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Optimization of experimental and modelling parameters for the differentiation of beverage spoiling yeasts by Matrix-Assisted-Laser-Desorption/Ionization–Time-of-Flight Mass Spectrometry (MALDI–TOF MS) in response to varying growth conditions

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ABSTRACT

The growth of spoiling yeasts in beverages results in reduced quality, economic and image losses. Therefore, biochemical and DNA-based identification methods have been developed but are mostly time-consuming and laborious. Matrix-Assisted-Laser-Desorption/Ionization–Time-Of-Flight Mass Spectrometry (MALDI–TOF MS) could deliver discriminative peptide mass fingerprints within minutes and could thus be a rapid and reliable tool for identification and differentiation. However, routine analysis of yeasts by MALDI–TOF MS is yet impaired by low reproducibility and effects of different physiological states of organisms on the reliability of the identification method are still controversial.

The aim of this study was to optimize sample preparation and measurement parameterization using three spoilage yeasts (*Saccharomyces cerevisiae var. diastaticus*, *Wickerhamomyces anomalus* and *Debaryomyces hansenii*). The influence of environmental or physiological parameters including oxygen availability, different nutrients, cell density and growth phase were analysed and revealed small differences in mass fingerprints. Yeasts grown in the presence or absence of oxygen were precisely differentiated along these differences in mass fingerprints and a crude classification of growth phase was possible. Cell concentration did not affect the spectra distinctly, neither qualitatively nor quantitatively, and an influence of available nutrients could not be measured in each case. However, core mass peaks remained constant under all tested conditions enabling reliable identification.

1. Introduction

In the beverage industry, culture yeasts are used as starters for fermented drinks such as beer, wine, sake or cider. However, 'wild yeasts' can contaminate beverages or starter cultures, which can lead to spoilage of the end product (Priest, 1981). Spoilage is often characterized by the production of off-flavour e.g. phenolic, acidic, fatty acid and estery notes as well as hazes, turbidity and ethanol loss (Lawrence, 1988), can occur at infection levels as low as one wild yeast per 10^7 culture yeasts (Thurston, 1986), and leads to considerable economic losses for the beverage industry (Fleet, 2006).

A reliable identification of yeasts with a high spoilage potential facilitates risk evaluation and could provide useful information regarding possible sources of contamination. For this reason, species-specific biochemical and DNA-based identification methods have been developed (Fernandez-Espinar et al., 2006), which are widely applied but mostly time-consuming, laborious or require considerable expertise and therefore are inapplicable for an industrial application.

Matrix-Assisted-Laser-Desorption/Ionization–Time-Of-Flight Mass Spectrometry (MALDI–TOF MS) has been demonstrated to be a versatile method to analyse macromolecules from biological origin (Fenselau and Demirev, 2001), is already commonly employed in clinical diagnosis (Cassagme et al., 2013; Dhiman et al., 2011; Goyer et al., 2012; Marklein et al., 2009; Marvin et al., 2003; Stevenson et al., 2010; van Veen et al., 2010), and represents a promising tool for the rapid and reliable identification of yeasts.

For a MALDI–TOF MS analysis, cells are prepared and co-crystallized with matrix in a way that yields a sufficient number of medium-sized ions (2–20 kDa) in the mass spectra. The identification of microbiological samples by this method relies on the acquisition of mass fingerprints and the subsequent comparison of the obtained data with a database. As, commercially available

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databases are predominantly populated by clinical microorganisms, for our purposes it was necessary to extend existing databases and include contamination and spoilage associated yeast samples previously isolated by various beverage producing companies.

Dozens of sample preparation protocols for bacteria profiling are available in the literature. Such protocols differ in particular steps of the procedure, including different combinations of extraction solvents (Domin et al., 1999), physical methods to improve the cell wall disruption by microbeads (Fagerquist et al., 2005), thermolysis (Vargha et al., 2006) or sonication (Easterling et al., 1998) and enzymatic treatments up to 20 h (Giebel et al., 2008). However, in comparison to bacteria, yeast cells exhibit a higher cell wall stability, which complicates protein extraction and solubilization. For bacterial cells it has been previously reported that the mass spectra can be affected by the growth medium, period of incubation, pH and the cell preparation method used (Cohen and Chait, 1996; Valentine et al., 2005; Wunschel et al., 2005). Additional literature mainly focuses on the limitations of MALDI–TOF MS technique to identify microorganisms correctly (Marklein et al., 2009; Putignani et al., 2011; van Veen et al., 2010). However, the effect of differences in the physiological state of yeast cells on the reliability of the identification is less explored.

A major problem is that the acquired spectra always exhibit complex features, because protein signals can be contaminated by several chemical and/or physical processes during sample preparation or the measurement itself (Gras et al., 1999; Satten et al., 2004). Such interfering signals can be produced by electronic disturbances and fragments of material with rapid fluctuations randomly varying over small mass ranges (Mantini et al., 2007) or chemical noise, which is influenced by sample preparation and contaminations, temperature in the flight tube and software signal read errors (Tong et al., 2011), resulting in a baseline drift or background noise. Consequently, a very sensitive and accurate peak detection method, able to correctly separate protein peaks from noise, is required (Diamandis, 2004). Numerous data preprocessing techniques have been proposed including baseline correction, smoothing/denoising, data binning, peak alignment, peak detection and sample normalization (Jeffries, 2005; Tong et al., 2011; Yang et al., 2009). In this study, we employed LIMPIC (linear MALDI–TOF MS peak indication and classification), a computational method for the detection of protein peaks from multiple linear-mode MALDI–TOF MS data. In contrast to common commercially available identification software, the procedure to decompose the mass spectrum in signal, baseline and noise is improved (Mantini et al., 2007).

As there exists an essential lack of knowledge regarding the reliable identification of yeasts using MALDI–TOF MS, the aim of this study was to determine the effects of sample preparation methods crucial to sensitivity, reproducibility and mass accuracy as well as the measurement parameterization on the identification of spoilage yeasts. For this purpose three yeast species, *Saccharomyces cerevisiae var. diastaticus*, *Wickerhamomyces anomalus* and *Debaryomyces hansenii*, commonly associated with spoilage incidents were used. Additionally, the influences of varying culturing conditions including availability of oxygen, different nutrients and growth phases on protein mass signatures were analysed in order to evaluate the effect on the identification and to search for biomarkers that enable a classification according to these culturing conditions of a specific yeast was grown in.

2. Materials and methods

2.1. Strains

Three beverage spoiling yeasts, belonging to the species *S. cerevisiae var. diastaticus* (TMW 3.236) (Jespersen and Jakobsen, 1996; van der Aa Kuhle and Jespersen, 1998), *W. anomalus* (TMW 3.237) (Kurtzman, 2011; Timke et al., 2008) and *D. hansenii* (TMW 3.238) (Deak and Beuchat, 1996; Suzuki et al., 2011), kindly provided by the Research Centre Weihenstephan for Brewing and Food Quality (Freising–Weihenstephan, Germany), were used in this study.

2.2. Optimization of the preparation method

To optimize the sample preparation, yeasts were grown from −80 °C, 80% glycerol (Gerbu, Heidelberg, Germany) stocks on YPG (casein peptone: 10 g/L (Merck, Darmstadt, Germany); yeast extract: 5 g/L (Carl Roth, Karlsruhe, Germany); glucose: 20 g/L (Gerbu); pH 6.5 ± 0.2) and YM (yeast extract: 3 g/L; malt extract: 3 g/L (AppliChem, Darmstadt, Germany); soybean peptone: 5 g/L (Oxoid, Hampshire, England); glucose: 10 g/L) agar plates (15 g/L agar–agar (BD, Le Pont de Claix, France)) at 30 °C. All media were sterilized for 20 min at 121 °C. Sugars were sterilized separately and added aseptically to the media after cooling to approx. 50 °C. Single colonies were used to inoculate 15 mL of both growth media followed by an aerobic incubation overnight at 30 °C in 50 mL flasks (Schott Duran, Wertheim, Germany) sealed with cotton plugs (No. 18, Zefa, Harthausen, Germany) on a rotary shaker at 180 rpm (Witeg Labortechnik, Wertheim, Germany). Subsequently, another 15 mL of the respective growth medium were inoculated with 1% of the overnight culture and incubated as described above.

Cultured on at least three different days to obtain biological replicates, all samples were spotted as triplicates onto the MALDI stainless polished steel target (Bruker Daltonik, Bremen, Germany) for technical replication. Different strategies for rapid cell wall lysis were employed:

2.2.1. Direct transfer (D)

Biological material of a freshly grown single colony was smeared directly onto a spot as a thin film using a sterile toothpick.

2.2.2. On target extraction (FA)

The smeared spot (*cf.* D) was overlaid with 1 μL 70% formic acid (Fluka, Steinheim, Germany) and dried at room temperature.

2.2.3. Extraction (E)

900 μL liquid culture were transferred into a 1.5 mL tube (Sarstedt, Nümbrecht, Germany) and centrifuged at 11.800 × *g* (Mini Centrifuge MCF-1350, LMS, Tokyo, Japan) for 2 min; the supernatant was discarded and the pellet was mixed thoroughly (Vortex Genie 2, Scientific Instruments) with 300 μL deionized water. Afterwards 900 μL absolute ethanol (VWR International, Darmstadt, Germany) were added and mixed. The tube was centrifuged at 11.800 × *g* for 2 min, the supernatant was discarded and the pellet was air dried for minimum 30 min until the solvent evaporated completely. Dependent on the pellet size, 10–50 μL 70% formic acid, i.e. approx. 10 μL per 100 μg dried pellet, were added and mixed thoroughly until the extract was resuspended. This was followed by the addition of an equivalent volume of acetonitrile (ACN) (Carl Roth) and mixed again. The mixture was centrifuged at 11.800 × *g* for 2 min and subsequently 1 μL of the supernatant was spotted onto a MALDI target and dried at room temperature.

2.2.4. Extraction without centrifugation (EZ)

Cells were prepared as described in (E), except the last centrifugation step, so that a suspension was spotted directly onto the MALDI target.