Chapter 3
Sample Preparation Methods for the Rapid MS Analysis of Microorganisms

Shobha Devi, Anren Hu and Yen-Peng Ho

Sample preparation is the most critical steps in microbial analysis to generate accurate and informative data. Well-designed methods are required to enable unambiguous and sensitive identification of microbial cells/biomarkers from complex sample mixtures. However, there is no standard protocol for sample preparation because the microbial samples are complex. Moreover, sample preparation strategies depend on the type, source, abundance, and physical properties of samples. Traditional procedures have been used to collect, isolate, and identify pathogens from different samples. Furthermore, biochemical, serological, and molecular biology methods have been employed for the definitive identification of microbial isolates. These established methods are often time-consuming and labor-intensive. Mass spectrometry (MS) has become one of the main tools to accomplish the rapid identification and quantification of microbial cells/biomarkers. To simplify the complexity of the samples and improve the detection of low-abundance microbial cells/biomarkers, sample preparation methods that can selectively enrich target analytes and simultaneously eliminate interferences are greatly desired prior to MS analysis. In response to this challenge, numerous sample treatment techniques have been developed. In this chapter, we describe the sample preparation methods for the identification of microorganisms by MS, including techniques of enrichment, cell lysis, and separation of microbial cells/biomarkers. Two types of procedures for the microbial sample preparation can be used for the MS identification. In one sample preparation procedure, intact cells in the form of suspension are analyzed after they are enriched or cultivated. In the other procedure, extraction, enrichment, and/or separation of biomarkers from the cells are carried out prior to MS analysis. The workflow of these approaches is outlined in Fig. 3.1.

Y.-P. Ho (✉) · S. Devi
Department of Chemistry, National Dong Hwa University, 97401 Hualien, Taiwan
e-mail: ypho@mail.ndhu.edu.tw

A. Hu
Institute of Medical Biotechnology, Tsu-Chi University, Hualien, Taiwan

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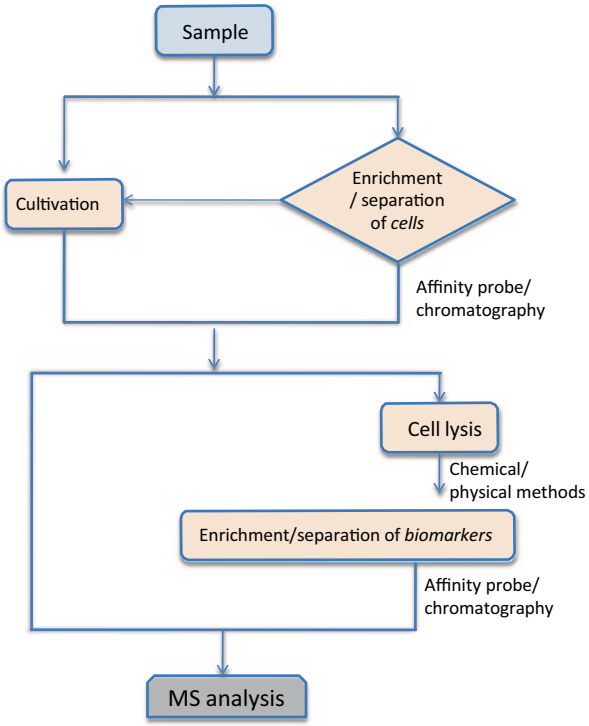

Fig. 3.1 Workflow of sample preparation approaches for microbial analysis. *MS* mass spectrometry

Enrichment Methods

Various strategies have been developed for the enrichment of microbial samples. The enrichment methods involving non-covalent, covalent interactions, and immunoassays are discussed in this section. In general, affinity enrichment steps include binding of targets, washing, and elution. A general scheme is provided in Fig. 3.2. Microbial cells/markers are isolated and concentrated after the incubation of the sample solution with affinity probes. The enriched cells are lysed or directly mixed with a matrix-assisted laser desorption/ionization (MALDI) matrix solution and subjected to mass spectrometry (MS) analysis. Moreover, biomarkers obtained from the enriched cells may be concentrated and separated prior to MS analysis

Enrichment of Microbial Cells/Biomarkers Involving Non-covalent Interactions

A simple concentration step prior to MS analysis may improve the detection and identification of microorganisms from complex biological mixtures. Affinity meth-

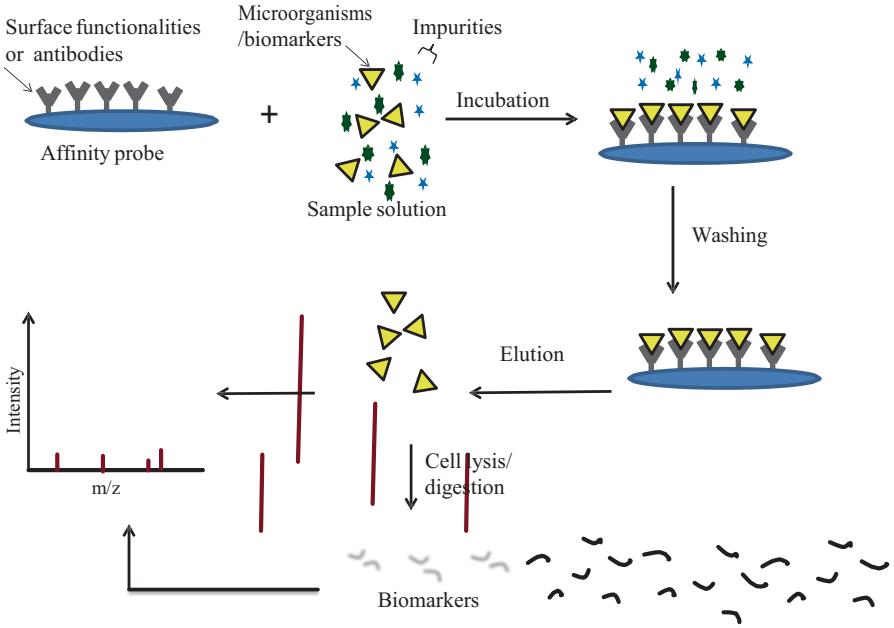

Fig. 3.2 General scheme for the enrichment of microbial cells or biomarkers with affinity probes.

ods have been developed for effective cleaning and enriching microorganisms from complex matrices.

Non-covalent binding between the cells/biomarkers and the functionalities of the affinity surface may arise from columbic (electrostatic) interactions, hydrogen bonding, or hydrophobic interactions.

Cells

Various affinity probes have been used to concentrate and purify the bacteria of interest. One such probe for the selective enrichment of bacteria using lectins has been previously reported (Bundy and Fenselau 1999). Lectins are glycoproteins that have selective affinity for carbohydrates. These molecules have the capacity to interact with bacteria through non-covalent interactions involving hydrogen bonding, hydrophobic, and van der Waals' interactions with various carbohydrates (lipopolysaccharides and peptidoglycans) located on the cell surface. The selectivity of lectins for a particular carbohydrate can be used as a probe for selective isolation of bacterial species. For example, the lectin, concanavaline A (Con A), is reactive with various Gram-negative bacteria due to its binding to lipopolysaccharides on the cell surface. Further improvement of this technique was achieved using wheat germ agglutinin (WGA)-lectin bound to an affinity membrane. WGA-lectin probes have been employed for the enrichment of *Escherichia coli* and *Salmonella typhimurium* from milk, urine, and processed chicken samples (Bundy and Fenselau 2001).

Surface-activated glass slides with immobilized lectins have been reported to selectively capture bacteria *(E. coli)* and bacterial spores *(Bacillus cereus* and *Bacillus subtilis)* (Afonso and Fenselau 2003).

Numerous magnetic nanoparticles (MNPs) of various sizes, shapes, and compositions have been used as affinity probes to selectively concentrate trace amounts of bacteria from complex biological and food samples. Nanoparticle (NP)-based magnetic separation has been reported to perform exceptionally well for pre-concentration, isolation, and enrichment of microorganisms in comparison to other common separation techniques. Microbial cells often bind with NPs through electrostatic or hydrophobic interactions. Generally, the procedure involves addition of functionalized NPs into cell suspensions and incubation of the suspensions for the efficient attachment of NPs to the microorganisms. Then the NP-microbial cell conjugates can be isolated by magnetic separation and subjected to MS analysis.

Various carbohydrates have been recognized as receptors for the attachment of pathogens (Sharon 2006). Silica-coated MNPs (Fe_3O_4@SiO_2) modified with D-mannose have been employed to concentrate *E. coli* strain ORN178, which possesses mannose-specific receptor sites (El-Boubbou et al. 2007). Pigeon ovalbumin (POA), a phosphoprotein containing high level of galactose units, also serves as an affinity probe to enrich bacterial cells through disaccharide–protein interactions. POA-immobilized Fe_3O_4@Al_2O_3 MNPs have been used for the selective enrichment of *E. coli* and *Pseudomonas aeruginosa* from urine samples at a concentration as low as 4×10^4 cells/mL, corresponding to 10^2 cells deposited on the matrix-assisted laser desorption/ionization (MALDI) plate (Liu et al. 2008, 2009).

Vancomycin-modified Fe-Pt MNPs have been reported for selective enrichment of vancomycin-resistant enterococci and other Gram-positive bacteria *(Staphylococcus aureus* and *Staphylococcus epidermidis)*. The selective interaction between MNPs and bacterial cell walls (expressing D-Ala-D-Ala as the terminal peptides) is through multiple hydrogen bonding (Gu et al. 2003).

Vancomycin-immobilized MNPs were employed for the selective enrichment of Gram-positive bacteria from urine samples. The optimal detectable concentration was 7.4×10^4 and 7.8×10^4 CFU/mL for *S. aureus* and *Staphylococcus saprophyticus*, respectively (Lin et al. 2005). Vancomycin-modified NPs have also been utilized to concentrate Gram-positive bacteria from tap water prior to MALDI-MS. The optimal detection limit was found to be 5×10^2 CFU/mL (Li et al. 2010). The architecture and orientation of vancomycin on the surface of silica-encapsulated Fe_3O_4-NPs and the overall surface coverage have been found critical in mediating fast and effective interaction between the NPs and the pathogen cell wall. Only one orientation/architecture in a series of modified NPs led to the efficient and reproducible capture of several important pathogenic bacteria (Kell et al. 2008).

Anion-exchange MNPs also served as affinity probes to separate/concentrate bacterial cells. The positively charged NPs may interact with bacteria (generally

carrying negative charges). Commercially available anion-exchange MNPs or microparticles have been used to enrich various bacterial species from tap water and reservoir water. The detection limit was 1×10^3 CFU/mL and the analytical time was around 2 h (Guo et al. 2009; Li et al. 2009). Cationic ionic liquid-modified magnetic nanoparticles (CILMS) have been used for the enrichment of pathogenic bacteria from blood samples (Bhaisare et al. 2014).

Cultivation of captured bacteria may further improve the detection limit. Pathogens in milk/pudding/coffee have been captured with magnetized zirconium hydroxide, directly cultured without colony isolation, and then analyzed using MALDI and LC-MS/MS. The limit of detection for *Enterococcus faecalis* spiked into milk was down to a level as low as 32 CFU/mL (Chen et al. 2012). The synthesis of both positively and negatively charged NPs with various functionalities including Fe_3O_4, SiO_2, TiO_2, ZrO_2, polyethyleneimine (PEI), and polyacrylic acid (PAA) to concentrate target Gram-negative and Gram-positive bacteria has been reported. The capture efficiency of all MNPs was examined. The affinity of Fe_3O_4@PEI for each of the bacterial species was attributed to its polymer structure having more positive charges on the surface. The capture mechanism of selective binding of negatively charged TiO_2 NPs with bacteria (also negatively charged) may include not only electrostatic attraction but also covalent and hydrogen bonding (Reddy et al. 2014).

The size effect of magnetic particles (nano- vs. micro-sized particles) on the capture efficiency of microorganisms may require further studies. Nano-sized particles have greater surface area to volume ratio and better dispersion properties. Micro-sized particles have higher surface coverage of functionalities. Both types of particles have been successfully applied to cell enrichment.

Biomarkers

Affinity chromatography is a powerful tool for the concentration of microbial proteins and has potential applications for diagnosis and even for therapy. Lectin affinity chromatography was used for capturing glycosylated proteins, which are potential virulence factors, from many parasites including apicomplexan (Fauquenoy et al. 2008). Surface proteins related to pathogenesis of *Mycoplasma hyopneumoniae* have been labeled with biotin and affinity captured for MS characterization (Reolon et al. 2014). Isotope-coded glycosylation-site-specific tagging has been employed to enrich low abundant *N*-glycosylated proteins from a complex bacterial extract (Kaji et al. 2003).

Immobilized MNPs can also be used for the selective enrichment of biomarkers. 12-Hydroxy octadecanoic acid-modified $BaTiO_3$ NPs have been used to extract hydrophobic compounds, including phospholipids and membrane proteins from *E. coli* (Kailasa and Wu 2013). Oleic acid capped $Mg(OH)_2$ NPs have been used as extracting and concentrating probes for hydrophobic proteins from *E. coli* and *B. subtilis* prior to MALDI-MS analysis (Kailasa and Wu 2012).

Surface-enhanced laser desorption/ionization (SELDI-MS) technology plays an important role in rapid identification of biomarkers from various microorganisms. SELDI is an affinity-based method in which the protein mixture is adsorbed onto various chemically or biochemically modified surfaces such as anion exchange (AX), cation exchange (CX), and hydrophobic or immobilized metal ion affinity chromatography (IMAC) surfaces. Some proteins in the sample bind to the surface while others are removed by washing. After washing, the adsorbed proteins are mixed with matrix and allowed to crystallize. Binding of the proteins to the surface itself acts as a separation step and the proteins can be easily analyzed. Pathogenic Gram-negative bacteria *Francisella tularensis* has been analyzed employing AX, CX, and IMAC (loaded with copper ions) chip surfaces. These approaches enable the discrimination among the different species and the subspecies of Francisella (Lundquist et al. 2005; Seibold et al. 2007). The *Campylobacter* species have been analyzed using SELDI-MS with hydrophobic and IMAC protein-chip array surfaces (Kiehntopf et al. 2011).

SELDI-MS based on protein-chip arrays have been utilized for the selective and rapid identification of *S. aureus* from other non *S. aureus* species (Yang et al. 2009). Furthermore, the protein chips have also been employed for the rapid identification of antibiotic resistance of *E. coli* (Dubska et al. 2011).

Enrichment of Cells/Biomarkers Involving Covalent Interactions

Cells

Titania-coated magnetic iron oxide (Fe_3O_4@TiO_2) NPs have been employed to enrich five Gram-negative bacteria including *E. coli* O157:H7, *uropathogenic E. coli, Shigella sonnei, P. aeruginosa*, and *Klebsiella pneumonia* and three Gram-positive bacteria *(Listeria monocytogenes, S. saprophyticus*, and *S. aureus)* (Chen et al. 2008). The Fe_3O_4@TiO_2 NPs exhibited much higher capture capacities towards Gram-negative bacteria. The outer membrane of Gram-negative bacteria contains lipopolysaccharide, as a major component, whereas most Gram-positive bacteria lack lipopolysaccharides. Therefore, the metal oxide-coated MNPs interacted weakly with the Gram-positive bacteria but strongly with the Gram-negative bacteria through metal coordination bonding with the phosphate groups on the lipopolysaccharides. A titanium-based bacterial chip has been utilized to capture pathogenic bacteria. This reusable bacterial chip can be directly used as a MALDI target plate for the rapid and sensitive bacterial analysis in MALDI-MS (Gopal et al. 2013).

Biotinylation of cell surface proteins can be used for cell enrichment. This approach has been employed by covalent modification of the membrane proteins with a cleavable reactive ester derivative of biotin (sulfo-*N*-hydroxysuccinimide-SS-biotin). Then, the biotinylated cells were enriched with streptavidin-coated resin (Scheurer et al. 2005).

Biomarkers

Enrichment of biomarkers through covalent interactions may benefit from the stability of the bonding at different salt concentrations, pH values, and temperatures. IMAC can be used to concentrate biomolecules based on the metal ion chelation with the biomolecules. IMAC is highly useful for the enrichment of phosphopeptides from complex mixtures using various metal ions such as Ga(III) and Fe(III). For instance, this method has been employed to enrich phosphopeptides from *Saccharomyces cerevisiae* (Gruhler et al. 2005).

Covalent chromatography has been applied to the enrichment of thiol-containing proteins from *E. coli* lysate. Initially, the free thiol groups were derivatized with the 2,2-dipyridyl disulfide reagent. Then, the peptides were passed through a thiopropyl sepharose resin column, which facilitated the exchange of the disulfide-modified peptides with the thiol groups of the column (Wang and Regnier 2001). Covalent chromatography and IMAC (loaded with copper) have been used to selectively capture cysteine and histidine containing peptides respectively from *E. coli* cell lysate (Wang et al. 2002). Disulfide containing membrane proteins have been enriched from *Pseudomonas putida* using a similar approach (An et al. 2011).

The magnetic microspheres have been employed for specific enrichment of *F. tularensis* glycoproteins. The capture was based on the irreversible reaction of the hydrazide groups of microspheres with the aldehydes generated by the oxidation of the cis-diol groups of glycoproteins. Biomarkers containing various functional groups (carboxyl, amino, sulfhydryl, etc.) can also be conjugated with these microspheres through covalent bonding (Horak et al. 2012).

Organomercurial agarose beads have been developed to enrich cysteine containing peptides from yeast cell lysates. A significant increase of proteins that were suitable for identifying yeast proteins has been observed in MS analysis (Raftery 2008).

Biomarkers may be easily tagged through the modification of the reactive functional groups such as amino, carboxylic, hydroxyl and thiol groups. The most popularly used chemical affinity tag for the enrichment of biomarkers is the biotin tag, which can be attached to various biomarkers through covalent bonding. Enrichment of biotinylated proteins can be done by both avidin and streptavidin immobilized affinity chromatography. Various research groups have employed biotinylation to enrich different target proteins. Biotinylation has been employed to enrich and detect membrane proteins from *Deinococcus radiodurans* (Goshe et al. 2003) and *P. aeruginosa* (Blonder et al. 2004).

Immuno-Affinity Enrichment of Microbial Cells/Biomarkers

Although the interaction involved in immuno-affinity methods is basically non-covalent in nature, it appears to be unique enough to warrant separate discussion. Immunological methods have been extensively used for the studies of pathogenic microorganisms since the late 1950s. Traditional immunoassays are time-consuming

and in some cases the antibody cross-reactivity with food and biological matrices may lead to false-positive results and/or a worse detection limit. Immuno-affinity MS is a more specific affinity approach capable of selectively targeting and characterizing protein biomarkers. In MS-based immunoassays, proteins are affinity retrieved from biological samples via surface-immobilized antibodies, and are then detected via MS analysis.

Cells

Micro-sized magnetic beads coated with antibodies (against a chosen microbial species) can bind to target microbial cells in complex biological samples. The magnetized beads are easily dispersed in solution because of their small sizes (2–5 μm in diameter) and can be retrieved using a magnetic field. Sample debris and nontarget organisms and molecules are removed by washing.

This method enriches specific microbial species, while the magnetic separation involving non-covalent interaction described in Section 1.1 is mostly nonspecific. Magnetic beads coated with antibodies that were specific to the antigen of *Salmonella choleraesuis* have been applied for the isolation/concentration of *S. choleraesuis* from river water, human urine, and chicken blood (Madonna et al. 2001).

Immunoglobulin (IgG) functionalized magnetic Au-NPs and Pt-NPs have been utilized to selectively enrich targeted bacteria from sample solutions (Ho et al. 2004; Ahmad and Wu 2013).The captured bacteria with the beads or particles were directly applied onto the MALDI target plates prior to MS analysis.

Captured bacteria can be lysed after the magnetic separation. The released proteins are digested and the peptides are analyzed using LC-MS. An antibody against *Bacillus anthracis* spores were immobilized on IgG magnetic beads for the immunocapture of intact spores prior to their detection using multiple reaction monitoring (MRM)-MS (Chenau et al. 2011). Immuno-LC-MS/MS has also been used for the selective isolation and detection of *Yersinia pestis*. Optimizing the immunocapture conditions for the enrichment of intact bacterial cells from complex matrices improved the detection limit to 2×10^4 CFU/mL in milk or tap water and in soil (Chenau et al. 2014).

Biomarkers

Staphylococcal enterotoxin B (SEB) is a potent bacterial protein toxin responsible for food poisoning, as well as a potential biological warfare agent. An affinity probe has been prepared by immobilizing anti-SEB antibody on the surface of paratoluene-sulfonyl-functionalized magnetic beads. Immobilization and affinity capture procedures were optimized to maximize the density of anti-SEB IgG on the surface of magnetic beads and the amount of captured SEB. MALDI-MS detection of the enriched SEB from different matrices, such as cultivation media of *S. aureus* strains and raw milk samples (Schlosser et al. 2007).

Enrichment of microbial toxins including ricin, SEB, and botulinum neurotoxins (BoNT) has been performed using multiplex-immuno-affinity purification. Specific monoclonal antibodies for each of the four toxins were selected from a pool of antibodies, the selected antibodies allowed for the specific and simultaneous capture of toxins. This assay enabled unambiguous identification of toxins in complex food matrices with a detection limit of 500 fmol. Additionally, it allowed for the rapid differentiation of closely related BoNT sero- and subtypes ((Kull et al. 2010).

Cell Lysis

Microbial cells need to be lysed to release intracellular components prior to MS analysis. Various cell lysis strategies are available depending on the sample types and target biomarkers.

Gram-negative and Gram-positive bacteria differ from each other due to the difference in composition of the cell walls. The complexity of the microbial sample, limited availability of the sample, and need for rapid identification have prompted the development of cell lysis methodologies which can be coupled to MS techniques. Various cell lysis techniques are available for the release of intracellular constituents, including chemical (acids, detergents), enzymatic (lysozyme), physical (bead milling, ultra-sonication, French press, freeze-thawing, osmotic shock, corona plasma discharge), and the combined methods.

Chemical Methods

Strong organic acids, bases, alcohols, and detergents/surfactants have been employed in microbial sample preparations, due to their high efficiency in disrupting membranes and solubilizing proteins. Different cell types require different buffer formulations. Conditions such as pH, salt concentration, and temperature are considered to be important parameters in the sample preparation protocols.

Trifluoroacetic acid (0.1% TFA) can effectively extract proteins from both Gram-positive/negative bacteria (Nilsson 1999). Comparison of various solvents involving water, 40% ethanol, 0.1% TFA, and various solvent mixtures indicated that 0.1% TFA could yield the most MALDI-MS peaks (Ruelle et al. 2004a; Liu et al. 2007). Extraction of proteins from *E. coli* has been achieved using a range of solvents with varying polarity. The different combinations of extraction solvents, involving 10:45:45 formic acid (FA)/methanol/water, 17:33:50 FA/isopropylalcohol/water, 17:33:50 FA/methanol/water, 33:67 acetonitrile (ACN)/water, and 0.1:99.9 TFA/water, have also been examined. Among these, the second and the third solvent combinations provided the highest number of signals from *E. coli* extract (Domin et al. 1999).

Bacillus anthracis is the etiological agent of anthrax in humans/animals (Demirev and Fenselau 2008; Lasch et al. 2009). Numerous low molecular-weight proteins can be readily extracted from the spores of *B. anthracis* and related species. Many of these proteins have been identified as small acid-soluble spore proteins (SASPs), due to their basic nature. They can be selectively solubilized in acids and easily protonated to provide strong signals when ionized by MALDI or electrospray ionization (ESI). SASPs have been proposed as candidate biomarkers capable of discriminating between various Bacillus spores. Solvents that have been reported to extract SASPs include 10 % TFA (Castanha et al. 2007), 50 % acetic acid (Fenselau et al. 2007), 30 % ACN/40 % FA (Dickinson et al. 2004), 5 %ACN/TFA (70:30, v/v) (Dybwad et al. 2013), and 1N HCl (Hathout et al. 2003).

Various types of detergents/surfactants have been reported for microbial cell lysis to extract proteins. Detergents break the structure of cell membranes by disrupting the lipid–lipid, protein–lipid, and protein–protein interactions. Detection of proteins with high molecular weight up to 140 kDa has been achieved by mixing nonionic surfactant (1.0 mM *N*-octyl-B-D-galactopyranoside) with bacterial cells on the MALDI target (Meetani and Voorhees 2005). The release of proteins from *E. coli* has been achieved by a lysis buffer containing 2 % Triton, which facilitated the identification of many proteins (Chong et al. 1997). The most commonly used detergent is sodium dodecyl sulfate (SDS), which is very efficient in cellular lysis and protein solubilization. However, it poses a major impediment for LC-MS/MS experiments. In particular, the presence of SDS hinders the resolving power of reverse phase LC. The introduction of SDS into a mass spectrometer during electrospray may lead to ion suppression or contamination inside the ion source. Therefore, the detergents used for lysis must be removed using suitable cleanup methods. Detergent cleanup methods including trichloroacetic acid precipitation, chloroform/methanol/water extraction, a commercial detergent removal spin column method, and filter-aided sample preparation (FASP) have been investigated. These methods have been compared by lysing the bacterial samples in a sample buffer containing 2 % SDS. The FASP method outperformed the other three SDS cleanup methods. An additional fractionation step enhanced the number of proteins identified from bacterial cell lysates by 8–25 % (Sharma et al. 2012). Melittin, a lytic peptide, shows lytic activities against bacteria and mammalian cells. The leucine-substituted analogs of melittin exhibited selective lysis of *E. coli* and *Bacillus megaterium* (Pandey et al. 2010).

Guanidine HCl has been reported to be very efficient in cell lysis with minimal amount of bacterial sample. Comparative study on effective small-scale cell lysis using guanidine HCl and trifluoroethanol (TFE) indicated that guanidine HCl gave better cell lysis results for complex microbial samples. Furthermore, comparison of lysis techniques including sonication and modified guanidine lysis showed that a higher number of proteins were identified using guanidine HCl (Thompson et al. 2008).

Lysozyme is commonly used for cell lysis to enhance the release of proteins from microbial cells. The results of the lysozyme treatment on the *Enterococcus* species indicated that the most reproducible MALDI-MS profile was achieved by treating

the bacteria with lysozyme for 30 min. It was noted that the isolates exposed to lysozyme have more intense peaks when compared to the untreated isolates. The lysozyme-treated cells yielded fewer peaks in the low-mass range (900–1400 Da), but the whole-mass range was increased up to 10 kDa (Giebel et al. 2008). An increase in the number of peaks for *S. aureus, Streptococcus haemolyticus*, and *Streptococcus pyogenes* (Gram-positive bacteria) has been achieved by the treatment of lysozyme combined with sonication.

Gram-positive bacteria contain a thick peptidoglycan layer in the cell wall which may impede the release of analyte molecules from the intact cells during MALDI analysis. When these Gram-positive bacteria were exposed to lysozyme, digestion of peptidoglycan might occur and yield the additional higher-mass signals in the spectra (Smole et al. 2002). In contrast, a decrease in the MALDI-MS quality after the lysozyme treatment has also been observed for both Gram-positive (Vargha et al. 2006) and Gram-negative bacteria (Williams et al. 2003). Decrease in signal quality might be attributed to the introduction of additional components to the analyzed mixture.

Physical Methods

Physical methods are also shown to be efficient in disrupting microbial cells. Various physical methods such as micro-beads disruption, ultra-sonication, freeze/thaw cycle, thermolysis, and corona plasma discharge have been employed for efficient cell lysis. A simple and inexpensive method for the disruption of small volumes of bacteria and yeast is by suspending glass beads within the sample and vortexing the sample repeatedly. The beads disrupt cell membranes through shear forces, grinding between the beads, and collision with the beads. *Campylobacter* or *Streptococcus* cells have been successfully disrupted with zirconia/silica beads (Fagerquist et al. 2005; Teramoto et al. 2007).

Thermolysis also induces cell lysis. For example, *F. tularensis* cell lysis has been achieved by heating the sample at 65 °C for 2 h (Lundquist et al. 2005) prior to SELDI-MS analysis. Effective release of high-mass biomarkers from *Bacillus* spores has been achieved using wet heat treatment.

Wet heat treatment has been performed for 3–30 min by two techniques using either a screw-cap tube submerged in a glycerol bath at 120 °C or an eppendorf tube submerged in a water bath at 100 °C. Both techniques were successful in releasing high-mass biomarkers (Horneffer et al. 2004). Osmotic lysis and French press have been utilized for *Shewanella oneidensis* cell lysis (Brown et al. 2010). Reproducible patterns of spectral markers and increased sensitivity have been achieved for *Cryptosporidium parvum* employing freeze-thaw cell lysis procedure (Magnuson et al. 2000).

Ultrasonic vibrations at a frequency of 25 kHz is commonly used for cell disruption. The duration of ultrasound needed depends on the cell type, sample size, and cell concentration.

Ultra-sonication has been successfully applied for the lysis of *E. coli* to detect high-mass proteins with higher signal-to-noise ratio (Easterling et al. 1998).

Nonthermal plasma or corona treatment of microorganisms at room temperature and ambient pressure produced unique biomarkers when analyzed by mass spectrometers (Birmingham et al. 1999). Atmospheric pressure nonthermal plasmas rapidly lysed bacterial spores *(B. subtilis)* after exposing the spores to ionized gas for a few minutes (Birmingham 2006). Barrier discharges have also been utilized as cell lysis devices (Pineda et al. 2000). Corona plasma discharge showed potential to benefit the MALDI-MS profiling performance with rapid sample treatment. For example, detection of signals of *B. cereus* spores required only 3 s of treatment with corona plasma discharge (Victor Ryzhov and Catherine 2000). The treatment of intact cells deposited on the MALDI target with corona plasma discharge of 15 s was found to be useful for the signal enhancement. This effect was more notable in the analysis of Gram-positive bacteria because of their higher cell wall stability. MS analysis of plasma-treated bacterial spores revealed new biomarkers in the mass spectra, which were undetectable in the spectra of unprocessed samples (Birmingham et al. 1999).

Separation of Cells and Biomarkers

Intact Cells

Capillary electrophoresis (CE) is a separation technique based on the differential migration rates of charged species in an applied electric field. Microorganisms carry charged or chargeable groups (amino acids and carbohydrates) on their outer surface; therefore, CE can be used to separate intact bacterial, viral, and fungal cells. CE allows for the rapid and efficient separation of microorganisms with least consumption of sample and reagents. CE analysis of *Tobacco mosaic* virus and *Lactobacillus casei* using a fused-silica capillary coated with methyl cellulose has been carried out (Hjerten et al. 1987). Off-line coupling of CE to MALDI-MS may be used for microbial identification. Separation and designation of two distinct subcomponents of cow pea mosaic virus were done by capillary zone electrophoresis and MALDI-MS analysis of the capsid proteins (Liang and Schneider 2009). CE coupled off-line with desorption electrospray ionization (DESI) MS has been employed for the separation/identification of *E. coli* and *S. cerevisiae* (Petr et al. 2009).

Field flow fractionation (FFF) can also be used for microbial cell separation. In the FFF technique, a field (may be gravitational, centrifugal, thermal-gradient, electrical, magnetic, etc.) is applied perpendicular to the fluid flow, causing particles to migrate with different velocities. Fields of sedimentation, diffusion, and electrical diffusion are manipulated to optimize the separations of microbes. Separation of *Pseudomonas putida* and *E. coli* has been achieved by hyperlayer FFF. Fractions of the whole cells were collected after the separation at different time intervals, dif-

ferent sizes, and possibly different growth stages of bacteria. The bacterial analysis by FFF/MALDI-MS has been completed in 1 h (Lee et al. 2003). Hyperlayer flow FFF coupled online with inductively coupled plasma MS has been employed for the separation/analysis of *S. oneidensis* (Jackson et al. 2005).

Biomarkers

CE is also useful for the separation of biomolecules. The selective proteotypic-peptide analyses of protein digests obtained from various bacterial cell (Gram positive/Gram negative) extracts were successfully performed using CE-MS. Minor bacterial species present in the complex mixture at even 1% relative abundance could be identified with high confidence (Hu et al. 2007). CE time of flight (TOF)-MS has also been found to be applicable in separation and identification of metabolites including amino acids, amine, nucleotides, sugars, lipids, and other substances from various microbial cells (Garcia et al. 2008). Tryptic peptides from whole cell lysates have been separated and analyzed by CE-ESI-MS/MS for the sensitive and specific identification of β-lactamases in multidrug-resistant Gram-negative bacterial species *(K. pneumoniae, E. coli*, and *Enterococcus cloacae)*. Analysis of clinical isolates identified the presence of β-lactamase peptides (OXA-48 and KPC) in all of the carbapenemase positive samples (Fleurbaaij et al. 2014).

LC is the most widely used method for the separation of large variety of microbial biomarkers. LC-MS has been employed for the separation and detection of protein biomarkers from *E. coli* (Ho and Hsu 2002). A multidimensional LC-MS method has been used for the rapid determination of bacterial proteins to identify *B. anthracis* strains (Krishnamurthy et al. 2007). Reproducible intact protein markers identified using the LC-MS approach were used to correctly identify unknown pathogens at the species (Everley et al. 2008) and strain level (Everley et al. 2009). Multidimensional LC separation coupled with MS/MS has been employed to obtain the whole-cell proteome, providing insight into the pathogenesis of *Streptococcus pneumonia* (Sun et al. 2011). In order to identify biomarkers for *Ruegeria lacuscaerulensis*, tryptic digests of low molecular weight proteins have been separated and identified by the shotgun nano-LC-MS/MS approach (Christie-Oleza et al. 2013). *Acinetobacter baumannii* DU202, a clinical isolate, exhibits resistance to many antibiotics, including imipenem, tetracycline, ampicillin, and chloramphenicol. The proteomes of the cytoplasm, cell wall, and membrane of *A. baumannii* DU202 have been analyzed by LC-MS/MS. Combining the proteomic analysis with genome sequence data provided the comprehensive picture of antibiotic resistance in this strain (Lee et al. 2014). Identification of β-lactam resistance in *A. baumannii* has been achieved by shotgun proteomics and nano-LC-MS. Various antibiotic-resistant proteins, including AmpC, β-lactamase, and carO, have been successfully identified in clinical-resistant strains of *A. baumannii* (Chang et al. 2013).

Gas chromatography-based separation methods for the identification of bacterial metabolites are well established. The maximum molecular weight of compounds

that can be analyzed when coupled to MS is < 1000 Da. GC-MS and direct-infusion MS methods have been employed to produce specific and discriminant metabolite profiles from different yeast mutants (*S. cerevisiae* strains) (Mas et al. 2007). The analysis of volatile compounds generated by bacteria may be a possible alternative method for the identification of pathogenic bacteria. The rapid detection of *L. monocytogenes* from milk has been achieved by extracting, separating, and detecting the volatile organic compounds (VOCs) by headspace-solid phase micro-extraction coupled to GC-MS. The limit of detection was found to be $1–1.5 \times 10^2$ CFU/mL of cells in milk (Tait et al. 2014). High-resolution pyrolysis gas chromatography/MS selected ion monitoring technology has also been employed to detect *L. monocytogenes* from food products (beef and milk) (Li et al. 2014). The identification of VOCs produced by microorganisms also assisted in determining the bacterial infections. Differentiation between a methicillin-resistant and a methicillin-sensitive isolates of *S. aureus* was possible due to the significant differences between the produced VOCs compounds including 1,1,2,2-tetrachloroethane, 2-heptanone, and 1,4-dichlorobenzene (Boots et al. 2014). Needle trap GC-MS has been employed for the enrichment and analysis of VOCs in *E. coli* and *P. aeruginosa* (Zscheppank et al. 2014).

Sample Preparation for MALDI-MS

Currently, most of the rapid microbial analyses are based on MALDI techniques. Selecting an optimal matrix is a crucial step in developing all of the sample preparation protocols for the MALDI-MS. The MALDI matrices that are frequently used for microbial analysis are α-cyano-4-hydroxycinnamic acid (CHCA), ferulic acid (FA), and sinapinic acid (SA). It has been demonstrated that the use of a different matrix for the same kind of sample led to a significant change in the MALDI mass spectrum. For example, the MALDI mass spectra of *E. coli* obtained with CHCA and a mixture of SA/4-methoxycinnamic acid showed significantly different signals (Demirev et al. 1999). CHCA is one of the most common matrix for bacterial identification.

Analysis of bacterial cells using CHCA yielded better signal-to-noise ratio and sensitivity and more number of intense signals in the lower mass range (Ryzhov and Fenselau 2001; Shaw et al. 2004; Ruelle et al. 2004b). Presence of more signals in the lower mass range may be due to the formation of doubly protonated ions (Pineda et al. 2003). SA is a matrix of choice for the analysis of high-mass proteins (Ryzhov and Fenselau 2001; Moura et al. 2008). Increased signal-to-noise ratio and intensity of signals and improved spectral quality due to the decrease in peak broadening were also found with SA (Ochoa and Harrington 2005). In comparison to SA and CHCA matrices, FA has been reported to be better for the detection of high-mass biomarkers from Gram-positive/Gram-negative bacteria (Madonna et al. 2000). This phenomenon may be attributed to the higher tolerance to salts and contaminants with FA. 2,5-Dihydroxybenzoic acid (DHB) is a matrix suitable

for the analysis of carbohydrates and small peptides. MALDI profiling with this matrix produced a lower degree of peak broadening (Ryzhov and Fenselau 2001), increased reproducibility (Jones et al. 2003), and higher MALDI-MS/MS fragment ions (Demirev et al. 2005).

2-(4-Hydroxyphenylazo)-benzoic acid was employed as a matrix for MALDI-MS analysis of *Rhizobium* cells, which yielded a maximum number of signals in the 1–10 kDa range (Mandal et al. 2007). Ionic liquid matrices (ILMs) based on SA and DHB in conjugation with various bases including aniline, dimethyl aniline, diethylaniline, dicyclohexylamine, and pyridine have been reported for the analysis of intact bacteria in MALDI-MS (Abdelhamid et al. 2013).

The choice of matrix solvents in sample preparation protocols for the MALDI-MS is also an essential step. Solvent volatility was frequently modified in order to achieve fast or slow crystallization. Large biomolecules require slow crystallization to have more time to incorporate into the matrix crystal. The homogeneity of the matrix layer containing small crystals can be achieved by fast solvent evaporation. Bacterial cell lysate or extract was often mixed with the suitable composition of matrix solvent to enhance the MS mass range and signal intensities. For example, an *E. coli* suspension mixed with the CHCA matrix in 0.1% TFA/ACN (60:40) generated peaks only in the low-mass region, whereas more peaks in the high-mass range (over 10 kDa) were observed when the matrix was dissolved in ACN/isopropanol/0.1% TFA (49:49:2) (Ruelle et al. 2004b). Acetonitrile was found in almost all the matrix solvent compositions because of its excellent solubilization capability both for matrices and peptides/proteins. In addition, several co-matrices have been employed in sample preparation protocols to modulate the sample crystallization properties or to suppress the ionization of interferences. For example, addition of crown ether (at 0.01 M concentration) to the 5-chloro-2-mercaptobenzothiazole matrix has been reported to produce higher sensitivity and better spectral reproducibility in the analysis of Gram-positive bacteria (Evason et al. 2000).

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