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Wine yeasts identification by MALDI-TOF MS: Optimization of the preanalytical steps and development of an extensible open-source platform for processing and analysis of an in-house MS database

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ABSTRACT

Saccharomyces cerevisiae is the most important yeast species for the production of wine and other beverages. In addition, nowadays, researchers and winemakers are aware of the influence of non-*Saccharomyces* in wine aroma complexity. Due to the high microbial diversity associated to several agro-food processes, such as winemaking, developing fast and accurate methods for microbial identification is demanded. In this context, MALDI-TOF MS mass fingerprint provides reliable tool for fast biotyping and classification of microorganisms. However, there is no versatile and standardized method for fungi currently available. In this study, an optimized sample preparation protocol was devised for the biotyping of yeasts of oenological origin.

Taking into account that commercially available reference databases comprise almost exclusively clinical microorganisms, most of them bacteria, in the present study a database of yeasts isolated from vineyards and wineries was created, and its accuracy was tested using industrial and laboratory yeast strains. In addition, the implementation of a program for MALDI-TOF MS spectra analysis has been developed as an extensible open-source platform for MALDI data processing and analysis with statistical techniques that has arisen from our previous experience working with MALDI data. The software integrates two R packages for raw MALDI data preprocessing: Continuous Wavelet Transform (CWT)-based algorithm and MassSpecWavelet. One of the advantages of the CWT is that it can be directly applied to a raw spectrum, without prior baseline correction.

Mass fingerprints of 109 *S. cerevisiae* strains and 107 non-*Saccharomyces* isolates were generated by MALDI-TOF MS upon optimized sample preparation and instrument settings and analyzed for strain, species, and genus-level differentiation. As a reference method, for *S. cerevisiae* differentiation at strain level, the analysis of the polymorphism in the inter-delta region was chosen. The data revealed that MALDI-TOF MS can be used for the rapid and accurate identification of *S. cerevisiae* and non-*Saccharomyces* isolates at genus and species level. However, *S. cerevisiae* differentiation at strain level was not successfully achieved, and the differentiation among *Metschnikowia* species was also difficult.

1. Introduction

Yeast classification requires a multistep approach through the study of cell morphology and physiology (Yarrow, 1998), followed by the use of one or more molecular techniques such as ribosomal DNA sequencing of the D1/D2 and ITS regions, among many others (Fell et al., 2000; Kurtzman, 1998; Kurtzman et al., 2011; Kurtzman and Robnett, 1998). However, intraspecific discrimination of yeasts into specific subgroups (biotypes, serotypes, etc.) or the distinction of individual strains is needed in many instances. For these purposes many molecular methods (Cogliati et al., 2015; Guerra et al., 2001; Pérez et al., 2001; Querol et al., 1992a, b; Schuller et al., 2004; Waltimo et al., 2001) and non-molecular methods can be employed (Buzzini et al., 2007; Gekenidis et al., 2014; Stopiglia et al., 2014; Teanpaisan et al., 2008). These techniques offer very high accuracy and discriminative power; however, they are impractical to perform on a routine basis due to their high costs and time-consuming procedures. At this respect, MALDI-TOF Mass Spectrometry has emerged during the last years as a cost-effective

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technique for microbial identification also even at sub-species level.

While the idea of differentiating bacteria through mass spectrometry dates back to 1981 (Wieten et al., 1981), typing by intact-cell MALDI-TOF MS was initially described for bacterial species in 1996 (Holland et al., 1996). The technique is based on the identification of characteristic protein patterns, mainly ribosomal proteins, derived from microbe composition followed by direct interrogation of updated databases for proper species identification. Today, MALDI-TOF MS provides an easy-to-use, rapid and reliable proteomic alternative to established phenotypic techniques such as the Vitek2 system, widely used in hospitals and other clinical-related centers (Davies et al., 2012; Seng et al., 2009; Stübiger et al., 2016; Tani et al., 2012). The possibility of identifying intra-specific strains by MALDI-TOF has been demonstrated for several clinically important bacteria, such as *Haemophilus influenzae* Type b isolates (Månsson et al., 2015); *Staphylococcus aureus* (Østergaard et al., 2015), *Escherichia coli* (Książczyk et al., 2016) or *Clostridium difficile* strains (Kiyosuke et al., 2015). Regarding yeasts, they are a vast group of organisms with relevance in clinical, agronomical, biotechnological and ecological fields. Currently, about 1500 different species of yeast are known (Kurtzman et al., 2011), of which only a few are presented in manufacturer MALDI-TOF MS databases (MALDI Biotyper mass spectral database - Bruker Daltonics, Germany), most of clinical concern (Goyer et al., 2012; Lévesque et al., 2015; Marklein et al., 2009; van Veen et al., 2010).

One of the most important advances in winemaking has been the selection of cultures of *S. cerevisiae* for their use as inocula in controlled fermentation processes at industry. Strain differentiation among yeast strains of oenological concern is of importance due to the great variability existing in fermentative, technological and sensory properties among industrial *S. cerevisiae* strains. In this context, the development of rapid and accurate methods for yeast identification and typing in wine industry is in the spotlight of oenological research, since they will be really helpful for identification of potential novel inocula, for the study of the yeast diversity associated to different samples and also in the study and confirmation of the implantation of inoculated strains in fermentation processes.

MALDI-TOF seems to be well established for clinical isolates (Alanio et al., 2011; Brun et al., 2013; Chalupová et al., 2014). However, taking into account that commercially available reference databases comprise almost exclusively clinical microorganisms, most of them bacteria, additional information of a variety of yeasts should be acquired for accurate and rapid identification of yeast strains isolated from different environments. Putignani and co-workers analyzed spectra from 303 clinical yeast isolates obtaining 11.6% discordant results, indicating high analytical performance and being able to discriminate patterns even for strain typing of some species (Putignani et al., 2011). Accordingly, in perhaps the largest study referred to date, a diverse collection of 1192 yeast and yeast-like clinical isolates was tested by Bader et al., obtaining the correct identification in at least 95.1% of the isolates (Bader et al., 2011). By contrast, Agustini et al. evaluated a yeast collection of 845 environmental yeast strains, showing that 32.3% strains were not identified due to the absence of a reference spectrum, indicating the existence of incomplete databases for non-clinical yeasts (Agustini et al., 2014).

To determine the efficiency of the analytical procedures (culture preparation, MS conditions, protein extraction, matrix selection, matrix application, etc.) needed in MALDI-TOF MS analysis for microbial identification, it is necessary to evaluate modifications proposed in the general protocols. The issue of standardization was preliminarily addressed with regard to bacteria (Evason et al., 2000, 2001; Goyer et al., 2012; Keys et al., 2004; Valentine et al., 2005; Williams et al., 2003). The adaptation of these defined methods sharply reduced the variation among spectral profiles of isolates being analyzed; improving the accuracy and reliability of MALDI-TOF MS for bacterial identification in inter- and intra-laboratory comparison studies (Keys et al., 2004; Mellmann et al., 2009; Wunschel et al., 2005).

Although innovative and greatly informative, the fast, reliable and accurate identification of yeast isolates by MALDI-TOF MS technology implies the utilization of preanalytical steps optimized for bacteria that even today, are still under evaluation for their suitability for yeasts (Cassagne et al., 2013; Fraser et al., 2016; Goyer et al., 2012; Relloso et al., 2015; Usbeck et al., 2013). Contrary to bacteria, fungal cell wall is more rigid and is usually based on glucans, mannoproteins and chitin, rarely on glucans and cellulose. 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.

This paper explores the utility of the MALDI-TOF MS technique for differentiation of a panel of *S. cerevisiae* and non-*Saccharomyces* strains pertaining to different oenological origins of Spain. The extensive MS dataset acquired in this study provides information on the robustness of MALDI-TOF MS biotyping identification. In order to confirm the identity of the yeast isolates obtained from vineyards, the identification by MALDI-TOF MS was compared with the identification based on 26S rDNA sequence. In the present study, we sought to further enhance the accuracy of the procedure in identifying oenological yeast species, in part shared by other investigators (De Carolis et al., 2014; Goyer et al., 2012). The procedure for spectra acquisition was optimized in the most important variables concerning sample processing: protein purification, sample application, MALDI plate, matrix, etc. (Table 1). Then, the same method was utilized during the in-house database construction process.

2. Materials and methods

2.1. Strains and general media

One-hundred and nine *Saccharomyces cerevisiae* strains (Table S1) and 107 non-*Saccharomyces* isolates (Table S2), originally isolated from wineries from three Spanish Designations of Origin (DO) (DO Ribera del Duero, DO Rueda and DO Tierra de León) were used in this study and deposited in CYC (Complutense Yeast Collection, Complutense University of Madrid, Spain). Commercial and collection strains used as reference in the study are listed in Table S3.

Sabouraud-Chloramphenicol Agar (Oxoid) was routinely used for *S. cerevisiae* isolation and Lysine Agar (Oxoid) was used for the isolation of non-*Saccharomyces* strains (Belda et al., 2016a). A suitable diluted aliquot of grape must, obtained from healthy grape bunches, was spread onto agar media plates and incubated at 28 °C for 48–72 h. Discrete colonies were isolated, and then restreaked on the same medium to obtain pure cultures that were cryopreserved and included in a yeast collection. The yeast collection was conserved at − 80 °C in a cryoprotective medium that was developed as follows: 0.1% glucose, 1.5% casein peptone, 0.5% proteose peptone, 0.5% sodium chloride, 0.2% yeast extract, 0.1% sodium citrate, 0.1% sodium bisulfite and 15% glycerol.

Yeast Malt Agar (YMA) was routinely used for cultivation. YMA was 1% glucose, 0.5% proteose peptone n° 3, 0.3% yeast extract, 0.3% malt extract and 2% agar. The medium was sterilized at 121 °C.

Yeast Proteose Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich) was routinely intended for cultivation in DNA extraction procedures. YPD was 2% glucose, 2% proteose peptone n° 3, 1% yeast extract. The medium was dispensed in 2 ml Eppendorf tubes and sterilized at 121 °C.

2.2. Molecular identification of yeast isolates

Non-*Saccharomyces* isolates, and eleven isolates (listed in Table S1) of *S. cerevisiae* that were inadvertently isolated from lysine agar (intended for the isolation of non-*Saccharomyces* yeasts), were identified according to the partial sequencing of the 26S large subunit rRNA gene (Belda et al., 2016a). Total genomic DNA was extracted from yeasts grown in YPD using the isopropanol method (Querol et al., 1992a, b), and the DNA for sequencing was amplified by using an

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