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Preliminary evaluation of infrared spectroscopy for the differentiation of *Brettanomyces bruxellensis* strains isolated from red wines

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ABSTRACT

The objective of this study was to evaluate different infrared spectroscopy methods in combination with chemometrics for the differentiation between *Brettanomyces bruxellensis* strains. These methods of discrimination were applied to intact yeast cells of *B. bruxellensis* strains and on wines spoiled by the same strains. Eleven wine isolates of *B. bruxellensis* were evaluated for volatile phenol production in red wine and their genetic diversity was determined by Restriction Endonuclease Analysis–Pulsed Field Gel Electrophoresis (REA-PFGE). Fourier transform mid-infrared (FTMIR) spectroscopy was used to obtain spectral fingerprints of the spoiled wines. Attenuated total reflectance (ATR) was used to obtain spectral fingerprints from the intact cells of the 11 *B. bruxellensis* strains. The groupings from the genetic fingerprints obtained with REA–PFGE were used as reference firstly for comparison with the groupings observed with the FTMIR spectral fingerprint of the wines and secondly for the FTIR-ATR spectral fingerprints from the whole cells. Results indicated that ATR-IR spectra obtained by scanning whole cells of *B. bruxellensis* could be useful for rapid strain typing in comparison or complementary to molecular techniques and FTMIR spectra from wines provide a useful resource for the discrimination between *B. bruxellensis* contaminated wines.

1. Introduction

Under poorly controlled winemaking conditions, a large variety of spoilage yeasts can produce unfavourable compounds that could potentially decrease wine quality (Loureiro and Malfeito-Ferreira, 2003). One such group of spoilage yeasts belongs to the genus *Brettanomyces* (or *Dekkera* the sporogenous teleomorph). These species are highly adapted to proliferate in red wine and can have detrimental effects on the organoleptic quality of wines. Among a range of spoilage compounds produced by this genus (Aguilar-Uscanga et al., 2003; Fugelsang et al., 1993; Mansfield et al., 2002; Snowdon et al., 2006), the formation of volatile phenols, specifically 4-ethylphenol and 4-ethylguaiacol, is seen as the most important indicator of *Brettanomyces* activity in red wine (Chatonnet et al., 1992, 1995, 1997; Joseph and Bisson, 2004; Loureiro and Malfeito-Ferreira, 2003). The concentrations of volatile phenols vary in wine and the phenolic off-flavours perceived are typically described by terms such as 'medicinal', 'barnyard-like', 'inky', 'sweaty leather' and 'Band-aid' (Chatonnet et al., 1992; Rodrigues et al., 2001). These yeasts are particularly well adapted to survive the winemaking process due to their relative resistance to the sulphur dioxide (SO_2) concentrations normally used in wine, superior ethanol tolerance and growth in nitrogen limited conditions (Licker et al., 1998).

Recent years have seen higher incidence of *Brettanomyces* contaminated wines and consequently numerous identification methods for this slow-growing yeast followed (Cocolin et al., 2004; Egli and Henick-Kling, 2001; Ibeas et al., 1996; Mitrakul et al., 1999; Renouf et al., 2006). With the molecular identification techniques that allow for inter- and intra-species identification of *Brettanomyces* (Curtin et al., 2005; Miot-Sertier and Lonvaud-Funel, 2007), it became evident that more information is required regarding the diversity of *Brettanomyces* strains that occur during winemaking.

Preliminary studies focusing on strain diversity regarding volatile phenol production by different strains of *B. bruxellensis* isolated from South African red wines indicated that an alternative, rapid and reliable approach to strain differentiation was needed. In order to detect possible variation in standard parameters of wines spoiled by isolates of *B. bruxellensis*, Fourier transform infrared (FTIR) spectroscopy, which facilitates the quantification of important wine parameters, was applied. FTIR spectroscopy relies on the principle of detecting molecular vibrational frequencies in the mid-infrared (MIR) region of the electromagnetic spectrum. Each organic molecule has characteristic frequencies of absorption in the mid-infrared region dependent on the presence of different functional groups in the molecule. The region 929 cm^{-1} to 1600 cm^{-1} captures a substantial

Abbreviations: *p*-CA, *p*-coumaric acid; FA, ferulic acid; 4-EP, 4-ethylphenol; 4-EG, 4-ethylguaiacol; 4-VP, 4-vinylphenol; 4-VG, 4-vinylguaiacol; MVDA, multivariate data analysis; FTMIR, Fourier transform mid-infrared; ATR, attenuated total reflectance; REA–PFGE, restriction endonuclease analysis–pulsed field gel electrophoresis.

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amount of characteristic chemical information and is referred to as the 'fingerprint' region (Smith, 1999). Since the MIR absorbance spectrum contains information about the chemical composition of a substance, calibrations to quantify specific individual components have to be developed for each. The global calibrations for the FTMIR currently include the standard wine parameters (e.g. pH, VA, ethanol, malic acid, lactic acid etc.) as quantifiable compounds. In addition to the quantified wine parameters obtained from such spectral analysis, FTMIR spectra produced from scanning a wine could also serve as a spectral fingerprint of a wine at a given time. This has already been used as a rapid screening tool of the fermentation profiles of wine yeasts and shown to differentiate between wines made with different commercial *Saccharomyces cerevisiae* strains (Nieuwoudt et al., 2006; Osborne, 2007). Additionally, FTIR spectroscopy has also been investigated as a tool for rapid differentiation of bacterial and yeast strains (Essendoubi et al., 2005; Naumann et al., 1991; Ngo-Thi et al., 2003; Wenning et al., 2002).

The primary goal of this study was to evaluate the potential of infrared spectroscopy as a discriminating tool for *B. bruxellensis* wine isolates. The first objective was to investigate FTIR transmission spectroscopy on spoiled wines from 11 *Brettanomyces* isolates. The second objective was to determine if FTIR-attenuated total reflectance (FTIR-ATR) when applied to whole cells could discriminate between the *Brettanomyces* isolates. Multivariate data analysis was applied on all generated data.

2. Materials and methods

2.1. Yeast strains and maintenance

The *B. bruxellensis* strains used in this study (SA1, SA29, SA33, SA35, SA36, SA39, SA45, SA47, SA59, SA60 and SA61) were all from the IWBT laboratory culture collection (Institute for Wine Biotechnology, Stellenbosch University, South Africa) and were isolated from various wines from the Stellenbosch wine-producing region. Yeasts were maintained on YPD agar medium (glucose 20 g/L, yeast extract 10 g/L, peptone 10 g/L, agar 25 g/L) containing cycloheximide 50 mg/L (Sigma-Aldrich, Steinheim, Germany), chloramphenicol 30 mg/L (Roche, Mannheim, Germany) and kanamycin 30 mg/L (Roche). Incubation occurred over a period of 5-11 days at 30 °C.

2.2. Strain identification and molecular differentiation

Two molecular techniques were applied to differentiate the strains among the 11 isolates of *Brettanomyces*, namely Pulsed Field Gel Electrophoresis (PFGE) and Restriction Endonuclease Analysis–Pulsed Field Gel Electrophoresis (REA-PFGE). These methods were performed as previously described by Miot-Sertier and Lonvaud-Funel (2007).

2.3. Small scale wine fermentations

All strains used in this study were evaluated for volatile phenol production in a Shiraz red wine. The wine was sampled after alcoholic fermentation (AF). Prior to inoculation, the wine was pre-treated. The natural microbial flora was inhibited with the addition of 200 mg/L dimethyldicarbonate (DMDC, Velcorin®) whereafter the wine was kept at 8 °C for one week before filtration (0.45-μm filters; Millipore, USA). The wine was additionally diluted with sterile distilled water to lower the final ethanol concentration from 14.5% to approximately 10%. Standard wine analyses were done on the wine before inoculation with the various *B. bruxellensis* isolates (Table 1). The concentrations of volatile phenols of the wine are also given in Table 1. The free measurable hydroxycinnamic acid precursors were measured at 1.6 mg/L and 1.7 mg/L for *p*-coumaric acid (*p*-CA) and ferulic acid (FA), respectively. The *p*-CA concentration was adjusted by spiking 10 mg/L *p*-CA to the wine. All *Brettanomyces* strains were pre-cultured in YPD media containing 10 mg/L *p*-CA for 5 days at 30 °C. Wines were inoculated with ~10^4 cells/mL in 100 mL of wine and were incubated (without agitation) at 30 °C. Samples were drawn for microbial analysis after 7, 14 and 21 days, respectively. Analysis of volatile phenols and standard wine parameters were performed at day 0 and day 21. Experiments were done in triplicate.

Table 1
Standard wine measurements and volatile phenol concentrations of the wine before inoculation with *Brettanomyces bruxellensis* isolates.

Standard parameters		Volatile phenols (ug/L)	
pH	3.69	4-vinylphenol	327
Volatile acidity	0.43 g/L	4-vinylguaiacol	Nd
Total acidity	4.54 g/L	4-ethylphenol	Nd
Malic acid	0.07 g/L	4-ethylguaiacol	Nd
Lactic acid	0.56 g/L		
Residual sugar	0.6 g/L		
Ethanol	9.79 %v/v		
Glycerol	10.42 g/L		

nd = not detected.

2.4. FTMIR spectral analyses of the wine

FTMIR (transmission) spectra were generated by using a FOSS Winescan FT120 instrument (software version 2.2.1) equipped with a purposely built Michelson interferometer (FOSS Electric A/S, Hillerød, Denmark). Wine samples were first centrifuged and then filtered by vacuum pump prior to analysis. Duplicate spectra were acquired in the spectral range of 4992.25 to 929.778 cm^{-1} for each sample and the spectra were averaged for data processing. Samples were preheated to 40 °C in a heater block before analysis. Each spectrum is based on an average of 20 repeat scans at 4 cm^{-1} intervals under fixed instrument settings as described by the supplier of the spectrometer (Winescan FT120 Type 77110 and 77310 Reference manual, FOSS Electric, Denmark, 2001). The background absorbance of water was corrected by doing a zero setting using the FOSS Zero liquid S-6060 prior to the sample and so obtaining a zero-beam spectrum. FTIR data are recorded as absorbencies.

2.5. Standard wine analysis

Quantified chemical data were obtained from the FTMIR analysis to indicate the distribution of standard wine measurements listed in Table 1. These components are important to monitor as they can indicate major variation between samples originating from the different *Brettanomyces* strains. The conversion of the absorbance spectra into quantifiable results was achieved with the commercially available calibrations referred to as global calibrations (provided with the Winescan FT120 instrument). These global calibrations are constructed on the basis of a partial least squares (PLS) regression. Absorbance at selected wave numbers (cm^{-1}) or groups of wave numbers are used to generate a regression algorithm which best fit the reference values in the data set (Patz et al., 2004).

2.6. Volatile phenol analysis

The method used for volatile phenol analysis entailed sample preparation by means of ether extraction followed by Gas Chromatography–Flame Ionisation Detection (GC–FID) analysis. A volume of 50 μL of the internal standard (200 mg/L 3,4-dimethylphenol in absolute ethanol) was added to 15 mL of the sample and extracted with 2 mL of diethyl ether (BDH, Lancashire, UK) for 30 min on a rotary mixer. A volume of 2 μL of the organic phase was injected into a Hewlett Packard HP 5890 Series II Gas Chromatograph (Avondale, PA, USA) fitted with a FID. The column employed was a HP-INNO Wax