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

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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

livingcells. 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 ℃.

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 ℃. 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℃ 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'-TGAGGTCAAACTTTGAG ; probe : 5'-FAM-CCAGACGCTGCCTGCTTCTA-TAMER-3'(Rao et al., 2015). The qPCR conditions were as follows: 95 ℃ for 10 min and 40 cycles of 95 ℃ for 15 s and 60 ℃ 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

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 ℃ 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 ℃, 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 ℃ 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 ($\Delta Ct = Ct_{dead, av} - Ct_{living, av}$) 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 ($\Delta Ct = Ct_{dead, av} - Ct_{living, av}$) 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.

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}.

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

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

1. Andreas N, Anne K and Camper. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiology Letters 2009;291: 137-142.

2.Andorrà I, Esteve-Zarzoso B, Guillamón JM and Mas A. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. International Journal of Food Microbiology 2010;144 (2): 257 - 262.

3.Chen SQ.Detection of Saccharomyces cerevisiae in juice by real time PCR. Science and Technology of Food Industry 2013;34(7): 319-321, 349 (in Chinese).

4.Chen SQ, Pang B, Cai XF, Yi J, Guo ZL, Fu PB et al. Identification of spoilage yeasts isolated from food by real-time PCR. Food and Fermentation Industries 2013, 39(7): 185-187 (in Chinese).

5.Chen SQ, Cai XF, Pang B, Han Y, Yi J, Ren Y et al. Rapid identification of eosinophils yeast isolated from honey by real-time PCR method. Educational Technology Expo 2016;270(3): 72-73, 82 (in Chinese).

6.Duarte A, Botteldoorn N, Coucke W, Denayer S, Dierick K and Uyttendaele M. Effect of exposure to stress conditions on propidium monoazide (PMA)-qPCR based Campylobacter enumeration in broiler carcass rinses. Food Microbiology 2015;48: 182 - 190.

7.Gemma A, Mariana F, Jordi M and Francesc C. Viable quantitative PCR for assessing the response of Candida albicans to antifungal treatment. Applied Microbiology and Biotechnology 2013;97 (1): 341 - 349.

8.Harrison E, Muir A, Stratford M and Wheals A. Species-specific PCR primers for the rapid identification of yeasts of the genus Zygosaccharomyces. FEMS Yeast Research 2011;11 (4): 356-365.

9.Liang NJ, Dong J, Luo LX and Yong L. Detection viable Salmonella in lettuce by propidium monoazide real time PCR. Journal of Food Science 2011;76 (4): 234 - 237.

10.Marco V, Marisa M, Giuseppe C, Julien B and Lucilla L. Use of Propidium monoazide for the enumeration of viable Brettanomyces bruxellensis in eine and beer by quzantitative PCR. Food Microbiology 2014;42: 196 - 204.

11.Nocker A, Cheung CY and Camper A. K. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. Journal of Microbiological Methods 2006;67 (2): 310-320.

12.Olivieri C, Marota I, Rollo F, and Luciani S Tracking plant, fungal, and bacterial DNA in honey specimens. Journal of Forensic Sciences 2012;57 (1): 222-227.

13.Park YK, Koo MH and Oliveira IM. Biochemical characteristics of osmophilic yeasts isolated from pollens and honey. Bioscience, Biotechnology, and Biochemistry 1996;60 (11): 1872-1873.

14.Ramon D. Trends in wine microbiology. Microbiologia 1997;13(4): 405-411.

15.Rao H, Cai XF, Fu PB, Chen SQ, Pang B, Guo ZL, et al. The oligonucleotides and real time PCR kit for identification of Z. rouxii. Chinese patent. ZL 2013 1 0683581.9 ［P］2015-04-01 (In Chinese).

16.Rawsthorne H, Dock CN and Jaykus LA. PCR-Based Method Using Propidium Monoazide to Distinguish Viable from Nonviable Bacillus subtilis Spores. Applied Environmental Microbiology 2009;75(9): 2936 - 2939.

17.Snowdon JA and Cliver DO. Microorganisms in honey. International Journal of Food Microbiology 1996;31 (1-3): 1-26.

18.Tian Q, Feng JJ, Hu J and Zhao WJ. Selective detection of viable seed-borne Acidovorax citrulli by real-time

PCR with propidium monoazide. Scientific Reports 2016; 14 (6): 35457. doi: 10.1038/srep35457.

19.Udomsil N, Chen S, Rodtong S and Yongsawatdigul J .Quantification of viable bacterial starter cultures of Virgibacillus sp. and Tetragenococcus halophilus in fish sauce fermentation by real-time quantitative PCR. Food Microbiology 2016; 57, 54–62.

20.Yang X, Badoni M and Gill CO. Use of propidium monoazide and quantitative PCR for differentiation of viable Escherichia coli from E. coli killed by mild or pasteurizing heat treatments. Food Microbiology 2011;28: 1478-1482.

21.Zhou B, Liang T, Zhan Z, Liu R, Li F and Xu H . Rapid and simultaneous quantification of viable Escherichia coli O157: H7 and Salmonella spp. in milk through multiplex real-time PCR. Journal of Dairy Science 2017;100(11): 8804-8813.

22.Zhu RG, Li TP, Jia YF and Song LF. Quantitative study of viable Vibrio parahaemolyticus cells in raw seafood using propidium monoazide in combination with quantitative PCR. Journal of Microbiological Methods 2012;90(3): 262-266.

Tables of Shiqiong Chen's paper:

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

Number	Name	Origin
1	Honey of Sophora japonica	Beijing
2	Acacia Honey	Beijing
3	Urapon White Honey	Heilongjiang
4	Hua Lin Beehive Honey	Beijing
5	Vitex Honey	Beijing
6	Yuanma Vitex Honey	Beijing
7	Jiaoyan Acacia and Linden Honey	Beijing
8	Pure Honey	Beijing

Table 2. The standard curves parameters tested by QPCR and PMA-QPCR of *Z. rouxii* cultured by 8 kinds of different honey solutions

No. and treatment		QPCR					PMA-QPCR				
		Slope	Intersection	R^2	LTD（CFU/ml）		Slope	Intersection	R^2	LTD（CFU/ml）	E(%)
1	W	-3.70	46.15	0.964	10^3		-3.71	86.40	0.970	10^4	85.93
	H	52.57									
2	W	-3.36	44.63	0.989	10^3		-3.86	98.27	0.957	10^4	81.59
	H	48.79									
3	W	-2.97	44.65	0.983	10^3		-3.10	117.34	0.992	10^4	110.17
	H	44.93									
4	W	-3.10	44.484	0.988	10^4		-4.37	69.66	0.974	10^5	
	H	51.94									
5	W	-3.06	43.40	0.997	10^3		-3.27	102.21	0.997	10^4	
	H	45.49									
6	W	-3.18	43.37	0.995	10^3		-3.45	94.88	0.990	10^4	
	H	47.66									
7	W	-3.20	43.16	0.989	10^3		-3.09	110.55	0.994	10^4	
	H	47.34									
8	W	-3.24	43.66	0.994	10^3		-3.20	105.24	0.993	10^4	
	H	43.12									
		-3.12	39.38	0.992	10^3		-3.45	95.06	0.994	10^4	
		44.98									
		-3.46	41.79	0.992	10^3		-4.12	74.95	0.988	10^4	
		48.95									
		-3.71	44.72	0.979	10^3		-3.75	84.80	0.968	10^4	
		46.76									
		-3.52	43.18	0.981	10^3		-3.92	79.90	0.967	10^4	
		49.04									
		-2.96	41.35	0.979	10^3		-3.22	104.47	0.999	10^4	
		45.14									
		-3.29	43.57	0.994	10^3		-3.28	101.65	0.964	10^4	
		45.99									
		-2.76	39.377	0.9382	10^3		-3.102	110.07	0.933	10^4	
		44.87									
		-2.98	41.254	0.9862	10^3		-3.100	110.05	0.958	10^4	
		45.27									

Note: the concentration of *Z. rouxii* cells in all honey solutions were 10^7 CFU ml^{-1}. The “H” 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:

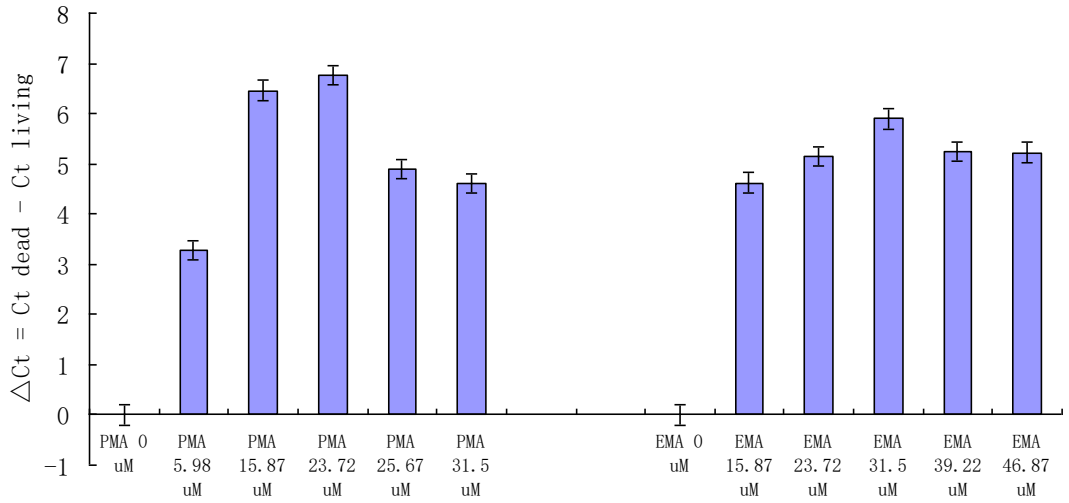

Figure 1. The optimized PMA and EMA concentration and the comparison of PMA and EMA effects for distinguishing between living and dead cells of *Z. rouxii* by PMA/EMA combined with real time PCR method

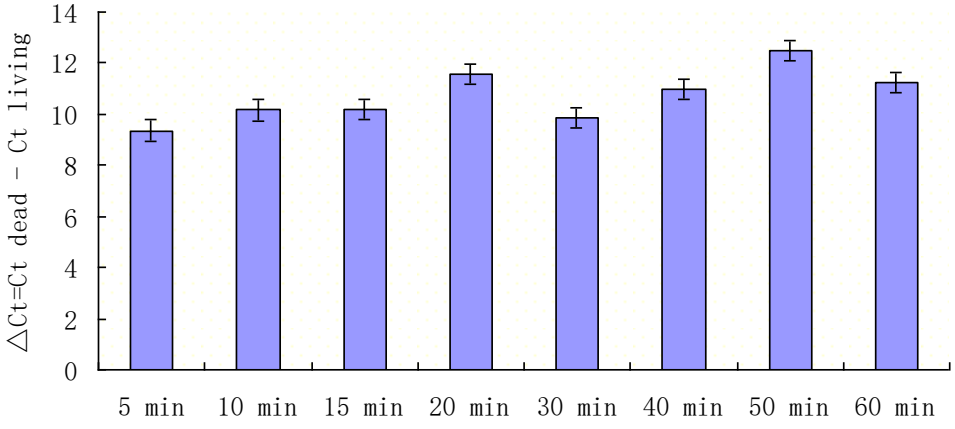

Figure 2. The optimized exposure time of PMA treatment for *Z. rouxii* (the cells concentration was 2 ×10^7 CFU ml^{-1})

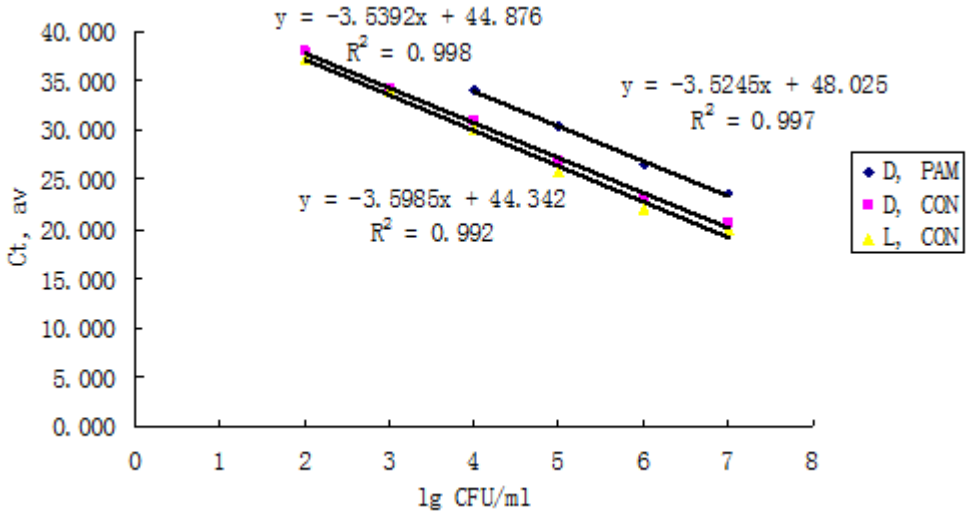

Figure 3. The standard curve of PMA combined with real time PCR to detect dead and living Z. rouxii cultivated by MEA medium. Note: "D, PMA" meant the *Z. rouxii* cells were with both heat and PMA treatment; "D, CON" meant the *Z. rouxii* cells were with only heat treatment; "L, CON" meant the *Z. rouxii*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