Detection of Viable Honey *Zygosaccharomyces rouxii* using DNA Binding Dyes and Real-Time PCR

**Keywords:** PMA-QPCR; *Zygosaccharomyces rouxii*; detection; honey

**ABSTRACT**
Accurate and rapid quantitative measurement of viable *Zygosaccharomyces rouxii* from honey solutions is the key to control *Z. rouxii* in honey and its products. In this study, the research adopted propidium monoazide bromide/ethidium monoazide bromide combined with the real-time PCR method to optimize the PMA-QPCR procedure and to detect the limits of viable cells both cultivated in Malt Extract Agar medium and 8 different kinds of 50% honey solutions. The results are as follows: (1) The optimal PMA and EMA concentrations were 23.72 and 31.50 μM respectively. The effect of PMA was superior to EMA. (2) The optimal exposure time with PMA treatment was 20 min. (3) For *Z. rouxii* cultured by MEA medium, the detection limit of real time PCR (QPCR) was $10^3$ CFU ml$^{-1}$, and that of PMA-QPCR was $10^4$ CFU ml$^{-1}$. (4) When 50% Honey solution prepared with 8 different kinds of honey was utilized to culture *Z. rouxii*, the detection limit by QPCR was still $10^3$ CFU ml$^{-1}$; and that by PMA-QPCR in 7 kinds of honey solution remained the same ($10^4$ CFU ml$^{-1}$) too. (5) Under the conditions established in this experiment, the standard curve showed a good linear relationship with the cells concentration cultured either by MEA or 50% honey solution in the range of $10^3$ CFU ml$^{-1}$ - $10^7$ CFU ml$^{-1}$, and had quite good correlation coefficient, slope and intercept, and most of them also had good amplification efficiency. This shows that the established method is not only suitable for the detection of *Z. rouxii* in MEA, but also suitable for the detection of *Z. rouxii* in honey solution. This study provides a more promising method for the detection of live *Z. rouxii* in honey and its products.
INTRODUCTION

Zygosaccharomyces rouxii (Z. rouxii) is a very common kind of osmotolerant yeast. It often exists in high sugar and high salt foods (Snowdon and Cliver, 1996; Olivieri et al., 2012; Park et al., 1996). Z. rouxii was found in honey (Chen et al., 2016). Chen et al. (2016) isolated 60 strains of yeast from honey and identified 21 strains belonging to Z. rouxii by a real-time PCR method. Z. rouxii can result in the corruption of honey and its products. Therefore, Z. rouxii adversely affects the shelf life and quality stability of honey and its products.

Traditionally the cultivated method can be adapted to detect Z. rouxii existing in honey, while it is time-consuming and laborious, and has been unable to meet the emergency requirements for real-time supervision of honey and its products. It generally takes 1-2 weeks (Ramon, 1997). Based on the advance of molecular biology, real-time PCR was developed for rapid identification of Z. rouxii in honey (Chen et al., 2016). The real-time PCR technology has the advantages of rapidness, sensitivity and specificity (Chen, 2013; Chen et al., 2013; Harrison et al., 2011). Chen et al (2016) have established a real-time PCR method (QPCR) for the fast identification of Z. rouxii, which the identification time was shortened from 1-2 weeks to about 5 hours. However, the DNA in dead cells can exist for a long time, the method, like most traditional nucleic acid testing methods, cannot distinguish between dead and live cells so that the overestimation was prone to happen. Propidium monoazide (PMA) and ethidium monoazide bromide (EMA) are nucleic acid binding dyes. PMA or EMA can penetrate through damaged cell walls or membranes of dead yeast or bacteria and form covalent cross-linking with DNA molecules under visible lights. The cross-linked DNA with PMA or EMA is unable to process PCR amplification. The incorporation of PMA/EMA with real time PCR (PMA/EMA-QPCR) assays can detect only live cells, but not dead cells, thus the overestimation was remedied (Nocker et al., 2006; Andreas et al., 2009; Rawsthorne et al., 2009; Liang et al., 2011; Yang et al., 2011; Zhu et al., 2012; Marco et al., 2014; Duarte et al., 2015; Tian et al., 2016; Udomsil et al., 2016; Zhou et al., 2017). There is a difference between PMA and EMA. EMA can partially penetrate into living cells, resulting in loss of genomic DNA, but PMA, partly due to carrying more charges, does not easily enter...
living cells. In most cases, the detection results with PMA treatments are more authentic than those with EMA treatments. But there are also exceptions. For example, Andorrà et al. (2010) compared the detection results of live yeast in wine treated by PMA and EMA and found that there was no difference in their study. In Gemma’s research (2013), the detection results for live *Candida albicans* with EMA treatments were more precise than those of PMA treatments at their tested concentration 50 μM.

In order to establish a fast detection method, for the first time this study coupled real-time PCR with PMA or EMA treatments on detecting of living *Z. rouxii* cultured by both MEA medium and honey solution, compared the effects of PMA and EMA, optimized PMA/EMA concentrations and the exposure time of PMA treatment. This research provides a promising detection method for viable cells for *Z. rouxii* in honey.

**MATERIALS AND METHODS**

**Strains and culture conditions**

*Zygosaccharomyces rouxii* standard strain (CGMCC 2.1915) was bought from Culture Collection Center of Chinese Academy of Sciences and was grown in Malt Extract Agar (MEA) medium (3% Malt Extract Powder, 0.3% soy peptone, 1.5% agar) for 48 h at 28 °C.

**Optimization of the PMA/EMA-QPCR procedure**

PMA (Biotium, Inc., USA) was dissolved in 20% Dimethyl Sulfoxide (DMSO) whereas EMA (Biotium, Inc., USA) was dissolved in water, then their concentrations were adjusted to 2 mM by adding distilled water. Both stock solutions were stored in the dark at -20 °C. Different dyes concentrations were analysed: for PMA 0.0, 5.98, 6.38, 15.87, 23.72, 25.67 and 31.50 μM, respectively; for EMA 0.0, 15.87, 23.73, 31.50, 39.22 and 46.88 μM, respectively. The best effects of PMA and EMA were compared. The light source was a 650 W halogen lamp (Jinshan, Tianjin Lamps and Lanterns Factory, China). A box constructed with refractory walls was used for the sample tubes. The halogen lamp was placed 15 cm from the 1.5 ml Eppendorf polypropylene tubes containing the cell solution resuspended in...
500 µl of sterilized water. The optimized PMA concentration was used. And the incubation time was 10 min in the dark. The exposure time was 5 min (5 min × 1 times), 10 min (5 min × 2 times), 15 min (5 min × 3 times), 20 min (5 min × 4 times), 30 min (5 min × 6 times), 40 min (5min × 8 times), 50 min (5min × 10 times), and 60 min (5 min × 12 times), respectively. The samples were exposed to the light for 5 min, with an interval of 1 min on ice to prevent overheating. All these parameters were tested against viable and dead cells, with and without dyes treatments. Dead cells were obtained from 48 h culture with the same volume of distilled water and heated at 90°C for 20 min. The lack of cell viability was confirmed by plate count with MEA medium. Cells without PMA/EMA treatment were used as controls to evaluate the effect of both dyes.

**DNA extraction and real-time PCR amplification**

Genomic DNA was extracted using the yeast genome DNA extraction kit (TIANGEN BIOTECH Co., LTD, Beijing) according to the manufacturer’s instruction. The DNA concentration and quality were estimated by a NANODROP (NanoDrop ND-1000, USA). In all cases, real-time PCR was performed in an ABI 7300 real-time PCR system (Applied Biosystems, USA). The real-time PCR were carried out in a total volume of 20 µL containing 25 to 50 ng of DNA template, 10 µL TaqMan Gene Expression Master Mix (Applied Biosystems, USA), 0.2 µM forward primer, 0.2 µM reverse primer and 0.1 µM of *Z. rouxii* specific probe. The sequences of the primers and probe were the same as our previous studies as the following: primer-F: 5’-CCACGATAGTCGTATTAGG-3’; primer-R : 5’-TGAGGTCAAACTTTTGAG ; probe : 5’-FAM-CCAGACGCTGCTGCTTCTA-TAMER-3’(Rao et al., 2015). The qPCR conditions were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s, which had been optimized in our previous studies (Chen, et al., 2013; Chen, et al., 2016).

**Standard Curve**

Standard curves were established using a 2-days *Z. rouxii* culture. DNA was obtained by the yeast genome DNA extraction kit (TIANGEN BIOTECH Co., LTD, Beijing) according to the

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manufacturer’s instruction as step 2.2. The initial amount of yeast cells was determined by plate counts method to be $10^7$ CFU ml$^{-1}$. Consequently, serial logarithmic dilutions of the initial DNA stock solution in Tris buffer (10 mM Tris, pH 8.0) were prepared. Standard curve was performed by duplicate on a ABI 7300 system.

**Detection of Z. rouxii cultured in honey solution by PMA-QPCR**

Due to the complex ingredients of honey samples, this experiment studied the effect of honey on the detection results of PMA-QPCR method. All 8 kinds of honey samples were purchased from various supermarkets. The details are shown in table 1. Seven of them were from Beijing and one from Heilongjiang. Each kind of honey samples was used to make 2 bottles of 100 ml of 50% (w/v) honey solution (one of them was used as control). All the solutions were sterilized at 121 ºC for 20 minutes, cooled to room temperature and then inoculated into 3 ml (w/w) of CGMCC 2.1915 suspension ($10^7$ CFU ml$^{-1}$). The same volume of sterile water was added into the bottles used as the control. After cultivating for 2 days at 28 ºC, four tubes of the cells suspension of Z. rouxii from each kind of honey solution were taken (500 µl tube$^{-1}$). They were equally divided into two groups (2 tubes group$^{-1}$): group QPCR and group PMA-QPCR. And each group was arranged to two subgroups: subgroup H and subgroup W. For subgroup H, the 50% honey solution was not removed; for subgroup W, the solution was centrifuged firstly, then the supernatant was discarded and 500 µl of sterile water was added in the precipitation. Subsequently, DNA was extracted as described in section 2.2, and the DNA concentration and quality were measured by NANODROP. After adjusting the DNA concentration, the extracted DNA was 10-fold serially diluted to $10^8$, and the qPCR amplification or PMA-qPCR was performed. The same treatment and dilution had been duplicated.

**Plate count**

To determine the actual correspondence between molecular data and culture counts, 0.1 ml of appropriate dilutions of samples for PMA-QPCR and heat treatment assays were spread on the duplicates of MEA plates (Luqiao Corporation, Beijing, China). On the same day, the
molecular analysis were carried out. The Plates were incubated at 28 °C for 48 - 120 h. The colony number was determined by using a colony counter (Scan 100, Interscience).

**Statistical analysis**

Mean values and standard deviations were calculated on the basis of two independent experiments, each performed in duplicate. Microsoft Office Excel 2007 (Microsoft Corporation, USA) was employed to determine the equations of standard curves, correlations and curve values (slope and intersection). The amplification efficiency was calculated from the slopes of the equations.

**RESULTS AND DISCUSSION**

**Optimization of the dyes treatment**

For PMA, when the final concentration was 23.72 μM, ΔCt (ΔCt = Ctdead, av – Ctliving, av) reached the highest value, which was 7.70. For EMA, when the final concentration was 31.50 μM, ΔCt reached the highest value, which was 5.90 (Figure 1). Therefore, the optimized concentration of PMA and EMA were 23.72 μM and 31.50 μM respectively. But even at the optimized concentration of EMA (31.50 μM, ΔCt 5.90), the effect wasn’t as good as that at the optimized PMA concentration (23.72 μM, ΔCt 7.70). Thus, the effect of PMA was superior to EMA for detecting viable *Z. rouxii*. This was consistent with the results of a similar study (Zhu et al, 2016). Partially due to carrying more positive charges, it was less likely for PMA to enter into living cells, only enter into the damaged membranes of dead cells by heat treatment, so that the amplification of dead cells were inhibited. Therefore, PMA is more suitable to detect viable cells of *Z. rouxii*.

When the samples were exposed to the light for 20 min (5 min × 4 times), ΔCt (ΔCt = Ctdead, av - Ctliving, av) was 11.55 (Figure 2). It was suitable for detecting viable cells of *Z. rouxii*. In addition, under the condition of 50 min exposure time (5 min × 10 times), ΔCt reached the highest value, which was 12.48. But considering the efficiency, as well as issues such as excessive heat, this study selected 20 min (5 min × 4 times) as the optimal exposure time.

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The detection limit of the viable cells of Z. rouxii by PMA-QPCR

The results of viable plate count showed that the concentration of the suspension was $10^7$ CFU ml$^{-1}$. The experimental results demonstrated the Ct value was linear with the logarithm of Z. rouxii cells concentration (lg CFU ml$^{-1}$), whether or not treated by PMA or by heat (Figure 3). In this paper, samples were identified to be positive when Ct<35, suspicious when 35≤Ct<40, negative when Ct≥40 (Undetermined results in the ABI 7300 system). The standard curve equation for Z. rouxii without heat and PMA treatment was $y = -3.5985x + 44.342$ (Figure 3, L, CON). The detection limit of the cells concentration was $10^3$ CFU ml$^{-1}$. The Ct value at this limit was 33.93. The standard curve amplification efficiency was 90%; R$^2$ was 0.992; the corresponding slope was -3.5985. The standard curve equation for Z. rouxii with only heat treatment was $y = -3.5392 x + 44.876$ (R$^2$ =0.998) (Figure 3, D, CON). The detection limit was $10^3$ CFU ml$^{-1}$, and the corresponding Ct value was 34.25. The standard curve amplification efficiency was 92%; R$^2$ was 0.998; the corresponding slope was -3.5392. The standard curve equation for Z. rouxii with PMA and heat treatment was $y = -3.5245x + 48.025$ (Fig. 3, D, PMA). The detection limit was $10^4$ CFU ml$^{-1}$, with Ct value of 34.11. The standard curve amplification efficiency was 92%; R$^2$ was 0.998; the corresponding slope was -3.5245.

The above results revealed that there was no significant difference between live and dead cells when detecting Z. rouxii only by QPCR method without PMA treatment. So the common QPCR couldn’t distinguish between dead or live cells of Z. rouxii. However, when the cells concentration of Z. rouxii was less than $10^4$ CFU ml$^{-1}$, dead cells would be fully inhibited and thus couldn’t be detected by PMA-QPCR. So, the PMA-QPCR method can be applied to reduce the possibility of false positive results caused by dead cells.

The influence of honey on the Z. rouxii detected by the PMA real-time PCR method

The genomic DNA of the standard strains of Z. rouxii, which were cultured by 8 kinds of different honey solutions, with two kinds of treatments (H and W, the same as that mentioned in the materials and methods section), were extracted and 10-fold serially diluted to $10^{-8}$. 

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QPCR and PMA-QPCR were performed in duplicate. Standard curves were obtained according to the average Ct value and lg CFU ml\(^{-1}\). The slopes, intersections and R\(^2\) were showed in Table 2. It was indicated that for Z. rouxii cultured by all the 8 kinds of solution, whether detected by QPCR or PMA-QPCR, the linearity of the standard curves was set for the concentration between 10\(^3\) and 10\(^7\) cells ml\(^{-1}\). All the standard curves had good R\(^2\), slopes and intersections.

For QPCR detection, the concentration limits of Z. rouxii cultured by 7 of the 8 kinds of solutions were all 10\(^3\) CFU ml\(^{-1}\). And the detection limits of the PMA-QPCR of Z. rouxii cultured by 7 of the 8 kinds of solution were all 10\(^4\) CFU ml\(^{-1}\). Only the detection limit of the No. 2 honey was special, its QPCR limit was 10\(^4\) CFU ml\(^{-1}\), and that of PMA-QPCR was 10\(^5\) CFU ml\(^{-1}\), which meant there might be PCR inhibitors in this honey. Table 2 indicated that for most of the tested honey solution when the cells concentration was less than 10\(^4\) CFU ml\(^{-1}\), all the dead cells would be inhibited when by PMA-QPCR detection. This was the same result as the previous section.

In addition, based on the slopes of the standard curves, the amplification efficiency of each standard curve was calculated (Table 2). Among all the 32 standard curves, the amplification efficiencies of 14 were in the range of 90%-110%, which were the successful QPCR according to the basic principle of QPCR. And except for the H treatments of No. 2, No. 5 and No. 6, which had relatively lower amplification efficiency (69.66%, 74.95% and 79.90%), and W treatment of No. 8, which had relatively higher amplification efficiency (which was 130.24%) , the amplification efficiency of all the other standard curves were ranged from 80% to 120%, which were acceptable. It was indicated that No. 2, No. 5, No. 6 and No. 8 honey might contain PCR inhibitors, especially in the H treatments of No. 2, No. 5 and No. 6 honey. So, in later PMA-QPCR detection, if the Z. rouxii in honey solution were washed by sterile distilled water before DNA extraction step, the PCR inhibitors might be removed out. Because the QPCR amplification efficiency of W treatment of No. 8 was still 130.24%, which was higher than acceptable range, there might be certain abnormal factors or errors. It should be repeated in the subsequent experiments to find out the factors that lead to abnormal

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results.

To sum up, in the 8 tested honey samples, the components of 3 kinds of honey samples had a slight influence on the method of PMA-QPCR detection of Z. rouxii, the reason possibly there were PCR inhibitors in these honey samples. For the other 5 kinds honey, the amplification efficiencies were between 80% and 120%, which were acceptable. It was indicated that the method established in this paper is feasible for the detection of Z. rouxii in honey solution. And how to remove PCR inhibitors will continue to be studied.

DISCUSSION

In this study, the method of PMA combined with real-time PCR was first applied to detect viable cells of Z. rouxii from honey. It overcame the drawbacks of traditional DNA molecular detection methods, which couldn’t distinguish the dead and viable cells and might lead to overestimated results. Our method with potential application values, which is able to control the quality of honey and its products more efficiently.

This study optimized the exposure time under the 650 W halogen lamp to be 20 minutes (5 min×4 times). The optimal time of illumination obtained in this study was different from the other literatures (Rawsthorne et al., 2009; Andorrà et al., 2010; Zhu et al., 2012; Gemma et al., 2013; Udomsil et al., 2016). The reasons probably were the difference of halogen lamps, illumination distances, and strains types and medium. This study also optimized the PMA and EMA concentrations to be 23.7 uM and 31.50 M, respectively, which was different from the other literatures (Andorrà et al., 2010; Gemma et al., 2013). Though the PMA and EMA were both bought from Biotium, the difference might be caused by the differences of production batch of PMA and EMA, transportation and storage conditions, strains, and the culture medium, etc.

This study laid a good foundation for the rapid detection of the living cells of Z. rouxii in honey.
REFERENCES

18. Tian Q, Feng JJ, Hu J and Zhao WJ. Selective detection of viable seed-borne Acidovorax citrulli by real-time Citation: Shiqiong Chen et al. Ijsrm.Human, 2018; Vol. 8 (3): 261-274.


Tables of Shiqiong Chen’s paper:

**Table 1. Information about the 8 kinds of honey samples used in this research**

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Origin</th>
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<td>1</td>
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<td>2</td>
<td>Acacia Honey</td>
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<td>Urapon White Honey</td>
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<td>Hua Lin Beehive Honey</td>
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<td>6</td>
<td>Yuanma Vitex Honey</td>
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<td>7</td>
<td>Jiaoyan Acacia and Linden Honey</td>
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<td>Pure Honey</td>
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Table 2. The standard curves parameters tested by QPCR and PMA-QPCR of *Z. rouxii* cultured by 8 kinds of different honey solutions

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<tr>
<th>No.</th>
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<tr>
<td></td>
<td>Slope</td>
<td>E(%)</td>
<td>Intersect</td>
<td>R²</td>
<td>LTD (CFU/ml)</td>
<td>Slope</td>
<td>E(%)</td>
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<td>and treatment</td>
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<td>W</td>
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Note: the concentration of *Z. rouxii* cells in all honey solutions were 10⁷ CFU ml⁻¹. The “H” meant that the honey solution in the culture was not removed before DNA extraction. “W” meant that
the honey solution was centrifuged firstly, then the supernatant was discarded and 500 µl of sterile water was added in the precipitation before DNA extraction. “LTD” meant the lowest cells concentration which can be detected.

Figures of Shiqiong Chen manuscript:

**Figure 1.** The optimized PMA and EMA concentration and the comparison of PMA and EMA effects for distinguishing between living and dead cells of *Z. rouxii* by PMA/EMA combined with real time PCR method

**Figure 2.** The optimized exposure time of PMA treatment for *Z. rouxii* (the cells concentration was $2 \times 10^7$ CFU ml$^{-1}$)

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Figure 3. The standard curve of PMA combined with real time PCR to detect dead and living Z. rouxii cultivated by MEA medium. Note: “D, PMA” meant the Z. rouxii cells were with both heat and PMA treatment; “D, CON” meant the Z. rouxii cells were with only heat treatment; “L, CON” meant the Z. rouxii cells were with neither heat nor PMA treatment.