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Analysis of ribosomal RNA stability for the detection and quantification of wine yeast through RT-qPCR

MASTER THESIS

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Master's Degree in Fermented Beverages

Faculty of Oenology

UNIVERSITAT ROVIRA I VIRGILI

Tarragona
September 01, 2016

Analysis of ribosomal RNA stability for the detection and quantification of wine yeast through RT-qPCR

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ABSTRACT

Some wine yeasts in VBNC state have an important role during alcoholic fermentation, affecting the final wine, thus it is important to detect and quantify them. Advances are being achieved on culture-independent techniques to obtain the total number of live cells, but nowadays there is not a target able to quantify all live cells, and not the dead ones. The aim of this study is to determine the capacity of rRNA to be a viable cell marker, through the analysis of its stability in lysed cells. First, the effect of different lysis treatment was tested in a strain of *S. cerevisiae* and three strains of non-*Saccharomyces*. Treatments with high temperatures, ethanol and antimicrobial DMDC lysed the yeast completely, but the antibiotic cylcloheximide and the different cellular disruption methods (mechanical pressure, sonication and freezing-thawing) did not lyse them. After that, we quantified the rRNA during 48h after lysing the cells (with heat and DMDC). In order to do this quantification, the RNA was extracted and through RT-PCR it was transformed into cDNA, which was quantified with qPCR. The results suggested that rRNA is stable during 48h after cellular lysis, but statistical tests are needed to determine the results significance. At the same time, it was determined that it is necessary to do a lysis treatment before the RNA extraction protocol used here to obtain a real quantification. To sum up, it seems that rRNA is not a good cellular viability marker in the used wine yeast. Therefore, it is necessary to keep looking for a marker to quantify viable cells.

Keywords: *Saccharomyces*, *Hanseniaspora*, *Starmerella*, *Torulaspora*, culture-independent techniques, cell lysis, VBNC, DMDC, heat, qPCR.

RESUM

Algunes espècies de llevats vínics en forma viable però no cultivable tenen un paper important durant la fermentació alcohòlica, afectant al producte vínic final, per això és important detectar-les i quantificar-les. S'estan fent avenços en usar tècniques independents de cultiu per obtenir el nombre total de cèl·lules viables, però encara no s'ha trobat una molècula diana per quantificar totes les cèl·lules vives, i no quantificar les mortes. L'objectiu d'aquest estudi és determinar la capacitat del rRNA com a marcador de cèl·lules viables, mitjançant l'anàlisi de la seva estabilitat en cèl·lules lisades. Primer, es va provar l'efecte de diferents tractaments de lisi en una soca de *S. cerevisiae* i tres de no-*Saccharomyces*, i mentre les altes temperatures, l'etanol i l'antimicrobià DMDC lisaven totalment aquests llevats, l'antibiòtic cicloheximida i els diferents mètodes de disrupció cel·lular (pressió mecànica, sonicació i congelació-descongelació) no els lisaven. Tot seguit es va determinar la quantitat de rRNA durant 48h després de lisar les cèl·lules mitjançant altes temperatures i DMDC. Per fer la quantificació es va extreure el RNA, que mitjançant RT-PCR es va passar a cDNA, que es va quantificar usant qPCR. Els resultats suggereixen que el rRNA és estable durant 48h després de la lisi cel·lular, a falta de fer tests estadístics per determinar la significança dels resultats. Paral·lelament, es va determinar que s'ha de fer un tractament de lisi abans del protocol d'extracció de RNA usat en aquest estudi per obtenir una quantificació real. En conjunt, sembla que el rRNA no és un bon marcador de viabilitat cel·lular en els llevats vínics usats. Per tant, s'han de continuar buscant un marcador per quantificar cèl·lules viables.

Paraules clau: *Saccharomyces*, *Hanseniaspora*, *Starmerella*, *Torulaspora*, tècniques independents de cultiu, lisi cel·lular, VBNC, DMDC, alta temperatura, qPCR.

1. INTRODUCTION

Microorganisms have an important role in the industry of fermented products (i.e. wine), as they perform the fermentation. Thus, in those products the microbial control is necessary, not only to detect contaminants, but also to monitor the production process in order to improve it. We need to develop good detection and quantification methods to study and understand the population dynamics of fermentation.

The techniques used to detect and quantify microorganisms can be classified in culture-dependent and culture-independent. Culture-dependent techniques detect cells capable of growing on a specific medium, thus they cannot do a good estimation of the population, as a high percentage of microorganisms in nature are non-culturable in known medium (Hugenholtz, Goebel, & Pace, 1998). Culture-independent techniques target a cell component that does not vary through the cells physiological state (Luca Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013), such as immunologic or genetic material, so they can detect the cells despite their grown abilities. Those techniques have several advantages in front of the culture-dependent techniques: they are faster, more specific, more sensitive and more accurate (Cocolin et al., 2013).

Moreover, as they do not depend on the cell physiological state, they can detect Viable But Non-Culturable (VBNC) cells, which are metabolically active cells that cannot undergo cellular division on a medium in which they can grow under standard conditions (Oliver, 1993). Normally, cells turn to that state in response to adverse environmental conditions, so it can be considered a survival strategy (Cocolin et al., 2013). The importance of detecting them is based on their potential to return to a culturable state (Lleo, Pierobon, Tafi, Signoretto, & Canepari, 2000) and their potential metabolic activity, through which they can affect the ecosystem: produce a disease, produce spoilage or provide good qualities to the product. Therefore, culture-independent techniques can be used as a tool to understand better the true microbial diversity, allowing a precise study of microbial populations (Cocolin et al., 2013).

In order to detect VBNC cells, one of the biggest challenges of culture-independent techniques is finding a target that is present and non-variable in live cells, and disappear with the cell is dead. DNA has been a very used target, because it does not change according to the physiological characteristics, but it is very stable after death (Allmann et al., 1995; Andorrà, Monteiro, Esteve-Zarzoso, Albergaria, & Mas, 2011; Bleve, Rizzotti, Dellaglio, & Torriani, 2003; Wang, Esteve-Zarzoso, Cocolin, Mas, & Rantsiou, 2015), even it can be stable thousands of years (Cocolin et al., 2013). Therefore, its presence does not mean that it comes from a viable cell, and targeting DNA can result on an overestimation of the population (Wang et al., 2015).

RNA seems to be less stable than DNA (Andorrà et al., 2011; Hierro, Esteve-Zarzoso, Gonzàlez, Mas, & Guillamón, 2006; Wang et al., 2015), for this it is becoming a wide-used target for detecting viable cells population. Messenger RNA (mRNA) is turned over rapidly in viable cells, so it could be a good indicator of cell viability (Bleve et al., 2003). However, there are some problems that difficult the use of this molecule: it is present

at low quantities in the cells and it is very unstable. Those characteristics can lead to problems of degradation during extraction and manipulation, causing underestimation of the cell population (Hierro et al., 2006). Moreover, it varies according to the physiological state of the cell, which can cause either underestimation or overstimation. It has been studied widely in bacteria, but no such much in fungi (Bleve et al., 2003).

Ribosomal RNA (rRNA) represents the 80-85% of the total RNA inside yeast cells (von der Haar, 2008), and it seems to be more stable than mRNA, but less than DNA (Hierro et al., 2006). These are the reasons why it has been proposed as a target for culture-independent techniques to detect total cell population. However, as the number of ribosomes approximately reflects the rate of protein synthesis, its quantity seems to depend on the cell's physiological state and the population growth stage (Hierro et al., 2006), which can influence on the accuracy of quantification. Moreover, its stability seems to be species-dependent (Andorrà et al., 2011). This topic has been poorly studied, despite its great importance, as it could be a very good target to quantify total viable population.

Wine fermentation and processing are environments in which is important the detection and quantification of total cells. When analysing its ecology by culture-dependent techniques, different yeast species are recovered at the initial stages, but at mid and late stages, only *Saccharomyces cerevisiae* strains are detected (Schütz & Gafner, 1993). On 2000, the ecology of the process was analysed by culture-independent methods, and they found that other yeast species (non-*Saccharomyces*) were detected in mid and late stages of the fermentation (L. Cocolin, Bisson, & Mills, 2000). This indicated that yeasts in VBNC state could be present during the fermentation, and that some of those yeasts may have a contribution to the kinetics of yeast growth during alcoholic fermentation (Divol & Lonvaud-Funel, 2005; Fleet, 2008).

These findings increased the interest to investigate the alcoholic fermentation ecology, and opened new fields of study. On the one hand, many reports focused on studying the contribution of those species to the final wine product. On the other hand, some other articles focused on finding techniques to detect and quantify those species. Overall, the objective was to find the relation between the type of cells and the affection of their metabolism to the final wine. Those studies help to potentiate the good characteristics (such as the aromatic profiles and the complexity) and eliminate the bad ones (stuck or slugglish fermentation and wine spoilage) produced by metabolism of VBNC non-*Saccharomyces* populations (Lleixà et al., 2016). They can be a good tool for wine industry: on the one hand, they can help wineries to monitor the fermentation and detecting yeasts that produce spoilage in a faster and more reliable way, because there are some spoilage microorganisms that grow very slow or cannot grow on culture media. On the other hand, they can help to improve wine quality by modifying wine flavour or improve product quality (Mills, Johannsen, & Cocolin, 2002).

From 2000 until now, several culture-independent techniques have been applied to monitor the wine fermentation, all of them targeting genetic material. The first studies (Cocolin et al., 2000; Mills et al., 2002) used Polymerase Chain Reaction Denaturing

Gradient Gel Electrophoresis (PCR-DGGE) and Reverse Transcription-PCR-DGGE (RT-PCR-DGGE), qualitative techniques that targets DNA and RNA, respectively. On 2003, Phister and Mills started to use quantitative PCR (qPCR), which differs from PCR (which only can detect the genetic material it amplifies) in the fact that it can quantify the initial genetic material of the sample, and this can be correlated with the quantity of cells (Phister & Mills, 2003). Hierro et al. were the first to target rRNA using RT-qPCR (Hierro et al., 2006). Andorrà et al. used the chemicals EMA and PMA in a technique that determine only the cells that have intact membrane, targeting the DNA (Andorrà, Esteve-Zarzoso, Guillamón, & Mas, 2010). Andorrà et al. applied FISH targeting DNA and RNA. This technique is not PCR-based, and can give information of the morphology, number and spatial distribution of the cells, but it has a lower detection limit than qPCR (10^4 cell/mL) (Andorrà et al., 2011). There were more studies following or combining those techniques, and the majority detected viable no-*Saccharomyces* at mid and late stages of fermentation.

qPCR is a very promising technique to quantify DNA and RNA of known microorganisms, because it allows to enumerate the population of each yeast species during the fermentation (Hierro et al., 2006). Although it requires specific primers, which only allows to quantify the known microorganisms (Andorrà et al., 2010), it has some advantages: it can process a large number of samples (Bleve et al., 2003) and it has a high perception limit, which allows to detect a species that represents less than 1% of total population.

It is important to differentiate the states of the cell (such as live, VBNC and dead with the genetic material protected from degradation), because they give important information. On the one hand, it indicates us the cell's influence on the environment (as it varies according to the cells metabolic state (Blazewicz, Barnard, Daly, & Firestone, 2013)). On the other hand, it gives information of the presence of cell stresses (as they induce the formation of VBNC cells (Andorrà et al., 2011)). In order to use rRNA as a target to discriminate the number of cells that exist in each state is necessary to know what does the presence of rRNA in the ecosystem reflects (Cocolin & Mills, 2003). We can do it by evaluating the RNA stability of lysed cells, which seems to be species-dependent (Andorrà et al., 2011), so it has to be studied on different strains. Moreover, it is necessary to confirm if rRNA concentration depends on the physiological state of the cell (Hierro et al., 2006), and if it is a good target to distinguish between the living and lysed states. Those tests must be done before continuing targeting RNA as the molecule to assess total concentration of cells, and there are only a few assays doing it.

As lysed cells rRNA seem to be degraded at different rates depending on the lytic process (Wang et al., 2015), different lysis treatments have to be tested. According to this, it would be interesting to test treatments mimicking the alcoholic fermentation conditions that yeast have to face when they become non-culturable, or lysed. The main findings about this topic say that interactions among yeasts can be affected by different factors, such as nutrient concentration (C and N as the main limiting factors), fermentation factors (oxygen, temperature) and yeast metabolites (ethanol, medium-chain fatty

acids, killer toxins) (Albergaria, Francisco, Gori, Arneborg, & Gírio, 2010; Goddard, 2008; Salvadó, Arroyo-López, Barrio, Querol, & Guillamón, 2011; Wang, Mas, & Esteve-Zarzoso, 2016).

Until now, there have been three studies assessing rRNA stability in wine yeast cells. One of them showed that RNA is more stable than mRNA, but less stable than DNA after a heat treatment (Hierro et al., 2006). Another stated that the RNase treatment degraded the rRNA, but a boiling treatment had different effects depending on the species (Andorrà et al., 2011). Finally, another study showed that an ethanol treatment caused a partial degradation of rRNA (Wang et al., 2015). There is a need of further investigation to get more knowledge about the topic.

Moreover, it is interesting to test some extrinsic factors, such as the presence of dimethyl dicarbonate (DMDC), which is used as a cold sterilization agent in wine to lyse the yeasts before inoculating lactic acid bacteria to perform the malolactic fermentation. It is authorized in EU as a food additive for wines in concentrations up to 200 mg/L (EFSA 2015).

The main objective of this study was to determine the stability of rRNA after two different inactivation treatments applied on four different species, one *S. cerevisiae* and three non-*Saccharomyces*. In order to achieve that, on the one hand we determine treatments able to lyse the cells. In this case, cells were considered lysed if they were non-culturable in YPD media after the treatments we performed. On the other hand, to quantify RNA, it was necessary to find a technique to measure RNA concentration. Finally, we determined the DNA and rRNA stability after two lysis treatments.

2. MATERIAL AND METHODS

2.1. General protocol

This study was designed according a main workflow, as explained in Figure 1. It consisted on submitting defined quantities of cells from YPD culture to lysis treatments. We obtained cell pellets at different time points after the treatment, and measured the quantity of DNA and RNA. Moreover, we designed three assays to get preliminary information: (1) To determine the growth curve to set the more appropriate stage of the population dynamics for the treatments. (2) To test the potential of cell lysis of several treatments in order to choose the most effective. (3) To raise the qPCR standard curves to estimate the cell population.

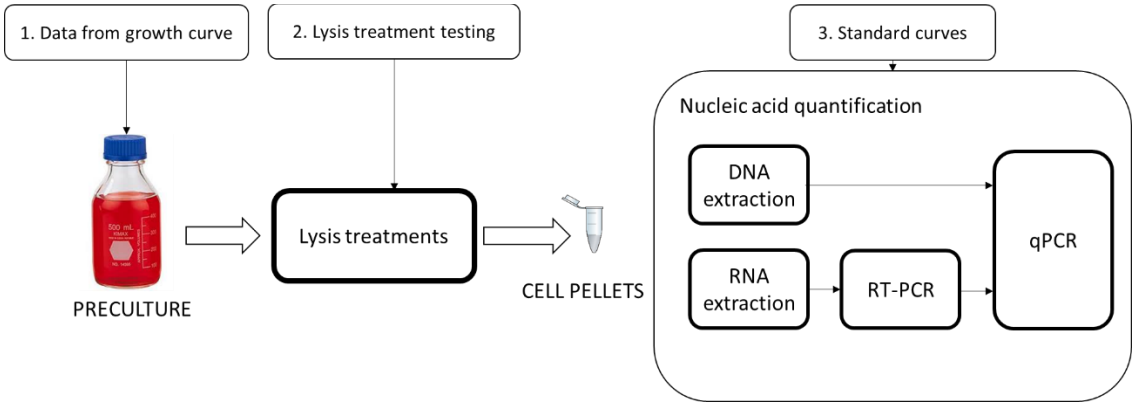

Figure 1. Workflow of the study.

2.2. Yeast strains

The yeast strains of *S. cervisiae* NSa, *Hanseniaspora uvarum* CECT13130 and *Starmerella bacillaris* NSc were natural isolates from wines and were maintained in our group collection (Wang et al., 2016). The yeast strain *Torulaspora delbruekii* com (Biodiva) was a commercial strain from Lallemand Inc. (Canada). They were identified by 5.8S-ITS analysis (Esteve-Zarzoso, Belloch, Uruburu, & Querol, 1999).

All the experiments were performed using these four strains. They were pre-cultured by growing overnight in YPD medium (1% yeast extract, 2% peptone and 2% glucose, w/v pH 6.2) at 28°C before use.

2.3. Species-specific growth curve

We used cells from the beginning of the stationary phase for all of the tests performed. The growth curve of each species was determined to set this point. Each strain was cultivated in YPD medium for several days at 28°C with 150 rpm. We monitored the cell concentration by measuring the OD value (600nm) with the spectrophotometer Ultrospec™ 2100 *pro* (Amersham Biosciences, UK) and by microscope counting, and the

culturable cell concentration by plating on YPD-agar plates (2% glucose, 2% fructose, 1% yeast extract, 1.7% agar). We monitored the cell growth every 3h from 0 to 36h, and every 24h from 36h to 200h. We did each growth curve in duplicate, and we used sterile YPD as a negative control.

2.4. Lysis treatments

The inactivation treatments were tested for *S. cerevisiae*. Yeasts were cultivated in YPD media, and pellets were obtained by centrifugation of three cell concentrations: 10^6, 10^7 and 10^8 cell/mL. Pellets were washed by suspension in 1 mL of Milli-Q water, and then centrifuged, and submitted at four types of treatment:

1. Heat-shock treatments. We resuspended the pellets on 1 mL of water, and put in a water bath at 95^{o}C for four different time intervals: 5, 10, 20 and 30 minutes.

2. Treatments using two different antimicrobial compounds (cycloheximide and Dimethyl dicarbonate). (a) The pellets were resuspended in 1 mL of two different solutions of cycloheximide (Sigma chemical, St Luis, Missouri): 1 µg/mL and 2 µg/mL. (b) The pellets were resuspended in 1 mL of water, and 1µL and 10µL of dimethyl dicarbonate (DMDC) (as Velcorin™ (Santa Cruz Biotechnology, Dallas, Texas)) were added to obtain final concentrations of 10000 and 1000ppm. In both cases, the suspensions were kept at 4^{o}C for 24h.

3. Ethanol treatments. We resuspended the pellets in 1 mL of ethanol solutions in water with three different concentrations (70%, 80% and 90%) and let them at room temperature for 24h.

4. Physical lysis treatments. We resuspended the pellets in 1 mL of water and transferred into 2-mL conical-bottom microcentrifuge tubes. In cases a, b, c and d those tubes had 1g of 0.5-mm-diameter glass beads. Then, we subjected them to five different combinations of freeze-thawing, mechanical pressure (using mini-bead-beater) and sonication treatments. (a) 5 minutes of shaking using a mini-bead beater (Biospec Products Inc., Bartlesville Oklahoma). (b) -20^{o}C for 10 minutes, room temperature (RT) for 10 minutes and 5 minutes of mini-bead beater treatment. (c) Two cycles of -20^{o}C for 20 minutes and 28^{o}C for 10 minutes followed by 5 minutes of mini-bead beater treatment. (d) Two cycles of -80^{o}C for 20 minutes and RT for 20 minutes plus 5 minutes of mini-bead beater treatment. (e) Treatment of sonication (39% vibration amplitude (Amp)) using Vibra-Cell™ (Sonics & Materials Inc., Newtown Connecticut) for 15 minutes. (f) Two cycles of -20^{o}C for 20 minutes plus 28^{o}C for 10 minutes plus a sonication treatment (39% Amp) during 15 minutes.

The inactivation of *S. cerevisiae* was determined on YPD-agar and YPD-broth. After the lysis treatments, the pellets were washed using Milli-Q water, and resuspeded on 1 mL

of distilled water. 100µL of the suspensions were plated on YPD-agar and in 5 mL of YPD-broth, which were incubated at 25-28ºC during 48h. Both determinations were done in duplicate. Then, on YPD-agar plates we checked if there were colonies, and in YPD-broth we determined the increase of the medium turbidity. With these methods, we determined if the cells were culturable. As a comparison to these lysis treatments, we prepared a positive control, which was a sample without any treatment.

We performed three inactivation treatments for *H. uvarum*, *S. bacillaris*, *T. delbrueckii*, following the procedure explained before: (1) Heat-shock of 5 minutes in a water bath at 95ºC. (2) (a) Antimicrobial treatment with 1 µg/mL of cycloheximide, and kept at 4ºC for 24h. (b) Antimicrobial treatment with DMDC (10000 ppm) at 4ºC for 24h. (3) Ethanol toxicity with 70% of ethanol, and maintained at room temperature for 24h.

2.5. Lysis, harvest and storage of the lysed cells

Finally we used two treatments: heat shock and 10000 ppm of DMDC. The cell concentration was the one they get at stationary phase. After the treatment, the pellets were washed and resuspended in 1mL of Milli-Q water. They were maintained at 25-28ºC during 48h, and one sample was harvested every 12 hours from time 0. One sample was harvested before doing the treatment. For all the samples, the suspension was centrifuged and the pellet was fast-frozen on liquid nitrogen, and then stored at -80ºC for futher quantification of the genetic material. One sample was diluted 10-fold and stored at -80ºC in case of the need of RNA quantification. The whole assay was done in triplicate, but we only analysed one of the samples, so further analysis of the other samples is required to have significant conclusions.

The effectiveness of the inactivation treatment was measured by plating 100µL of the suspension from the sample not-treated and one sample from the control sample (0h after each treatment) in YPD-agar plates. We incubated these plates in duplicate at 28ºC during 2 days, and determined if colonies grew on the plates.

2.6. Nucleic acid extraction and quantification

2.6.1. DNA extraction

DNA was extracted from the pellets stored at -80ºC using DNeasy Plant minikit (QIAGEN, Valencia, California). We followed the procedure of another study (Hierro et al., 2006), with the following modifications: after the physical lysis using mini-bead beater, we changed the centrifugation at 10000 rpm for 1 minute by centrifugation at 14000 during 2 minutes (4ºC) to recuperate all the supernatant. The extracted DNA was stored at -20ºC.

2.6.2. RNA extraction

RNA extraction was performed using the samples 10-fold diluted (for the samples of lysis treatments (2.5)) and the non-diluted samples (from the samples of the standard curve (2.7)) stored at -80^{o}C, using PureLink RNA mini kit (Invitrogen, Carlsbad, California). To improve the extraction performance, we resuspended the pellet in 600 µL of a mixed solution with 10% mercaptoethanol in **lysis buffer**. The suspension was poured in a 2-mL conical-bottom microcentrifuge tube with 1g of 0.5-mm-diameter glass beads. It was shaken for 5 minutes using a mini-bead beater, and the liquid was recovered in a sterile RNase-free 1.5 mL tube, which was centrifuged at 14000 rpm for 5 min (4^{o}C) to recuperate the supernatant. Then, we extracted the RNA from the supernatant using the PureLink kit following the manufacturer's instructions. To remove the DNases, we added a DNase: instead of adding 700µL of buffer **WB1** to the column, we added two times of 350 µL of buffer **WB1**. Between them, 80µL of a solution with 70µL RDD buffer and 10µL DNase (RNase-Free DNase Set, Qiagen, Valencia, California) was added to the column and incubated at room temperature for 15 minutes. The extracted RNA was stored at -80^{o}C. All the reagents in bold were from the PureLink RNA mini kit.

2.6.3. Reverse Transcription PCR

The RNA was converted into cDNA using PCR reaction described by Hierro et al. (2006). In the samples from the lysis treatment (2.5), a 1:20 dilution of RNA was used. We did one negative control for each sample, replacing Superscript II RNase reverse transcriptase (Invitrogen, Carlsbad, California) by water. The cDNA was stored at -20^{o}C.

2.6.4. Quantitative PCR

Quantitative PCR (qPCR) was used to analyse the DNA and cDNA samples. In the samples from the lysis treatment (2.5), 1:50 dilution of the cDNA was done. The quantitative PCR reaction was done following Hierro et al. (2006), but replacing the SyberGreen with Power SybrGreen Master Mix (Applied Biosystems, Foster City, California). The primers used were YEASTF and YEASTR (Hierro et al., 2006). The amplifications were carried out in optical-grade 96-well plates by 7300 Real Time PCR System (Applied Biosystems, Foster City, California). Each sample was analysed in duplicate, and one sample of DNA was the positive control in all the qPCR performed. Water was used as the non-template control (NTC). The negative controls done in the RT-PCR were analysed too to ensure that there was no DNase contamination in the samples that came from RNA.

2.7. Standard curves

All the strains were cultivated in YPD media from the pre-culture. We obtained pellets of several cell concentrations (from 10 cell/mL to 10^8 cell/mL) by centrifugation, and we washed them with Milli-Q water. Those pellets were fast-frozen using liquid nitrogen

and stored at -80ºC, and used to quantify the nucleic acids. For the standard curves, we plotted the $\log_{10}$(cel concentration) against the Cycle Threshold (C_T).

We obtained the plot from the tendency line of the graph. It follow this equation: $y = ax + b$, in which y corresponds to the C_T value and x to the $\log_{10}$(cell concentration). a corresponds to the slope, which represents the difference between the C_T values of correlative cell concentrations, and the ideal is -3.32. The correlation coefficient (r^2) represents how well the experimental data fit the regression line. Its absolute value should be above 0.99, which indicate that the plot is lineal. Moreover, the efficiency is calculated following the formula $\%\ of\ efficiency = ((10^{-1/slope})-1)x100$. It symbolizes the percentage of the template amplified at the end of each cycle. The optimal is an amplification efficiency of 90-105% (BIO-RAD Laboratories, 2006).

2.8. Statistical analysis

The results obtained from the qPCR are in form of C_T values. We transformed them into cell concentrations by the DNA standard curve for each strain. We considered the DNA concentration is stable, therefore we make it the reference to evaluate the rRNA stability. In some cases we defined the rRNA stability with a rRNA/DNA ratio. This ratio is defined by the cell concentration obtained from the rRNA quantification divided by the cell concentration obtained from the DNA quantification, and it reflects the difference between RNA concentration and DNA concentration through the cell concentration of each one. We plotted the RNA and DNA concentration corrected by cell concentration against the time point after the finishing of the treatment. In some cases, we plotted the RNA/DNA ratio in a graph.

As we did not analyse the stability of RNA and DNA in triplicate, we could not do further statistical test. But in further work to obtain significant conclusions we will have to do statistical analyses of variations by One-Way ANOVA to calculate the value of F and significance, using IBM SPSS Statistics. The time, the cell concentrations, the RNA/DNA ratios and C_T values will be used directly for variation analysis.

3. RESULTS

3.1. Growth curve

The growth of each species was monitored by OD, microscope counting and viable cell determination (using YPD-agar plates). The growth curve of *S. cerevisiae* is shown in Figure 2, and the other species present similar tendencies but with small differences in times to reach different phases and cell concentrations (Table 1).

Table 1.Growth curves values. The errors obtained by statistical study of duplicates are indicated after "±".

		S. cerevisiae	*H. uvarum*	*S. bacillaris*	*T. delbrueckii*
Time 50% maxim OD		9 h	9 h	12 h	15 h
Time maxim OD		15 h	12 h	15 h	18 h
Maxim population	**cell/mL**	$1.01x10^8$ ±$5.66x10^5$	$3.11x10^8$±0	$2.66x10^8$±$8.54x10^7$	$2.03x10^8$±$8.84x10^6$
	cfu/mL	$7.45x10^7$±$4.95x10^6$	$8.68x10^7$±$1.10x10^7$	$2.05x10^8$±$3.39x10^7$	$1.52x10^8$±$2.30x10^7$
OD-cell concentration Standard curve	**Equation**	$y = 5x10^{-8}x + 0.0129$	$y = 2x10^{-8}x + 0.0047$	$y = 3x10^{-8}x + 0.0053$	$y = 4x10^{-8}x + 0.0113$
	Regression coefficient	0.9975	0.9994	0.9953	0.9982

This curve indicated the time that takes the population to arrive to stationary phase in controlled conditions using YPD media (indicated with the arrow in Figure 2). The fastest strain was *H. uvarum*, followed by *S. cerevisiae* and *S. bacillaris*, and the slower was *T. delbrueckii* (Table 1). Moreover, the maximum cell concentration for each strain in those conditions was found out, being above 10^8cell/mL for all of them, the highest was *H. uvarum*, and the lowest was *S. cerevisiae*. Moreover, the OD standard curves, which relates the OD value with the cell concentration for each species, are different for each species.

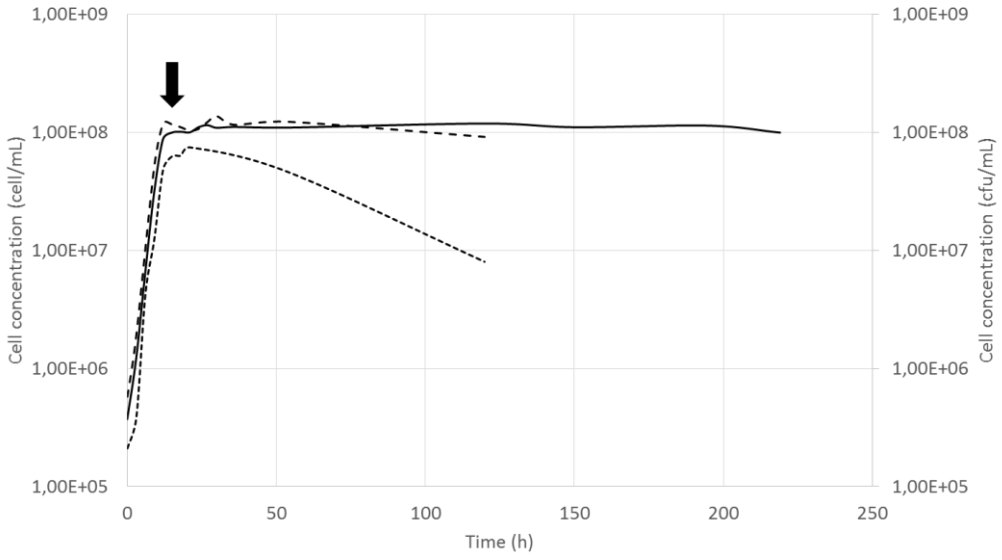

Figure 2. Growth curve of *S. cerevisiae*. The arrow indicates the beginning of the stationary phase. The OD (—)and microscope counting (- - -) are indicated in cel/mL (left axis) and the viable cell determination (┄) is indicated in cfu/mL (right axis).

3.2. Testing lysis treatments

We tested different treatments to know the effectiveness in producing the lysis of different *S. cerevisiae* populations. We performed a positive control, a not-treated sample. All the positive controls showed growth on YPD-agar plates and on YPD-broth for all the cell concentrations (data not shown).

The heat shock consisted on submitting the cells to a temperature of 95°C for different time intervals (5, 10, 20 and 30 minutes). From Table 2 we can see that neither colonies grew on the plates of YPD-agar or turbidity increased on YPD-broth regardless the cell concentration and the heating time. So, the heat treatment was very effective to produce cell impairment to grow in optimal media (YPD) and we can consider that induced the nonculturable state of the cells (although most likely it produced also the cell lysis). Therefore, a temperature of 95°C for 5, 10, 20 or 30 minutes is a good treatment to lyse *S. cerevisiae* cells in concentrations of 10^6-10^8 cell/mL.

Table 2. Culturability of *S. cerevisiae* in YPD-agar and YPD-broth after different treatments: (1) 95°C for different time intervals. (2a) and (2b) Different antimicrobial compounds for 24h at 4°C. (3) Ethanol for 24h in different concentrations. N: no growth (no colonies observed in YPD-agar, and no increase of turbidity observed in YPD broth). Y: growth (more than 300 colonies observed in YPD-agar, and increase of turbidity observed in YPD-broth). DMDC: Dimethyl dicarbonate.

Medium	Cell concentration (cell/mL)	(1) Heat-shock treatment				(2a) DMDC		(2b) Cycloheximide		(3) Ethanol treatment		
		5 min	10 min	20 min	30 min	1000ppm	10000ppm	10^{-6}g/L	$2{\cdot}10^{-6}$g/L	70%	80%	90%
YPD-agar	$1x10^6$	N	N	N	N	N	N	Y	Y	N	N	N
	$1x10^7$	N	N	N	N	N	N	Y	Y	N	N	N
	$1x10^8$	N	N	N	N	N	N	Y	Y	N	N	N
YPD-broth	$1x10^6$	N	N	N	N	N	N	Y	Y	N	N	N
	$1x10^7$	N	N	N	N	N	N	Y	Y	N	N	N
	$1x10^8$	N	N	N	N	N	N	Y	Y	N	N	N

When the test involved chemicals (DMDC, cycloheximide and ethanol), previously to the inoculation of the cells to YPD those were span down and washed, so the compound was absent.

We tested the lysis capacity of *S. cerevisiae* cells with two different antimicrobial compounds. On one hand, treatments with the two DMDC ($C_3H_6O_3$) concentrations induced lack of culturability either on YPD-agar or YPD-broth (Table 2). On the other hand, the same cell concentrations treated with cycloheximide ($C_{15}H_{23}NO_4$) showed colonies in YPD-agar and increased turbidity in YPD-broth. These results were similar to the ones obtained in the control samples, whereas the results of DMDC are similar to the ones obtained with heat-shock treatment. This reflects that *S. cerevisiae* cells cannot grow after the cells are in contact with DMDC (1000 and 10000 ppm) for 24h, but can grow after 24h contact with cycloheximide (10^6 and $2x10^6$).

S. cerevisiae cells were also exposed to increasing ethanol concentrations (70%, 80% and 90%) for 24h at room temperature, and then plated on YPD-agar and YPD-broth. All the ethanol concentrations inhibited the formation of colonies in YPD-agar, confirmed by lack of turbidity increase in the YPD-broth, regardless the cell population tested (Table 2). Thus, concentrations of ethanol between 70% and 90% inhibited the culturability in YPD of *S. cerevisiae* populations.

Table 3. Culturability of *S. cerevisiae* in YPD-agar and YPD-broth after mechanical, ultrasound and freeze-tawing treatments. Y: growth (more than 300 colonies observed in YPD-agar, and increase of turbidity observed in YPD-broth). Number: colonies observed from plating 100µL. Treatments: A. 5 minutes of mechanical pressure B. -20ºC for 10 minutes, room temperature (RT) for 10 minutes and 5 minutes of mechanical pressure. C. Two cycles of -20ºC for 20 minutes and 28ºC for 10 minutes followed by 5 minutes of mechanical pressure. D. Two cycles of -80ºC for 20 minutes and RT for 20 minutes plus 5 minutes of mechanical pressure. E. Treatment of sonication for 15 minutes. F. Two cycles of -20ºC for 20 minutes plus 28ºC for 10 minutes plus a sonication treatment for 15 minutes.

Medium	Cell concentration (cell/mL)	A	B	C	D	E	F
YPD-agar	$1x10^6$	3	1	1	Y	Y	Y
	$1x10^7$	Y	43	Y	Y	Y	Y
	$1x10^8$	Y	Y	Y	Y	Y	Y
YPD-broth	$1x10^6$	Y	Y	Y	Y	Y	Y
	$1x10^7$	Y	Y	Y	Y	Y	Y
	$1x10^8$	Y	Y	Y	Y	Y	Y

Treatments A, B and C, which have the common factor of mechanical pressure using a mini-beat beater treatment, showed a decrease on culturability for cell concentrations of 10^6 cell/mL, but for YPD-broth it was still culturable (if we compare with the control results) (Table 3). For higher cell concentrations the treatment did not have any effect. This means that mechanical pressure had an effect on *S. cerevisiae* cells, but not enough to block the growth on high cell concentrations. Comparing these treatments, the frezze-thawing did not increase the efficiency of the mechanical pressure. This is supported by the difference between B and C, which showed that the increase of the number of freeze-thawing cycles did not increase the treatment performance. Moreover, a decrease of the freezing temperature (C and D comparing treatments) did not increase the cell lysis.

Samples treated with sonication (E and F) did not decrease the turbidity when compared with the control samples: colonies were present in YPD-agar plates, and turbidity appeared in YPD-broth (Table 3). Moreover, exposing the cells to a freeze-thawing cycle before the sonication did not increase the inactivation of cells at the tested concentrations. However, comparing the results from A and E treatments, a difference in culturability of YPD-agar was observed, thus, for the conditions tested the mechanical

pressure treatment was more efficient than the sonication in inactivating the *S. cerevisiae* cells.

Overall, none of the physical treatments tested inactivated a suspension with 10^8 cell/mL of *S. cerevisiae*. However, mechanical pressure had higher effect than sonication. Moreover, we found that increasing the number of freeze-thawing cycles or the interval between the temperatures did not increase the capacity to lyse the cells.

Table 4. Culturability of three yeasts species in YPD-agar and YPD-broth after three treatments: (1) Heat shock (95ºC for 5 minutes), (2) DMDC (10000 ppm for 24h at 4ºC), (3) ethanol (70% ethanol for 24h at room temperature). The control of DMDC treatment consisted on treating the sample with cycloheximide (10^6g/L for 24h at 4ºC). N: non growth (no colonies observed in YPD-agar, and no increase of turbidity observed in YPD-broth). Y: growth (more than 300 colonies observed in YPD-agar, and increase of turbidity observed in YPD-broth). nd: non-determined. DMDC: dimethyl dicarbonate

		Hanseniaspora uvarum		*Stamerella bacillaris*		*Torulaspora delbrueckii*	
Type of treatment	**cell concentration (cell/mL)**	**YPD-agar**	**YPD-broth**	**YPD-agar**	**YPD-broth**	**YPD-agar**	**YPD-broth**
(1) Heat-shock treatment	**$1x10^6$**	N	N	N	N	N	N
	$1x10^7$	N	N	N	N	N	N
	$1x10^8$	N	N	N	N	N	N
Control heat treatment	**$1x10^6$**	Y	Y	Y	Y	nd	nd
	$1x10^7$	Y	Y	Y	Y	nd	nd
	$1x10^8$	Y	Y	Y	Y	Y	Y
(2) Antibiotic treatment	**$1x10^6$**	N	N	N	N	N	N
	$1x10^7$	N	N	N	N	N	N
	$1x10^8$	N	N	N	N	N	N
Control antibiotic treatment	**$1x10^6$**	Y	Y	Y	Y	nd	nd
	$1x10^7$	Y	Y	Y	Y	nd	nd
	$1x10^8$	Y	Y	Y	Y	Y	Y
(3) Ethanol treatment	**$1x10^6$**	N	N	N	N	N	N
	$1x10^7$	N	N	N	N	N	N
	$1x10^8$	N	N	N	N	N	N
Control ethanol treatment	**$1x10^6$**	Y	Y	Y	Y	nd	nd
	$1x10^7$	Y	Y	Y	Y	nd	nd
	$1x10^8$	Y	Y	Y	Y	Y	Y

After having positive results of the treatments on *S. cerevisiae*, we tested some of the treatments to other species, which gave the same results than *S. cerevisiae* (Table 4). This was also confirmed by the positive controls, which showed culturability in all the situations. Even cycloheximide, did not lyse the other strains. Therefore, submitting cell concentrations of 10^6-10^8 cell/mL to 95ºC for 5 minutes, to 70% ethanol for 24h and to 10000 DMDC for 24h at 4ºC inactivate the cells of *S. cerevisiae*, *H. uvarum*, *S. bacillaris* and *T. delbrueckii*.

3.3. DNA standard curves

A total of 4 individual standard curves were constructed with different strains grown on YPD. For each strain, we obtained pellets of concentrations between 10^1-10^8 cell/mL, and qPCRs were performed. The C_T value was plotted against the cell concentration to obtain the standard curve, and thus the correlation coefficient and the efficiency were calculated.

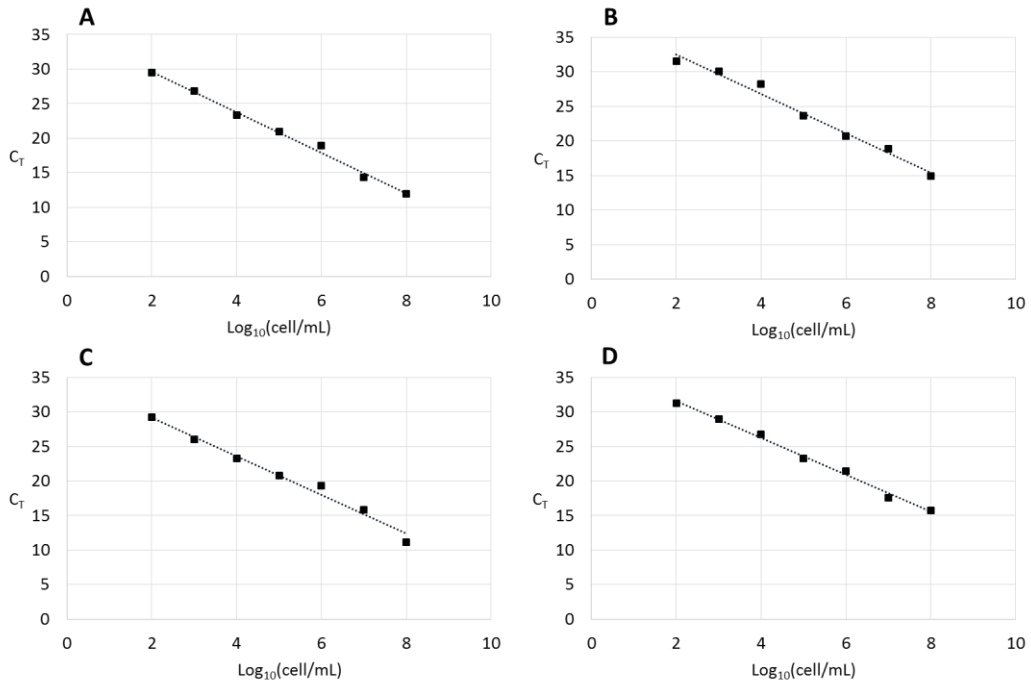

Figure 3. DNA standard curves. A. *S. cerevisiae*. B. *H. uvarum*. C. *T. delbrueckii*. D .*S. bacillaris*. C_T: Cycle threshold

In Figure 3 the standard curves and the tendency lines of different species are shown. The C_T corresponding to 10^1 was the same as the C_T of the NTC, so 10^2 cell/mL was the lower limit of determination. The main paramters of the qPCR of *S. cerevisiae* were the best of all the speices (Table 5): the regression coefficient was above 0.99 (very good linearity), although the slope and the efficiency were slightly higher than the ideal one. Overall, the standard curve was good between 10^2 and 10^8 cell/mL.

Table 5. Plot equations, correlation coefficients and efficiencies of DNA standard curves of the four species

	S. cerevisiae	***H. uvarum***	***T. delbrueckii***	***S. bacillaris***
Standard curve	y = -2.9311x + 35.483	y = -2.8482x + 38.217	y = -2.8044x + 34.81	y = -2.6647x + 36.89
Regression coefficient	0.9929	0.9818	0.9825	0.9935
Efficiency	119%	124%	127%	137%

The slope of the other three species was a little higher than that of *S. cerevisiae* (Table 5). This suggests that all the species have a lower difference of C_T between the dilutions than the ideal, and therefore amplifies more percentage of template at the end of each

cycle, as we can see from increased efficiencies. According to the regression coefficients, *S. bacillaris* had the best, and the ones from *H. uvarum* and *T. delbrueckii* were acceptable. Therefore, we obtained good standard curves between cell concentrations 10^2-10^8 for all the species.

3.4. Quantifying the rRNA

A total of 4 individual standard curves were constructed with the different species grown on YPD in the same way than for DNA standard curves, but with an additional step, the reverse transcription to get the cDNA. Negative controls of the RT-PCR were used to exclude DNA contamination in the RNA-extracted samples. All the negative controls had C_T values similar to the NTC, which means that the samples were not contaminated with DNA (data not shown). The results obtained did not allow to plot good standard curves, due to poor extraction in low cell concentrations or due to saturation in high cell concentrations (results not shown).

Table 6. Plots, correlation coefficients and efficiencies of the RNA standard curves of the four species.

	S. cerevisiae	***H. uvarum***	***T. delbrueckii***	***S. bacillaris***
Standard curve	y = -3.4935x + 31.96	y = -3.9844x + 41.463	y = -8.5608x + 63.645	y = -2.9944x + 35.793
Regression coefficient	0.8982	0.7667	0.9993	0.7143
Efficiency	93%	78%	31%	93%

The values of table 6 reflect that the curves done with *S. cerevisiae*, *H. uvarum* and *S. bacillaris* had very low regression coefficients, which meant that the values did not fit a linear plot. For *T. delbrueckii*, the slope and the efficiency were too low, so the difference among C_T was too high. From those results we could conclude that we could not construct good standard curves for RNA for these species. Moreover, note that for concentrations 10^7 and 10^8 cell/mL in several species there was saturation (data not shown), and those were the concentrations more interesting for our work, because these are the cell concentrations at the beginning of the stationary phase.

To solve these poor results, the next approach was to dilute the samples from a known concentration: we did successive 10-fold dilutions of the cDNA from the 10^7 cell/mL and the 10^8 cell/mL and plotted the C_T against the cell dilution (Figure 4). The parameters of these graphs were very good and similar, but there was saturation in cell concentrations higher than 10^6 cell/mL (data not shown). The fact that both curves were similar reflected that the saturation was not produced before the qPCR. All the tests were done with the species *S. cerevisiae*.

The next step was to adjust the DNA quantity to prevent the saturation, and >100-fold dilution was needed. We plotted on a graph the qPCR results of 100-fold dilutions of the cDNA obtained from extractions of 10^1-10^8 cell/mL (Figure 5). No saturation at high concentrations was observed, although, the detection limit was the 100-fold dilution of

10^4, which could be an inconvenient if low cell concentrations were present in our samples. However, the slope of the standard curve with concentrations from 10^4 to 10^8 cell/mL was -5.5577, and thus this method is not acceptable for RNA quantification.

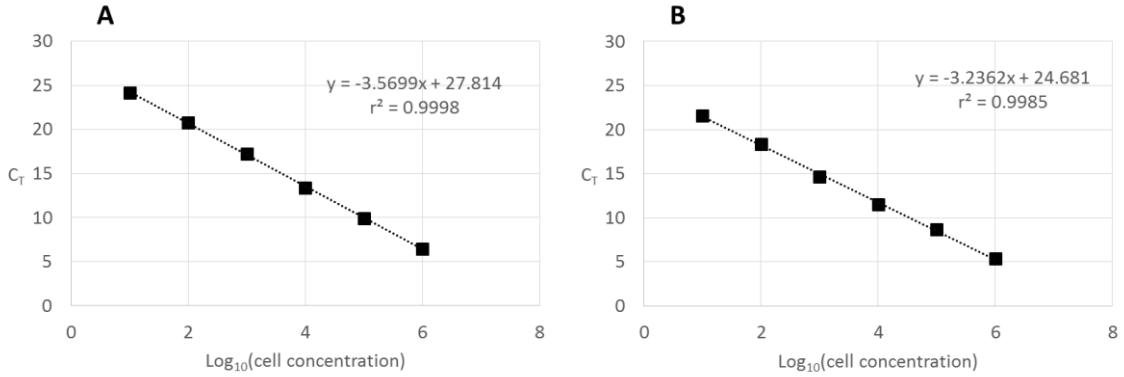

Figure 4. RNA standard curves of 10-fold diluted cDNA obtained from different cell concentrations. A. 10^7 cells/mL. B. 10^8 cells/mL. C_T: cycle threshold. r^2: regression coefficient.

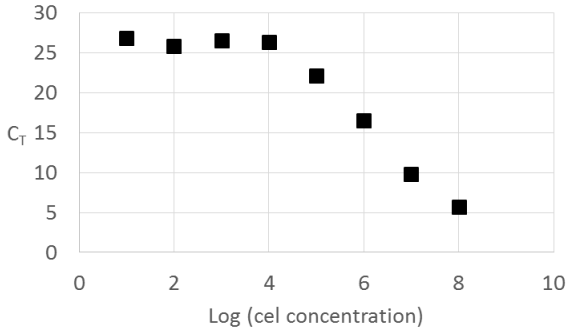

Figure 5. Representation of the results of 100-fold dilution of cDNA obtained from sucessive cell concentrations (10^1-10^8 cell/mL). The x axis represent the cell concentration from which we have diluted 100-fold the cDNA. C_T: cycle threshold.

Therefore, we used DNA standard curves to do the RNA quantification. As normally the RNA concentration inside the yeast is 10^3 fold higher than the DNA concentration, samples were diluted 10000-fold before doing the qPCR to have the C_T values inside the linear plot of the standard curve. The dilutions were done in three steps: 10-fold dilution of the cell pellet, 20-fold dilution of the RNA and 50-fold dilution of the cDNA, as those dilutions give results that reflect the non-diluted ones (Figures 4 and 5).

Table 7. Ct and DNA concentration (expressed as equivalent cell concentration) of RNA quantifications through two protocols. B. 10-fold dilution of cell pellet. D. 10-fold dilution of the cell pellets plus 20-fold dilution of the RNA plus 50-fold dilution of cDNA.

	C_T value		**Cell concentration (cell/mL)**	
Time after treatment	**B**	**D**	**B**	**D**
0 h	3.662	21.909	$1.33x10^{10}$	$2.21x10^{8}$
12 h	3.801	18.817	$2.07x10^{10}$	$2.13x10^{9}$
24 h	3.967	15.038	$4.43x10^{10}$	$3.75x10^{8}$
36 h	3.481	19.323	$2.54x10^{10}$	$1.17x10^{8}$
48 h	4.080	15.155	$1.74x10^{8}$	$9.21x10^{7}$

The comparison between two dilutions showed that the C_T values of 10^{-1} dilution (B in Table 7) were outside the DNA linear plot, while those of 10^{-4} dilution (D in Table 7) were inside the curve. The similarity of the results in the 10^{-1} dilution indicated saturation.

Therefore, 10000-fold dilutions and transforming the data with the standard curve of DNA was the best option to quantify the RNA concentration of the cells.

3.5. Stability of rRNA and DNA after heat shock and DMDC-lysed cells

Quantifying rRNA and DNA during 48h after the treatments assessed the stability of both nucleic acids. The plating of a non-treated sample and a treated sample confirm that the treatments lysed the cells (data not shown).

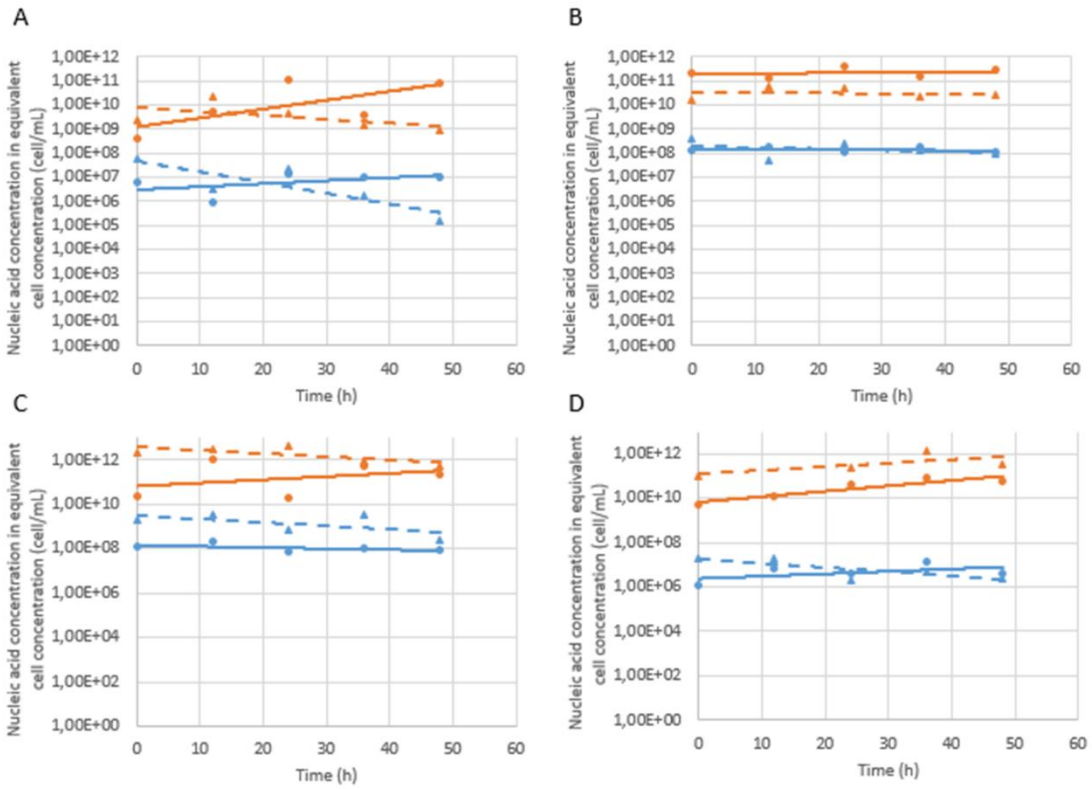

Figure 6. DNA and RNA concentration using cell concentration equivalent during 48h. The markers represent the points analysed and the lines its tendency. Orange: rRNA concentration. Blue: DNA concentration. Straight line and dots: heat treatment. Dashed line and triangles: antibiotic treatment. A. *S. cerevisiae*. B. *H. uvarum*. C. *S. bacillaris*. D. *T. delbrueckii*.

In Figure 6A we can see the results of *S. cerevisiae*. The DNA concentration was stable during the 48h, after the heat-shock treatment and the DMDC treatment. The rRNA concentration showed the same dynamics, which means that is stable along the time too.

For *H. uvarum*, the DNA concentration was stable during 48h after the heat-shock and DMDC treatments (Figure 6B). The rRNA results showed too a very stable pattern. Note that the rRNA concentration for samples treated with heat-shock were always higher than the treated with DMDC, however we cannot know if the differences are significant without doing an statistical test.

The DNA concentration of *S. bacillaris* was stable during 48h after each of the treatments (heat-shock and DMDC) (Figure 6C). The rRNA results showed the same dynamics than DNA ones, thus both nucleic acids followed the same pattern.

The DNA quantification of *T. delbrueckii* (Figure 6D) cells after heat and DMDC treatments showed that it was stable during the 48h after the treatments. The rRNA concentrations were stable, and the samples from heat-shock and DMDC treatments show similar patterns. All this suggested that the rRNA and DNA of *T. delbrueckii* cells did not degrade during 48h after the treatments tested.

In all the species, the rRNA concentration was higher than the DNA one. *S. cerevisiae* had the more variable rRNA/DNA ratios, and *H. uvarum* had the less variable ones. Moreover, these two species and *S. bacillaris* maintained ratios between 10^1-10^4, but *T. delbrueckii* had ratios between 10^3-10^6 (data not shown), which meant that the difference between RNA and DNA concentration is bigger in this specie. Moreover, in point 0h normally there was less difference between rRNA and DNA concentrations.

Overall, for all the species the results suggested that rRNA is as stable as DNA during the 48h following a heat-shock or DMDC treatment. However, we need to analyse the triplicates to demonstrate the repeatability, and do a statistical test in order to extract a conclusion from those results.

3.6. Comparation of RNA extraction from treated and untreated samples

An untreated sample was analysed from each species. In Figure 7 there are the results of the untreated sample and heat-treated one. We can observe that there was a big difference on the rRNA/DNA ratios: the non-treated sample had a lower ratio than the treated one, which meant that less RNA was quantified for the non-treated sample. Even in some cases the ratio was negative, which meant that there was more DNA quantified than RNA quantified. The cell culture was the same, and we obtained growth on the untreated sample and non-growth at the treated one on YPD.

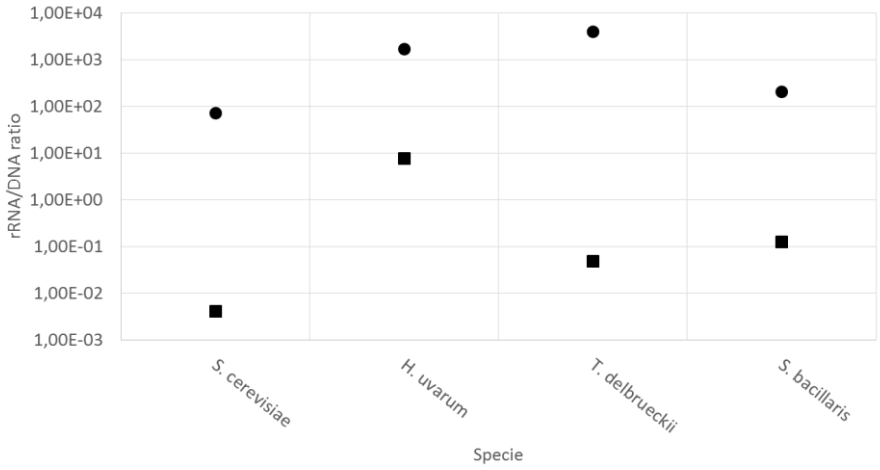

Figure 7. Representation of rRNA/DNA ratio of untreated (■) and treated (time 0 heat treatment) (▲) samples of different species.

4. DISCUSSION

Ribosomal RNA (rRNA) was proposed as a target of culture-independent techniques to quantify and detect the total population of viable cells in an ecosystem (which include culturable and VBNC cells). It was chosen because it seems to have the characteristics of a good target: stability in live cells and less stability than DNA in death cells. However, those characteristics have not been proved, and it is extremely necessary to evaluate the stability of RNA in lysed cells to be able to use RNA as a marker of viable cells. To do this we tested different lysis treatments and checked the rRNA stability after applying some of them.

In order to detect different status of cellular pre-lysis, we tested different ways of cellular recuperation and different lysis treatments. On the one hand, we tested how the lysed cells recuperate on solid and liquid media (YPD-agar and YPD-broth). As explained before, we considered lysed the cells that did not grow on both media. On the other hand, we tested the effects of certain treatments to the cell, to know if they cause different cell status. Here we will discuss the effect of each treatment to the cells.

The heat produced as a result of the Crabtree effect modifies the fermentation environment, giving an advantage to *S. cerevisiae* over non-*Saccharomyces* species, facilitating its imposition (Goddard, 2008). Thus, high temperatures can be a factor that inhibits the culturability of non-*Saccharomyces*, for this we were interested in studying its effects. In this study, all the species were lysed in an irreversible way when submitted to 5 minutes at 95^{o}C. This is explained because high temperature disrupt enzyme and membrane functions, which cause stop cell function (Madigan, Martinko, Stahl, & Clark, 2012).

Ethanol is a product of the alcoholic fermentation, and it is suggested as a factor that influences on the imposition of *S. cerevisiae* over non-*Saccharomyces* yeast. As it can inhibit the culturability of those cells, its action was investigated in this study. The results pointed out that 70% of ethanol during 24h lyses the yeast cells, conclusion that is supported with the bibliography, which says that ethanol at high concentrations (>60%) kill yeast by denaturalizing enzymes, affecting the lipids of the membrane and damaging mitochondrial DNA (Madigan et al., 2012; You, Rosenfield, & Knipple, 2003).

DMDC and cycloheximide are antimicrobial compounds known in the wine industry in order to lyse wine yeast. DMDC is added to the alcoholic fermentation product in order to sterilize it before inoculating lactic acid bacteria to help to impose them to do malolactic fermentation. Cycloheximide is used to select certain yeast population when monitoring the fermentation using culture-dependent methods. For those reasons we want to test their lysis capacity on the species used in this assay. In this study, DMDC lysed the cells in an irreversible way. Its mechanism of action is related with the inactivation of microbial enzymes, through methoxycarbonylation of nucleophilic groups of the proteins, mainly glycolytic enzymes (alcohol dehydrogenase and glyceraldehyde-3-phosphate-dehydrogenase) (EFTA, 2015). On the other hand, cycloheximide did not lyse the cells. It is an inhibitor of the elongation phase of the

protein synthesis, and acts interacting specifically with the cytoplasmic ribosomes of eucariotic cells (Madigan et al., 2012).

The difference between the effect of both antibiotics could be that DMDC has a fungicidal activity, which blocks the growth in an irreversible way, and cycloheximide is fungistatic, so it only blocks the growth when it is in contact with the cells (Rich, 1959). As we removed the antibiotic before plating, the cells submitted to cycloheximide recovered the metabolism and grew, while the cells submitted to DMDC did not. This makes sense with the fact that DMDC is very unstable in aquous solution, because it breaks down almost immediately after addition, producing methanol, carbon dioxide and other compounds. Therefore, its action has to continue after it disappears in order to have an effect (*EFSA,* 2015). Moreover, it seems that cycloheximide has no effect on the respiration and fermentation (Rich, 1959). For all of this we did not use it in further study.

The physical treatments we tested consisted on combinations of mechanical pressure, freeze-thawing and sonication. The objective of all those techniques was to disrupt the cell wall, with the aim to lyse it, and kill the cell.

When we added a freeze-thawing step to sonication and mechanical treatment, it did not increase its lysis efficiency. This treatment consisted on freezing the intracellular and extracellular water (which forms ice crystals), and thawing it at room temperature or 37^{o}C (which make the crystals to contract). This causes the cells to swell and then break, so they lyse for mechanical and osmotic shock and deshidratation (Park, Grant, Attfield, & Dawes, 1997). In the study it seemed that a lower freezing temperature is less effective in lysing the cells. This could be because when using higher temperatures, the freezing rate is slow, and bigger crystals are formed, which can damage the cell more that small crystalls formed using low temperatures (fast freezing rate). Moreover, rapid thawing allows melting to occur faster than cell growth, reducing cell death (Deller, Vatish, Mitchell, & Gibson, 2014).

Sonication treatments consisted on treating a suspension of cells with ultrasound, which produces cavitation bubbles in the liquid media. Those bubbles collapse when reach a critical size, causing a release of energy that can damage cells (Gao, Hemar, Ashokkumar, Paturel, & Lewis, 2014) through modification of cellular activity, puncturing of the cell wall and increase sensivity to heat. This treatment had no effect on the culturability of the tested species. This is consistent with bibliography which says that *S. cerevisiae* VL1 cells are not destructed by sonication. However, ultrasounds damages the cell wall and the cytoplasmic membrane, which affects the internal structure of the cell so they are more sensible to be lysed by other factors, such as temperature (Ciccolini, Taillandier, Wilhem, Delmas, & Strehaiano, 1997).

The mechanical pressure was more effective on lysing the cells than sonication, but none of the combination lysed all the *S. cerevisiae* cells. This treatment was done by using a mini-bead beater, which shakes the cells at great velocity inside of a tube with beads,

causing collisions between the beads and the samples, which lyse the cell wall. The velocity and time of the treatment were not sufficient to lyse all the cells.

Those three treatments have more effect on mammalian and bacterial cells, but not yeast cells, and this is because of the differences in cell wall characteristics. The cell wall of yeast consists mainly of mannoproteins and β-linked glucans (Gao et al., 2014), and normally are wider than the bacterial cell wall (mammalian cells does not have cellular wall). Thus, mammalian and bacterial cells are easier to lyse through a physical method.

The rRNA quantification of a non-treated and a heat-treated sample showed a unexpected pattern for all the species. We expected rRNA quantity to be equal or lower in the treated sample than in the non-treated, as is not expected that the cell produce rRNA after its lysis. However, the RNA/DNA ratio show that rRNA quantity in non-treated cells was lower (10^{-2}-10^{1}<10^{2}-10^{4}), and sometimes even negative. As in yeast cells the ratio RNA/DNA is normally 10^{3}-10^{4}, the results from treated samples are more consistent. The bad correlations obtained in the RNA standard curves, constructed from live cells, support this.

Therefore, we propose one theory to explain those results, related with the extractions performance. The protocol of RNA extraction has a step of cellular lysis through mechanical pressure treatment, and after the extraction, we performed a RT-PCR with the primer NL-4, which targets a zone of 26S rRNA. The hypothesis suggest that when the cell lyses, the ribosomes denaturalises, and the 26S rRNA is more accessible to the primer during the RT-PCR, which leads to obtain a higher quantity of cDNA, and more RNA quantified. As we have seen before that mechanical pressure does not kill the cells, we can extrapolate that the procedure to kill the cells before the extraction was no efficient, and not all the rRNA denaturalise, which leads to an underestimation of the rRNA, because the primer cannot bind all 26S rRNA sequences. Therefore, we have to improve the extraction procedure in order to obtain a real quantification of rRNA, which can be improved by the use of lysed cells. But it is necessary to do more tests to confirm the hypothesis.

The results concerning the stability of DNA match with the bibliography and are clear: DNA is stable 48h after cell lysis. The results obtained from the rRNA stability show that rRNA concentration is stable 48h after heat-shock treatment of 5 minutes at 95^{o}C and of 10000 ppm of DMDC. It has to be pointed out that only one replica was analysed, so it is necessary to consider the triplicates to get conclusions. Here, these results are compared with other studies of rRNA stability.

Hierro et al. tested the rRNA stability using 10^{5} cell/mL treated at 60^{o}C during 20 minutes, and a 99% decrease in rRNA concentration 24h after the loss of cell viability was observed (Hierro et al., 2006). In our study, a similar treatment was applied, but with some modifications. We used higher temperature and less time, and higher cell concentration, which can influence the result.

The treatment used for Andorrà et al. was boiling (100^{o}C) a cell suspension during 10 minutes. After 24h, the rRNA decreased for *S. cerevisiae*, but for *Hanseniaspora*

guillermondii remained constant more than 96h (Andorrà et al., 2011). Our results do not match with the *S. cerevisiae* ones, and it could be due that they used longer time and higher temperature. But the results of *H. guillermondii* match with all our results. Moreover, this study tested a RNase treatment, which degraded the rRNA of both species of cells immediately. Wang did an ethanol treatment (75%), which produced a decrease of two orders of magnitude the cell concentration, but after 48h some rRNA existed (Wang et al., 2015).

Therefore, expecting to analyse the triplicates to have significant data, we can say that the rRNA is not degraded after 48h of being lysed by heat-shock and DMDC treatment. Therefore, rRNA is not a good target for culture-independent techniques, as it is not a good as indicator of the cell state. Moreover, working with RNA has some disadvantages, such its proclivity to contamination with RNA-degrading enzymes, which can cause issues of reproducibility, and the fact that its manipulation is more demanding than the DNA manipulation. Therefore, it is not appropriate to use rRNA quantification to quantify or detect in a sample the total viable cell population of the species tested.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Heat, ethanol and DMDC treatments lysed wine yeast cells, but mechanical pressure and sonication did not have the capacity to lyse those cells, even combined with freezing-thawing cycles. Therefore, it's questionable the adequation of the use of mechanical pressure to break the cell wall prior to rRNA extraction, because it does not lyse all the cells, and can cause an underestimation of rRNA concentration. Moreover, from comparing the rRNA quantity of non-treated and treated samples we have hypothesize that cellular lysis causes a desestructuration of ribosomes, which makes easier for the primer to arrive to its target, and to get a more accurate rRNA quantification. Following this line, RNA extraction protocol has to be performed from dead cells. Moreover, it seems that the rRNA of lysed cells by heat or DMDC treatments is stable 48h after the loss of viability. As a conclusion, we can suggest that rRNA is not a sensible marker of cellular viability.

After these conclusions, we would suggest two lines to follow: in the one hand, study better the rRNA extraction protocol, and design a new protocol in which the cells are killed before the RNA extraction. On the other hand, the possibility to use mRNA as a marker of cell viability has to be tested. The main hurdle of this molecule is that the expression is not constant and thus, it does not reflect the number of cells properly. Although some constitutive genes are used (actin, some DNA polymerases, etc) they are not completely constant during the cell life cycle and thus, nut completely appropriate.

AKNOWLEDGMENTS

Firstly, I would like to thank Albert Mas, my thesis supervisor, for integrating me in his research team, letting me participate in this project and guiding me through the process. I would also like to express my gratitude to Chunxiao Wang, for teaching me during the first days at laboratory, guiding me and solving problems and questions that emerged along the period.

I want to appreciate the contribution of the Universitat Rovira i Virgili for giving me funding to do this thesis. I want to render thanks to all the professors of the master of Fermented Beverages, who have taught me a lot of the wine world, and have transferred me the enthusiasm to this world. Moreover, I am very grateful to every person of the wine biotechnology team, who always were available to solve my questions and help me. Specially, I want to thank my labmates, who not only were always ready to help me, but they have embolden me to keep going on this project.

Finally, I want to thank my family and friends for supporting me during this entire year (with nocturnal car travels, English corrections, attempts of scientific divulgation speeches…) and encouraging me.

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