RAPID DETECTION AND IDENTIFICATION OF YEASTS IN MUST AND WINE BY FLOW CYTOMETRY AND FISH

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INTRODUCTION

In winemaking, natural grape juice fermentation is carried out by a succession of different yeast populations. The alcoholic fermentation is generally dominated by *Saccharomyces cerevisiae*, but in later stages of fermentation may appear the spoilage yeast *Brettanomyces bruxellensis* and other yeasts such as genera *Pichia*, *Kluyveromyces*, *Candida* or *Hanseniaspora*. Their detection is very important, for it gives to wine unpleasant flavours.

Both morphophysiological and molecular methods have been used to study the dynamics of yeast populations during wine fermentations [1-3]. While classical methodologies used to identify the species present are laborious, time-consuming and somewhat unreliable [4], genotyping methods, such as DGGE [5], RFLP [6, 7], RAPD [8, 9], MSP-PCR [10], electrophoretic karyotyping [11], and optimized interdelta sequence analysis [12] have been successfully applied to differentiate yeast species or strains.

In the last decade, fluorescence in situ hybridization (FISH) became the method of choice for the direct detection and identification of microorganisms [13, 14], since it combines the direct visualization with the reliability of molecular methods. In wine-related applications, this technique has been used both for the rapid monitoring of lactic acid bacteria [15, 16] and for the detection of the slow growing yeast *Brettanomyces bruxellensis* among others [17, 18].

The D1/D2 domains at the 5´ end of the 26S rRNA subunit provide an excellent basis to develop species-specific FISH probes for yeast, due to a high degree of interspecies sequence variation. These domains have been used for identification as well as in phylogenetic studies [18-20]. Due to the nucleotide sequence variability and to the large number of sequences available in public databases, this region provides an excellent basis to design species-specific FISH probes targeting the rRNAs of yeasts [17, 21, 22].

Flow cytometry (FCM) is a technique that permits the rapid, optical analysis of individual cells, and has many advantages over conventional cytometry. Firstly, since measurements rates of 1000 to 25000 cell sec^{-1} can be achieved (depending on the brand/model of the instrument), flow cytometric data sets often represent 10000 to 500000 cells for a given population. This leads to the relatively straight-forward acquisition of statically significant results (measurements made by light or fluorescence microscopy on at best a few hundred cells). By analyzing many more cells than would be possible by conventional cytometry, rare cells types are more likely to be detected. Secondly, since FCM uses very sensitive electronic detectors to measure the intensity of scattered light or fluorescence at a given wavelength, different intensities of light scatter/fluorescence can be distinguished. By calibrating an instrument with samples of known size or fluorescent intensity, it is possible to obtain quantitative measurements of sample heterogeneity. Thirdly, by using dichroic filters to optically separate light of different wavelengths, flow cytometric measurements can be made on several different characteristics of each cell. Such multiparametric measurements are useful because they allow to correlate the different characteristics and thus define subpopulations and/or distinguish between different cell types. Typical commercial flow cytometers allow 5-10 different parameters (e.g. size, protein content, DNA content, lipid content, antigenic properties, enzyme activity, etc) to be collected for each cell, allowing a multidimensional representation of a population to be obtained. Finally, since measurements are made on single cells, heterogeneity

within the population can be detected and quantified. Thus, flow cytometric analysis enables the investigator to build up a multidimensional representation of the individual cells within a population [23]. Furthermore, FCM is a rapid technique for cell-by-cell multiparameter analysis that is often used in combination with fluorescent labelling [24]. The forward-angle light scatter (FSC), the side-angle light scatter (SSC), and the fluorescence at selected wavelengths are measured. Subpopulations can be identified and distinguished when they differ in light scatter or fluorescence characteristics. Also, subpopulations can be physically selected (sorted) for further study. FCM in combination with fluorescent labeling is increasingly applied in microbiology. It is used in counting the total number of bacteria and in detecting specific strains by 16S rRNA sequence or by antigen expression. It is also used for characterizing and quantifying cellular physiological parameters such as DNA content, enzyme activity, respiration, membrane potential, intracellular pH, and membrane integrity [25-29].

In this study we used fluorescent oligonucleotide probes targeted to the D1/D2 region of the 26S rRNA of different yeast species known to be involved in the vinification, such as *Saccharomyces cerevisae* and *Brettanomyces bruxellensis*. The aim of this was that the species-specific FISH probes were now applied to samples of vinification. To achieve this goal, we have developed an optimized method for the detection and identification of yeasts in musts and wines by FCM and epifluorescence microscopy.

MATERIAL AND METHODS

Yeast strains

Reference strains from yeast species were used to test and validate species-specific DNA probes designed for FISH application. The reference strains were obtained from CECT (Colección Española de Cultivos Tipo, Spain). *Brettanomyces bruxellensis* CECT 1009, *Saccharomyces cerevisiae* T73, *Candida stellata* CECT 11046, *Hanseniaspora guilliermondii* CECT 11027, *Hanseniaspora uvarum* CECT 1444, *Kluyveromyces marxianus* CECT 1018, *Kluyveromyces thermotolerans* CECT 1962, *Pichia anomala* CECT 1114, *Pichia membranaefaciens* CECT 11982, *Torulaspora delbrueckii* CECT 11199.

Yeast culture conditions

Yeast strains were grown in five different media such as culture media, white must, white wine, red must and red wine, under continuous shaking (150 rpm) at 25°C. First, yeasts were growth in GPYA medium (containing 40 g l^{-1} glucose, 5 g l^{-1} peptone, 5 g l^{-1} yeast extract). Musts and wines were filtered by 0.22 µm previously to yeasts inoculation. Yeasts were inoculated in musts and wine previously described at concentration of 10^6 cfu ml^{-1}. The cultures were incubated for 3 days.

Oligonucleotide probes

Specific oligonucleotide probes for *in situ* hybridization previously designed were used to target the rRNA for each species, and a universal probe for eukaryotes. The probes were labelled at the 5´ end with fluorochrome 6-Fam (carboxyfluoresceine), synthesized by MWG-Biotech (Ebersberg, Germany), and then tested for specific binding against the 16S rRNA of the yeast listed in Table 1.

Table 1. Sequences of fluorescein-labelled oligonucleotide probes targeting the different yeast species 26S rRNA D1/D2 region.

Probe	Position	Sequence (5´→3´)	Specificity	Reference
EUK516	502-516	ACCAGACTTGCCCTCC	Eukaryotic	[30]
Sce	D527	TGACTTACGTCGCAGTCC	*S. cerevisiae*	[18]
26S-D.brux.5.1	879-898	CTTACTCAAATCCCTCCGGT	*B. bruxellensis*	[31]

Fluorescence in situ *hybridization*

Cells permeabilization

Cells in exponential growth phase (OD_{600} = 0.5) or in wine and must samples were harvested by centrifugation (10 min 12000 rpm), and washed twice with 1 ml sterile phosphate-buffered saline solution (PBS) at pH 7.4 (130 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4). After, there were added different Triton X-100 solutions (0, 0.5, 2.5, 5 mg ml^{-1} Triton X-100 (Sigma) in buffer PBS). Solutions of permeabilization were incubated at 40°C for 10 min. Treatments were stopped by washing with PBS.

Cells fixation

Permeabilized cells were fixed with 4% (v/v) paraformaldehyde at 4°C for 4 h. Fixed cells were stored by adding a 1:1 mixture of PBS and 96% (v/v) ethanol at -20°C.

Hybridization of cells

Previously to hybridization, stored cell were washed twice with PBS. Approximately 10^6 of fixed and permeabilized cells were hybridized in 1 ml of hybridization buffer (0.9 M sodium chloride, 0.01 % w/v sodium dodecyl sulphate, 20 mM Tris-HCl) with two different concentration of probes, 1.5 and 5 ng μl^{-1} respectively. Furthermore, we tested different hybridization temperatures (40°C, 43°C and 46°C) and different times of incubation (2, 3 hours and overnight).

After incubation, the hybridization mixture was gently removed with several millilitres of the washing buffer (20 mM Tris-HCl pH 7.2, 0.9 M NaCl, 0.1% SDS) and incubated for 30 minutes at a temperature 5°C over the hybridization temperature.

Analytical methods

Epifluorescence microscopy

Fluorescence of yeasts was detected with a Leica DMRB microscope fitted for epifluorescence microscopy with a 100 W mercury lamp high pressure bulb and Leitz filter blocks I3 (blue light exciter BP 450-490 nm, beamsplitter RKP 510 nm, emitter LP 520 nm). Colour photomicrographs were obtained using Adobe photoshop coupled to image capture system. The exposure times used were 0.10-0.30 s for phase contrast photomicrographs and 10-120 s for epifluorescence photomicrographs.

Flow cytometric analysis

Analysis were performed with a flow cytometer Coulter Epic XL-MCL (Beckman Coulter Inc., Miami, Florida, USA), equipped with a laser of 488 nm. 10000 cells from sample were counted for analysis.

RESULTS

Optimization of hybridization conditions

Hybridization conditions were evaluated by epifluorescence microscopy. The highest fluorescence signal was observed when cultures came from the exponential phase in culture media, because of their high cellular rRNA contents. It is necessary to consider that false negatives or weak signals could be obtained when small numbers or insufficient accessibility of the target molecules are present [32], or because of poor permeability of fixed cells.

Application of permeabilization step in the method was favorable, since there was a greater fluorescent signal in permeabilized yeasts. The worst fluorescent signal was obtained in no permeabilized cells. Permeabilized cells with different concentrations of Triton X-100 showed no difference in the fluorescence signal. The fluorescence signal was higher in cells permeabilized

with 2.5 g l^{-1} and 5 g l^{-1}, with no differences between the latter two. For this reason it has been established in the method a permeabilization step with 2.5 g l^{-1} of Triton X-100 for 10 minutes at 40°C.

As to the temperature of hybridization we observed no significant differences in fluorescent signal in yeasts incubated with probes at three different temperatures. No differences were observed in the hybridization conditions of the three fluorescent probes. Therefore, as temperature of hybridization was set to 46°C and wash temperature was set 51°C, for all three fluorescent probes.

In relation to the concentration of probe used, best results have been observed using a probe concentration of 5 ng μl^{-1}, since it was a higher fluorescence observed in these yeasts.

Respect to time of incubation in hybridization has established in three hours, obtaining better fluorescence signal in yeasts hybridized during this time period.

Evaluation of probe specificity

The specificity of the EUK516, Sce and 26S-D.brux.5.1 oligonucleotide probes was evaluated by performing *in situ* hybridization experiments with pure cultures of *S. cerevisiae* and *B. bruxellensis*. The results show that probes hybridized exclusively to their respective target 26S rRNA (Table 2), resulting in clear-out identification of individual cells. There were no cross-reactions, false negatives, probe precipitation or unspecific probe bindings. Figure 1 shows whole-cell hybridization of some pure cultures of the strains tested. One advantage of the FISH techniques and FCM is that two or more specific probes can be used in the same preparation. Labelling these probes with different fluorochromes allows a visual discrimination between different species.

Table 2. Studied species and results of whole-cell hybridisation with oligonucleotide probes.

	Reaction with probe		
SPECIES	**EUK516**	**Sce**	**26S-Dbrux.5.1**
S. cerevisiae	+	+	-
B. bruxellensis	+	-	+
C. stellata	+	-	-
H. guilliermondii	+	-	-
H. uvarum	+	-	-
K. marxianus	+	-	-
K. thermotolerans	+	-	-
P. anomala	+	-	-
P. membranaefaciens	+	-	-
T. delbrueckii	+	-	-

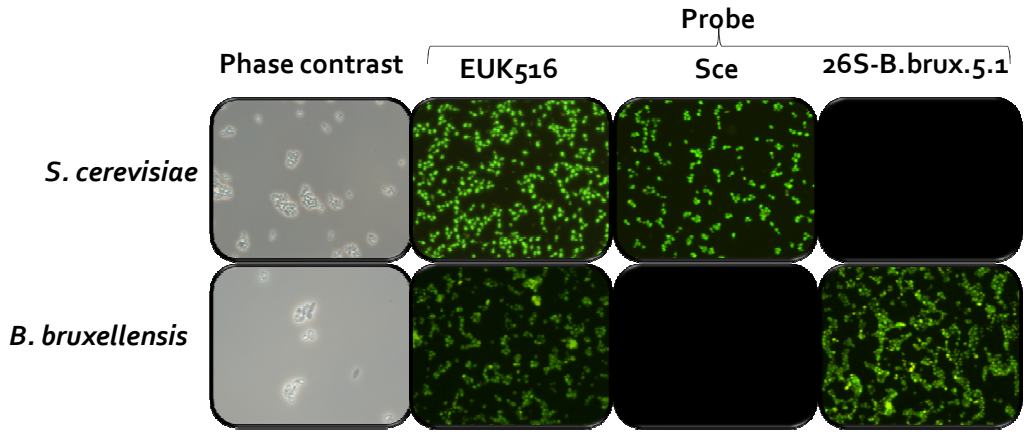

Figure 1. Epifluorescence photomicrographs of yeasts hybridization of pure cultures in culture media.

Detection and identification of yeast from winemaking samples by FCM

We evaluated the ability of detection of the labelled yeasts by FCM at different winemaking samples such as white and red must, white and red wine, and compared to yeast in culture media. Due to different factors such as cell cycle phase, cell wall thickness or stress level, some cells can present low fluorescence signal.

By epifluorescence microscopy, there were no differences detected in the fluorescence intensity of cells among different samples, but by FCM there were detected significant differences in this parameter. The higher ratios of fluorescent cells were obtained in samples of white wines, and the lower ratios were obtained for reds (Figure 2).

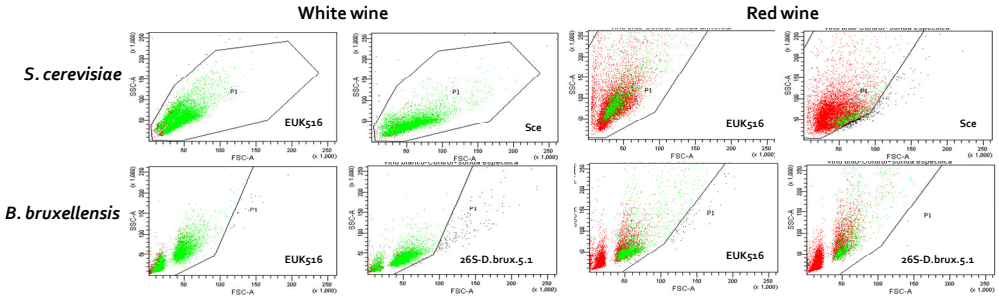

Figure 2. Some examples of FCM dot plots illustrating the identification and counting of S. cerevisiae and B. bruxellensis in white and red wines.

DISCUSSION

In situ hybridization with fluorescent rRNA-targeted oligonucleotide probes represents the most powerful technique for the direct *in situ* identification of single yeast cells in complex microbial ecosystems, such as grapes, must and wine. The possibility of combining probes of different specificity to detect a particular species or genus is an interesting feature of this technique [16, 33].

The identification of the microbiota in grapes, must or wine using traditional methods is laborious and requires several days or weeks. We demonstrated the potential of fluorescent probes for more rapid identification, by epifluorescence microscopy and by FCM. The FISH protocol represents an excellent and rapid method to solve different oenological problems such as detection of species related to wine spoilage or production of toxic compounds, quality control of wines before bottling,

and detection of yeast population dynamics in fermentations. From an oenological point of view, this technique makes it possible to study the population dynamics of the targeted species during the winemaking process.

FISH coupled with microscopy detection Is a commonly applied technique for analysis of specific microorganisms [34]. However, visual counting is difficult and time consuming. The accuracy of this quantification approach is relatively low [35], taking into account the bias associated with interobserver variability. In recent years, the combination of FISH with FCM detection (FISH-FCM) appeared to be a very high throughput method for identification of specific microorganisms [36-38].

Future developments must include increasing the fluorescent signal per ribosome in all species and in all media. The use of alternative fluorochromes must be evaluated too for multiple labelling of different species in a single sample.

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Summary

Flow cytometry (FC) can be considered a useful method for microbiological quality control in wineries, and for the investigation of the growth dynamics of microorganisms in wine. It offers a number of advantages, among them a high speed (and a concomitant detection of thousands of individual cells), high precision, simultaneous measurements of multiple cellular parameters, possibility of detecting the presence of heterogeneous populations (e.g. different species simultaneously), preservation of cell viability and cellular functions, and easiness to use. In this work, we have adapted previous fluorescence in situ hybridization (FISH) protocols for yeasts to FC, optimizing at the same time a liquid hybridization protocol. For this, we used specific fluorescein-labelled oligonucleotide probes targeted to the D1/D2 region of the 26S rRNA of different yeast species known to occur frequently in wine, both spoilage and beneficial. To optimize the hybridization protocol, we tested different parameters such as yeast growth, fixation and permeabilization of cells, hybridization parameters, and others. Then, the potential of using FC in combination with fluorescent probes for rapidly detection and identification of yeasts in laboratory media, musts and wines (reds and whites) was confirmed. One important advantage is that the hybridized yeasts in liquid can be now examined under a microscope (FISH) and/or by FC. Hybridization with fluorescent probes is extremely specific, which facilitates the identification and counting of the different species of yeasts in the same wine with a single analysis.