Analysis of ribosomal RNA stability in dead cells of wine yeast by quantitative PCR

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ABSTRACT

During wine production, some yeasts enter a Viable But Not Culturable (VBNC) state, which may influence the quality and stability of the final wine through remnant metabolic activity or by resuscitation. Culture-independent techniques are used for obtaining an accurate estimation of the number of live cells, and quantitative PCR could be the most accurate technique. As a marker of cell viability, rRNA was evaluated by analyzing its stability in dead cells. The species-specific stability of rRNA was tested in *Saccharomyces cerevisiae*, as well as in three species of non-*Saccharomyces* yeast (*Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Starmerella bacillaris*). High temperature and antimicrobial dimethyl dicarbonate (DMDC) treatments were efficient in lysing the yeast cells. rRNA gene and rRNA (as cDNA) were analyzed over 48h after cell lysis by quantitative PCR. The results confirmed the stability of rRNA for 48h after the cell lysis treatments. To sum up, rRNA may not be a good marker of cell viability in the wine yeasts that were tested.

Keywords: Cell lysis; Dimethyl dicarbonate (DMDC); Heat; Wine yeasts.

1INTRODUCTION

Microorganisms are the main actors in wine industry. Consequently, microbial analyses are critical for understanding fermentation process, for detecting spoilage microorganisms, and for further controlling or improving wine quality (Mills et al., 2002). In the last century, culture-independent methods have been developed to detect and quantify the main microorganisms (Cocolin et al., 2013; Hierro et al., 2006). However, it is an issue to consider whether these methods could accurately detect viable microorganisms without including dead cells.

During wine fermentation, microorganisms undergo a series of stresses (decreases in nutrients, the appearance of toxic new compounds such as ethanol, and interactions with other microorganisms) that challenge the viability of yeast cells (Wang et al., 2016). Due to these challenges, live cells can exist in different states; it is important to detect and quantify all live cells, because they can affect the progress of alcoholic fermentation and the final wine quality (Fleet, 2008). Specifically, the Viable But Not Culturable (VBNC) state has been defined as metabolically active cells that cannot undergo cellular division in growth medium (Oliver, 1993). But VBNC cells are included in viable cells because they have the potential of resuming growth and achieving full metabolic activity, which can affect the wine-making process. In wine, the presence of cells in the VBNC state has been attributed to the use of fungistatic and bacteriostatic compounds, such as SO_2 (Divol and Lonvaud-Funel, 2005), as well as to the interactions between different wine yeasts (Wang et al., 2016).After removing the two factors by resuscitation assays, VBNC yeast cells can recover to the normal state (Salma et al. 2013; Wang et al., 2016). Therefore, VBNC state can be considered as a transition state of yeast from culturable cells to dead cells (Branco et al., 2015; Wang et al, 2016). During this transition process, yeast cells lose the ability to form colonies, and the permeability of cell membrane change as well as the intracellular pH (Andorrà et al. 2010;

Branco et al. 2015; Wang et al. 2015). Therefore, due to the lack of growth of VBNC cells and dead cells, how to distinguish them without underestimating or overestimating dead cells is the question. It cannot be realized by traditional culture-dependent methods without resuscitation; the only alternative is to find culture-independent methods that includeVBNC cells and exclude dead cells when quantifying the total viable population. These types of techniques can detect cells despite their growth abilities, and they are faster, more sensitive, and more accurate (Cocolin et al., 2013). Therefore, these culture-independent methods can be used as a tool to better understand the true microbial diversity and allowing the accurate study of microbial populations (Cocolin et al., 2013). Since 2000, several culture-independent techniques have been used to monitor wine fermentation; all of these techniques target genetic material. It was found that some wine yeasts not recovered on culture plates were detected when using culture-independent techniques (Cocolin et al. 2000). This opened a new field of study, focused on knowing not only the conditions that cause the loss of culturability but also ways to detect non-culturable cells and differentiate them from dead cells. Among these techniques, quantitative PCR (qPCR) is promising because it can detect and quantify the population of each yeast species during wine fermentation (Hierro et al., 2006). Although it requires specific primers, which allows quantification of only the targeted microorganisms (Andorrà et al., 2010), qPCR has some advantages: it can be used to process a large number of samples (Bleve et al., 2003), and it is highly sensitive, which allows the quantification of yeast from 1 or 10 cells/mL to 10^8cells/mL depending on yeast species.

To detect all viable cells, culture-independent techniques have to target cell components that are stable in viable cells but absent in dead cells. DNA is a common target used in most culture-independent analyses because it does not change in different physiological cell states; in fact, it is very stable in dead cells (Allmann et al., 1995; Andorrà et al., 2011; Bleve et al., 2003), even for thousands of years (Cocolin et al., 2013). Therefore, its presence does not

imply the existence of viable cells, and targeting DNA results in an overestimation of the viable population (Wang et al. 2015). Messenger RNA (mRNA) is turned over rapidly in viable cells, so it could be a good indicator of cell viability (Bleve et al. 2003). Unfortunately, mRNA is normally present in low quantities, and it is unstable and varies among different physiological states, which would affect the accuracy of quantification if it is used as a target, resulting in either the underestimation or overestimation of the cell population (Hierro et al., 2006). Ribosomal RNA (rRNA) represents the 80-85% of the total RNA in yeast cells (Von der Haar, 2008); furthermore, it seems to be more stable than mRNA in dead cells but less stable than DNA (Fontaine and Guillot, 2003). For these reasons, rRNA has been proposed as a target for culture-independent techniques to detect the total viable cell population. This topic has been poorly studied, despite its great importance, as rRNA could be an excellent target for use in quantifying the total viable population.

To use rRNA as a target for differentiating live and dead cells, it is necessary to understand what rRNA reflects in a given ecosystem (Cocolin and Mills, 2003). Until now, limited studies have focused on the evaluation of rRNA stability in lysed wine yeast cells (i. e. dead cells of wine yeast after lysis treatment). Hierro et al. (2006) reported that in cells lysed by heat treatment, the D1/D2 domains of 26S rRNA was more stable than mRNA but less stable than DNA encoding the 26S rRNA. Other studieshave reported that the stability and the rate of degradation depend on the lysis treatment applied (ethanol and boiling treatments have different effects) (Andorrà et al., 2011; Wang et al., 2015) and the yeast species (Andorrà et al., 2011). Therefore, based on those results, the aim of this study is to test rRNA stability in lysed cells of different wine yeast species (*Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Torulaspora delbrueckii*), with the stability of corresponding rRNA gene as comparison. These species were treated using different lysis methods: heat shock and dimethyl dicarbonate (DMDC). DMDC is an antimicrobial

compound used as a cold sterilization agent in wine to lyse yeasts before inoculation with lactic acid bacteria for malolactic fermentation. It is authorized in the EU for use as a food additive for wines in concentrations up to 200mg/L (EFSA Panel on Food Additives and Nutrient Sources Added to Food (ANS)).

2 MATERIAL AND METHODS

2.1 Yeast strains

The wine yeast strains *S. cerevisiae* NSa, *H. uvarum* CECT13130 and *S. bacillaris* NSc are natural isolates from wines and are maintained in our group collection (Wang et al., 2016). The yeast strain *T. delbruekii* Com (Biodiva) is a commercial strain from Lallemand Inc. (Canada). All the experiments were performed using these four strains. The strains were pre-cultured overnight in YPD medium (1% yeast extract, 2% peptone and 2% glucose, w/v, pH 6.2) at 28°C before use.

2.2 Assays of lysis treatments

Inactivation treatments were tested for the four yeast strains. Samples of three cell concentrations (10^6, 10^7 and 10^8cells/mL) were pelleted by centrifugation. After washing with Milli-Q water, the pellets were suspended in 1 mL of Milli-Q water and submitted to two types of treatments: heat shock and DMDC (Velcorin^{TM}, Santa Cruz Biotechnology, USA). Although different times of heat shock (5, 10, 20 and 30min) were tested at 95°C, 5min was enough to lyse all the cells. For DMDC, the common dosage used in wine is 0.25g/L, and here two different concentrations (1.25g/L and 12.5g/L) were tested at 4°C for 24h. The latter concentration lysed all cells. Cells were considered lysed when no growth was seen on YPD agar and in YPD broth (the assessment of OD value) after 48h.

2.3 Harvesting of lysed cells at different time points

Cells were collected at the beginning of the stationary phase and submitted to heat shock (95ºC for 5min) or DMDC treatment (12.5g/L at 4ºC for 24h). After receiving the treatments, cells were pelleted and washed to stop the influence from treatments. Treated cells were maintained in water suspension at 28°C for 48h, and the samples were harvested at 0 (control, immediately after the treatments), 12, 24, 36 and 48h after the treatments. Pellets from all lysed cells were fast-frozen using liquid nitrogen and stored at -80°C for further quantification of the genetic material. The entire assay was done in triplicate.

Before each lysis treatment, cells were collected in seventeen Eppendorf tubes, fifteen used as above assay, one for untreated sample, and one for the control sample (0h after each treatment). The effectiveness of the inactivation treatment was checked by plating 100µL of the suspension from the untreated samples and the control samples on YPD-agar plates. These plates were incubated in duplicate at 28ºC for 2 days, and no colonies grew on the plates, except for the untreated samples.

2.4 Nucleic acid extraction and quantification

2.4.1 DNA extraction

DNA was extracted from the pellets stored at -80°C using a DNeasy Plant mini kit (Qiagen, USA), following the procedure from Hierro et al. (2006). The extracted DNA was stored at -20°C.

2.4.2 RNA extraction

RNA extraction was performed on 10-fold diluted samples that were stored at -80°C, using a PureLink RNA mini kit (Invitrogen, USA). To improve the extraction performance, a mixture of 10% mercaptoethanol and lysis buffer from the kit was used with 1g of 0.5-mm-diameter glass beads to break up cells in a mini-bead beater. The manufacturer's instructions were followed to extract RNA with an added procedure of DNase treatment, which included a

15-min treatment with 70µL of RDD buffer and 10µL DNase (RNase-Free DNase Set, Qiagen, USA) at room temperature. The RNA was stored at -80°C.

2.4.3 Reverse Transcription

RNA (1:20 dilution)was converted into cDNA using the PCR reaction described by Hierro et al. (2006). A negative control for each sample was generated by replacing the Superscript II RNase reverse transcriptase (Invitrogen, USA) with water. The cDNA was stored at -20°C.

2.4.4 qPCR

A 7300 Real Time PCR System (Applied Biosystems, USA) was used to perform qPCR reactions. The qPCR reactions were carried out with the primers YEASTF and YEASTR according to the procedures from Hierro et al. (2006), but SyberGreen was replaced with Power SybrGreen Master Mix (Applied Biosystems, USA). Samples (rRNA gene and cDNA) were analyzed in duplicate. And for all reactions, water was used as non-template control (NTC), and one sample of DNA was used as the positive control. cDNA samples were used at a 1:50 dilution, and negative controls from RT-PCR were analyzed to check the background signal of each sample, which confirmed the effectiveness of the DNase treatment.

2.5 Standard curves

Cell pellets were collected from a series of concentrations (from 10cells/mL to 10^8cells/mL) obtained by 10-fold dilutions. After washing with Milli-Q water, these pellets were fast-frozen using liquid nitrogen and stored at -80°C; then, DNA was extracted as described in 2.4.1, and further quantified by qPCR (2.4.4). The $\log_{10}$(cell concentrations) was plotted against the cycle threshold (C_T) values to obtain a standard curve for each yeast species.

2.6 Statistical analysis

The results obtained from qPCR are in the form of C_T values. To interpret the results, they were transformed into cell concentrations by using the DNA standard curve of each strain, and the obtained values were corrected according to the dilution used for the rRNA samples. The corrected cell concentrations were used directly for further variation analysis. The variations in rRNA gene/rRNA within treatments and between treatments were analyzed by one-way ANOVA using IBM SPSS Statistics 22.

3 RESULTS AND DISCUSSION

As the aim of this study was to evaluate the stability of two molecules that are present at very different concentrations in the cells and between species (Wang et al., 2015), we have normalized the results according to the standard curves built based on DNA samples. rRNA had to be diluted by 3 or 4 orders of magnitude to achieve C_T values similar to those of rRNA gene. As a result, in all the analyzed samples, the rRNA gene values were 10^7-10^8cells/mL, whereas the rRNA presented a 10^4-fold higher value than the rRNA gene molecule. The high value of rRNA represent that yeast cells contain many copies of rRNA, and the copy number was around 10^2-10^3 and varied among yeast species with *S. cerevisiae* lower than the other three species (data not shown). The high copies of rRNA would help to increase the sensitivity of qPCR targeting rRNA, whereas it also increases the difficulties to balance the quantification accuracy caused by high dilution fold.

The results of the quantification of rRNA gene and rRNA in dead cells showed very high stability of both molecules in the four analyzed species over 48h. Regarding the stability of rRNA gene and rRNA, the statistical analysis results coincided with the quantification results, with only *H. uvarum* and *T. delbrueckii* showing significant differences in their rRNA gene concentrations after DMDC treatment (Table 1). Although the statistical tests showed significant differences in some rRNA gene samples, the concentrations observed were always

within the same concentration order of magnitude. These results seem consistent with the previous assumption that DNA is highly stable (Cocolin et al., 2013).

The stability of rRNA after heat treatment has been reported before. Hierro et al. (2006) submitted a population of *S. cerevisiae* (10^5 cells/mL) to 60°C for 20min. The RT-qPCR analysis revealed that after 24h, there was a 99.9% decrease in the rRNA concentration. Andorrà et al. (2011) submitted cell suspensions of *S. cerevisiae* and *Hanseniaspora guilliermondii* to 100°C for 20min. According to the data of flow cytometry, the rRNA of *S. cerevisiae* was degraded after 24h, while the rRNA of *H. guilliermondii* was stable for at least 240h. In our study, after treatment for 5min at 95°C, the rRNA from the four analyzed species was stable for 48h. These results agreed with our results for *H. uvarum*, but not for *S. cerevisiae*. The varying results among the studiescould be caused by different quantification method used and different times and temperatures used for heat treatment.

Unlike heat treatment, this is the first report of the stability of rRNA gene and rRNA after DMDC treatment. It appears that both treatments are equally effective in lysing cells, and no differencesin nuclear acids between the treatments were found in *S. cerevisiae* or *T. delbrueckii* (Table 1). *S. bacillaris* presented significant differences in rRNA gene and rRNA quantification between the two treatments (Table 1); treatment with DMDC yielded a higher recovery of both molecules (data not shown). *H. uvarum* also presented significant differences in the amount of rRNA quantified between treatments, but in this case, the concentrations quantified after heat treatment were higher.

Previous studies have reported that rRNA is degraded after cell lysis, although this depends on the yeast species, the quantification method and the treatment. Therefore, rRNA could be a good target for viable cell quantification and potentiallya good indicator of the cell state. Our results do not support this idea, however, as it seems that rRNA is more stable than previously thought. Therefore, targeting rRNA cannot be proper for detecting viable cells of

the species tested because quantifying rRNA would include dead cells, similar to using DNA quantification.

After seeing these results, it would be interesting to assess rRNA stability over long periods of time after cell lysis. Corresponding results will be helpful for assessing the application possibility of this methodology as a control to avoid spoilage during aging and storage. However, long-term rRNA stability would not be applicable to fast processes, such as alcoholic fermentation during wine production. This process is very fast, and stability for more than 48h is a challenge for the quick quantification of microorganisms involved in wine fermentation. Moreover, working with RNA has some disadvantages, such as its proclivity to contamination by RNA-degrading enzymes, which can cause limited reproducibility, and the fact that its manipulation is more demanding than DNA manipulation. Moreover, Hierro et al. (2006) reported that the rRNA concentration seems to depend on the cell's physiological state and the population growth stage, which can influence the accuracy of quantification. Therefore, it would be interesting to test other molecules to find a better target.

We would consider testing the possibility of using mRNA as a marker of cell viability, as it has been proven to be less stable than rRNA in some organisms (Fontaine and Guillot, 2003). However, the main hurdle of using this molecule is that most genes are not continuously expressed during the cell life cycle, so mRNA does not accurately reflect the number of cells (Nadai et al., 2015). Some studies have used mRNA targeting different genes. Bleve et al. (2003) used actin mRNA, which encodes a very conserved protein; in their study, actin mRNA was detected in several yeasts after 24h of heat treatment for populations of 10^8cells/mL. Vaitilingom et al. (1998) applied reverse transcriptase PCR to detect microorganisms with protein synthesis elongation factor, which also encodes one of the most abundant proteins, and this factor is constitutive. Nadai et al. (2015) found some genes (*ALG9*, *FBA1*, *UBC6* and *PFK1*) that are expressed continuously during fermentation,

independent of perturbations to the experimental conditions when sulfite is added to the wine. Therefore, future studies could focus on testing genes that are universal and constant during the cell cycle but that are unstable after cell lysis to detect or even quantify total viable cells.

In summary, the rRNA stability was shown in dead cells of four wine yeast species from two lysis treatments. The results challenge our previous ideas regarding rRNA stability, and rRNA is not a good marker of cellular viability in the set conditions of this study. Therefore, when rRNA is used as an intracellular target for determining live cells, more caution and tests should be taken to avoid overestimating live cells.

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325 Table 1. Variation analysis of the quantification value of rRNA and rRNA gene in/between
326 heat treatment (H) and DMDC treatment (A). (1)-(4) showed the variation in either
327 treatments, and (5)-(8) showed the variation between treatments and molecules. * and **
328 means significance level <0.05 and <0.01 separately.
329

	S. cerevisiae	***H. uvarum***	***T. delbrueckii***	***S. bacillaris***
(1) rRNA gene H	0.452	0.159	0.95	0.831
(2) RNA H	0.974	0.599	0.599	0.6
(3) rRNA geneA	0.327	0.048*	0.013*	0.323
(4) RNA A	0.776	0.651	0.755	0.418
(5) rRNA geneH-A	0.19	0.32	0.972	0.002**
(6) RNA H-A	0.581	0.007**	0.76	0.001**
(7) rRNA gene-RNA H	0.001**	0.005**	0.005**	0.007**
(8) rRNA gene-RNA A	0.012**	0**	0.013*	0**