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Characterization of Yeasts and Filamentous Fungi using MALDI Lipid Phenotyping

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ABSTRACT

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) becomes the method of choice for the rapid identification of microorganisms (i.e. protein biotyping). Although bacterial identification is already quite advanced, biotyping of other microbes including yeasts and fungi are still under development. In this context, lipids (e.g. membrane phospholipids) represent a very important group of molecules with essential functions for cell survival and adaptation to specific environments and habitats of the microorganisms. Therefore, lipids show the potential to serve as additional molecular parameters to be used for biotyping purposes. In this paper we present a molecular characterisation of yeasts and filamentous fungi based on the analysis of lipid composition by MALDI-MS (i.e. *MALDI lipid phenotyping*). Using a combination of Principal Component Analysis (PCA) and Hierarchical Clustering we could demonstrate that this approach allowed a classification and differentiation of several groups of yeasts (e.g. *Saccharomyces*) and filamentous fungi (e.g. *Aspergillus*, *Penicillium*, *Trichoderma*) at the species/strain level. By analysing the MALDI lipid profiles we were able to differentiate 26 closely related yeast strains, for which discrimination *via* genotypic methods like AFLP in this case are relatively more elaborate. Moreover, employing statistical analysis we could identify those lipid parameters (e.g. PCs and LPCs), which were responsible for the differentiation of the strains, thus providing insights into the molecular basis of our results. In summary, MALDI lipid phenotyping represents a suitable method for fungal characterization and shows the potential to be used as companion tool to genotyping and/or protein biotyping for the characterization and identification of yeasts and fungi in diverse areas (e.g. environmental, pharmaceutical, clinical applications, etc.).

1. Introduction

Fungi and yeasts represent a very important group of microorganisms as they can be found almost everywhere in nature showing adaptation to a variety of different environments. Some species are of particular importance for mankind, particularly in the fields of biotechnology, medicine, food safety and pest control. In biotechnology, the control and analysis of microbial flora is of great importance, for e.g. food production and fermentation processes (e.g. alcoholic beverages). For clinical purpose, fast and reliable identification of microorganisms is very important for successful treatments (e.g. to decide the most efficient antibiotic therapy). Identification of yeasts is (traditionally) based on morphological, physiological and biochemical attributes which proved to be slow, expensive and often may lead ambiguous results (Lopandic et al., 2006; Moothoo-Padayachie et al., 2013; Wuczkowski et al., 2003). To ensure exact identification, several DNA-based molecular methods are applied for several years, but they are also expensive, time consuming and they need significant expertise (Moothoo-Padayachie et al., 2013). The same is true for spectroscopic approaches which also were used for the discrimination of yeast strains (Moothoo-Padayachie et al., 2013). Identification of filamentous fungi ("moulds") in the mycology laboratory is traditionally based on penotypic identification, which requires experienced mycologists for microscopical observation of morphological parameters. For that, sporulating structures must be present mostly, which require time for growth and interpretation. As a consequence, correct identification is especially complicated in non-sporulating (so-called “sterile”) cultures. Sometimes, the isolates are morphologically similar but belong to genetically different species. In some cases, these species can be separated

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by different coloured colonies, but extended inoculation will be necessary for the formation of these pigments (Clark et al., 2013; Lau et al., 2013). Molecular methods are also used for identification of filamentous fungi, depending on the species difficulties may arise from problems with cell lysis or by the presence of PCR inhibitors. Moreover, additional 5 to 7 days will be required for DNA-based identification (Cassagne et al., 2011).

Thus, there is a need for simple and economic methods for the reliable identification of fungi and yeasts on a molecular basis. In the last years, several mass-spectrometry (MS) based methods for the identification of microorganisms have been developed. Among them, MALDI-MS has proved to be the most reliable tool (Chalupová et al., 2013; Ling et al., 2014). Protein profiling by MALDI-MS is already used in clinical microbiology laboratories for several years for routine identification of bacteria and clinically relevant yeasts based on diagnostic systems (hard- and software) (Benagli et al., 2011; Buchan and Ledeboer, 2013; Cherkaoui et al., 2010; Clark et al., 2013; Maier et al., 2008; Mellmann et al., 2008; Rosenvinge et al., 2012). Especially ribosomal proteins are present in high concentration in the cells and are therefore preferentially recorded. However, potential problems may arise from ribosomal modifications caused by different growth conditions, a factor that must be evaluated if protein extracts are used for analysis (Reich et al., 2013). Cost and time savings were noted in comparison to conventional identification systems dropping down the costs from several $ to cent level per sample (Dhiman et al., 2011; Tan et al., 2012). Closely related species which could not be identified by biochemical tests were separated via MALDI-MS (Bader et al., 2011).

Some studies were already carried out on MALDI-MS identification of filamentous fungi and yeasts in the clinical field (Alanio et al., 2011; Buchan and Ledeboer, 2013; De Carolis et al., 2012a; Lau et al., 2013; Marklein et al., 2009; Nobrega de Almeida et al., 2015; Posteraro et al., 2013; Santos et al., 2010; Stevenson et al., 2010; Theel et al., 2011), but there is a big potential of this method also for other fields like phytopathology, microbial contamination and microbial biotechnology (Chalupová et al., 2012). MALDI-MS may be also used for antimicrobial susceptibility testing of yeast and fungal isolates (De Carolis et al., 2012b; Marinach et al., 2009; Ngai et al., 2011; Shields et al., 2012). Regarding bacteria, protein extracts can be easily prepared “on-target”, whereby a small amount of biomass (usually ~ 1 μL inoculation loop) is applied directly on the MALDI target plate followed by an extracting agent (e.g. formic acid) and a suitable matrix (e.g. α-cyano-hydroxycinnamic acid, CHCA). This fast procedure works quite well for bacteria but usually leads to bad results with fungi and moulds due to their more protective cell wall structures (Klimek-Ochab et al., 2011). Thus, the preparation of protein extracts is necessary, whereby several more or less time-consuming procedures have already been evaluated (Cassagne et al., 2011; Chalupová et al., 2013). Consequently, there is great potential to develop more simple and better reproducible methods for MS-based identification of fungal organisms. Since lipids represent very important structural and functional components of microbial cells, their lipid composition can be used for chemotaxonomic purposes. So far, most approaches in this direction focussed on fatty acid profiling by gas-chromatography mass spectrometry (GC-MS) (Jeffery et al., 1997; Olsen and Jantzen, 2001). However, this technique needs hydrolyzation of the lipid molecules to release fatty acids for analysis, whereby the structural information of native lipid molecules (e.g. membrane glycerophospholipids) is lost. The potential for using intact lipid molecules for bacterial characterization was first explored using electrospray mass spectrometry (ESI-MS) (Smith et al., 1995). More recently, reversed phase HPLC coupled to ESI-MS/MS was found to be suitable for phospholipidome analysis of lipid extracts from yeasts (Buré et al., 2013). However, these approaches are rather time- and cost-extensive approaches, which have never been put into praxis so far. In contrast, recently MALDI-MS has been shown to by a convenient method for the identification of bacteria using lipid composition in a very simple way (Cody et al., 2015; Lay et al., 2012; Voorhees et al., 2013). Consequently, the same types of MALDI instruments, which are already used in many clinical laboratories can easily be adapted for lipid profiling of microorganisms.

In our present study we have evaluated the potential of MALDI-MS based lipid profiling for the *molecular characterisation of yeasts and fungi* in comparison to protein biotyping and DNA-based techniques (i.e. AFLP). We used amplified fragment length polymorphism analysis (AFLP) to study the genetic diversity of the strains, a method which allows to discriminate among *Saccharomyces* species at strain level (Lopandic et al., 2007). Our approach was assisted by multivariate data analysis. We performed Principal Component Analysis (PCA) followed by Hierarchical Clustering in order to identify those parameters, which are mainly responsible for the molecular differentiation of the yeast strains. Our results indicate, that adaption to the specific habitats of the different yeast species and strains is accompanied by distinct membrane lipid compositions, especially of membrane phospholipids (PLs) like phosphatidylcholine (PC) and lyso-PC (LPC). Thereby, we could demonstrate that recording of the lipid profiles allowed a differentiation between closely related yeast strains, which could not be achieved using the protein composition of the cells alone.

2. Experimental section

2.1. Samples

All fungi and yeasts were obtained from the ACBR microbial culture collection at the Department of Biotechnology (DBT), University of Natural Resources and Life Sciences, Vienna. Tables 1 and S1 give an overview of the different strains used in this study.

2.2. Reagents

2,4,6-trihydroxyacetophenone (THAP), 6-aza-2-thiothymine (ATT) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). α-cyano-4-hydroxycinnamic acid (CHCA) and 9-aminoacridine (9AA) was obtained from Bruker Daltonics (Bremen, Germany) and Acros Organics (Thermo Fisher Scientific, NJ, USA), respectively. Acetone (AcOH), acetonitrile (ACN), ethanol (EtOH), formic acid (FA), isopropanol (ISO), methanol (MeOH), and ultrapure water (UHQ) were obtained from Merck (Darmstadt, Germany). All chemicals were obtained in the highest purity grade available for analytical purposes.

2.3. Cell culture

The yeasts were grown on malt extract agar plates (20g malt extract, 1g peptone, 20g glucose, 20g agar in 1L H_2O) for two days at 30°C. For measurement of lipid extracts, filamentous fungi were grown on the same agar for one week at room temperature. In order to prepare protein extracts we followed Bruker standardized liquid culture method (Bruker Daltonics, 2012), strains were grown in liquid malt extract medium (20g malt extract, 1g peptone, 20g glucose in 1L H_2O) at room temperature with constant rotation (Minirotator RS-24, Biosan, Riga, Lettland).

2.4. Genetic analysis

Yeast DNA was isolated and purified according to the protocol of the MasterPureTM Yeast DNA Purification Kit (Epicentre, Madison, Wisconsin, USA). Genetic variability of the *Saccharomyces* hybrids was estimated by means of the amplified fragment length polymorphism (AFLP) technique using the AFLP™ Microbial Fingerprinting kit of Applied Biosystems (Foster City, CA, USA) as described by Lopandic et al. (2007) and Pfliegler et al. (2014). The restriction and ligation steps as well as the preselective amplification were performed following the manufacturer’s recommendations. One primer pair, *EcoRI-AC*-FAM/*MseI*-C, was used for selective amplification. The fragments generated