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Research review paper

Identification of fungal microorganisms by MALDI-TOF mass spectrometry

Jana Chalupová [a,1], Martin Raus [a,1], Michaela Sedlářová [b], Marek Šebela [a,*]

[a] Department of Protein Biochemistry and Proteomics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic
[b] Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-783 71 Olomouc, Czech Republic

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a reliable tool for fast identification and classification of microorganisms. In this regard, it represents a strong challenge to microscopic and molecular biology methods. Nowadays, commercial MALDI systems are accessible for biological research work as well as for diagnostic applications in clinical medicine, biotechnology and industry. They are employed namely in bacterial biotyping but numerous experimental strategies have also been developed for the analysis of fungi, which is the topic of the present review. Members of many fungal genera such as *Aspergillus*, *Fusarium*, *Penicillium* or *Trichoderma* and also various yeasts from clinical samples (e.g. *Candida albicans*) have been successfully identified by MALDI-TOF MS. However, there is no versatile method for fungi currently available even though the use of only a limited number of matrix compounds has been reported. Either intact cell/spore MALDI-TOF MS is chosen or an extraction of surface proteins is performed and then the resulting extract is measured. Biotrophic fungal phytopathogens can be identified via a direct acquisition of MALDI-TOF mass spectra e.g. from infected plant organs contaminated by fungal spores. Mass spectrometric peptide/protein profiles of fungi display peaks in the *m/z* region of 1000–20 000, where a unique set of biomarker ions may appear facilitating a differentiation of samples at the level of genus, species or strain. This is done with the help of a processing software and spectral database of reference strains, which should preferably be constructed under the same standardized experimental conditions.

Contents

Abbreviations: AFST, antifungal susceptibility testing; CA, caffeic acid; CHCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; FA, ferulic acid; HABA, [2-(4-hydroxyphenylazo)]benzoic acid; IC/IS, intact cell or intact spore; IGS, intergenic spacer; ITS, internally transcribed spacer; LSU, large subunit; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; SA, sinapinic acid; SSU, small subunit; TFA, trifluoroacetic acid.

* Corresponding author. Tel.: +420 585634927; fax: +420 585634933.
E-mail address: marek.sebela@upol.cz (M. Šebela).

[1] Both authors contributed equally.

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1. Introduction

Microfungi are described as a group of eukaryotic organisms such as molds, rusts and yeasts plus fungi-like microorganisms (belonging to the taxonomic ranks Protista, Chromista and Myxomycota), which are no longer classified in the kingdom Fungi. A broad spectrum of these microorganisms with miscellaneous ecology, physiology and morphology covers important producers of enzymes, organic acids, pharmaceuticals, alcohols or antibiotics. Many of them also synthesize harmful toxins causing human and animal diseases. As natural recyclers of organic plant material, fungal plant pathogens have a negative impact on agriculture (Bennett, 1998; Cannon and Sutton, 2004; Santos et al., 2010). Studies on fungal organisms are complicated because of an inadequate comprehension of the whole fungal speciation connected with population biology, ecology, evolution and phylogeny. As regards to the detection of human/animal and plant mycoses and identification of the causal agents, standard biological methods become insufficient in many cases. They are often time consuming and tend to fail.

Since the advent of mass spectrometry (MS), numerous identification methods for microorganisms based on profiling of cell surface proteins have been described. They include namely the intact cell or intact spore mass spectrometry (IC/IS MS), but also rely on an initial extraction of proteins by acidified solvents (Welham et al., 2000) or with the help of a bead beating prior to the MS analysis (Hettick et al., 2008a,2008b). MS measurements with bacterial cells evolved hand in hand with the development of MS itself (Meuzelaar and Kistemaker, 1973). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as one of the most reliable tools for fast and easy identification, differentiation and classification of microorganisms. IC/IS MALDI-TOF MS operates with unique mass spectrometric profiles (fingerprints) acquired by the desorption of specific peptide/protein biomarkers from the cell/spore surface of a particular pathogen (Fenselau and Demirev, 2001). Based on results and experience gained in bacterial identification, IC/IS MALDI-TOF MS has also been introduced for a differentiation of microscopic fungi. Contrary to bacteria, fungal cells are larger in size and their cell wall is more rigid. It is usually based on glucans and chitin, rarely on glucans and cellulose (in the distinct phylogenetic lineage of fungi-like Oomycota). Mannoproteins are also major cell wall components, especially in yeasts (Carlile et al., 2001). Taking this into consideration, modified approaches had to be developed as regards to the procedure of sample preparation, selection of a proper matrix compound, sample deposition techniques etc.

A review article describing characterization of filamentous fungi by MALDI-TOF MS appeared in 2010 (Santos et al., 2010) but the text naturally does not cover yeast analysis. More recently, Havlicek et al. (2012) summarized current trends in MS-based microbial diagnostics with a special focus on the instrumentation (fungi were included but only marginally). In 2013, two comprehensive reviews appeared (Clark et al., 2013; Posteraro et al., 2013), which emphasized the use of MALDI-TOF MS for the analysis of fungi in clinical microbiology laboratories. However, other applications than those related to medicine were not included. The present review deals with MALDI-based identification of fungi in various branches of science and diagnostics. Standard biological methods of determining fungal species are briefly discussed together with their limitations, which provide a space to be filled up with mass spectrometric strategies. There are first attempts of fungal identification mentioned together with a further progress illustrated on specific examples. A special attention has been paid to potential applications in biotechnology, medicine and phytopathology.

2. Biological methods of fungal strains identification

The main goal in all fields of diagnostics is to identify the origin of a human, animal or plant disease in such a way, which is fast, reliable and effective. Basic methods for the detection of fungal pathogens are based on host specificity, disease symptoms and microscopic characters. Although the host specificity and disease symptoms can preliminary help to estimate a causal agent, signs usually change during the disease progression and thus they can give unclear information (Carlile et al., 2001; Doohan, 2005). Because of considerable variability in fungal morphology, microscopy still remains an indispensable tool for identifying individual species. Microscopic techniques, which are commonly used for this purpose, include observation and evaluation of different infectious structures and reproductive organs (sexual and asexual spores) as regards to the color, shape and surface. Together with light microscopy, scanning electron microscopy can be used to reduce the rate of misdiagnose. Evaluation of different shapes of spores (e.g. spherical, oval, ovate, with or without papillae) or various branching of spore-carrying structures (monopodial, sympodial, dichotomous) requires experts and specialists with a practice in identification of fungal agents (Sedlář et al., 2009). In certain cases, fungi are isolated and grown in culture media, which brings the possibility of evaluating physiological characteristics such as colony color or growth rate (Santos et al., 2010). For example, yeasts show a limited morphology, which complicates their identification. On the other hand, yeast cells produce many metabolites, which can be used for biochemical tests and metabolomic profiling. Immunological aspects are taken into account in many cases when no morphological characters are visible. Pathogens can be identified and quantified using species-specific antibodies coupled with a fluorescent dye or enzyme. Enzyme-linked immunosorbent assay was applied for the detection of mycelium of a rice spoilage agent *Humicola lanuginosa*. Similarly, *Botrytis cinerea* was found in grape juice (Carlile et al., 2001). However, immunological methods work reliably only at the genus level and sometimes it may be difficult and expensive to generate the required antibodies.

Molecular biology methods are highly specific. They benefit from a variability in DNA sequences, which allows determining and differentiating closely related species or strains and detect pathogens at early stages of host infection with no visible signs. Specific genetic features such as host resistance can also be recognized (McCartney et al., 2003). In contrast to the morphology-based examinations, these methods are independent of operator's experience. The existence of conserved genes on one side and different DNA sequence regions, which are unique for individuals, on the other side makes this approach suitable for analyzing both common and different phylogenetic features. Data derived from ribosomal DNA (rDNA) sequences are often used for classification and identification of fungi. From this point of view, 18S ("small subunit", SSU), 5.8S and 28S ("large subunit", LSU) gene sequences coding for ribosomal RNA are interesting (Martin and Rygiewicz, 2005). Their significance for fungal identification are the following: 1) the 18S gene has varied enough during evolution and can help to determine the taxonomic kingdom and reveal the phylogenetic aspects and relationships of fungal classes; 2) the 28S gene is more variable and it has been used in classification at levels from genus to phylum; 3) the 5.8S gene does not contain much information but is still useful, e.g. for the identification of ascomycetes, basidiomycetes and zygomycetes. The regions between the 18S, 5.8S and 28S genes on rDNA are not highly conserved and constitute internally transcribed spacers 1 and 2 (ITS1 and 2), respectively, while regions beyond the rDNA genes are known as externally transcribed spacer and intergenic spacer (IGS), the latter separating rDNA copies. By amplification of the

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