Food Microbiology 28 (2011) 1483–1491

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence *in situ* hybridization, flow cytometry and quantitative PCR

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ARTICLE INFO

Article history:
Received 30 November 2010
Received in revised form
29 July 2011
Accepted 7 August 2011
Available online 12 August 2011

Keywords:
Wine
Culture-independent methods
RNA stability
Yeast population dynamics

ABSTRACT

Traditionally, it was assumed that non-*Saccharomyces* (NS) yeasts could only survive in the early stages of alcoholic fermentations. However, recent studies applying culture-independent methods have shown that NS populations persist throughout the fermentation process. The aim of the present work was to analyze and quantify *Saccharomyces cerevisiae* (Sc) and *Hanseniaspora guilliermondii* (Hg) populations during alcoholic fermentations by plating and culture-independent methods, such as fluorescence *in situ* hybridization (FISH) and quantitative PCR (QPCR). Species-specific FISH probes labeled with fluorescein (FITC) were used to directly hybridize Sc and Hg cells from single and mixed cultures that were enumerated by epifluorescence microscopy and flow cytometry. Static and agitated fermentations were performed in synthetic grape juice and cell density as well as sugar consumption and ethanol production were determined throughout fermentations. Cell density values obtained by FISH and QPCR revealed the presence of high populations (10^7–10^8 cells/ml) of Sc and Hg throughout fermentations. Plate counts of both species did not show significant differences with culture-independent results in pure cultures. However, during mixed fermentations Hg lost its culturability after 4–6 days, while Sc remained culturable (about 10^8 cells/ml) throughout the entire fermentation (up to 10 days). The rRNA content of cells during mixed fermentations was also analyzed by flow cytometry in combination with FISH probes. The fluorescence intensity conferred by the species-specific FISH probes was considerably lower for Hg than for Sc. Moreover, the rRNA content of Hg cells, conversely to Sc cells, remained almost unchanged after boiling, which showed that rRNA stability is species-dependent.

1. Introduction

The transformation of grape must into wine involves the coexistence and succession of different yeast species. The microorganisms present on the berry surfaces are composed mainly of non-*Saccharomyces* (NS) yeasts which predominate during the early stages of the alcoholic fermentation. These are soon overtaken by the growth of *Saccharomyces cerevisiae* (Sc) which dominates the mid to final stages of the fermentation (Fleet and Heard, 1993; Fleet, 2003). This typical growth pattern has long been accepted and established mainly by plating methods. Indeed, more recent studies have questioned this pattern after using molecular methods that reveal the persistence of NS populations throughout the fermentation process (Fernández et al., 1999; Cocolin et al., 2000; Andorrà et al., 2010; Zott et al., 2010). The causes of the early displacement of NS wine yeasts are still controversial. Previously, it was thought that this was mainly due to the lower tolerance of NS species toward the increasingly adverse conditions (low pH values, high levels of ethanol and organic acids, nutrient depletion, etc.) established in the medium as the fermentation progresses (Fleet and Heard, 1993). More recently, the dominance of Sc has been attributed to other factors such as growth arrest mediated by cell-to-cell contact mechanisms (Nissen et al., 2003) and the secretion of toxic compounds (Pérez-Nevado et al., 2006; Albergaria et al., 2010). However, most of these studies have been carried out by using classic plating methods which are laborious, time-consuming, somewhat unreliable (Giraffa, 2004) and which only detect culturable populations.

Molecular techniques have been developed and used to control microbial growth and to characterize the microflora of different processes and environments. These methods are generally faster, more specific, more sensitive, and more accurate and allow the precise study of microbial populations and their diversity (Justé

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doi:10.1016/j.fm.2011.08.009

et al., 2008). Molecular techniques can be used to identify or genotype microorganisms previously grown on a culture media (commonly known as culture-dependent techniques), or they can be applied directly to a sample (known as a culture-independent techniques) (Rantsiou et al., 2005). The application of culture-independent methods gives a better understanding of the true microbial diversity, avoiding the biases that growth and isolation by enrichment platting might introduce. Furthermore, culture-dependent techniques can underestimate the size and diversity of a given population because they do not account for non-culturable populations, such as sublethally injured and/or viable but non-culturable (VBNC) cells, which may fail to grow on plates and are common in wine (Millet and Lonvaud-Funel, 2000). Underestimating VBNC and/or sublethally injured populations can be extremely important in some situations (e.g. food contaminants and/or pathogens) since these cells are still metabolically active (Oliver, 2005; Mills et al., 2008) and can recover growth under certain conditions. The difference between the total population of a microorganism and its culturable subpopulation within a given environment corresponds to its VBNC subpopulation and indicates the stress effect induced by that environment on the physiological state of the cells. For this reason, to better analyze a microbial population both molecular and plating methods should be used.

Several culture-independent techniques have been developed and used for detecting and quantifying wine yeast species (Cocolin et al., 2000; Mills et al., 2002; Phister and Mills, 2003; Hierro et al., 2006, 2007; Andorrà et al., 2008, 2010). One of the most promising of these methods, due to its simplicity and rapidity, is the fluorescence *in situ* hybridization (FISH). This technique combines the simplicity of microscopic observation with the specificity of DNA/RNA analysis. Furthermore, most of the molecular techniques do not provide information regarding the morphology, number and spatial distribution of cells within a given environment. In theory, the FISH technique can detect single cells but in practice the detection limit is often 10^4 cells/ml since enumeration is usually carried out by hemocytometry. This limitation can be overcome by concentrating the samples prior to hybridization and counting (Blasco et al., 2003). Another limitation of using hemocytometry is that it does not have sufficient automation for high throughput sample analysis (Amann et al., 2001). This can be solved by using flow cytometry (FC) in combination with FISH for the selective enumeration of mixed microbial populations, which allows a high resolution and highly automated analysis (Amann et al., 1990). The main advantage of this technique is its sensitivity since it can detect one cell in a million. FC in combination with FISH probes has been used to identify and analyze mixed microbial populations (Amann et al., 1990; Wallner et al., 1993; Rigottier-Gois et al., 2003).

Xufre et al. (2006) developed 26S rRNA gene probes for identification of numerous wine-related yeast species, including Sc, *Candida stellata*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii* (Hg), *Kluyveromyces thermotolerans*, *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Pichia membranifaciens* and *Pichia anomala*. Stender et al. (2001) detected the slow growing yeast *Dekkera bruxellensis*. These authors, however, used cultivation and isolation steps prior to applying the FISH technique and thus those results did not account the overall cell population but rather just the culturable cells.

The aim of the present work was to use plating and culture-independent methods based on RNA (FISH) and DNA (QPCR) to analyze Sc and Hg populations. These methodologies were used for the direct quantification and identification of Sc and Hg populations in single cultures and mixed fermentations performed on synthetic media and on a simulated wine fermentation. FC in combination with FISH probes was used to quantify the relative fluorescence intensity of hybridized cells of Sc and Hg during the course of mixed fermentations and also to determine the stability of the rRNA content of the cells.

2. Materials and methods

2.1. Yeasts strains, inoculum cultures and growth media

The yeasts strains used were Sc CCMI 885 (Culture Collection of Industrial Microorganisms, LNEG, Lisbon, Portugal) and Hg NCYC 2380 (National Collection of Yeast Cultures, Norwich, UK). Both strains were first isolated from Portuguese wines, Sc from Alentejo and Hg from Douro wine producing regions. Yeasts were maintained on YMPD-agar slants (1% dextrose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2% agar, w/v) and stored at 4 °C.

Inocula of Hg and Sc were prepared by transferring the biomass of one YMPD-agar slant (pre-grown for 48 h at 30 °C) into 50 ml of YMPD medium in 100 ml flasks which were incubated for 16 h at 30 °C and 150 rpm.

Single cultures of Sc and Hg were performed on YMPD media and mixed fermentations were performed on synthetic grape juice (SGJ) (D-glucose, 110 g/L; D-fructose, 110 g/L; L-(1)-tartaric acid, 6.0 g/L; L-(2)-malic acid, 3.0 g/L; citric acid, 0.5 g/L; YNB (yeast nitrogen base), 1.7 g/L; CAA (Casamino Acids), 2.0 g/L; $CaCl_2$, 0.2 g/L; arginine-HCl, 0.8 g/L; L-(2)-proline, 1.0 g/L; L-(2)-tryptophan, 0.1 g/L; pH 3.5), prepared as described by Pérez-Nevado et al. (2006).

2.2. Single cultures on YMPD medium

Single cultures of Sc and Hg were carried out in 500 ml flasks filled with 250 ml of YMPD medium. Each flask was inoculated with 1×10^4 cells/ml of the respective yeast strain and incubated without agitation at a constant temperature of 20 °C. Both fermentations were performed in duplicate and monitored by plate counting and by direct application of the FISH technique, using species-specific FITC-labeled probes and DAPI staining. Hybridized cells were counted in a Neubauer chamber using an epifluorescence microscope (Olympus BX-60, Tokyo, Japan).

2.3. Alcoholic fermentations

Two mixed fermentations (shaken and static) were performed in duplicate in 500 ml flasks filled with 250 ml of SGJ and inoculated with 1×10^5 cells/ml of each yeast strain (Hg and Sc). Shaken fermentations were conducted under constant agitation of 100 rpm in an orbital shaker (Unitron, Infors, Switzerland). All the experiments were conducted at a controlled temperature of 20 °C. The fermentations were monitored by taking daily samples to quantify cellular density and determine sugar consumption and ethanol production. The yeast population was determined by the classic plating method and also by FISH and QPCR.

To simulate the second part of the alcoholic fermentation, a commercial red wine was diluted with sterile distilled water until the ethanol concentration reached 30 g/l. The medium was supplemented with glucose and fructose to attain a final concentration of 55 g/l for each sugar and 3 g/l of yeast extract and thus simulate the average sugar concentration of a mid-point of a wine fermentation. This medium was then inoculated with 10^6 cells/ml of each strain (Sc and Hg) with inocula previously grown (for 16 h at 30 °C) on YMPD broth. The fermentations were conducted in duplicate with constant agitation (100 rpm) at a controlled temperature of 20 °C. Sugar consumption was monitored daily by both density measures and by HPLC. Samples were taken every 24 h until the end of fermentation (sugars below 2 g/l). Yeasts were monitored using plating counts, and by direct application of the FISH (with species-specific probes and DAPI) and QPCR techniques.