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High-Throughput Screening Technique for Microbiome using MALDI-TOF Mass Spectrometry: A Review

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Abstract : A rapid and reliable approach to the identification of microorganisms is a critical requirement for large-scale culturomics analysis. MALDI-TOF MS is a suitable technique that can be a better alternative to conventional biochemical and gene sequencing methods as it is economical both in terms of cost and labor. In this review, the applications of MALDI-TOF MS for the comprehensive identification of microorganisms and bacterial strain typing for culturomics-based approaches for various environmental studies including bioremediation, plant sciences, agriculture and food microbiology have been widely explored. However, the restriction of this technique is attributed to insufficient coverage of the mass spectral database. To improve the applications of this technique for the identification of novel isolates, the spectral database should be updated with the peptide mass fingerprint (PMF) of type strains with not only microbes with clinical relevance but also from various environmental sources. Further, the development of enhanced sample processing methods and new algorithms for automation and de-replication of isolates will increase its application in microbial ecology studies.

Keywords : MALDI-TOF, mass spectrometry, microbiome, environment

Introduction

Microorganisms are present throughout the environment and are crucial to a wide range of natural processes. They play a crucial role in the biodegradation of organic matter, detoxification of various pollutants and hence recycling of nutrients in the ecosystem through the biogeochemical cycles which are fundamental to the environment and for the survival of animals, plants and human life. Pathogenic microbes cause diseases in plants and animals, however, there are other groups of beneficial microbes that can prevent the growth of various harmful microbes. Certain microbes participate in the fermentation and production of various food products whereas the presence of certain other microbes in food causes spoilage. Moreover, the human body is home to various complex communities of bacteria that plays a significant role in various physical and biochemical activities. In conclusion, microorganisms are present throughout the environment and are significant to a wide range of natural processes. Thus, the identification and classification of microorganisms are crucial and cannot be ignored.

Conventional techniques for bacterial identification are culture-dependent morphological, physiological and biochemical assays which are labor-intensive and time-consuming.[1] Although molecular techniques such as 16S rRNA, DNA-DNA hybridization, polymerase chain reaction (PCR)-based methods and fluorescent in-situ hybridization (FISH) are popular, again, they require high expertise and are expensive and time-consuming.[2] Thus, rapid and unequivocal identification of microbes in real samples is a current important area of focus.

Mass spectrometry is an analytical technique in which compounds are ionized into charged molecules and the mass-to-charge (m/z) ratio is determined. With the development of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), the scope for the analysis of large biomolecules such as proteins has expanded. When compared to ESI-MS, MALDI-TOF-MS has a few

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advantages such as it creates singly charged ions, making data interpretation simpler and it does not require prior chromatographic separation.[3] Consequently, MALDI-TOF MS has emerged as a clear choice for large-scale proteomics work due to the high throughput and speed associated with complete automation.[4]

For the first time in 1996, the spectral fingerprints from whole bacteria were obtained by using three strains of

Table 1. Microbial detection techniques. Adapted and modified from Singhal et al. 2015.[10]

Detection method	Advantages	Disadvantages
Conventional; culture dependent identification by biochemical tests	· Sensitive · Inexpensive	· Lengthy and time-consuming process · Might require 24–48h
Immunological-based methods	· Faster than conventional methods · Can detect both contaminating organisms and their toxins	· Not as specific, sensitive and rapid as nucleic acid-based detection methods · Require large amounts of antigen · Developed for only a small number of microorganisms
Florescent in situ hybridization (FISH)	· Rapid detection and identification directly from slide smears · Fast and ease-of-use of conventional staining methods combined with the specificity of molecular methods	· Test limited by the availability of specific antigens for detection
Molecular based methods (i) Real-time PCR (ii) Multiplex-PCR (iii) Repetitive extragenic palindromic PCR (rep-PCR) (iv) Random amplification of polymorphic DNA (RAPD) (v) Amplified fragment length polymorphism (AFLP) (vi) Variable-number tandem repeat (VNTR) (vii) Multilocus sequence analysis and multilocus sequence typing (MLST) (viii) Pulsed-field gel electrophoresis (PFGE)	· Culturing of the sample is not required · Specific, sensitive, rapid and accurate · Closed-tube system reduces the risk of contamination · Can detect many pathogens simultaneously · Easy to perform · Cheap, rapid, and easy to perform · High discriminatory ability and reproducibility	· A highly precise thermal cycler is needed · Trained laboratory personnel required for performing the test · Discriminatory power and reproducibility are lower compared to PFGE and MLST · Labor-intensive and costly · Lack of reproducibility · Time-consuming and costly · Relatively costly and time-consuming
DNA sequencing	· 16S rDNA and 18S rDNA sequencing is the gold standards · Can identify fastidious and uncultivable microorganisms	· Trained laboratory personnel and powerful interpretation software are required · Expensive · Not suitable for routine clinical use
Microarrays	· Large-scale screening system for simultaneous diagnosis and detection of many pathogens	· Expensive · Trained laboratory personnel required
Loop-mediated isothermal amplification (LAMP) assay	· Can generate large copies of DNA in less than an hour · Easy to use · No sophisticated equipment is required	· Developed for only a small number of microorganisms yet
Metagenomic assay	· Useful for random detection of pathogens	· Data acquisition and data analysis are time-consuming · Trained laboratory personnel required
MALDI-TOF MS	· Fast · Accurate · Less expensive than molecular and immunological-based detection methods · Trained laboratory personnel are not required	· The high initial cost of the MALDI-TOF equipment · The limited resolution, and database discordances

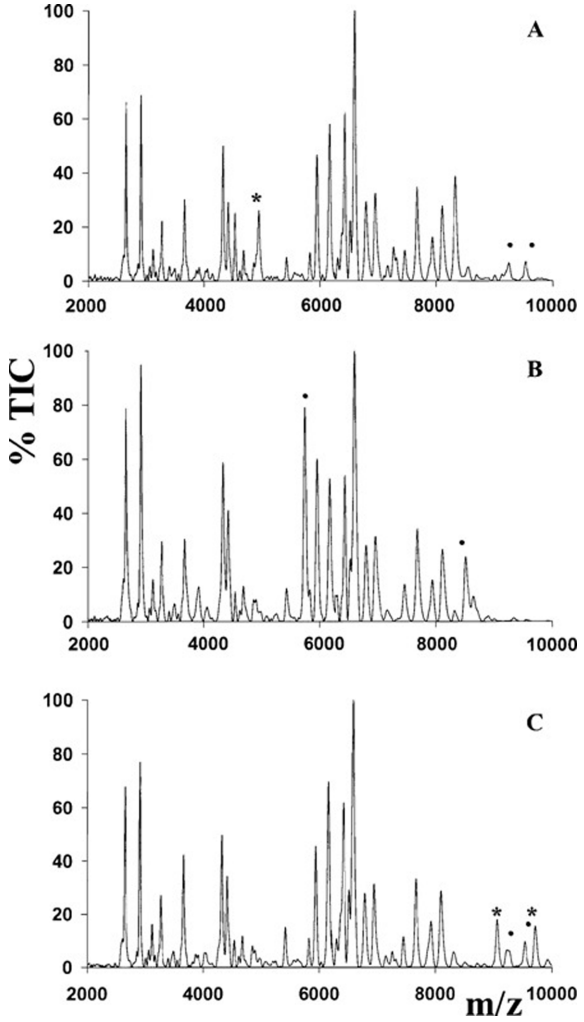

Figure 1. MALDI spectra of (A) Salmonella 2B5, (B) Acinetobacter 14B5 and (C) Escherichia coli 1B1 showing the potential genus- (•) and strain- (*) specific biomarkers. Reprinted with permission from Ruelle et al.[12] (2004). Copyright 2004 John Wiley & Sons.

Pseudomonas[5] and in the same year, spectral fingerprints of various *Bacillus* species were also acquired by using MALDI-TOF-MS.[6] Since then, a lot of focus has been placed on using MALDI-TOF to identify not only bacteria but also yeast and mold.[7] However, since the last decade, the technology has been utilized for routine analysis of microbes as it is rapid, reliable, cost-effective and has various other advantages over conventional techniques.[8] Some of the advantages and disadvantages of various microbial detection techniques have been shown in Table 1. Further, the technology has the potential to replace current identification methods in microbiology labs because it can identify a wide variety of microorganisms.[9] Thus, this review focuses on the comprehensive illustration of the use of MALDI-TOF-MS for microbial identification in various environmental fields.

To comprehend the microbial population, monitor the environment, and find potential pathogenic bacteria, it is essential to identify microorganisms from environmental sources. The ability to identify each microbe by its unique protein fingerprint has made MALDI-TOF-MS a significant advancement in the field of environmental proteomics. The identification of unknown microbe is carried out either by matching the abundance of the biomarkers with the proteome database or by comparing the PMF with the PMFs present in the database. Several conserved signals under various experimental conditions that can be used as potential biomarkers were observed by Wang et al.[11] Several other studies also reveal the specific conserved biomarkers which are unaffected by environmental factors can be used for bacterial identification as shown in the work by Ruelle et al.[12] (Figure 1). Although several in-house databases have been created,[13] however, the currently the two major databases are the MALDI BioTyper library by Bruker Daltonics, Inc. which uses Bruker Main Spectrum analysis (MSP) and the SARAMIS by bioMérieux which uses the bioMérieux SuperSpectrum approaches.[14, 15] The accuracy of identification of unknown microbes using MALDI-TOF MS depends on the database. Generally, the identification accuracy up to the species level is 90%,[16] however, the database should be regularly updated in order to enhance the identification of novel microbes.[17] Additionally, the sample culture, preparation, storage, and the type of matrix used affect the reproducibility of MS spectra obtained with MALDI.[8]

MALDI-TOF MS in microbial identification

Ecological and environmental studies

Commonly used methods for identification and classification involve 16S rRNA sequencing, gel electrophoresis techniques, rep-PCR and multilocus sequence typing (MLST).[18] However, such techniques are expensive, time taking and laborious. As an alternative, MALDI-TOF MS has been utilized for exploring the biodiversity of microorganisms by identifying and characterizing novel strains in different environments. For taxonomic classification, microorganisms are separated into genera, species and strains. MALDI-TOF MS has been proven to resolve intra- and inter-species classifications and distinguish very closely related species with high reliability. Dieckmann et al.[19] reported the difference in 1 bp out of 400 bp or 3-4 bp out of 1500 bp of the 16S rRNA gene sequence of *Pseudoalteromonas* sp. by their MALDI-TOF-MS spectra. Investigating the variety and composition of microbes requires careful observation of various habitats, diets, and surfaces. It is carried out sometimes to constantly check on the spread of harmful pathogens or occasionally to find the source of bacterial contamination. MALDI-TOF MS has

proven that it can be used to continuously monitor these settings and offer insightful data on the richness and composition of bacterial species. Zeng et al.[20] utilized a culturomics technique involving MALDI-TOF MS-based high-throughput colony screening technique and genome sequencing to identify one novel strain of Gemmatimonadetes out of 330 isolates from the streams in Northern Greenland. Another study reported that 8 biosurfactant-producing bacteria out of 234 isolates were characterized by MALDI-TOF MS and identified as *Proteus mirabilis, Alcaligenes faecalis*, and *Providencia alcalifaciens.*[21] Environmental water isolates of *Aeromonas* were also isolated and identified using MALDI-TOF MS by comparing m/z signatures with known strains. It was suggested in the work that this technique is useful for environmental monitoring due to its speed and capacity to handle a large number of samples.[22]

Some researchers are interested in learning more about the microbiota connected to distinct metal ores since bacteria play a significant role in the biogeochemical cycle of various elements. Nosalova et al.[23] conducted one such study to look at the microbiota of gold ore. Colony-forming units (CFU) within the range of 2.18×10^5 to 3.16×10^5 bacteria per 1 g of dry ore material were detected in cultivation studies. Results revealed that 89% of the 473 isolates from gold ore belonged to the genus *Acinetobacter, Microbacterium, Pseudomonas,* and *Rhizobium*. Another study conducted in the Rozalia gold mine in Hodrusa-Hamre revealed that the gold ores mainly consisted of bacteria from 18 different species including *Aerococcus, Pseudomonas, Rhizobium, Acinetobacter, Microbacterium, Acidovorax, Staphylococcus* and others.[24]

Environmental biotechnology and bioremediation

Environmental pollution is a major problem, and today eco-friendly remedial methods are a necessity. A potential strategy is a bioremediation, which uses microorganisms to decrease or remove toxic substances from the environment.[25] As a high-throughput method, MALDI-TOF MS is a useful tool for bioremediation research. With the use of this approach, site-specific microorganisms found in polluted settings may be quickly identified. A study conducted by Garcia Lara et al.[26], identified 25 bacterial strains using Bruker BioTyper (Bruker Daltonics) from DDT-contaminated sites. Among 25 strains, 4 were identified at the genus level whereas rest 21 strains were identified at the species level.

A category of environmental contaminants is hydrocarbons, and for successful biodegradation, it is crucial to isolate and characterize novel microorganisms with the capability to break down these pollutants. The diversity of the bacterial population in highly weathered oil-contaminated sites was studied using MALDI-TOF MS (Figure 2) and differentiation of the isolates was done by principal component analysis (PCA) and dendrogram analysis (Figure 3).[27] Microorganisms belonging to *Bacillus* species were identified with high extracellular biosurfactant activities capable of biodegrading weather hydrocarbons. Silva et al.[28] reported isolation and identification of 44 bacteria were isolated from compost and identified using the 16S rRNA gene sequencing and MALDI-TOF MS techniques. 36 bacteria were discovered using MALDI-TOF MS at the species level such as *Bacillus shackletonii* and *Klebsiella pneumoniae* or genus levels such as *Gordonia, Acinetobacter, Stenotrophomonas* and *Pseudomonas.* Silva-Jimenez et al.[29] also studied the bacterial diversity of seawater, surface water and marine sediments and identified 52 bacteria using Bruker BioTyper (Bruker Daltoics) that has a high potential to degrade and utilize pyrene as the sole energy source. The bacteria identified belonged to *Actinobacteria, Proteobacteria* and *Firmicutes.*

Additionally, without the necessity for culture, it is also feasible to identify the enzymes responsible for the degradation of chemical hazards. In research published in 2015, Lovecka et al.[30] gathered isolates from polluted areas in the Czech Republic where they were able to break down lindane, hexachlorobenzene, and DDT (dichlorodiphenyltrichloroethane). The amplification of the pesticide degradation genes linA and bphA1 allowed researchers to analyze the degradation processes carried out by microorganisms. MALDI-TOF MS was used to identify six out of seven isolates which were *Rhodococcus* sp., *Aeromonas* sp., *Stenotrophomonas* sp. and three *Bacillus* sp. which was also cross-checked with 16S rRNA sequencing results. Furthermore, the in-situ monitoring of the bioremediation approach may also be done using MALDI-TOF MS. Undoubtedly, this technique can be utilized for more exploration.

Agriculture and plant pathology

In order to protect crops and boost agricultural productivity, several researchers have used MALDI-TOF MS to identify plant growth-promoting bacteria and pathogenic fungi. Researchers at the Federal University of Lavras, Brazil, have identified endophytic bacteria from garlic roots as potential plant growth promoters for commercial use.[31] MALDI-TOF MS was used to analyze the microbial activities of 48 microorganisms, which included nitrogen fixation, phosphate production and siderophores production. The garlic roots contained *Burkholderia cepacian* and *Enterobacter cloacae.* Whereas Muthuri et al.[32] isolated a total of 43 endophytic bacteria with potential plant growth-promoting activities from bananas and analyzed them using MALDI-TOF MS. The identified isolates belong to *Bacillus, Enterobacter, Ewingella, Klebsiella Pseudomonas, Rahnella, Raoultella, Serratia, Yersinia* and *Yokenella*.

MALDI-TOF MS has been utilized to isolate and identify plant growth-promoting rhizobacteria (PGPR) in order to reduce heavy metal stress in plants. Pramanik et al.[33] reported the identification of *Klebsiella pneumoniae* K5 which has been reported to be highly resistant to

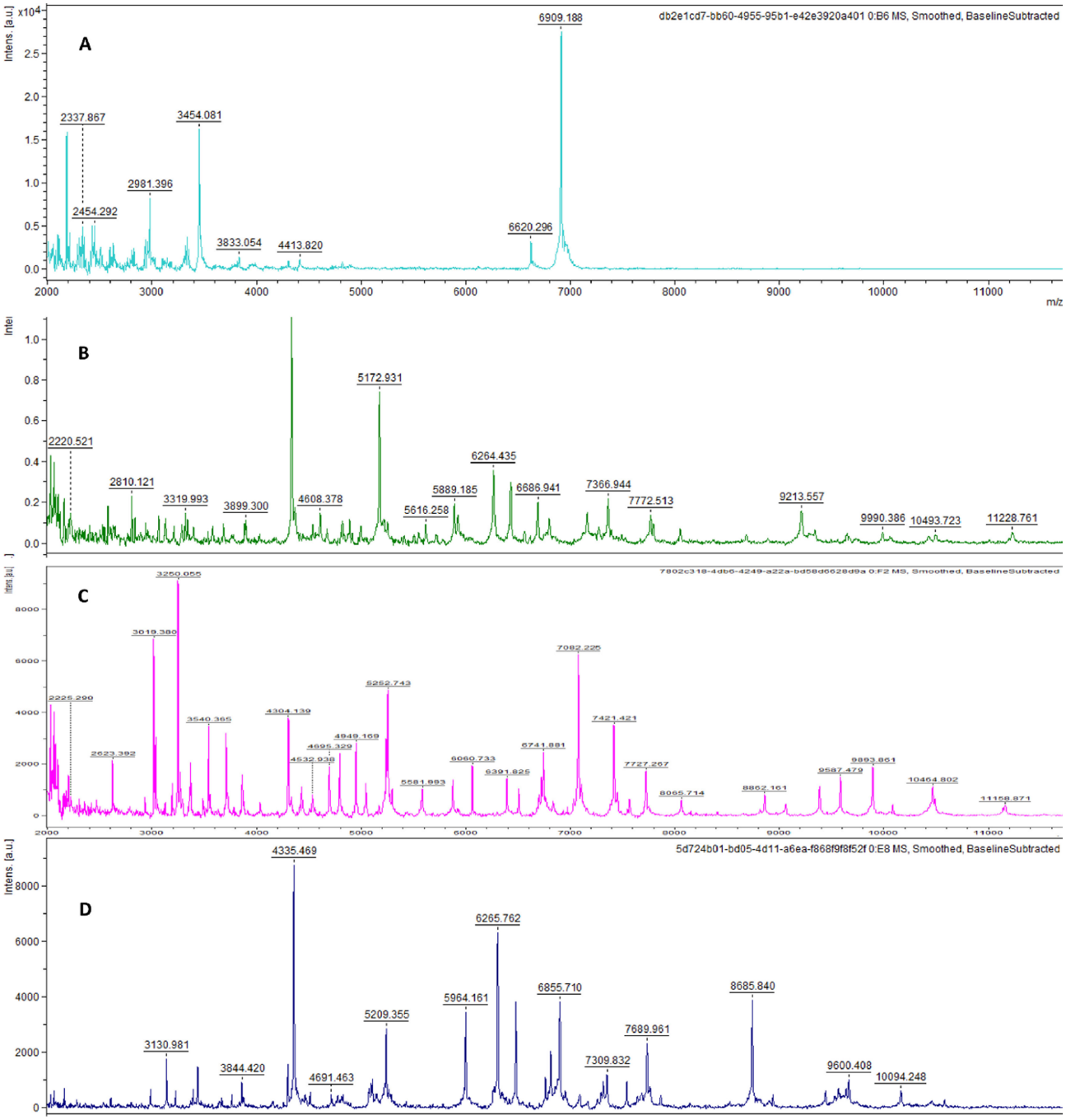

Figure 2. MALDI-TOF MS spectra of A) *Bacillus subtilis* (SA28), B) *Bacillus cereus* (SA17), C) *Bacillus licheniformis* (S33) and D) *Lysinibacillus boronitolerans* (SA3). Reprinted with from Alsayegh et al.[27] (2021).

cadmium stress with a value of 4 mg/ml. An easy MALDI-TOF MS-based novel method to identify and characterize zinc-solubilizing bacteria was also proposed by Costerousse et al.[34] Further salt-tolerant PGPR have been identified using MALDI-TOF MS for their use in agriculture to reduce stress in plants and promote growth.[35]

The identification of plant pathogens using MALDI-TOF MS technology has been established in several research. Plant pathogens infecting rice seedlings such as *Burkholderia gladioli pv. Gladioli, B glumae* and *Erwinia chrysanthemi pv. Zeae* have been identified using MALDI-TOF MS directly from sample extracts. Despite small spectral changes, MALDI-TOF MS analysis revealed that the extracts of infected rice seedlings generated peaks

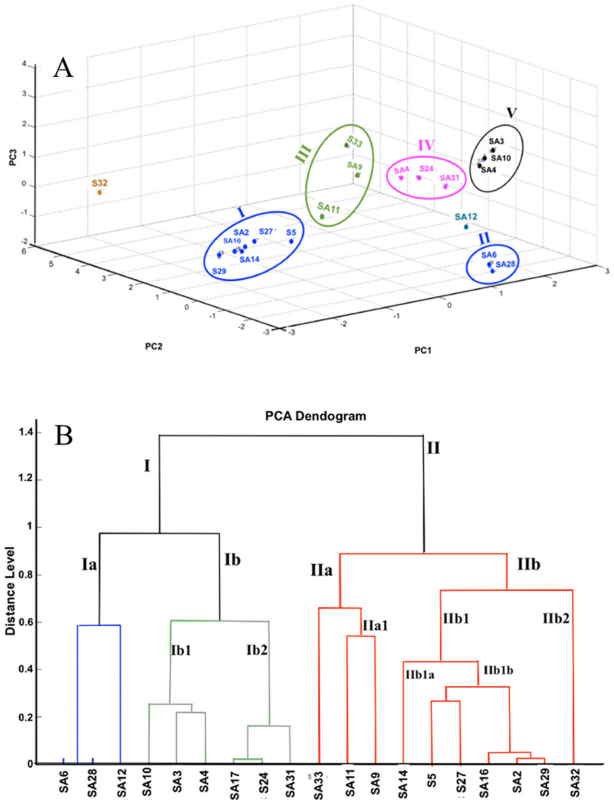

Figure 3. (A) Classification of studied strains using PCA plot (B) PCA dendrogram of the studied strains. Reprinted from Alsayegh et al.[27] (2021).

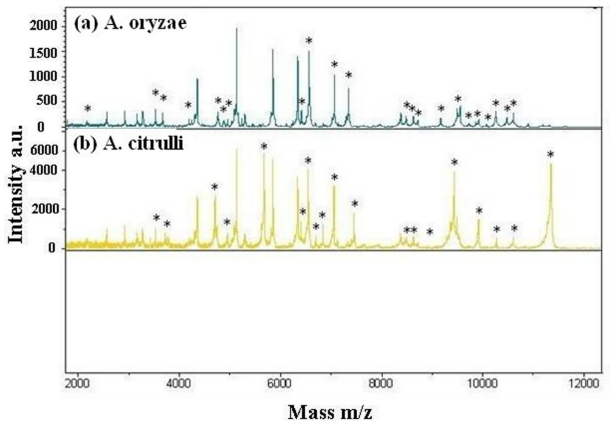

Figure 4. PMF obtains using MALDI-TOF MS for *A. oryzae* and *Acidovorax citrulli.* Reprinted from Wang et al.[38] (2012).

originating from bacteria with spectral peaks that had considerably high scores.[36] A novel strain *Pseudomonas grimontii*, which is potential pathogenic bacteria causing rot disease was identified using MALDI-TOF MS.[37] Wang et al.[38] differentiated two closely related plant pathogens which is even more difficult using the carbon source utilization process and ELISA. The two strains were identified as *Acidovorax citrulli* and *A. oryzae* using MALDI-TOF MS (Figure 4). Similarly, two closely related pathovars of *Xanthomonas oryzae* have also been identified in the same process.[39] These citations from the literature highlight the potential and broad application of MALDI-TOF MS in the study of plant growth-promoting and growth-inhibiting bacteria.

Food microbiology and water treatment

It is crucial to rapidly detect pathogenic microorganisms in order to maintain the safety and quality of food and drink items. There are several different ways that microorganisms are connected to food. They may be included in the production of food items, they might cause food to spoil, and they might even spread through food. By recognizing and separating distinct lactic acid bacteria, fermentative bacteria and foodborne pathogenic bacteria, MALDI-TOF MS technology can contribute to the study of food microbiology. *Brochothrix thermosphacta* is one of the major strains responsible for seafood and meat spoilage and has been characterized using MALDI-TOF MS.[40] The quality of raw milk has a significant role in influencing the quality of dairy products that are made. In order to understand the impact of protracted refrigeration on the changes in raw milk microbial ecology, Zhang et al.[41] investigated the quantities and kinds of psychrotrophic in raw milk with and without refrigerated enrichment (5 days at 7°C). Pseudomonas was observed to have the highest abundance among 119 isolates from fresh raw milk belonging to 12 genera and 23 species. Whereas, after refrigeration for 5 days, 127 isolates classified as 9 genera and 20 species were identified. This technique has further been used for the characterization of bacteria present in milk, yogurt and probiotics and bacteria responsible for the spoilage of milk and pork, biogenic amine-producing bacteria, and causative agents for seafood-borne bacteria.[10]

MALDI-TOF MS has been applied to the bacterial examination of drinking water, wastewater, and natural waterways for environmental monitoring. In a recent study, bacterial components in tap water and mineral water were identified. A total of 10 samples of mineral water from various brands and 11 samples of tap water from various localities were acquired.[42] *Alphaproteobacteria* were more prevalent in tap water than *Gammaproteobacteria*, which were often isolated from mineral water, resulting in differences in the bacterial diversity found in the two distinct forms of drinking water. Additionally, because some of the bacteria were only found during one season, the season in which the water was bottled also had an impact on the variety and abundance of bacteria. The study demonstrated that the MALDI-TOF MS is an effective approach for frequently checking the quality of water in the water business and may be used to determine the variety of bacteria in the water meant for human consumption.

Challenges and future perspectives

Although MALDI-TOF MS has been shown for its potential and applicability in routine analysis for microbial identification, however, till date it possesses certain limitations. *Bacteroides fragilis* group (BFG) isolated from various environmental samples including human and rat fecal matter, sewage from wastewater treatment plants, etc. were investigated by MALDI-TOF MS. The results showed variation in accuracy for hospital and untreated wastewater (20%), wastewater (40%)and human and rat fecal samples (100%). This could be due to the similarity of strains from various samples for which the MS database was optimized.[43] A study was conducted to explore the accuracy of identification by MALDI-TOF MS for 21 globally distributed species of *Burkholderia cepacia*. However, the accuracy for detection and characterization has been reported to be affected due to the high similarity between the species. Based on various studies for clinical as well as environmental samples related to the identification of the *B. cepacian* complex, the accuracy is significantly low for environmental strains as compared to clinical strains.[44-46]

The most essential step in identifying a species is comparing the PMF of an unknown isolate to reference mass fingerprints in a database, which necessitates a database to contain mass fingerprints of different strains of each species as well as reference mass fingerprints of all species of relevance. To enhance the spectrum depiction of the bacterial species, the sample preparation guidelines and spectral profile analysis (baseline subtraction, normalization, and others) need to be standardized. Insufficient spectral data in the public database is a major challenge today. However, the establishment of a drinking water library by Pinar-Mendez et al. is the most current and top-notch illustration of an internal database which is used as the alternative solution for the insufficient database. The incorporation of in-house databases into the public open-access databases and the development of user-friendly and cheaper software for analyses will further boost the credibility of the technology in near future.

Conclusions

MALDI-TOF MS has been demonstrated to be a workhorse in proteomics and can be a powerful tool in the investigation of environmental microbiology and bioremediation. Through comparison with molecular methods, several studies have proven the effectiveness of MALDI-TOF for the identification of environmental bacteria and concluded that this is an efficient and appropriate approach for environmental bacteria. With future advancements in reference bacteria database libraries, frequent implementation in environmental investigations should be promoted owing to time savings and cost-effectiveness. The method has been used with great accuracy and precision to identify a variety of bacteria, including those that are important for the environment and biotechnology such as those that degrade pollutants, biomineralize, fermentative, food- and water-borne diseases, produce coliforms and lactic acid, and relate to and promote plant development. Additionally, it also monitors environmental routines that improved in quick identification of bacteria present in water, air, and other surfaces.

There are still unexplored potential uses for MALDI-TOF MS technology. These applications may include the proteins and enzymes detection synthesized by bacteria for a particular environmental purpose, such as the synthesis of biosurfactants, minerals, or pollutants. The approach may be used to identify the relevant proteins/genes utilizing advanced bioinformatics techniques. Ultimately, there is a discrepancy between the taxonomic identity of bacteria and their prospective environmental functions. This gap must be closed through more research and development in order to maximize the technology's potential for environmental investigations and assure quick research breakthroughs through time and money savings.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

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