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THE IMPORTANCE OF PROTEIN FINGERPRINTS IN BACTERIAL IDENTIFICATION - THE MALDI-TOF TECHNIQUE

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Abstract

The available literary sources suggest the general applicability and benefits of the Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) in the field of microbiological identification including food quality and safety, and the clinical field. Due to its high reliability, MALDI-TOF might generally be the alternative to the sequence-based and serological-based methods. The essence of the technique is to map the unique protein pattern of microbes that contributes to characterizing a wide variety of microorganisms, including bacteria, fungi, and viruses. On the other hand, these applications only have reliable results under certain conditions (homogeneous infection, adequate cell count, appropriate separation technique). In this review, we focused on the application of MALDI-TOF MS for the environmental field where it has significant potential in the identification, differentiation, and categorization of environmental samples which includes (soil, water, and air), furthermore, some challenges, especially in case of the extreme conditions environment and summarize developments that have been enabled for routine application in the field of environment.

Keywords: microbial identification, environment, MALDI-TOF, protein pattern, mass spectrometry

INTRODUCTION

The identification of microorganisms is essential in different fields. Generally, this identification is made by morphological (e.g. cell shape), phenotypic (e. g. Gram staining), and genetic tests (e.g. polymerase chain reaction - PCR). The morphological and phenotypic tests are time-consuming, while the genetic tests require a high level of expertise and are quite expensive. Therefore, these techniques are not ideally suitable for routine identification and alternatives would be welcome for the rapid and low-cost identification of microorganisms (Rychert, 2019).

New technologies for accurately and rapidly identifying bacteria are essential in various fields of applied microbiology. Mass spectrometry is an alternative solution for identifying and typing. This analytic technique can analyze the mass-to-charge ratio of numerous biomolecules, such as peptides and proteins (Simke et al., 2022). Matrix-Assisted Laser Desorption Ionization (MALDI) is currently used for this purpose. The essence of the MALDI measurement is that the molecules of the examined sample are ionized with the contribution of an auxiliary material (matrix) that can absorb the excess laser energy used for ionization (Beavis and Chait, 1989). The analytes are embedded in the crystal of the matrix, which transfers the laser energy to the molecules of the analyte (Batoy et al., 2008). During the process, the macromolecule-matrix complexes from the test sample are released (desorption phase), and then the resulting molecular ions are delivered to the analyzer under high vacuum and accelerating voltage (Fig. 1).

The first description of using MALDI-TOF mass spectrometry technology for bacterial biomarkers was published in 1975 (Anhalt and Fenselau, 1975). Still, it took a long time to introduce this technology in routine microbiology. Over and above in 2004, the first complete database for bacterial identification was reported (Keys et al., 2004). At the same time, it was quickly recognized that this technique is also suitable for examining the unique protein pattern of microbes (Jones et al., 2003). The MALDI-TOF MS is now a widely used technique to characterize a wide variety of microorganisms, including bacteria, fungi, and viruses (Giebe et al., 2010).

A multicenter evaluation study of the MALDITOF MS system for identifying gram-negative bacteria was performed, including a total of 2,263 isolates representing 23 genera and 61 species. The study showed that the MALDITOF MS system

correctly identified 99.8% at the genus and 98.2% at the species level (Faron et al., 2015).

Spanu et al. (2011) evaluated the application of MALDI-TOF MS for identifying the most relevant species of the *Staphylococcus* genus, using the rpoB gene sequencing method as a reference. Correct species identification was achieved in 99% of strains until the subspecies level. Handal et al. (2015) aimed to evaluate the reliability of identification by MALDI-TOF MS compared to 16S rRNA sequencing of the most common clinically relevant anaerobic bacteria, including *Bacteroides* spp., *Clostridium* spp., *Prevotella* spp., *Fusobacterium* spp., and gram-positive anaerobic cocci. Authors reported that the MALDI-TOF MS correctly identified about 95% of the anaerobes to the genus level, and 87% to the species level, with identification errors mainly among the non-fragile *Bacteroides* spp. and the gram-positive anaerobic cocci. MALDI-TOF proved to be a successful method for identifying anaerobes.

MATERIALS AND METHODS

Approach to the Methodological Concept

In recent years, MALDI-TOF MS can be found in routine laboratories and utilized as an alternative approach for identification. During the preparation process, the sample can practically be picked up with a sterile toothpick and prepared on a target plate, which should be covered with 1 µL formic acid. When dried, it is overlayed with 1 µL α -HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) matrix solution and left to dry again (Bizzini and Greub, 2010). After the crystallization of the matrix-analyte mixture on the target plate, it is targeted with short laser pulses, usually from a UV/Vis laser. The matrix absorbs the laser energy, leading to the desorption of the analytes, which are then vaporized and ionized into the gas phase. This matrix-assisted desorption and ionization of analytes lead to the formation of predominantly singly charged sample ions. The desorbed and ionized molecules are first accelerated by an electrostatic field and then ejected through a flight tube subjected to a vacuum until they reach the detector. The **t**ime **o**f **f**light (TOF) required to reach the detector depends on the mass (m) and charge (z) of the analyte and is proportional to the square root of m/z (Carlsohn et al., 2007). Thus, bioanalytics with different m/z that make up a complex sample are separated according to their TOF, creating a mass spectrum characterized by both m/z and ion intensity, corresponding to the number of ions. Based on this mass spectra information, a characteristic fingerprint can be recorded of organic matter that can be investigated typically between 2000 and 20,000 m/z. In this range, the signal-to-noise ratio is very stable and easily detectable (Wieser et al., 2012). Generally, MALDI produces singly charged (z = 1) ions, so the m/z value of the analyte corresponds to its mass plus cation adduct (Croxatto et al., 2012).

MALDI-TOF MS, that can measure peptides and other compounds to analyze their complex mixture, is an ideal method for measuring non-purified extracts and intact bacterial cells (Biotyper). It looks like a rapid, accurate, and cost-effective way of microbial characterization and identification compared to

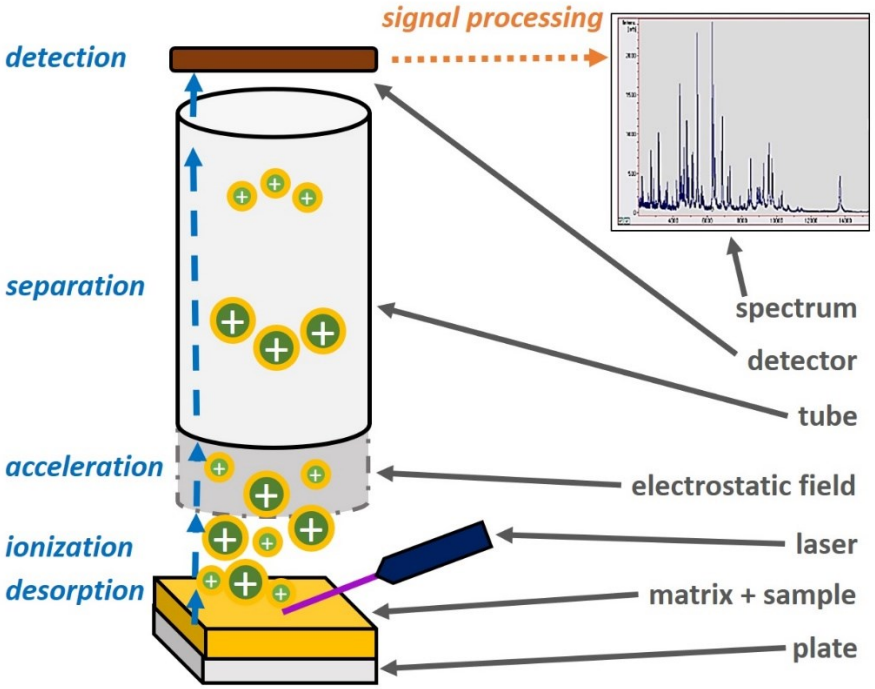

Fig.1 Overview of the investigation process. The sample–matrix complex is evaporated and ionized by laser irradiation. The ions are accelerated in an electric field and drift in a field-free pathway under a vacuum. During the flying, a separation between low-mass and high-mass ions occurs. The flight time depends on the length of the flight path, the ion's mass, its energy, and the value of charges.

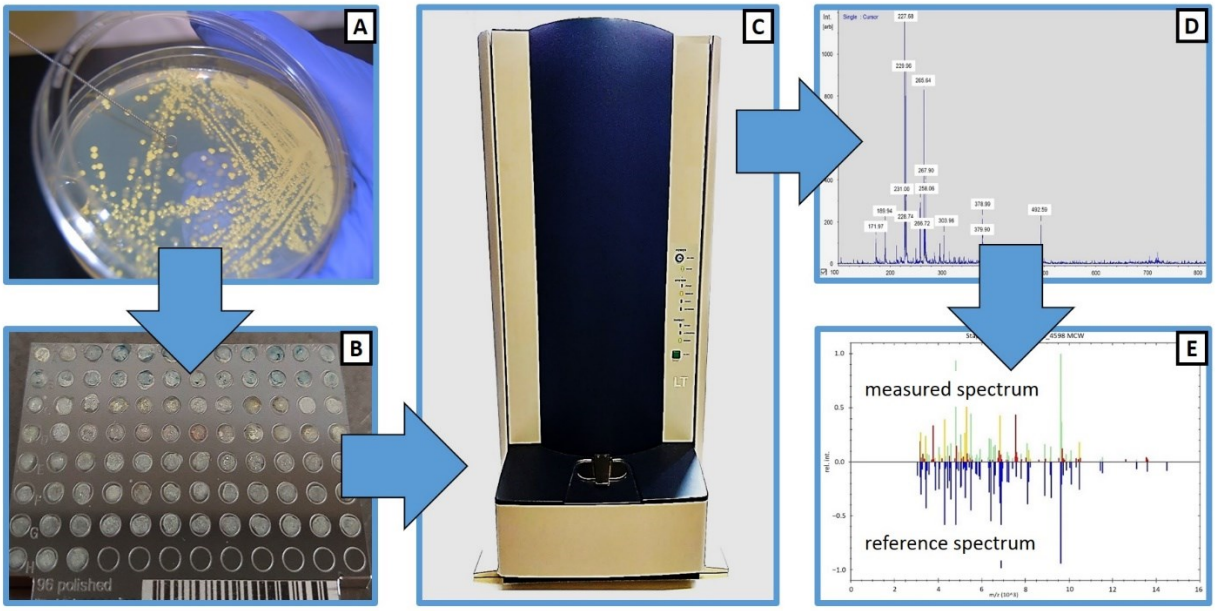

Fig.2 Workflow of sample preparation and profile analysis by MALDI-TOF Biotyper. (A) Sampling from the colony, (B) Preparation on MALDI Target plate, (C) Instrumental measurement, (D) Generating mass spectra, (E) Calculating MALDI-TOF profile spectra. During the measurement, the generally known peaks are identified, and their pattern is compared with the reference list in a database.

classical and molecular ways. During the measurement, mass spectral fingerprints generate from the sample's protein content, unique signatures for each microorganism at the species level (Fig. 2).

RESULTS

MALDI-TOF MS environmental microbiology applications

MALDI-TOF MS has been extensively applied and is still mainly used in the clinical field as shown by the number of published reviews (van Belkum et al., 2012) In the last years, hundreds of systems have been installed worldwide in clinical microbiology laboratories. The importance of rapidly identifying microorganisms involved in human infections and applying the right therapeutic is unquestionable. However, MALDI-TOF MS can also provide a significant contribution to environmental microbiology (Santos et al., 2016). The identification of microorganisms from environmental sources is necessary to understand the microbial community, for environmental monitoring, and to identify possible pathogenic microorganisms. MALDI-TOF MS has been an important advance in the field of environmental proteomics as the protein fingerprint of each microorganism can be used for identification. The identification can be performed by comparing the unknown protein profile to a database of reference profiles or by co-analyzing the unknown profile with profiles of known bacteria (Santos et al., 2016). Previous findings have described the application of MALDI-TOF MS in the identification of microorganisms in environmental samples such as sewage sludge, marine sponges, water, soil, roots, and the rhizosphere (Dieckmann et al., 2005; Lovecka, et al., 2015) For example, in the work by Emami et al. (2012) MALDI-TOF MS was used to characterize bacteria in ballast water. Thirty-six isolates were identified, at the genus level, and the results were similar to those obtained by 16S rRNA gene sequencing. For higher-quality spectra, the authors used cell lysates from actively growing colonies instead of crude cells and α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix that they found to be an important factor when trying to differentiate between closely related isolates.

In the same context, Štursa et al. (2009) and Ferreira et al. (2011) studied the application of MALDI-TOF MS for the identification and characterization of microorganisms in the rhizosphere of plants. In the former work, the authors built a database containing protein profiles of 56 species of fast-growing rhizobia and were able to identify large populations of isolates from nodules with 100% effectiveness. Furthermore, they concluded that MALDI-TOF MS is a very useful tool for diversity and ecological studies.

Application of MALDI-TOF MS in monitoring the microorganisms at specific stresses

MALDI-TOF MS can be used in the identification of proteins associated with specific stresses. When subjected to changes in environmental parameters, microorganisms change their protein expression profiles, as a response to overcome these changes. These differences in the protein expression profiles can be used as an indicator of environmental pollution. In the work by (Heim et al., 2003) MALDI-TOF MS was used to identify proteins produced by *Pseudomonas putida* due to iron limitation stress. The authors were able to identify 25 proteins that were up and downregulated due to iron deprivation. Lacerda, et al. (2007) used MALDI-TOF MS, and de novo

sequencing to identify proteins differentially expressed over time following exposure of a bacterial community to an inhibitory level of cadmium. Munoz et al. (2011) verified that MALDI-TOF MS analysis of whole cells is a powerful tool for studying the cultivable fraction of hypersaline environments. The authors used MALDI-TOF MS to classify bacterial isolates into 25 phenotypic clusters at 52% similarity, which was validated by 16S rRNA sequencing to indicate that each phenotypic cluster consisted of a homogeneous set of strains. In another work, by Donohue et al. (2007) MALDI-TOF MS was used for the speciation of unknown environmental water isolates of *Aeromonas* using the m/z signature of known strains of that microorganism. Due to its analysis speed and its capability for handling a large number of samples, the authors proffered that this technique could be useful for environmental monitoring.

Applications of MALDI TOF MS in the bioremediation field

The application of microorganisms to reduce or eliminate hazardous compounds from the environment, so-called bioremediation, is a promising approach as well (Vidali, 2001). MALDI-TOF MS can be used for the rapid screening and identification of site-specific microorganisms present in contaminated environments using their global protein expression (Singh, 2006). This allows researchers then to focus on specific microorganism species to evaluate their potential for degradation of chemical hazards. After evaluating their capability, the isolated microorganisms can be used for bioremediation of contaminated and polluted sites. As an example, in the works by (Uhlik et al., 2011; Lovecka et al., 2015) the identification of bacteria isolated from contaminated soil for bioremediation purposes was performed using MALDI-TOF MS. In both works, the bacteria were isolated from contaminated soil by using their ability to grow on a solid mineral medium with chemical hazards, pesticides, or biphenyl, as a sole carbon source (Santos et al., 2016). Afterward, the isolates were identified, some to the strain level, using MALDI TOF MS. The results obtained were in agreement with the results of 16S rRNA sequencing. However, in both studies, there were additional microorganisms that were not successfully identified by MALDI-TOF MS that may be due to their absence in the database. Nevertheless, the authors believe that MALDI-TOF MS is an important tool for bioremediation research as it allows the rapid and accurate identification of site-specific microorganisms. As the isolated microorganisms can grow using the hazardous exogenous compounds as the sole carbon source, they can act as potential degraders and therefore be used for on-site bioremediation. However, only the work by Lovecka et al. (2015) specifically studied the capability of the identified bacteria to degrade the contaminants by measuring the degradation of pesticides and the formation of degradation products.

Application of MALDI-TOF MS in Identification of Bacteria Isolated from Soil

A study by Garcia et al. (2021) aimed to measure bacteria indigenous to DDT-contaminated industrial sites in Salamanca City, central Mexico, as potential candidates for its bioremediation. By using ten-gram soil samples were suspended in 90 mL of saline solution 0.9% m/v and three dilutions were made (1:10, 1:100, 1:1000); 100 µL of each dilution were spread in Petri dishes with LB agar enriched with 5 mg/L DDT of technical grade, and incubated at 37 °C for 24 h under aerobic conditions. The colonies were purified by several transfers on the same medium. Among twenty-five isolates obtained and identified by MALDI-TOF MS, *Bacillus*, and *Pseudomonas* species were the most prevalent, accounting for 44% and 20% of all isolates, respectively. The following eight bacteria could grow in the minimum medium in the presence of a DDT concentration of at least 200 mg/L: *Lysinibacillus fusiformis*, *Bacillus mycoides*, *Bacillus pumilus*, *Bacillus cereus*, *Bacillus marisflavi*, *Bacillus megaterium*, *Lactobacillus adecarboxylata*, and *Serratia fonticola*.

Application of MALDI-TOF MS in the identification of Bacteria Isolated from wastewater

In an experiment done in 2017 by Jančová et al. (2020), in two seasons (summer and autumn), wastewater samples were obtained from six offtake points that are linked to the processing of various dairy products and are crucial for the drainage of wastewater, as it is known, milk and dairy products represent a suitable environment for the growth of microorganisms, that may influence, through their metabolic activity (positively or negatively), the product quality. by Using the MALDI-TOF MS and biochemical methods, identified 77 bacterial strains belonging to 19 bacterial genera out of 89 isolated colonies, plus three fungi – *Candida parapsilosis Metschnikowia pulcherrima*, and *Rhodotorula mucilaginosa* – were successfully identified. Approximately 10% of the isolated microorganisms were not identified by the use of the MALDI-TOF MS and biochemical methods. Out of 77 isolated and identified bacterial strains, 34 were Gram-positive, represented mainly by the genera of *Lactococcus* (35% of identified Gram-positive bacteria), *Staphylococcus* (21%), *Microbacterium* (12%), *Enterococcus* (9%), *Kocuria* (9%) and 43 Gram-negative mostly species of the genera *Acinetobacter* (35% of identified Gram-negative bacteria), *Pseudomonas* (19%), *Aeromonas* (14%), *Chryseobacterium* (9%), *Enterobacter* (7%) and *Klebsiella* (7%).

Application of MALDI-TOF MS in Identification of Bacteria Isolated from Seawater

The diversity of bacteria isolated from Qatari seawater by work Ashfaq, et. al., (2019), identified using MALDI–TOF MS includes *Halomonas aquamarine*, *Halomonas elongata*, *Pseudomonas fragi*, *Vibrio alginolyticus*, and *Vibrio fluvalis* (Table 1).

The match score between 2.3 and 3.00 shows highly probable species-level identification and between 2.0 and 2.29 represents genus-level identification and probable species level of identification. A score between 1.7 and 1.99 indicates probable genus-level identification. As shown in Table 1, only 5 out of 20 strains had scores in the lower (1.7–1.99) range, which means that 75% of the

Table 1 Identification of isolated strains from seawater samples using MALDI-TOF-MS (Ashfaq et al., 2019).

Identification	Score
Halomonas elongata	1.854
Halomonas aquamarina	1.78-2.07-2.08-2.04-2.07-2.02-1.96-2.01
Pseudomonas fragi	1.93-2.4-2.39-2.36-2.32-2.34-2.33
Pseudomonas stutzeri	2.47
Vibrio fluvalis	1.75
Vibrio alginolyticus	2.04

strains were identified up to species level, if we consider the 2.0-2.29 range as a species-level identification. *Halomonas* species belonging to the family of proteobacteria have been reported to be less frequent in coastal areas (Sorkhoh et al., 2010). However, it was noted that the species of *Halomonas* were found in most of the offshore and onshore samples tested in this research. Halomonas bacteria have ecological importance due to their oil-degrading capabilities (Sorkhoh et al., 2010), biomineralization potential through ureolytic activity (Arias et al., 2017), biofouling potential, and biofilm formation (Ivnitsky et al., 2010; Bereschenko et al., 2010; Zhang et al., 2011). *Pseudomonas fragi*; other bacteria obtained frequently from different seawater samples, is a psychorophilic Gram-negative, commonly found in temperate waters (Wang et al., 2017). Their ecological importance includes their ability to produce biofilm (Wirtanen and Mattila-Sandholm, 1994) and food spoilage, especially meat, fish, and other marine organisms (Tryfinopoulou et al., 2002; Ercolini et al., 2007).

Application of MALDI-TOF MS in Identification of Bacteria Isolated from Air

Pollution of air has been an emerging issue since the air we breathe carries contaminants, it can affect our health in various approaches. Exposure to bioaerosols, holding airborne pathogens leads to various infections, including lung cancer, bronchial asthma, hypersensitivity pneumonitis, toxic reactions, and various cardiac diseases (Gorny et al., 2002; Fracchia et al., 2006; Yassin and Almouqatea, 2010). For the prevention and control of air pollution caused by airborne bacteria, rapid, sensitive, and reliable detection techniques are required as well.

A study by Elbehiry et al. (2019) focused on using MALDI Biotyper (MBT) for rapid recognition of various microbial air pollutants. Five hundred air samples were collected from three localities, including Qassim University (150 samples), Al-Qassim hospitals (250 samples), and poultry slaughterhouses (100 samples). All air samples were collected by impactor air sampler from the indoor and outdoor environment. All samples were cultivated on nutrient and blood agar media for two days and a total of 129 isolates were purified for proteomic analysis using MALDI-TOF MS, then confirmed by quantitative polymerase chain reaction (qPCR). Altogether 119 (92.25%) isolates were identified by MALDI-TOF MS at the species level with a log (score) value $\geqslant$ 2. 000 whereas; 10 (7.75%) isolates were detected at the genus level with score values ranging from 1.7000 to 1.999. The MALDI-TOF MS was able to identify 93 (72.10%) gram-positive and 36 (27.90%) gram-negative bacterial isolates. The most common genera were *Staphylococcus* (n = 43, 33.33%), *Escherichia* (n = 16, 12.40%), *Enterococcus* (n = 15, 11.63%), and *Bacillus* (n = 15, 11.63%). *Staphylococcus aureus* and *Escherichia coli* were the most frequently identified species (n = 16, 12.40% for each). In general, had been detected 53 (41.10%) various bacterial species in hospitals, 41 (31.79%) in poultry slaughterhouses, and 35 (27.13%) in Qassim University, accordingly, the MALDI-TOF MS was positively adjusted for their fast and accurate identification.

In the same context, a study by Hernández et al. (2016), collected air samples in a biosafety level 2 laboratory of personal care products. Bacterial isolates were identified by (MALDI-TOF) mass spectrometry and when an organism could not be identified by referencing the appropriate database it was identified by analyzing 16S ribosomal deoxyribonucleic acid (rDNA) sequences. The identification results obtained are nineteen bacterial genera from all the sampled sites. Gram-positive genera isolated were *Agrococcus*, *Arthrobacter*, *Bacillus*, *Chryseomicrobium*, *Citricoccus*, *Corynebacterium*, *Exiguobacterium*, *Kocuria*, *Kytococcus*, *Microbacterium*, *Micrococcus*, *Planococcus*, *Planomicrobium*, *Sanguibacter*, and *Staphylococcus*. Gram-negative genera isolated were Pointibacter, Pseudomonas, Psychrobacter, and Skermanella. In total, 85% of bacterial genera were identified by MALDI-TOF mass spectrometry, and 15% of bacteria genera by 16S rDNA sequencing. Taxonomic analysis indicated that the genus Bacillus, represented by five species, was most common, followed by *Staphylococcus* with four species, and *Arthrobacter* and *Kocuria* with three species. This study and others show that accurate species identification is critical not only for the maintenance of environmental monitoring programs but also to mitigate the risk presented by potential human pathogens with antibiotic multi-resistance in indoor environments (Leung and Chan, 2006; Gálvez-Martín et al., 2012).

Identification of bacteria from extreme environments

A study by Kopcakova et al. (2014) aimed to analyze the ability of MALDI TOF MS to identify cultivable microflora from two waste disposal sites in the non-

ferrous metal industry. Despite the harsh conditions (extreme pH values and heavy metal content in red mud disposal sites from aluminum production or high heavy metal content in nickel sludge), relatively high numbers of bacteria were recovered. In both environments, the bacterial community was dominated by Gram-positive bacteria, especially actinobacteria. High-quality MALDI-TOF mass spectra were obtained but most bacteria isolates could not be identified using MALDI Biotyper software. The overall identification rate was lower than 20%; in two of the environments tested identification rates were lower than 10%. As a dominant bacterial species, *Microbacterium spp*. in drainage water from an aluminum red mud disposal site, *Bacillus spp*. in red mud samples from the same site, and Arthrobacter spp. from nickel smelter sludge were identified by 16S rRNA sequence analysis.

Edouard et al. (2012), who used the MALDI-TOF MS approach mainly for environmental isolates of *Propionibacterium* spp., reported identification rates as low as 18.7 % and proposed database enrichment for the reliable identification of environmental bacteria. Koubek et al. (2012) compared MALDI-TOF MS with two other methods for the taxonomic identification of bacterial isolates obtained from sediment samples contaminated with polychlorinated biphenyls. As with the other method, MALDI-TOF MS was capable of discriminating between four groups of isolates but was unable to identify them down to the species level. MALDI-TOF MS has been successfully applied to several taxa, e.g. *Arthrobacter* species (Vargha et al., 2006), *Leuconostoc* spp., *Fructobacillus* spp., and *Lactococcus* spp. (De Bruyne et al., 2011) or even for archaea or extremophiles (Krader and Emerson, 2004). In all these reports, however, MALDI-TOF MS was applied to pure cultures identified by the 16S rRNA-sequencing approach.

The application of MALDI-TOF MS in environmental microbiology remains limited. As the primary focus of the MALDI TOF MS-based methodology is directed toward medically important bacteria, reference database spectra expansion and refinement are needed to improve the ability of MALDI-TOF MS to identify environmental bacteria, especially those from extreme environments (Kopcakova et al., 2014).

CONCLUSION

MALDI-TOF MS can be a valuable tool in the field of environmental microbiology, it can be used, not only for microorganism identification and differentiation but also for the detection of protein or metabolite biomarkers that can be used as an indicator of the presence of a specific bacterium. This identification is important to study the bacterial community within the environment or to identify contaminant-degrading bacteria. Despite these advantages, MALDI-TOF MS reproducibility is influenced by culture conditions. Therefore, it is very important to use the same sample preparation protocol and to guarantee that the analyzed bacteria are all at the same growth stage. Additionally, improvements in the protein database should be made to include more environmental microorganisms and to take into account different protein profiles that can be obtained due to environmental stress, moreover, challenges due to the complexity of environmental samples and due to the diversity of environmental microorganisms. The reliability of any method of identification ultimately depends on the database.

However, future advancements such as the improvement of the protein databases and sample preparation will allow this technique to be implemented as a routine method for environmental microbiology, eventually replacing conventional methods.

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