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OPTIMIZATION OF A qPCR METHOD FOR THE DETECTION OF VIABLE *Saccharomyces* AND non-*Saccharomyces* CELLS DURING WINEMAKING

Virgil VRĂJMAȘU[1], Raluca-Ștefania RĂDOI-ENCEA[1, 2], Oana-Alina BOIU-SICUIA[1], Camelia Filofteia DIGUȚĂ[1], Florentina MATEI[1, 2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Mărăşti Blvd, District 1, Bucharest, Romania
[2]Transylvania University of Brașov, Faculty of Food Industry and Tourism, 140 Castelului Street, Brașov, Romania

Corresponding author email: camifilo@yahoo.com

Abstract

*The use of Saccharomyces and non-Saccharomyces (NS) during wine making is a new concept to keep the wines' local specificity. Different molecular tools were developed to quantify Saccharomyces yeast during winemaking, but for the NS several limitations were detected. In this regard, our work focused on the development of a qPCR method employing propidium monoazide (PMA) for the detection of NS viable cells. Very good correlation parameters and standard curves were obtained during the optimisation method for Saccharomyces reference strain versus NS belonging to Candida stellata and Torulaspora delbrueckii. The detection limit varied from 38 fg/µL to 49 fg/µL which corresponds to quantification limits of 70 CFU/mL to 1.03*10^2 CFU/mL. The optimised PMA-qPCR method can be considered as a rapid and suitable method for assessing the viable microbial count for both NS yeast species.*

Key words*: alcoholic fermentation, yeast, Saccharomyces, non-Saccharomyces, PMA, qPCR.*

INTRODUCTION

Spontaneous fermentation is an uncontrolled process, in which the alteration microorganisms could rapidly multiply and reach high levels quickly, which may negatively impact the quality of the finished product. The conventional practice of producing wines on an industrial scale with the use of *Saccharomyces* species involves controlled fermentation from all points of view, but, for a greater specificity, a possible direction might be the use of local yeasts from each geographical region, in addition to using grapes harvested from those areas (Radoi-Encea et al., 2023). Knight et al. (2015) delivered the concept of "microbial terroir", which implies that the microbial consortia that include *Saccharomyces* and non-*Saccharomyces* (NS) yeast, in a certain wine-growing area are specific to that certain area and are producing flavors typical of the area. NS yeasts, also known as wild yeasts, include diverse species, most of which belong to the genera *Hanseniaspora, Candida, Lachancea, Metschnikowia, Pichia, Torulaspora,* and *Zygosaccharomyces*. Although there are several PCR-based fingerprinting methods for typing *S. cerevisiae* strains, there are not so many available for different NS yeasts, and the main limitation has been the lack of sufficient genomic sequence data (Nisiotou et al., 2022). Meanwhile, the main disadvantage of DNA-based quantification methods is their inability to distinguish between viable and non-viable cells, as dead cells may also retain a significant amount of DNA.

By the use of the conventional cultivation-based methods wine-associated NS species can often be underestimated, due to variable growing rates of the different microorganisms in culture media or to the presence of viable but non-cultivable (VBNC) microorganisms. In this regard, cell viability could be defined in different ways, but it is mainly based on the presence of an intact membrane or to some metabolic activities. Some methods are taking advantage of dyes, such as propidium monoazide (PMA) or ethidiummonoazide (EMA), which are able to penetrate in membrane-compromised cells or in the dead cells and covalently bind to nucleic acid after photoactivation. Because the DNA that is covalently bound to

these dyes cannot be PCR amplified, only the DNA from viable cells, including the VBNC, can be detected and the application of quantitative PCR (qPCR) could specifically reveal viable cells (Rizzotti et al., 2015).
Our trials focused on the development of a PMA-qPCR method for the detection of NS viable cells during wine alcoholic fermentation, focusing on less studies NS yeast like *Wickerhamomyces anomalus*, *Candida stellata* and *Torulaspora delbrueckii*

MATERIALS AND METHODS

Microorganisms and yeast sample preparation

In this study were targeted one strain of *Saccharomyces cerevisiae* (EC 1118) and three non- *Saccharomyces* (non-S) isolated from local grapes and identified by molecular tool as *Wickerhamomyces anomalus* (synonym *Pichia anomala*) (MI 201), *Candida stellata* (MI 202) and *Torulaspora delbrueckii* (MI 203). These are stored in the microbial collection of USAMV of Bucharest, Faculty of Biotechnology. Their preparation follows several steps. After 24 hours cultivation in PDB medium (Difco Laboratoires, Franța) at 25ºC the viable cells were counted optical microscopy tool and verified by plating.
To prepare the dead yeast cells, 5 mL of fresh culture were kept for 1 hour at 80ºC and the viability was checked in the end.
For the qPCR test 1 mL of viable and dead yeast cells was centrifuged at 4000 rpm for 10 minutes at 4ºC; the sediment was re-suspended in 400 µL distilled water. PMA (propidium monoazide) was added under dark conditions, respectively 1 µL (20 mM stock solution) in 400 µL of sample, because PMA is know as a photo-reactive DNA-binding dye used in viability PCR. The incubation time was 10 minutes at room temperature, followed by twice photolysis treatment for 10 minutes and a centrifugation at 5000 rpm for 10 minutes; after that, the DNA extraction was performed.

DNA preparation

The DNA extraction followe the steps of the kit ZR Fungal/Bacterial MiniPrep™ (Zymo Research, SUA), under small modifications. The extracted DNA purity was measured by SpectraMax® QuickDrop ™ (Molecular Devices, SUA). Before the manipulation, the extracted DNA was stored a -20ºC.

Yeast quantification by qPCR

The quantification was performed in a RT-PCR (thermocycler) Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia).
The employed primers are described below. For *W.anomalus* Wu et al. (2017): W-CAR1-F (GCAATAGGGTTCCAAAAGTG) and W-CAR1-R (AGCCATTTCCACAACTTGA). For *C.stellata* Garcia et al. (2017).: CS1-F (AGTAACGGCGAGTGAACAGG) and CS1-R (GGCTATCACCCTCTATGGCG). For *T. delbrueckii* (Zott et al., 2010): Tods L2 (CAAAGTCATCCAAGCCAGC) and Tods R (TTCTCAAACAATCATGTTTGGTAG). For *S. cerevisiae* (Zott et al., 2010): SC (GAAAACTCCACAGTGTGTTG) and SC2 (GCTTAAGTGCGCGGTCTTG).
The reaction mix (final volume 25 µL) included the following: 12.5 µL Maxima SYBR Green qPCR Master Mix (2x), 0.125 µL Primer F (100 µM), 0.125 µL Primer R (100 µM), 7.25 µL water free Rnase and 5 µL DNA.
The amplification program for Tods L2/Tods R2 and SC1/SC2 primers, according to Zott et al. (2010) was the following: initial denaturation at 95°C/3 min; 40 cycles of denaturation at 95°C/15 sec + hybridisation at 60°C/60 sec + elongation at 72°C/30 sec.
The amplification program for W-CAR1-F/W-CAR1-R primers, according to Wu et al. (2017) was the following: initial denaturation at 95°C/3 min; 40 cycles of denaturation at 95°C/30 sec + hybridisation at 50°C/30 sec + elongation at 72°C/40 sec.
The amplification program for CS1-F/CS1-R primers, according to Garcia et al. (2017) was the following: initial denaturation at 95°C/3 min; 40 cycles of denaturation at 95°C/30 sec + hybridisation at 50°C/30 sec + elongation at 72°C/40 sec.
In the end, the temperature was raised with 1ºC at each 10 seconds, from 65°C to 95°C to get the right melting temperature (Tm) fo each specific qPCR.

The threshold cycle (Ct) calculation

The Ct was automatically defined by the use of Rotor-Gene Q Series software. According to the provider, to measure if the PMS has adequately inhibited the dead cells DNA amplification a ΔCt was calculated for each reference yeast strain, according to the following formula:

$\Delta Ct \text{ viable} = Ct_{\text{(viable, PMA treated)}} - Ct_{\text{(viable, non-PMA treated)}}$

$\Delta Ct \text{ dead} = Ct_{\text{(dead, PMA treated)}} - Ct_{\text{(dead, non PMA treated)}}$

The extracted DNA treated with PMA was decimally diluted to generate the standard curve. The PCR efficiency (E%) was calculated according to the following formula:

$E = [10^{-1/slope}-1] \times 100$, and the slope value was extracted form the standard curve according to $C_T = f(\log Q)$, where Q is equal with the DNA quantity for each reference strain (Figure 1). The DNA quantity for each yeast reference strain it was obtained by the extrapolation of the Ct value established on the standard curve.

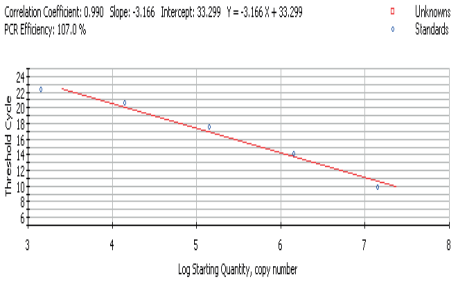

Figure 1. The slope calculation (CT versus log DNA)

RESULTS AND DISCUSSIONS

In a first step of the qPCR method optimisation, the thermic treatment efficiency was determined.

The thermic treatment efficiency was confirmed by CFU (colony formic unit) technique on PDA medium, by comparing the treated cell growth absence (thermal and thermal + PMA) with the viable cells growth treated and non-PMA treated. It was noticed o slight viability decrease after the use of the PMA on the viable cells (Table 1).

The fungal DNA extracted from the viable and dead cells, treated and non-PMA treated it was isolated by a simple and efficient method, using a routine commercial kit.

Table 1. The yeast cells viability treated and non-PMA treated

Samples	Log CFU/mL
W. anomalus viable, non-PMA	7.4
W. anomalus viable, PMA	7.3
W. anomalus dead, non-PMA	0
W. anomalus dead, PMA	0
C. stellata viable, non-PMA	7.6
C. stellata viable, PMA	7.0
C. stellata dead, non-PMA	0
C. stellata dead, PMA	0
T. delbrueckii viable, non-PMA	7.5
T.delbrueckii viable, PMA	7.3
T. delbrueckii dead, non-PMA	0
T. delbrueckii dead, PMA	0
S. cerevisiae viable, non-PMA	7.5
S. cerevisiae viable, PMA	7.1
S. cerevisiae dead, non-PMA	0
S. cerevisiae dead, PMA	0

The DNA quality and concentration were tested in comparison by two different tools: a SpectraMax® QuickDrop ™ (Molecular Devices, SUA) and agarose gel electrophoresis. The results are presented in Table 2; good quality and purity DNA was obtained, ready for the qPCR reactions.

Table 2. The purity and concentration of the exttacted DNA

Samples	Conc. (ng/µL)	A260	A 260 nm/280 nm
S. cerevisiae viable, non-PMA	55	0.042	1.854
S. cerevisiae viable, PMA	45	0.018	1.779
S. cerevisiae dead, non-PMA	13	0.020	1.501
S. cerevisiae dead, PMA	8	0.019	1.751
C. stellata viable, non-PMA	60	0.078	1.636
C. stellata viable, PMA	38	0.056	1.521
C. stellata dead, non-PMA	6	0.16	1.854
C. stellata dead, PMA	4	0.010	1.558
T. delbrueckii viable, non-PMA	55	0.076	1.751
T.delbrueckii viable, PMA	49	0.016	1.824
T. delbrueckii dead, non-PMA	14	0.015	1.752
T. delbrueckii dead, PMA	4	0.019	1.905
W. anomalus viable, non-PMA	51	0.071	1.854
W. anomalus viable, PMA	45	0.068	1.812
W. anomalus dead, non-PMA	14	0.027	1.675
W. anomalus dead, PMA	10	0.018	1.714

The PMA is a photoreactiv compound, extremely selective, which penetrates only the microbial dead cells which have a compromise membrane; this compromise membrane is stable binding thorough covalent strings to the DNA; in this way, the DNA extraction is inhibited, followed by a delayed or inhibited amplification by qPCR.

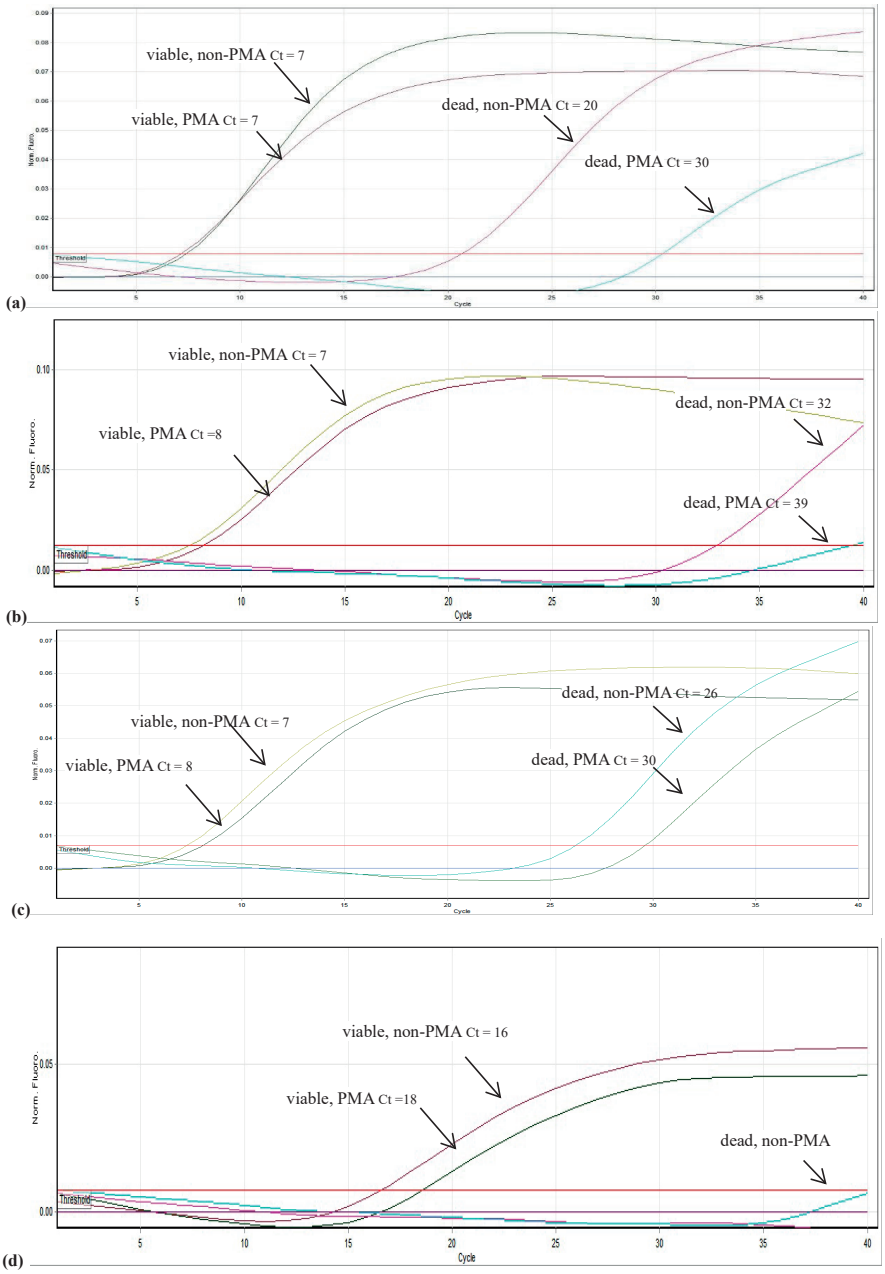

Figure 2. Schematic reprsentation of the PMA treatment in the viable and dead cells for different yeast strains. *S. cerevisiae* (a), *C. stellata* (b), *T. delbrueckii* (c), *W. anomalus* (d)

Depending on the reference strain, it was noticed that the viable PMA treated cells has a delayed Ct comparing to the viable non-PMA treated cells (Figure 2).

Moreover, it was noticed a significant delay of the non-PMA treated dead cells Ct comparing to the non-PMA treated viable cells (Figure 2). Depending on the yeast species, the PMA treated dead cells had a significant reduction of the Ct comparing to the non-PMA treated dead cells. In the case of *W. anomalus*, the Ct of the non-treated and treated dead cells could not be

detected (Figure 2d); much more experiments should be performed for the optimisation.
To figure out if the PMA has inhibited in a adequate manner the DNA amplification of the dead cells, it was calculated the ΔCt for each reference strain (Figure 3). According to the provider recommendations, the expected result for the viable cells is a ΔCt close to zero (+/-1).

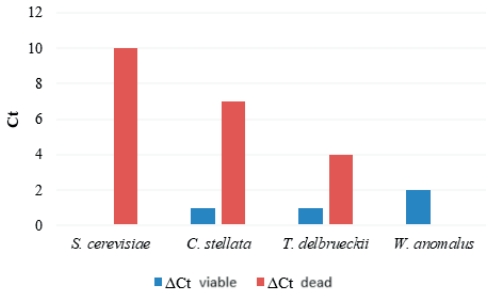

Figure 3. The effect of the PMA treatment on the Ct of the viable and dead cells

In our case, ΔCt for *S. cerevisiae, C. stellata* and *T. delbrueckii* it was 0, respectively 1, which lead to the conclusion that the PMA treatment did not affected the amplification of the viable cells' DNA. In the case of *W. anomalus* it was obtained a higher ΔCt, and new trials need to be performed for the protocol optimisation.In the case of the dead cells ΔCt, it should be higher than 4. In our trials, ΔCt for *S. cerevisiae, C. stellata* and *T. delbrueckii* reached the following values: 10, 7, respectively 4. This indicates that the PMA treatment has inhibited the amplification of the dead cells' DNA.
To generate the standard curve for each reference strain it was used genomic DNA from the PMA treated viable cells. The generated results are enclosed in Table 3. The standard curves' slopes were very similar (close to -3.4 ÷-3.5), which correspond to an amplification efficiency varying between 90.51% to 95.98%. It can be easily noticed that it was obtained a very good linear corelation (R^2), with values from 0.9908 to 0.9959.
Also, good results were obtained in the case of *S. cerevisiae, C. stellata* and *T. delbrueckii* for the detection and quantification limits, meaning that the detection limit varied from 38 fg/µL to 49 fg/µL which corresponds to quantification limits of 70 CFU/mL to $1.03*10^2$ CFU/mL. Similar results for the PMA-qPCR detection limit of *S. cerevisiae* were obtained by different groups (Xu-Cong et al., 2016; Navarro et al., 2020).

Table 3. Parameters of the DNA standard curves obtained by qPCR for *Saccharomyces* and non- *Saccharomyces* reference strains

Yeast species	Slope	Y intersection	R^2	Efficiency (%)	Detection limit	Quantification limit (CFU/mL)
S. cerevisiae viable, PMA	-3.5533	33.866	0.9948	91.17	45 fg/µL	$1.03*10^2$
C. stellata viable, PMA	-3.4221	33.806	0.9959	95.98	38.fg/µL	$9.6*10^1$
T. delbrueckii viable, PMA	-3.5724	35.247	0.9933	90.51	49 fg/µL	$7*10^1$
W. anomalus viable, PMA	-3.5467	33.92	0.9908	91.40	45 pg/µL	$7*10^3$

After the optimisation process, we figured out that the application of PMA-qPCR on wine samples could produce results in one working day, thus presenting a great advantage when compared with the 5-7 days necessary to obtain results from conventional culturing methods.
Similar results were reported by Rizzotti et al, (2015).

CONCLUSIONS

Several NS yeast are of high interest nowadays to obtain distinguished wines, with low alcohol content and specific aromatic profile. In our attempt, we tried to develop a rapid method to be able to supervise the development of *Saccharomyces* versus NS viable cells, which may contribute to the final character of the product. As dye, it was used propidium monoazide (PMA) which proved to be very effective on three put of the four studied species. The detection limit varied from 38 fg/µL to 49 fg/µL which corresponds to quantification limits of 70 CFU/mL to $1.03*10^2$ CFU/mL. In this regard, PMA-qPCR can be considered as a rapid and suitable method for

assessing the viable microbial count for *Candida stellata* and *Torulaspora delbrueckii* versus *Saccharomyces cerevisiae.* Further investigations are requested to optimise the method for other NS species, which are in minority by the end of the wine fermentation.

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