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Inactivation of Endogenous Pectin Methylesterases by Radio Frequency Heating during the Fermentation of Fruit Wines

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Abstract: Pectin methylesterase (PME) is a methyl ester group hydrolytic enzyme of either plant or microbial origin. Importantly, endogenous PMEs in fruits can catalyze the demethoxylation of pectin with a bulk release of methanol, largely impacting the fruit juice and wine industries. Here, we demonstrated radio frequency (RF) heating for inactivation of endogenous PMEs and investigated the relevant mechanisms underpinning enzymatic inactivation. The RF heating curve indicated that the optimal heating rate was achieved at an electrode gap of 90 mm (compared to 100 mm and 110 mm) and that the inactivation rate of the enzyme increases with heating time. RF heating exhibited better effects on enzymatic inactivation than traditional water heating, mainly by changing the secondary structures of PMEs, including α-helix, β-sheet, β-turn, and random coil. Moreover, fluorescence spectroscopy indicated changes in the tertiary structure with a significant increase in fluorescence intensity. Significantly, application of RF heating for inactivation of PMEs resulted in a 1.5-fold decrease in methanol during the fermentation of jujube wine. Collectively, our findings demonstrated an effective approach for inactivating endogenous PMEs during the bioprocesses of fruits.

Keywords: radio frequency; pectin methylesterase; inactivation; fruit wine; fermentation

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1. Introduction

Pectin methylesterase (PME, E.C. 3.1.1.11) belongs to class 8 of the carbohydrate esterase and catalyzes the specific hydrolysis of the methyl ester bond of pectin, which is one of the main components of the cell wall of plants [1]. This hydrolysis can alter the degree and pattern of methyl esterification and release a bulk of methanol [2]. PMEs are widely distributed in different plant tissues, such as roots, stems, leaves, and fruits [1,3]. Such endogenous PMEs play an important role in the growth and development of plants by regulating a variety of physiological or biochemical processes, including the synthesis or degradation of cell walls, cell-free pollen development, seed gemination, root extension, fruit softening, and disease resistance [1]. However, endogenous PMEs are usually undesirable enzymes in the fruit juice and wine industries because of the release of methanol during the hydrolysis of pectin. In the fermentation of fruit wines, excessive production of methanol can affect the product flavor and even cause food safety concerns. Thus, inactivating endogenous PMEs is crucial in the bioprocesses of fruit wines.

The activity of endogenous enzymes in fruits and vegetables can be inhibited through both chemical and physical approaches [1,4]. For example, some chemical inhibitors, such

as sulfites, ascorbic acid, and amino acids, have exhibited highly efficient inhibition of enzymatic activity of vegetables [1,5]. However, physical blanching, which inactivates enzymes by heating, is more cost-effective, healthier, and safer than chemical approaches, especially when used for processing of fruits and vegetables in food industries. One of the most traditional physical approaches is blanching with hot water. Although this approach can effectively inactivate enzymes, it usually alters the color and texture of fruits and vegetables [6], which decreases the value of products according to the preferences of consumers. Moreover, some soluble components might be lost in hot water, resulting in loss of substantial nutrients and discharge of a large amount of waste water [7].

Radio frequency (RF) heating is an innovative blanching technique relative to traditional techniques based on electro-technologies, such as ohmic heating, radiative or microwave dielectric heating, magnetic heating, and inductive heating [8]. Unlike traditional systems that transfer heat energy from a hot medium to a cooler product with large temperature gradients, RF heating enables electromagnetic energy to transfer into the product directly, initiating uniform volumetric heating through molecule–molecule frictional interactions [9]. Thus, RF heating is a promising technology for food processing because of its ability to rapidly and uniformly distribute heat, its deep-penetration capability, and reduced consumption of energy [10]. Recently, RF heating has been successfully applied for inactivation of enzymes with reduced loss of nutrients (e. g., vitamins B and C) and lower cost than traditional methods [11,12]. For example, RF heating has been observed to effectively inactivate lipoxygenase and peroxidase of green pea (*Pisum sativum* L.) [13]. Using RF heating, peroxidase was significantly inactivated in stem lettuce, resulting in better physiochemical properties and less cell damage than the traditional hot water blanching [11]. However, current research on its application for inactivation of PMEs in fruits and vegetables is rather limited.

In this study, we attempted to employ RF heating for inactivation of PMEs during the fermentation of red jujube wine and compared its effects with the those of traditional hot water heating. To further elucidate the relevant inactivation mechanisms, both secondary and tertiary structures of PME were investigated through circular dichroism spectroscopy and fluorescence spectroscopy analyses, respectively. Finally, the release of methanol was evaluated during the fermentation of red jujube wine in order to test the probability of application of RF heating in the fruit wine industry.

2. Methods and Materials

2.1. Raw Materials and Sample Preparation

Citrus pectin and PME were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ABF Ingredients (Darmstadt, Germany), respectively. All chemical reagents used in this study, including NaCl and NaOH, were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The PME solution was prepared by adding 0.5 g PME powder to 100 mL of deionized water. The fully mature and undamaged jujube (*Zizyphus jujuba* Mill.) used for wine fermentation was supplied by TIANKUNGUOYE Co., Ltd., Xinjiang, China. *Saccharomyces cerevisiae* BV818 was used for the fermentation of red jujube wine. We strictly followed the method used in our previous study [14] for jujube wine fermentation.

2.2. RF Heating Treatment

A GJG-2.1-10AJY RF system with a free running frequency of 27.12 MHz and a maximum power of 6 kW was employed in this study. To achieve real-time monitoring of the center temperature of samples, the RF system was equipped with a fluorescence fiber optic sensor. Three electrode gaps (90 mm, 100 mm, and 110 mm) were investigated and their effects on the uniformity and heating rate of RF treatment we evaluated in order to determine the optimal gap. Specifically, 20 mL of the PME solution was transferred into a 50 mL centrifuge tube located at the center of the bottom electrode plate. Before reaching the maximum of ~90 °C, the RF heating temperature was recorded at an interval of 20 s to evaluate the uniformity and heating rate. The system was turned off once the sample

temperature reached the given temperatures, and the heated samples were cooled shortly in ice water until they reached room temperature.

To evaluate the effects of RF heating on PME activity, 20 mL of the PME solution in a 50 mL tube was treated at the optimal electrode gap of 90 mm for 0 s, 20 s, 40 s, 60 s, 80 s, 100 s, 120 s, and 140 s. After treatment, samples were rapidly cooled in ice water until they reached room temperature for measurement of enzymatic activity. For water heating, the same sample system was heated in a water bath at a temperature of 90 °C. Once the center temperature of the samples reached 90 °C, the samples were kept in a water bath for 0 s, 20 s, 40 s, 60 s, 80 s, 100 s, 120 s, and 140 s. After heating for the given time, samples were immediately cooled in ice water until they reached room temperature for enzymatic activity measurement. Additionally, the red jujube used for the fermentation experiment was subject to the same RF treatment until the temperature reached 90 °C. Untreated red jujube was used as the control.

2.3. PME Activity Assay

PME activity was measured with reference to the method of Downie et al. [15], with some modifications. Briefly, 20 mL of 1% pectin-salt substrate (containing 0.1 mol/L NaCl) was incubated at 30 °C in a water bath, and the pH of the substrate solution was quickly adjusted to 7.5 using 1 mol/L NaOH or 0.05 mol/L NaOH. Then, 0.5 mL of PME solution was mixed with the substrate solution at 30 °C, and the pH of the reaction system was rapidly readjusted to 7.5 with 0.05 mol/L NaOH. After the pH reached 7.5, 10 µL of 0.05 mol/L NaOH was added. Time was measured until the pH of the solution reached 7.5. Calculations of PME activity unit (PMEU) and the relative PME activity (%) were performed as followed:

$$\text{PMEU}\ (\text{mmol/mL·min}) = \frac{0.01\ \text{mL}\ \times 0.05\ \text{mmol/mL NaOH}}{0.5\ \text{mL PME solution}\ \times\ \text{time}\ (\text{min})} \quad (1)$$

$$\text{R}_\text{A}\ (\%) = \frac{\text{PMEU}_1}{\text{PMEU}_0} \times 100\% \quad (2)$$

where R_A represents the relative enzymatic activity, PMEU_1 is the activity of treated PME solution, and PMEU_0 is the initial activity of untreated PME.

2.4. Spectroscopy Analyses of PME

Circular dichroism (CD) spectroscopy analysis of PME was performed with reference to protocol described by Liu et al. [16]. Specifically, CD spectra were scanned at the far UV range from 190 nm to 250 nm at a scanning speed of 5 nm/s at room temperature and recorded on Bio-Logic MOS-450 CD spectropolarimeter (Bio-Logic, Seyssinet-Pariset, France), where samples were contained in a quartz cuvette of 0.1 cm optical path length. The band width was set as 1.0 nm. The CD data were expressed in terms of the mean residual ellipticity (θ) in mdeg. The concentration of PME for the CD analysis was 5 mg/mL. The contents of secondary structures of PME were calculated according to the SELCON3 algorithms. The curves were generated with the Origin 9.0 (Original Corporation, Northampton, MA, USA) following a fast Fourier transform noise reduction routine to reduce impacts of most noisy spectra without distorting their peak shapes.

Fluorescence spectroscopy was used to analyze the tertiary structures of PME with reference to the method described by Liao et al. [17], with some modifications. Briefly, fluorescence spectra were detected in a HITACHI F-4500 spectrofluorometer (HITACHI, Tokyo, Japan), using a quartz cuvette of 1 cm optical path length at room temperature (25 ± 1 °C). All the samples were observed immediately after treatment. The concentration of PME solutions was 1 mg/mL. The PME solutions were excited at the maximum wavelength of excitation (λ_{ex} = 278 nm), and the emission spectra were recorded from 290 to 490 nm with a scanning speed of 100 nm/min. The gap between excitation and emission was set as 5 nm.

2.5. Determination of Methanol

Contents of methanol in the samples of fermentation broth were determined through gas chromatography (GC), with reference to the method described by Qin et al. [18], with some modifications. Briefly, the assay was carried out in a 7890B GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DB-Wax column (30 m × 250 μm × 0.25 μm, Agilent 122-7032) with an injection volume of 1 μL at an injection speed of 30.0 mL/min and a spilt ratio of 20:1. The initial temperature of the column was maintained at 40 °C for 2 min, increased to 80 °C at a rate of 5 °C/min, further increased to 200 °C at a rate of 20 °C/min, and finally maintained at 200 °C for 5 min. The carrier gas was nitrogen (N_2) at a constant flow rate of 0.5 mL/min. The temperatures of inlet and FID were 220 °C and 230 °C, respectively.

2.6. Data Analysis and Statistics

Every experiment or test in this study was performed in triplicate. All results were reported as means ± standard deviations (SD). Data obtained from different treatments were analyzed and compared by ANOVA and Tukey test at a significance level of 95% using SPSS Statistics 20 (the IBM Corporation, Armonk, NY, USA). Statistical images were generated and exported by Origin 9.0 (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. RF Heating Curve

The heating uniformity and rate considerably affected the inactivation efficiency of enzymes, as well as the physiochemical properties of products in food processing. However, RF heating uniformity is principally determined by dielectric properties, especially the sample position between the electrodes. Thus, the effects of three electrode gaps on the heating uniformity were investigated (Figure 1).

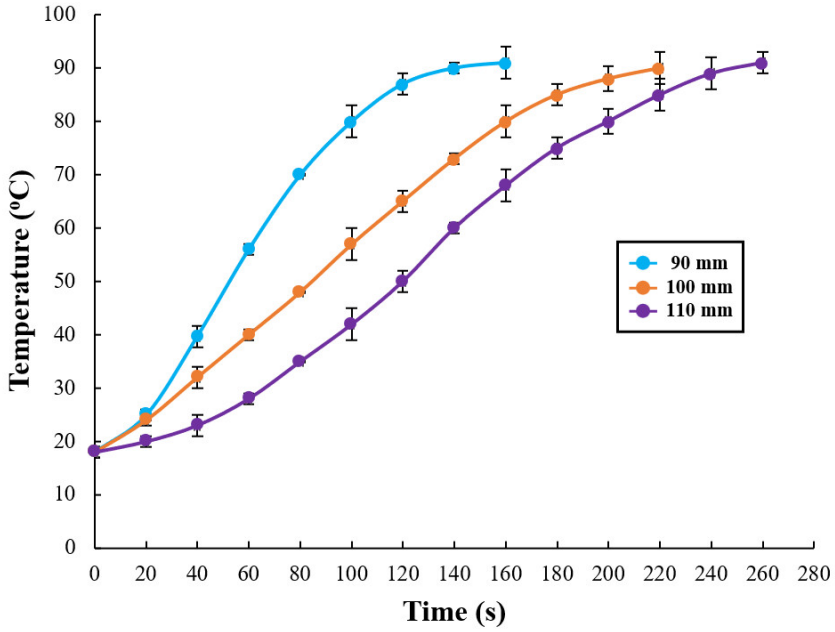

Figure 1. Temperature–time curve of RF heating of PME solution with three different electrode gaps.

With an electrode gap of 90 nm, the temperature of the PME solution reached 90 °C within 160 s. In contrast, the heating rate was lower when the electrode gap was set as 100 nm (220 s for 90 °C) or 110 nm (260 s for 90 °C). For all three electrode gaps, the RF heating rates of the PME solution slowed considerably after reaching 90 °C, which could be attributed to thermal losses, together with the evaporation of moisture at higher temperatures [19]. Moreover, the overall heating rate decreased with increased electrode gap, with

the best heating uniformity and rate with a gap of 90 nm. This pattern is consistent with results reported in previous studies [12,19,20]. Importantly, some observations revealed that different RF heating rates significantly affected the colors and textures of potatoes when blanched at the same temperature [12,20]. Considering the effects of RF heating rates on inactivation efficiency [11], an electrode gap of 90 nm was selected for PME inactivation.

3.2. Effects of RF Heating on PME Activity

To further confirm the inactivation efficiency of RF, traditional water heating was introduced. Both treatments indicated that relative activity decreased with prolonged heating time (Figure 2). However, the two treatments exhibited different patterns of inactivation efficiency. After RF heating for 140 s, the temperature of the PME solution rapidly increased from 18 °C to 90 °C, and the activity of the PME was fully inactivated. During this process, the relative activity almost did not change within the first 60 s of RF heating, whereas it dropped sharply afterwards when the temperature reached 60 °C. In contrast, the relative activity of the PME solution only decreased to 20.75% after water heating for 140 s at 90 °C, but this decrease was very dramatic in the initial phase and significantly slowed down after 20 s. This might be attributed to the unique composition of PME, which includes both thermolabile and thermostable fractions, where the former is easily inhibited at lower temperatures, but the latter could only be inactivated at higher temperatures [21]. It was also observed in a previous study that the minimum residual activity of PME reached 18% with conventional heating at 90 °C for 270 s, which indicates that PME is a thermally resistant enzyme [22]. Another reseaon might be that the components of the medium, such as sugars and salts, can stabilize the enzymatic structure and thus minimize the effect of exposure to heat [23].

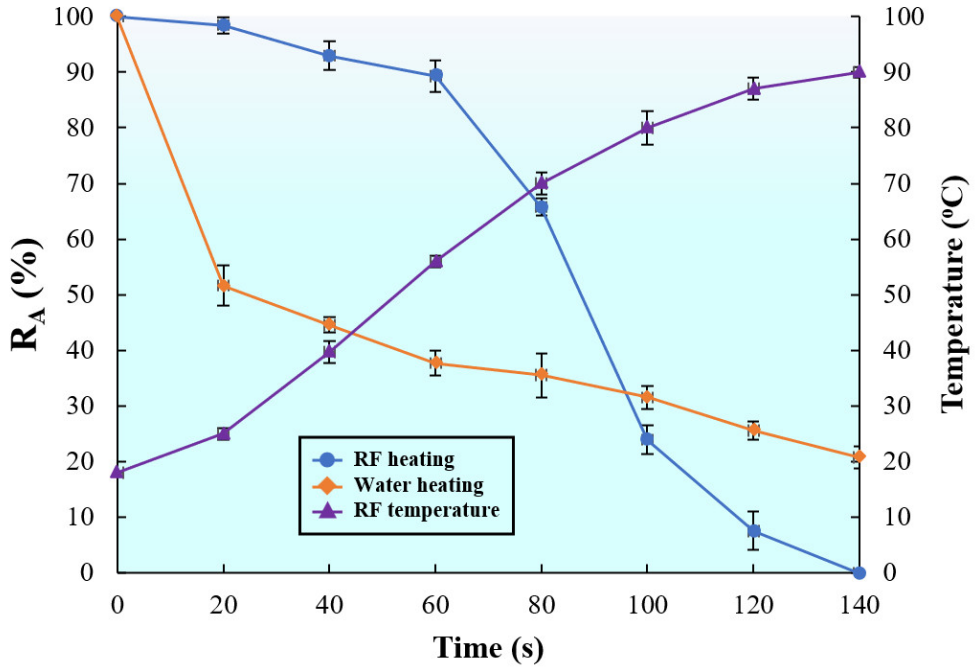

Figure 2. Comparison of effects on PME activity between RF heating and water heating. The electrode gap of the RF system was set as 90 nm. The temperature of the water bath was 90 °C.

The PME samples exhibited the same relative activity (~30%) under the two treatments at 90 s. However, the activity of the PME sample treated with RF was fully inactivated when the temperature reached 90 °C at 140 s, whereas that of the PME sample heated in water bath was still 23.92% at the same time and temperature, indicating that RF heating is more efficient than water heating. The high-efficiency inactivation of RF heating can also avoid a reduction in product quality (e.g., loss of some vitamins, acceptable food color, and texture) caused by high-temperature treatment for a long period of time [12,21]. Importantly, RF

heating achieved full inactivation of PME activity, which is preferable to treatment with pulsed electric fields, which inactivated 90% of PME activity in orange juice [24].

3.3. Mechanisms of RF Heating Inactivation of PME

It is well known that the functions and properties of enzymes are determined by their structures. Any alteration of conformation could lead to a change in enzymatic activity. To elucidate the mechanisms of RF heating inactivation, we examined the secondary structures of PME through CD spectroscopy. As shown in Figure 3a, the CD spectra of the PME solution had a negative absorption peak at 216 nm, which is a typical characteristic of β-type proteins [25]. The composition of the secondary structures of PME is listed in Table 1. The untreated PME (i.e., the control) was composed of 32.20% α-helix, 20.20% β-sheet, 20.90% β-turn, and 29.9% random coil. However, the percentages of β-turn and β-sheet reduced with RF heating time, and the content of α-helix remained constant, whereas that of random coil increased. Some observations have indicated that PME has a parallel β-helix architecture [26]. The decrease in β-turn and β-sheet contents could imply a conformation change in secondary structure. This could be because RF heating induces unfolding of the protein, leading to breaking of the hydrogen bond of β-helices and the transformation to α-helix or random coil, eventually leading to exposure of the hydrophobic group and further inactivation of the enzyme. Several studies support this assumption. For example, an investigation on effects of RF heating treatment on structural changes of soy protein revealed self-reassembly from random coil structure to β-sheet structure, indicating significant changes in the secondary structure [27]. Furthermore, the secondary structure of protein isolates in rice bran after RF treatment also changed with an increase in random coil and a decrease in β-sheet, α-helix, and β-turn [28].

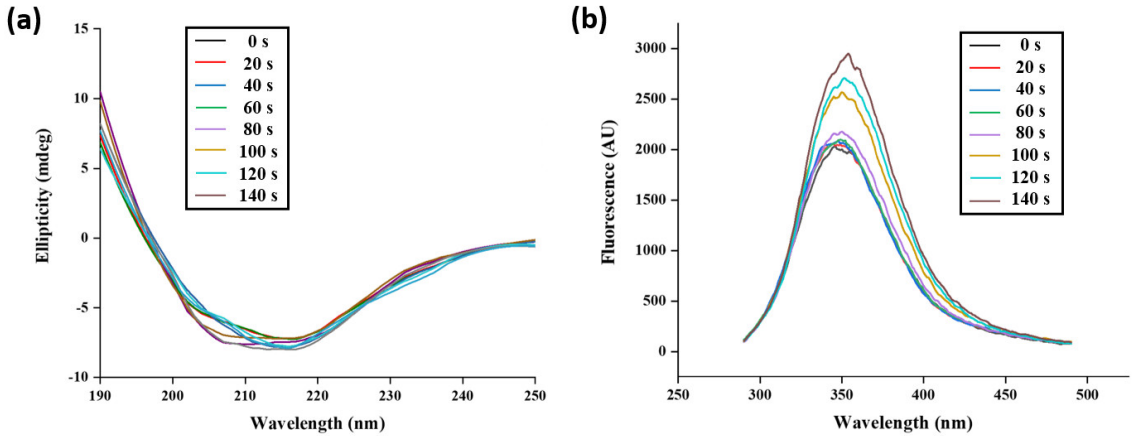

Figure 3. Spectroscopy curves of the PME solution after RF heating treatment. (**a**) Curve of CD spectroscopy and (**b**) curve of fluorescence spectroscopy. The electrode gap of the RF system was set as 90 nm. RF heating time was 0 s, 20 s, 40 s, 60 s, 80 s, 100 s, 120 s, and 140 s.

Table 1. Composition of the secondary structures of PME (%).

Time (s)	α-Helix	β-Sheet	β-Turn	Random Coil
0	32.2 ± 0.3	20.2 ± 0.1	20.9 ± 0.1	29.9 ± 0.5
20	32.4 ± 0.2	20.3 ± 0.4	21.0 ± 0.5	29.9 ± 0.1
40	33.6 ± 0.5	20.3 ± 0.2	20.7 ± 0.2	30.2 ± 0.2
60	33.1 ± 0.1	19.9 ± 0.4	20.3 ± 0.6	29.2 ± 0.5
80	31.3 ± 0.2	18.9 ± 0.1	19.3 ± 0.1	27.6 ± 0.2
100	32.5 ± 0.4	18.2 ± 0.6	19.5 ± 0.2	32.4 ± 0.1
120	33.1 ± 0.6	17.9 ± 0.5	18.5 ± 0.3	25.5 ± 0.2
140	32.4 ± 0.1	13.8 ± 0.2	17.6 ± 0.1	38.5 ± 0.5

Furthermore, change in the fluorescence intensity of tryptophan is highly correlated to the tertiary structure of protein; thus, a change in fluorescence intensity can reflect a conformation alteration [29]. To confirm the above inferences, we further investigated the tertiary structure of PME through fluorescence spectroscopy (Figure 3b). Generally, the fluorescence spectra of PME exhibited almost change when the RF heating time was less than 100 s, indicating a stable tertiary structure. However, the fluorescence intensity significantly increased with prolonged heating time, and the maximum absorption wavelength increased from 346 nm to 354 nm, indicating that the tryptophan of the PME was exposed from the non-polar environment to the external polar environment. This implies that RF heating might alter the tertiary structure and polarity of PME. Further confirmation could verify this implication by determining the protein structure.

3.4. Effects of RF Heating on the Release of Methanol during Fermentation

The methanol levels in fruit wines are mainly attributed to the specific raw materials and strictly subjected to legal limits; thus, source and quality control are extremely important for the wine industry [30]. To evaluate the efficiency of inactivation of endogenous PME during the fermentation process, the methanol levels of the red jujube wine were determined (Figure 4).

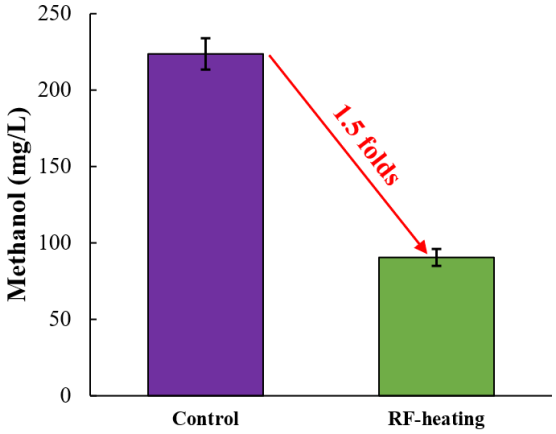

Figure 4. Comparison of methanol contents of the fermentation broth of red jujubes with and without RF heating treatment.

As a result, the methanol content (90.33 mg/L) in wine fermented with RF-treated jujube was reduced by 1.5-fold compared to the control (223.41 mg/L). Collectively, these findings revealed that treatment of red jujube with RF heating could significantly reduce the release of methanol during the fermentation process by inactivating endogenous PME of the raw materials [31]. This provides direct evidence of the application potential of RF heating in the fruit wine industry.

4. Conclusions

In the present study, we successfully demonstrated radio frequency (RF) heating for inactivation of endogenous PMEs and explored the relevant mechanisms underpinning the inactivation. The optimal heating rate was achieved at an electrode gap of 90 mm, and the inactivation rate of the enzyme was found to increase with prolonged heating time. Compared to traditional water heating, RF heating is more efficient for enzymatic inactivation. The mechanisms of inactivation could be involved in altering the secondary and tertiary structures of PME. Moreover, RF heating can significantly reduce the release of methanol in the fermentation of jujube wine. Collectively, our findings prove that RF heating is an effective approach for inactivating endogenous PMEs during the bioprocesses of fruits.

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