteins in 24-hour biofilm [13].
Salmonella typhimurium	One dimensional gel electrophoresis and high-performance liquid chromatography coupled to quadrupole time-of-flight (QTOF) mass spectrometer.	Identification of three unique proteins which were present in dimethyl sulfoxide (DMSO) treated extracellular biofilm matrix and absent in control extracellular biofilm matrix [14].
Vibrio cholerae	High-performance liquid chromatography and linear trap quadrupole (LTQ) mass spectrometer	Identification of Bap1 and RbmA proteins which were associated with the extracellular matrix of biofilm [15].
Streptococcus pneumoniae	Two-dimensional ultra-performance liquid chromatography coupled to quadrupole time-of-flight (QTOF) mass spectrometer	Identification of nine differentially expressed glycolytic enzymes (such as gapA, pgk, and pyk) during biofilm formation [16].
Salmonella typhimurium	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 [17].
Pseudomonas aeruginosa	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 elongation factor Tu, porin D, and 30s ribosomal protein) in the biofilm following treatment with Chromolaena odorata extracts under aerobic and anaerobic conditions [18].

MASS SPECTROMETRY IN PROTEOMICS

The key technology for the proteomics work is tandem mass spectrometry. The tandem mass spectrometry which is also known as MS/MS is an analytical technique that combines two or more mass analysers to increase their performance to analyse chemical samples including proteins and peptides. It has been widely employed to investigate proteomic expression in biofilms and extracellular matrix proteome. The MS/MS system consists of three parts namely ionization source, mass analyser, and detector. The sample needs to be introduced to an ionization source prior to isolation by the first analyzer of the mass spectrometer. The ionized sample molecule is fragmented by collision with an inert gas to produce product ions and neutral fragments which are then analysed by the second mass spectrometer.

Examples of tandem mass spectrometer include triple quadrupole, quadrupole-ion trap, quadrupole time-of-flight (TOF) and fourier transform ion cyclotron resonance (FTICR). Basically, these mass analysers differ in accuracy, resolution, mass range, and sensitivity [4]. 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 differentiate between ionized peptides of different m/z ratios. Mass range is the m/z range detected by a mass spectrometer. Sensitivity is defined as the ability of a mass spectrometer to discriminate molecular masses. Combination of two or more mass analysers produces hybrid mass spectrometers such as triple quadrupole, QTOF, quadrupole-ion trap, and TOF-TOF [4]. The schematic representation of QTOF mass spectrometer is shown in Figure 2. It is similar to a triple quadrupole where the last quadrupole is replaced by a TOF analyser. In QTOF mass spectrometer, ions are produced in the ion source. After passing through the gas stream, the ions enter the vacuum system and are separated according to their masses in Q1 and dissociated in Q2. The ions enter the orthogonal TOF analyser through a grid which is then pulsed into the reflector and onto the detector. In the TOF analyser, ions with the same mass but slightly different kinetic energies would reach the detector at the same moment. The mass-to-charge ratio (m/z) correlates with the time taken by an ion to arrive at the detector. It means that the lighter ions arrive at the detector first.

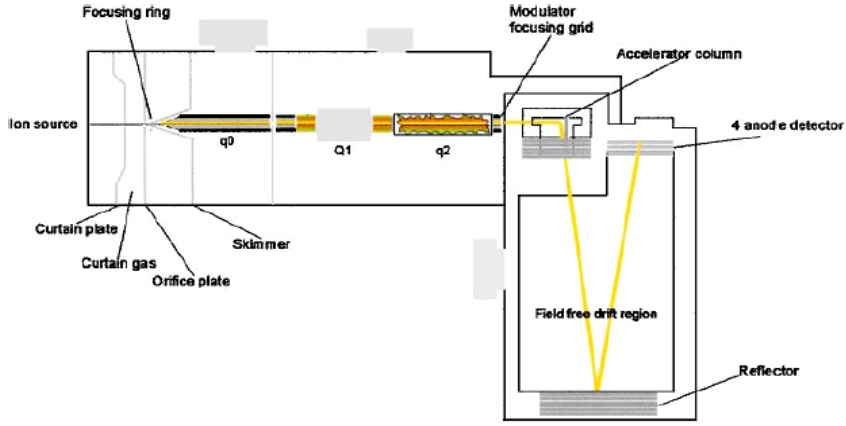

Figure 2: Schematic Illustration of Quadrupole Time of Flight Mass Spectrometer (Source by Mann *et al.*)

Two ionization methods mainly used in the biofilm studies using proteomics approach are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). 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 [20] have made a brief comparison between ESI and MALDI methods as follows: i) ESI ionizes molecules directly from the liquid phase. ESI-time-of-flight (TOF) combined with liquid chromatography has become a common method in the analysis of intact proteins because it allows high-accuracy mass determination ($\leq$ 50 ppm). Nonetheless, salts and detergents in the sample buffer, as well as contaminants, may suppress the analyte signal; ii) MALDI requires crystallization of molecules with ultraviolet-absorbing organic molecules (matrix molecules) prior to ionization. MALDI-TOF enables the determination of intact protein mass with acceptable accuracy ($\leq$ 500 ppm) and is less prone to salt components, detergents, and contaminants.

Fragmentation of precursor ions is crucial for tandem mass spectrometry and takes place between the first and second mass analysers. This step also ultimately defines the type of product ions in the mass analysis. According to [21], there are many techniques used to fragment the ions resulting in different types of fragmentation. For example, collision-induced

dissociation (CID) and surface-induced dissociation (SID). In brief, CID involves a 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. The CID is the most common activation method for tandem mass spectrometry [22]. Conversion of kinetic energy into internal energy following collisions between the precursor ions and inert gas target would induce decomposition of precursor ions with higher fragmentation probability [21]. Furthermore, the CID process is influenced by the relative masses of inert gas target and precursor ion. The larger precursor ions show lower internal energy for ion fragmentation through the CID. Several chemical bonds along the peptide backbone can be broken during the CID. The b and the y ions are commonly produced during the 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 possess arginyl or lysyl residues as their C-terminal residues. 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 begins with b1 for the N-terminal amino acid and is detected upward in molecular weight. Both b and y ion series determine the entire peptide sequence.

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). A 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. A 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 in order to increase sensitivity. Both precursor ion scan and neutral loss scan are not used in time-based mass spectrometers such as ion trap and FTICR.

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. In bottom-up proteomics, the peptide mass fingerprinting (PMF)

approach is often used for protein identification when the genome sequence information is available for the organism under study. If the genome sequence information of the studied organism is not available, protein identification can be performed using de novo sequencing [23]. In automated MS/MS analyses, the peaks are selected from the MS spectrum for fragmentation and MS/MS analysis thereby creating hundreds of high-resolution spectra. The experimentally collected MS/MS spectra are then compared with the theoretical MS/MS spectra of peptides available in the public protein databases and the score values are generated for the matching spectra. The PMF analysis is an ideal strategy to identify the proteins derived from the polyacrylamide gels. It may involve various bioinformatics tools such as Mascot, Profound, PeptideSearch, and PeptIdent. Many factors are taken into consideration during PMF analysis such as peak intensities, the accuracy of matching peaks, modified amino residues, missed or non-specific cleaved peptides, the origin of peaks, or background noise [24].

There are two common approaches for quantification of protein expression namely label-based and label-free protein quantification. In the label-based protein quantification, proteins are labelled with either isobaric tags for relative and absolute quantitation (iTRAQ) or with stable isotope labelling with amino acids in cell culture (SILAC), prior to the separation. The comparison of proteome expression profiles is carried out by the detection of the introduced label during MS analysis. This approach, however, requires high-cost isotope labels, specific software, and expertise for data analysis. On the other hand, the label-free approach is dependent on spectral counting or signal intensity measurement during MS/MS. Commonly, the amount of peptides selected for fragmentation correlates with the number 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.

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 conventional methods are widely accepted for the verification of a 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 the validation of multiple protein targets due to its high throughput and specificity. On the other hand, fluorescence-based ProQ Diamond assay is often used to simultaneously validate multiple phosphoproteins identified by MS/MS [25-27].

STRATEGIES FOR QUANTITATIVE PROTEOMICS ANALYSIS OF HETEROGENEOUS BIOFILM

Bacteria cells growing in biofilm growth mode are physiologically heterogeneous depending on their spatial location and chemical gradient of nutrients [7], making the quantitation of protein expression changes inconsistent and challenging [28]. The dynamic range of molecular expression in the heterogeneous biofilm has been addressed in the past few years. In a thick biofilm, the bacterial cells at the top of the biofilms exhibit a distinct pattern of gene expression, growth, and antibiotic tolerance as compared to those of bacterial cells at the bottom of the biofilm [28]. The high heterogeneity within the biofilm also contributes to different levels of metabolism and subproteome expression [29]. Moreover, the extensive genotypic and phenotypic heterogeneity in the biofilm of uropathogenic *E. coli* (UPEC) has hampered the consistent proteomic identification of surface proteins [30]. Across many published works on proteomic analysis of biofilms, the issue of biofilm heterogeneity is often neglected and is probably the important cause of the 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 population for sufficient protein samples and provide proteome information as an average of that for the entire biofilm [28]. According to [31], the average molecular expression level across the entire biofilm community does not account for physiological 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 [6-7, 10]. To avoid the inconsistent protein expression level, researchers may consider the following strategies for the quantitative proteomic analysis. Isolation of monolayer biofilm, laser capture microdissection (LCM), and flow cytometry (FCM) are useful for the proteomic study of biofilm subpopulations whilst subtractive proteomics is applicable to the whole biofilm population.

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 [6] 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 [28] and metabolic activity [29], these stages are suitable for quantitative proteomic analysis. The 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 [28]. Over a 24 h-period, the monolayer biofilm is unable to progress to a multilayer biofilm in the absence of monosaccharides. The findings demonstrated by [28] have concluded that a surface is not necessary for the creation of the biofilm state. Producing the monolayer biofilm using a monosaccharide-deficient medium is feasible, however, such a 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.

Laser Capture Microdissection

The laser capture microdissection (LCM) technique is a technique for the isolation of a specific subpopulation of cells of interest from the microscopic area. Williamson et al. [28] 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, the application of LCM in the proteomic study of heterogeneous biofilm is subjected to equipment availability.

Flow Cytometry

Flow cytometry (FCM) is a technique that makes microscopic particles pass through an interrogation point, where a laser beam impacts them and the light that the particles absorb, scatter, or emit due to their intrinsic or extrinsic physical properties are measured. It is another method that can be used to fluorescently label biofilm subpopulations [32]. Once these biofilm subpopulations are separated from the total biofilm biomass and have been studied by quantitative proteomics, the functions of these subpopulations can be better understood. Other works [33, 34, 35, 36] have also highlighted the importance of selective sampling of homogeneous biofilm subpopulations for the quantitative proteomics study.

Subtractive Proteome Profiling

Proteins expressed in biofilms are typically categorised into upregulated, downregulated, and unique expressions. On assumption that the biofilm sample is homogenous, most researchers often ignore the inconsistent pattern of protein expression. The analysis of upregulated and downregulated biofilm proteins deserves 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 proteins which are uniquely expressed in one state but not the other state are possibly not influenced by the 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 nuclear membrane proteome samples and dynamic range of protein expression [37, 38]. Yahya *et al*. [26] used the subtractive proteomic approach to identify 75 unique proteins which were present only in control S. Typhimurium biofilm and not in 32% dimethyl sulfoxide (DMSO)-treated biofilm including 30S ribosomal protein, 50 ribosomal protein L1, ATP synthase subunit alpha and glyceraldehyde-3-phosphate dehydrogenase. On the other hand, Husi et al. [39] used the subtractive proteomic approach to identify diagnostic cancer biomarkers from urine samples such as phospholipase A1 member A, histone H1.4, neuron navigator 2, and protein Daple. They found that such an analytical approach was useful to reduce the complexity of the urinary proteome and remove unrelated profiling data. Collectively, it could be

inferred that the subtractive comparison of protein profile for identification of the unique proteins is a feasible, straightforward, and time and cost-effective strategy to study the heterogeneous biofilms.

There is a need to use young or homogeneous biofilm population samples in quantitative proteomic studies. This is due to the fact that the early-stage biofilm is not influenced by biochemical heterogeneities in the biofilm [28-31, 40]. The proteome expression pattern in young or homogenous biofilm is consistent and is not associated with the mixture between monolayer and multilayer biofilms, thick microbial population, and chemical gradient of nutrients [6-7, 10]. In 2019, Bisht and Wakeman [41] have suggested several strategies to perform the quantitative proteomic study of heterogeneous biofilm population namely i) a combination of LCM and standard proteomic analysis, ii) pulsed stable isotope labelling by/with amino acids in cell culture (pSILAC) and iii) bio-orthogonal non-canonical amino acid tagging (BONCAT). The pSILAC approach requires the use of a stable isotope for labelling of amino acids of the heterogeneous biofilm population prior to exposure to a stressor like antibiotics. The expression of new proteins in the biofilm cells following the antibiotic exposure can be quantified under pulse and no pulse conditions. In the BONCAT method, differential expression of specialized tRNA synthases is determined in certain biofilm subpopulations. These tRNA synthases allow the incorporation of non-canonical amino acids within the labelled biofilm subpopulation so that researchers can identify specific biofilm subpopulation proteome relative to the whole biofilm population proteome. The BONCAT technology eases the identification of differentially expressed proteins of low abundance. Overall, the strategies discussed in this review could be used to study the global proteome expression in the heterogeneous biofilm more accurately. Investigation of the proteome expression in the biofilm could complement the standard antibiofilm screening works which are crucial to discover and develop the possible therapeutic intervention against a wide range of biofilm infections [42-50].

CONCLUSION

Mass spectrometry is an important tool to understand the overall aspects of the biofilm that mainly rely on proteomic changes. Due to the fact that the biofilm is a heterogenous microbial population, preparation of homogeneous biofilm fraction needs to be considered for the quantitative proteomic experiment. This is important to ensure the consistent proteomic expression data and reliable identification of differentially expressed biofilm proteins.

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