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Extraction Optimization and Antibacterial Property of Total Flavonoids from *Zizania Latifolia* Bracts and the Effect on Chilled Pork Preservation

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Abstract: *Zizania latifolia* is a widely distributed aquatic vegetable, and its bracts are an agricultural waste with a large proportion. In this study, Total Flavonoids of *Z. latifolia* Bracts (TFZB) were extracted with Ultrasound-Assisted Enzymatic Extraction (UAEE) for the first time, and the extraction process was optimized using Response Surface Method (RSM), and compared with other methods. Then, the antibacterial activity of TFZB extracted with optimized UAEE was assessed and the effects on chilled pork preservation were investigated. The optimal UAEE conditions for TFZB were: Cellulase dosage of 0.7%, enzymolysis time of 60 min, ultrasonic power of 704 W, and ultrasonic time of 47 min. Under these conditions, the TFZB extraction rate was up to 1.92%, which was significantly higher than those of other methods. The Scanning Electron Microscope (SEM) observation verified that the cytoskeletons of bracts had been severely damaged, resulting in a large release of TFZB. The infrared spectrum confirmed that TFZB has the typical characteristics that belong to natural flavonoids. Moreover, the TFZB extracted with optimized UAEE exerts significant antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* with Minimal Inhibitory Concentrations (MIC) of 2.5 and 1.25 mg/mL, respectively. Finally, the TFZB solutions were sprayed onto the surface of fresh pork, and the indexes of the sensory score, pH, Total Volatile Basic Nitrogen (TVB-N), Metmyoglobin (MetMb)%, and total count of bacterial colonies during the storage period of chilled pork were measured. The results indicated that TFZB at 1.0 mg/mL can significantly improve the sensory quality of chilled pork via inhibiting microbes, and better preserve the freshness of pork when compared with sodium benzoate at 5.0 mg/mL. The present investigation implied that the TFZB extracted with optimized UAEE can be used as a natural flavonoid alternative resource to produce environmental disinfection products and novel preserving agents for chilled pork preservation.

Keywords: *Z. Latifolia* Bracts, Total Flavonoids, Ultrasound-Assisted Enzymatic Extraction, Process Optimization, Antibacterial Property, Chilled Pork Preservation

Introduction

Zizania latifolia, belonging to the Gramineous family, is a perennial root-vert aquatic plant originally grown in China and Southeast Asia. The fruit of *Z. latifolia* also called wild rice was one of the 'six crops' in Zhou Dynasty over 3000 years ago, and was paid as a tribute to the royal family (Hassan *et al*., 2015). So far, the planting area of *Z. latifolia* in China has been up to 72000 hectares, with an annual production value of 40 trillion Yuan, making it the second major aquatic vegetable in China only after lotus roots.

Z. latifolia is a drug-food homologous plant, and swollen culm is the main edible part (Fig. 1b). *Z. latifolia* swollen culm has rich nutrients, including proteins, lipids, glucose, vitamins and minerals, and amino acids. As a local medicine, traditional Chinese medicine holds the opinion that *Z. latifolia* swollen culm is sweet and slightly cold, and has hot-

relieving, thirst-promoting, thirst-quenching, diuretic, dehumidifying, and obstruction-removing effects. Thus, it is reputed as 'ginseng in water' (Zhang *et al*., 2022).

Z. latifolia is a tall and large crop with a high yield, and the mass of bracts (Fig. 1a) accounting for 50-70% of total plant mass, and more than 5000 kg of fresh bracts are produced from 667 m^2 of bi-seasonal *Z. latifolia* (Xiao *et al*., 2022). In this way, the total annual production of *Z. latifolia* bracts produced only in Jiangsu Province is approximately 350000-490000 tons. In the harvest season, massive *Z. latifolia* bracts are optionally abandoned on the village roads and riverside to become a serious contaminant that causes plant diseases and insect pests. In addition, many bracts are even burned, leading to severe environmental pollution. Hence, comprehensive exploitation and utilization of *Z. latifolia* bracts are urgent problems for the development of *the Z. latifolia* industry. Currently, *Z. latifolia* bracts are mainly prepared into silages for feeding cattle, sheep, and other ruminants (Jaeyeul *et al*., 2022), which has been considered a desirable processing measure of agricultural wastes. However, this preliminary processing is limited by the low utilization rate. Agricultural wastes are also rich in functional components that can be exploited. Zhang *et al*. (2019) used a cellulase-assisted method to extract Total Flavonoids from Corn Bracts (TFCB) with an extraction rate of 1.28% and proved that TFCB possesses promising antioxidant activity and antibacterial effect (Zhang *et al*., 2019). Cui *et al*. (2018) found that the ethanol extracts of mulberry leaves significantly prolonged the warranty period of chilled meat from 3 to 6 days (Cui *et al*., 2018). Sumczynski *et al*. 2017) extracted flavonoids from the waste leaves of pomegranate with high yield (Sumczynski *et al*., 2017), and natural flavonoids from plants elicit beneficial effects, including lipid-lowering, free radical-scavenging, anti-oxidative and anticancer properties (Chu *et al*., 2018). These results bring us the implication that *Z. latifolia* bracts may be converted from 'waste' into 'treasure' by exploring functional flavonoids. Thus, in our previous study, the Total Flavonoids from *Z. latifolia* Bracts (TFZB) were extracted with Cellulase-Assisted Extraction (CAE), and we noted that TFZB possesses great antioxidant capacities and potential for strawberry preservation. It is suggested that TFZB can be used as an antioxidant and fruit freshening agent (Jiang *et al*., 2016). However, the yield of TFZB extracted with CAE was low (0.49±0.01%), which should be further improved.

Huang *et al*. (2009) extracted flavonoids from the leaves of *Eucommia ulmoides* and compared the yields with four extraction methods, including ultrasound-assisted microwave-assisted, enzyme-assisted, and ethanol extractions (Huang *et al*., 2009). It was found that ultrasound-assisted extraction brings the highest yield. The reason is that ultrasonic waves can generate a comprehensive effect of cavitation, vibration, crushing, and stirring in the liquid phase, which can crush cells, decrease grain size, and enlarge the contact area between the solid and liquid phases, thereby improving the penetrating ability of solvents and the dissolution of intracellular contents (Huang *et al*., 2009). Thus, in the present study, the efficient ultrasound-assisted extraction was combined with cellulase to extract TFZB for the first time, to improve TFZB yield.

Lipid oxidation and microbial growth can decrease the quality and shelf life of meat products (Gyawali *et al*., 2015). Phytochemicals are safer than chemical antiseptics, and it has been proven those phytochemicals have high antibacterial and antioxidative abilities (Naveena *et al*., 2008; He *et al*., 2016) and have gradually become the research highlights on antisepsis and fresh preservation of meat products. Li *et al*. (2020) found that the ethanol extracts of grape seeds inhibited microbial growth and propagation in sashimi, decreased the accumulation of Total Volatile Basic Nitrogen (TVB-N), histamine, and cadaverine, and prolonged the shelf life under cold storage by about 3 d (Li *et al*., 2020). Moon *et al*. (2018) evenly sprayed extracts from dandelion or gingko leaves to the surface of chilled venison and found that the treatments inhibited bacterial growth, decreased TVB-N and water loss, and protected colors, thereby prolonging the warranty period to 62 d when the extract concentration was 0.2 g/mL (Moon *et al*., 2018). Cui *et al*. (2018) sprayed ethanol extracts from mulberry leaves on the surface of chilled meat and found that the quality, pH, thiobarbituric acid reactant value, TVB-N, and microbial indices were all lower than those of the blank group during the storage period, and the warranty period of the chilled meat was prolonged from 3 to 6 days (Cui *et al*., 2018). The roles of these plant-derived ethanol extracts in meat preservation are ascribed to the active substances dominated by flavonoids and polyphenols. Hence, in the present study, the bacteriostasis of TFZB extracted under optimal conditions was evaluated and used for the preservation of chilled pork. The present contribution will give a new clue for fine processing and high-value reclamation of waste *Z. latifolia* bracts, and evidence for the development of flavonoids-based food preservatives.

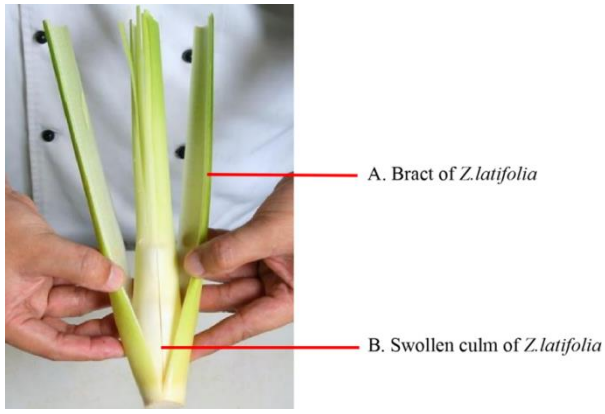

Fig. 1: The bract (A) and edible swollen culm (B) of *Z. latifolia*

Materials and Methods

Materials and Reagents

The bracts of *Z. latifolia* were collected from the peripheral markets and farmland roadside in Southeast Development Zone, Changshu, Jiangsu, China, between May and June 2021. The fresh pork was bought from Datang Agriculture Development Co., Ltd, Changshu, Jiangsu, China. The standard rutin with an analytical grade was purchased from Shanghai Yuanye Biotech Co., Ltd (Shanghai, China). Other chemically pure reagents and cellulase were bought from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The strains used here were all stored at a microbiology laboratory, School of Biology and Food Engineering, Changshu Institute of Technology, Changshu, Jiangsu, China.

Ultrasound-Assisted Enzymatic Extraction (UAEE) of TFZB

The collected fresh *Z. latifolia* bracts were cleaned, dried at 60°C constantly for 2 days, crushed, and passed through a 40-mesh. A certain amount of the bract powders was immersed in a disodium hydrogen phosphate-citric acid buffer solution (pH 5, solid-to-liquid ratio = 1: 40 g/mL). Then a certain amount of cellulase was added and incubated at 37°C for a certain time, followed by enzyme inactivation at 90°C for 10 min. After that, anhydrous ethanol was added to adjust the final ethanol concentration to approximately 60%. Finally, the total flavonoids were obtained after ultrasonic extraction at certain power for a certain time at 55°C, cooling, and centrifugation. Then, the total flavonoid content was measured to reckon the extraction rate (Jiang *et al*., 2016).

The extraction rate Y is:

$$Y(\%) = \frac{C \times V}{M} \times 100 \quad (1)$$

where:
C = The total flavonoids content in crude TFZB (mg/mL)
V = The total volume of the extract (mL)
M = The mass of the bract powders (mg)

Determination of Total Flavonoids

Total flavonoid content was measured with the $NaNO_2$-$Al(NO_3)_3$-NaOH method (Jiang *et al*., 2016). Specifically, 1 mL of extracting solution was put into a 10 mL colorimetric tube, then added 0.3 mL of 5% $NaNO_2$ solution, and shaken evenly. After placement for 6 min, 0.3 mL of 10% $Al(NO_3)_3$ solution was added. After 6 min, 4.0 mL of 1 m NaOH solution was added. The resulting solution was diluted with 60% ethanol to the scale mark and mixed evenly. After placement for 15 min, the absorbance at 510 nm was measured using a blank reagent as the reference. Then, the absorbance was substituted for the linear regression equation using rutin as the standard to calculate the total flavonoid content in crude TFZB. The linear regression equation and correlation coefficient R2 were:

$$A = 0.9671C + 0.0035 \ (R^2 = 0.998) \quad (2)$$

where, A is the absorbance; C is the content of total flavonoids; the linear range is 0 ~1 mg/mL.

Response Surface Methodology (RSM) Optimization

The Box-Behnken design of Design-Expert v.8.0.6.1 was adopted to optimize the UAEE of TFZB (Feng and Zhang, 2020). Based on the results of single-factor tests, a four-factor-three-level RSM test was designed and conducted using TFZB extraction rate as the response. The factors and levels were listed in Table 1.

Comparison of Extraction Methods

TFZB extracted with ethanol extraction, cellulase-assisted extraction, ultrasound-assisted extraction, and UAEE were compared to illustrate the contributions of ultrasonic irradiation and cellulase treatment. The bracts residue extracted by four methods were dried at 60°C to constant weight, respectively. An appropriate amount of residue was taken onto the dissociated mica with tweezers. After spraying gold coating, the surface of the residue was observed by a scanning electron microscope (SEM, Hitachi Regulus-8100) (Zhan *et al*., 2014).

Infrared Spectra Analysis

The characteristic peaks of TFZB extracted with optimized UAEE were observed under a Fourier transform infrared (FT-IR, Tianjin Gangdong Sci. and Tech. Co., Ltd., FT-IR 650) spectrometer. Then, 1 mg of TFZB powders and 400-500 mg of dried KBr powders were fully ground in an agate mortar and compressed into thin slices. Spectra within 4000-400 cm-1 were scanned (Chu *et al*., 2020).

Measurement of MIC

Two reported methods (Lee *et al*., 2015a; Zhang *et al*., 2012) were referenced with slight modification. Two grams of TFZB extracted with optimized UAEE were solubilized in 60% ethanol and added distilled water to the final concentration of 20 mg/mL. Into an aseptic 96-well plate, 100 μL of an aseptic beef extract peptone liquid medium was added to the 2nd to the 10th wells, and 100 μL of 20 mg/mL TFZB was added to the 1st and 2nd wells. Then, the 2nd well was evenly mixed and 100 μL of the mixture was sucked out and added into the 3rd well, which was then evenly mixed. Then, from the 3rd well, 100 μL of the mixture was sucked out and added to the 4th well. In this way, the 2-fold dilution was continued until reaching the 10th well. Then, 100 μL of the mixture in the 10th well was discarded. After that, the

10 wells were each added with 100 μL of a bacterial solution (*Escherichia coli* or *Staphylococcus aureus* at the logarithmic phase). The final concentrations of TFZB in the 10 wells were 10000 to 19.53125 μg/mL, respectively. Each treatment was conducted in triplicate. Then the plates were immediately sent to an enzyme labeling meter to measure the *OD*600 in each well, which was well recorded. After that, the 96-well plates were put into an incubator at 37ºC for 24 h. Then, the *OD*600 of each well was measured again. The difference in *OD*600 before and after culture in each well was *ΔOD*600. The lowest TFZB concentration that made $0 \leq OD600 \leq 0.05$ can be judged as the MIC.

Effects of TFZB on Chilled Pork Preservation

Preparation of TFZB Spray Liquids

TFZB were extracted with optimized UAEE, then, the extract was concentrated and freeze-dried until reaching constant weight. The dried flavonoids were solubilized in a small amount of 60% ethanol and diluted with distilled water into 0.5 and 1.0 mg/mL, respectively.

Preparation of Chilled Pork Samples

The warranty period of chilled pork is decided jointly by sensory, physicochemical, and microbial indices. Based on a reported method (Lee *et al*., 2015b), fresh foreleg pork was peeled and evenly cut into 240 pieces (35±5 g), which were then sprayed with different processing solutions (Table 2). Changes in five freshness indices during the storage period were observed, including sensory score, pH, TVB-N, MetMb %, and total count of bacterial colonies. Then, the effects of TFZB on chilled pork preservation were investigated.

In the freshness experiments, each processing solution was used to spray 12 samples (every 100 cm^2 was sprayed with 1 mL of a solution), 4 storage periods and 3 parallel experiments were set (Bellés *et al*., 2017). Thus, each group involved 48 samples. The processed pork samples were sealed in food-grade plastic preservative films (GB/T 10457-2021) and stored in a refrigerator at 4±0.5°C. Some samples were taken out after 0, 3, 6, or 9 days of storage and sent to the measurement of the sensory score, pH, TVB-N, MetMb%, and total count of bacterial colonies.

Sensory Assessment

Evaluators graded the sensory scores of the samples as per National Food Safety Standards -- Fresh (Frozen) Livestock and Poultry Products of China (GB2707-2016) and using a reported method (Lee *et al*., 2015b) according to the indices and standards in Table 3. The total score of different items combined was classified into ≥50, 40-49, 30-39, and <30, which accorded with the first, second, third, and low-grade fresh meat, respectively.

Sensory evaluation experiments were conducted in the Food Sensory Experiment Laboratory. During the experiments, an appropriate number of samples were put into clean white porcelain trays and observed under natural illumination in terms of color, shape, and smell.

pH Measurement

The method from China National Food Safety Standard-Food pH Measurement (GB 5009.237-2016) was adopted with some modifications. Specifically, 10 g of a pork sample was minced and added to 30 mL of aseptic deionized water. After oscillation for 10 min, the mixture was centrifuged, and the supernatant was collected for measuring the pH value. Before each measurement, the pH meter was corrected in a potassium hydrogen phthalate (pH 4.0) and phosphate buffer solution (pH 6.86) or the standard solutions certified by the Chinese government and awarded with a standard substance certificate. The evaluation criteria (Suman *et al*., 2010) were: pH 5.8-6.2, 6.3-6.6, and >6.7 corresponding to the fresh, semi-fresh, and bad meat, respectively.

TVB-N Measurement

TVB-N was measured according to the first method (semimicro nitrogen detection method) in China National Food Safety Standard-Measurement of Volatile Basic Nitrogen in Foods (GB 5009.228-2016). Specifically, 20 g of pork samples were put into a sealed beaker flask, which was then mixed with 100 mL of water and shaken to make the sample disperse evenly in the solution. After soaking for 30 min, the solution was filtered. Firstly, 10 mL of filtrate and 5 mL of MgO solution were mixed and infused into the reaction chamber, which was then heated to start distillation. The distilled liquid collection bottle was added with 10 mL of boric acid solution and 5 drops of mixed indicating solution (methyl red ethanol solution: Bromocresol green ethanol solution = 1: 5). After distillation, the collection bottle was taken off and titrated with 0.01 m HCl until the mixed solution in the bottle turning purple. A blank group was also set.

TVB-N concentration in the samples was measured according to Eq. (3):

$$X = \frac{(V_1 - V_2) \times c \times 14}{m \times (V / V_0)} \times 100 \quad (3)$$

where:

X : TVB-N concentration in a sample, mg/100 g
V1 : Volume of standard HCl solution consumed by the sample solution during titration, mL
V2 : Volume of standard HCl solution consumed by the blank group during titration, mL
c : Concentration of the standard HCl solution, mol/L
14 : Equivalent mass of nitrogen used to titrate 1.0 mL of standard HCl solution [c(HCl) = 1.000 mol/L]
M : Mass of sample, g
V : Volume of filtrate, mL
V0 : Total Volume of sample and solution, mL
100 : Conversion factor

Table 1: The factors and levels of RSM for the UAEE of TFZB extraction

	Factor			
Level	X_1: Dosage of cellulase (%)	X_2: Enzymolysis time(min)	X_3: Ultrasonic power (W)	X_4: Ultrasonic time (min)
-1	0.6	50	600	40
0	0.7	60	700	50
1	0.8	70	800	60

Table 2: Processing solutions

Group	Processing solution
Blank	Sterile water
Control	5.0 mg/mL sodium benzoate solution
Test group 1	0.5 mg/mL TFZB solution
Test group 2	1.0 mg/mL TFZB solution

Table 3: The sensory evaluation of chilled pork and grading standard

	Score			
Indices	9-10	6-8	4-5	1-3
Color	Shiny, uniformly red	Slightly dark color	Dark red with white	Dark red with severe whiteness
Smell	Inherent smell of fresh pork, no peculiar smell	Slight inherent smell of of fresh pork, no peculiar smell	Slight peculiar smell	Smell of rotting
Resiliency	Depressions immediately recovering after compression by fingers	Depressions recovering at a certain time after compression by fingers	Not easy recovery compression by fingers	No recovery after compression by fingers
Viscosity	Wet appearance, but not slimy	Slightly slimy	Slimy	Very slimy
Texture	Clear	Slightly blur	Very blur	Unclear
Water exudation	No	Slight	Much	Very much

Based on National Food Safety Standards -- Fresh (Frozen) Livestock and Poultry Products (GB 2707-2016), TVB-N ≤15 mg/100 g, 15 mg/100 g < TVB-N ≤20 mg/100 g, and TVB-N >20 mg/100 g were considered as the fresh, semi-fresh, and bad meat, respectively.

MetMb % Measurement

The methods from (Krzywicki, 1982; Yu *et al*., 2020) were adopted with slight modification. Thirty grams of pork were minced, and 5 g of which were added with 5 mL of 0.04 m phosphate buffer solution (pH 6.8), then fully homogenized and centrifuged at 10000 r/min for 15 min. After that, the supernatant was filtered through a 0.22 μm water-phase filter. Then, the absorbances at 700 nm (A_{700}), 572 nm (A_{572}), and 525 nm (A_{525}) were detected, respectively.

MetMb % was calculated according to Eq. (4):

$$MetMb\% = \left[1.395 - \frac{A_{572} - A_{700}}{A_{525-A_{700}}}\right] \times 100\% \quad (4)$$

The criteria were: MetMb % ≤30%, 30% < MetMb % ≤70%, and MetMb % >70% corresponded to the fresh, semi-fresh, and bad meat, respectively.

Total Number of Bacterial Colonies Detection

Total counts of bacterial colonies in pork samples (each 25 g) were measured according to GB 4789.2-2016 and expressed in the logarithm form (l g CFU/g). The evaluation criteria were (lg CFU/g) ≤4, 4 < (lg CFU/g) ≤6, and (l g CFU/g) >6 indicating fresh, semi-fresh, and bad meat, respectively (Cheng *et al*., 2016).

Statistical Analysis

All tests were repeated three times. Data were statistically tested by ordinary one-way ANOVA (Analysis of Variance) of multiple comparisons by using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, USA). P<0.05 means significant differences and the results are expressed as means ± SD (standard deviation). The results of the Box-Behnken experiment design were obtained by Design-Expert v.8.0.6 (Stat-Ease, Inc, USA) for statistical analysis, P<0.05 and P<0.01 indicate significant and highly significant differences, respectively.

Results

Single-Factor Tests

During the extraction, the TFZB extraction rate significantly rose as the cellulase dosage increased and maximized to 1.51% when the dosage was 0.7%, but significantly dropped when the dosage exceeded 0.7%. (Fig. 2a).

When the enzymolysis time was prolonged from 50 to 60 min, the TFZB extraction rate significantly rose to reach the maximum value of 1.77% at 60 min, and then decreased significantly (Fig. 2b).

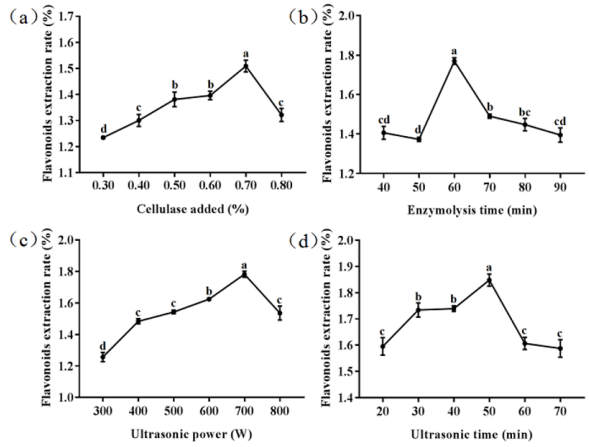

Fig. 2: Effects of single factors on the extraction rate of TFZB. (a) Cellulase dosage (b) Enzymolysis time (c) Ultrasonic power (d) Ultrasonic time. Different letters in lowercase refer to significant differences ($P < 0.05$ or $P < 0.01$)

As the ultrasonic power rose within 300~400 W, the TFZB extraction rate climbed significantly, but increased first slowly and then considerably within the power of 400~500 W, with a maximum of 1.78% at 700 W (Fig. 2c). After that, the extraction rate declined significantly with a further increment of ultrasonic power at 700~800 W.

The TFZB extraction rate was significantly improved as the ultrasonic time was prolonged within 20~30 min, but was nearly unchanged within the ultrasonic time of 30~40 min (Fig. 2d). The extraction rate was significantly raised when the ultrasonic time was extended from 40 to 50 min, but was significantly reduced within 50~60 min, and then stabilized (60~70 min). The TFZB extraction rate maximized to 1.85% at the ultrasonic time of 50 min.

RSM Optimization

Test Design and Results

Based on the results of the single-factor tests, the Box-Behnken design was conducted. The response was TFZB extraction rate (Y), and the four independent variables were cellulase dosage (X_1), enzymolysis time (X_2), ultrasonic power (X_3), and ultrasonic time (X_4). Each independent variable was set with low, medium, and high levels, which were encoded with -1, 0, and 1, respectively (Table 1). Tests based on the Box-Behnken design were conducted (Table 4). Results of the analysis of variance were listed in Table 5.

Modeling and Significance Test

A quadratic multifactor regression equation of TFZB extraction rate over the coded independent variables was established by Design-Expert 8.0.6.1:

$$Y = 1.92 + 0.027X_1 - 0.037X_2 + 0.014X_3 - 0.10X_4 + 7.5\times10^{-3}$$
$$X_1X_2 - 2.5\times10^{-3}X_1X_3 + 0.035X_1X_4 - 0.055X_2X_3 - 0.085X_2X_4$$
$$-0.040X_3X_4 - 0.28X_1^2 - 0.41X_2^2 - 0.36X_3^2 - 0.18X_4^2$$

where:

Y = TFZB extraction rate (w/w, %)
X_1 = The dosage of cellulase (w/w, %)
X_2 = Enzymolysis time (min)
X_3 = Ultrasonic power (W)
X_4 = Ultrasonic time (min)

The results of Table 5 and the 2D contours presented in Fig. 3 showed that the model was $P<0.0001$, indicating that the response is significant and linearly correlated with the four independent variables. The P value of lack of fit was 0.2046 (>0.05), suggesting that the fitting of the present equation was good, and can be used to predict and analyze TFZB extraction. $R^2 = 0.9755$ suggested that 97.55% of the tests can be interpreted by the equation, further confirming that the optimal conditions were appropriate. Then, the model $Y = f(X_1, X_2, X_3, X_4)$ was sent to analyze the variance. The linear term X_4 and quadratic terms X_1^2, X_2^2, X_3^2, and X_4^2 all very significantly affected the response, and the linear term X_2 and the interaction item X_2 X_4 (Fig. 3) significantly affected the response, but the effects of other items were not significant.

Validation Experiments

Based on the optimization of the regression equation, the model predicted that the coding values of the four parameters corresponding to the maximum extraction rate of TFZB by UAEE were 0.03, -0.02, 0.04, and -0.29, respectively. The corresponding actual value was cellulase dosage of 0.703%, enzymolysis time of 59.820 min, ultrasonic power of 703.700 W, and ultrasonic time of 47.139 min. Given the practical operability, the optimal extraction conditions were modified: Cellulase dosage of 0.7%, enzymolysis time of 60 min, ultrasonic power of 704 W, and ultrasonic time of 47 min. Under these conditions, confirmatory tests were repeated 3 times, and the average extraction rate of TFZB was 1.922±0.024%. The absolute value of error from the theoretically predicted value (1.932%) is less than 1%, and the difference examined by the t-test is not significant ($P>0.05$).

Comparison of Extraction Methods

The extraction rate and flavonoids content of TFZB extracted with the methods of ethanol extraction, cellulase-assisted extraction, ultrasound-assisted extraction, and UAEE were shown in Fig. 4. The extraction rates of TFZB were 0.35±0.01, 0.49±0.01, 1.32±0.02%, and 1.92±0.02%, respectively, and the corresponding flavonoids contents were 132±1.04, 133±1.21, 142±1.25 and 150±1.25 mg/g. Compared with the other methods, the UAEE significantly improved the extraction rate of TFZB and the content of total flavonoids in TFZB (Fig. 4).

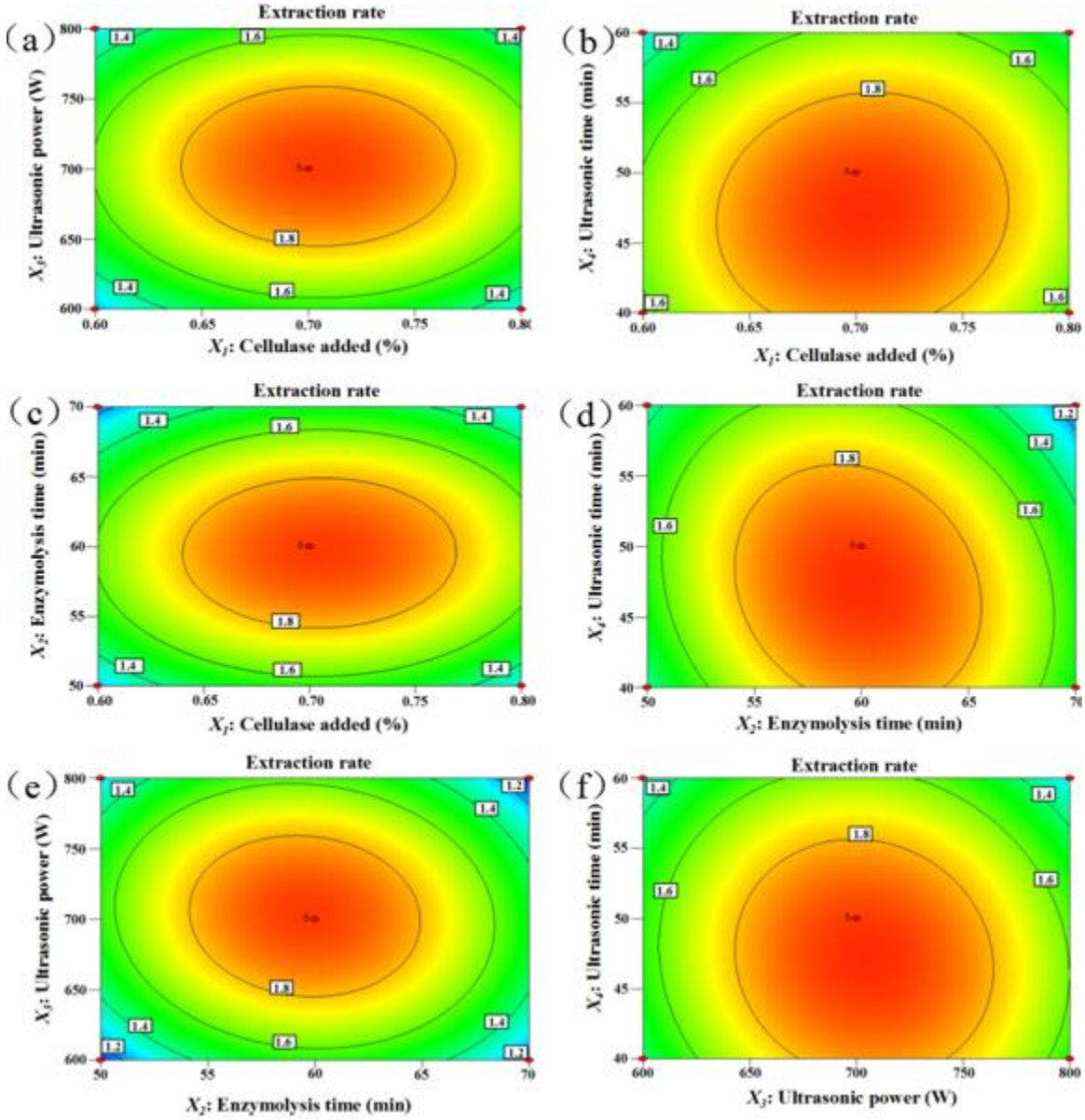

Fig. 3: The 2D contours of RSM. (a) Cellulase dosage versus Ultrasonic power; (b) Cellulase dosage versus Ultrasonic time; (c) Cellulase dosage versus Enzymolysis time; (d) Enzymolysis time versus Ultrasonic time; (e) Enzymolysis time versus Ultrasonic power; (f) Ultrasonic power versus Ultrasonic time

Table 4: The combinations and results of the Box-Behnken design

	Independent variable				
Run	X_1/(%)	X_2/(min)	X_3/(W)	X_4/(min)	Y/(%)
1	0	0	0	0	1.95
2	0	0	1	-1	1.52
3	-1	1	0	0	1.12
4	1	1	0	0	1.19
5	0	1	1	0	1.05
6	0	1	0	1	1.14
7	0	-1	0	1	1.28

Table 4: Continuous

8	-1	0	0	-1	1.56
9	0	0	0	0	1.89
10	-1	-1	0	0	1.28
11	1	0	0	-1	1.63
12	0	1	-1	0	1.17
13	0	0	1	1	1.29
14	0	0	-1	-1	1.38
15	0	-1	1	0	1.27
16	0	-1	-1	0	1.17
17	0	-1	0	-1	1.33
18	0	0	-1	1	1.31
19	1	0	-1	0	1.23
20	0	0	0	0	1.87
21	1	0	1	0	1.26
22	-1	0	0	1	1.24
23	0	0	0	0	1.97
24	-1	0	-1	0	1.26
25	-1	0	1	0	1.30
26	1	-1	0	0	1.32
27	1	0	0	1	1.45
28	0	1	0	-1	1.53
29	0	0	0	0	1.90

Table 5: The ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F value	P-value prob > F	Significance [a]
Model	2.000	14	0.140	39.820	< 0.0001	**
X_1	8.533×10^{-3}	1	8.533×10^{-3}	2.380	0.1451	n.s.
X_2	0.017	1	0.017	4.710	0.0477	*
X_3	2.408×10^{-3}	1	2.408×10^{-3}	0.670	0.4261	n.s.
X_4	0.130	1	0.130	35.750	< 0.0001	**
X_1X_2	2.250×10^{-4}	1	2.250×10^{-4}	0.063	0.8058	n.s.
X_1X_3	2.500×10^{-5}	1	2.500×10^{-5}	6.975×10^{-3}	0.9346	n.s.
X_1X_4	4.900×10^{-3}	1	4.900×10^{-3}	1.370	0.2618	n.s.
X_2X_3	0.012	1	0.012	3.380	0.0875	n.s.
X_2X_4	0.029	1	0.029	8.060	0.0131	*
X_3X_4	6.400×10^{-3}	1	6.400×10^{-3}	1.790	0.2028	n.s.
X_1^2	0.510	1	0.510	143.240	< 0.0001	**
X_2^2	1.060	1	1.060	296.970	< 0.0001	**
X_3^2	0.840	1	0.840	234.650	< 0.0001	**
X_4^2	0.210	1	0.210	57.880	< 0.0001	**
Residual	0.050	14	3.584×10^{-3}			
Lack of fit	0.043	10	4.306×10^{-3}	2.420	0.2046	
Pure error	7.120×10^{-3}	4	1.780×10^{-3}			
Cor total	2.050	28				

a: ** P<0.01 highly significant; * P<0.05 significant; P>0.05 not significant (n.s.)

Table 6: ΔOD_{600} of *Escherichia coli* and *Staphylococcus aureus* suspension with TFZB at different mass concentrations

Mass concentration of TFZB (μg/mL)	ΔOD_{600} of *E. coli* suspension	ΔOD_{600} of *S. aureus* suspension
10000.00	0.024±0.001	0.022±0.000
5000.00	0.030±0.002	0.027±0.001
2500.00	0.048±0.001	0.030±0.001
1250.00	0.063±0.003	0.040±0.001
625.00	0.068±0.003	0.051±0.001
312.50	0.069±0.001	0.057±0.002
156.25	0.069±0.002	0.060±0.001
78.13	0.070±0.003	0.068±0.003
39.06	0.069±0.001	0.070±0.002
19.53	0.070±0.002	0.070±0.001

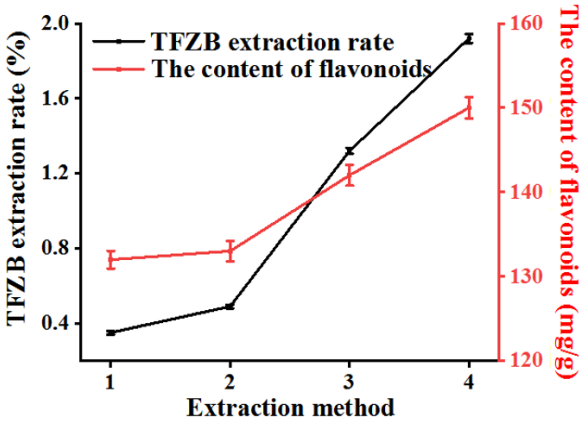

Fig. 4: TFZB extraction rate and the content of flavonoids by four extraction methods. 1: Ethanol extraction; 2: Cellulase-assisted extraction; 3: Ultrasound-assisted extraction; 4: UAEE

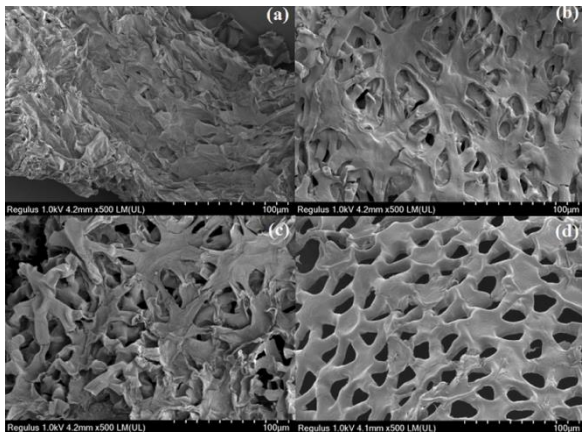

Fig. 5: SEM images of extraction residue from bracts of *Z. latifolia*

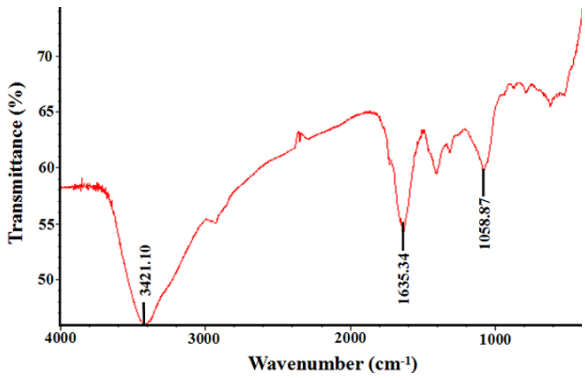

Fig. 6: Infrared spectra of TFZB

The SEM images of extraction residue of *Z. latifolia* bracts under different extraction methods were presented in Fig. 5, it can be noted that single ethanol treatment has little damage to the skeleton fiber structure (Fig. 5a). When combined with enzymatic hydrolysis (Fig. 5b) and ultrasonic irradiation (Fig. 5c), the bracts cytoskeletons were notably damaged, some fiber structures were broken, and different pore sizes appeared on the cytoskeleton. Surprisingly, when the two technologies were combined based on ethanol extraction, the bracts cytoskeletons were severely damaged, and a uniform large aperture appeared on the skeleton (Fig. 5d).

FT-IR Analysis

The nuclear parents of natural flavonoids often contain alkoxyl, methoxyl, isopentenoxyl, and hydroxyl groups, and show characteristic vibration peaks at 3100-3460, 1600-1640, and 1372, 1242, 1058 cm^{-1} on infrared spectra (Yu *et al*., 2017). The infrared spectrum of TFZB extracted with optimized UAEE showed vibration peaks at 3421.10, 1635.34, and 1058.87 cm^{-1} (Fig. 6), indicating the extracts have the typical characteristic peaks of natural flavonoids.

Antibacterial Ability Analysis

With Gram-negative *E. coli* (ATCC 25922) and Gram-positive *S. aureus* (ATCC 25923) as the tested bacterial strains, we detected the ΔOD_{600} of TFZB at different mass concentrations (Table 6).

When the TFZB mass concentration was ≥2.5 mg/mL, the ΔOD_{600} of the *E. coli* suspension was (0.048±0.001) <0.05, and when the TFZB mass concentration was ≥1.25 mg/mL, the ΔOD_{600} of the *S. aureus* suspension was (0.040±0.001) <0.05. The MICs of TFZB over *E. coli* and *S. aureus* were 2.5 and 1.25 mg/mL, respectively.

Effects of TFZB on Chilled Pork Preservation

Sensory Score

The sensory score is one of the key indices to judge the quality of chilled pork and directly affects the selling of products. The effects of TFZB on the sensory index of chilled pork were shown in Fig. 7a. The sensory scores of all processed samples decreased with the prolonging of storage time (Fig. 7a). Specifically, the decreasing rates of sensory scores in the blank group and the test group 1 were faster compared with the control group (sodium benzoate) and the test group 2, and the sensory scores of the test group 2 on day 3, 6 and 9 were all significantly higher compared with those of the control group. These results indicated that TFZB at a certain concentration can improve the smell, color, resilience, viscosity, and texture of chilled pork, thereby enhancing its acceptability of chilled pork. For instance, with the sensory second-grade freshness (score 40) as the boundary, the blank group was over-standard on day 3, and the sensory scores of test group 1 on day 3 were exactly 40 and mostly were over-standard after day 3. The control group and test group 2 were over-standard on days 6 and 9, respectively.

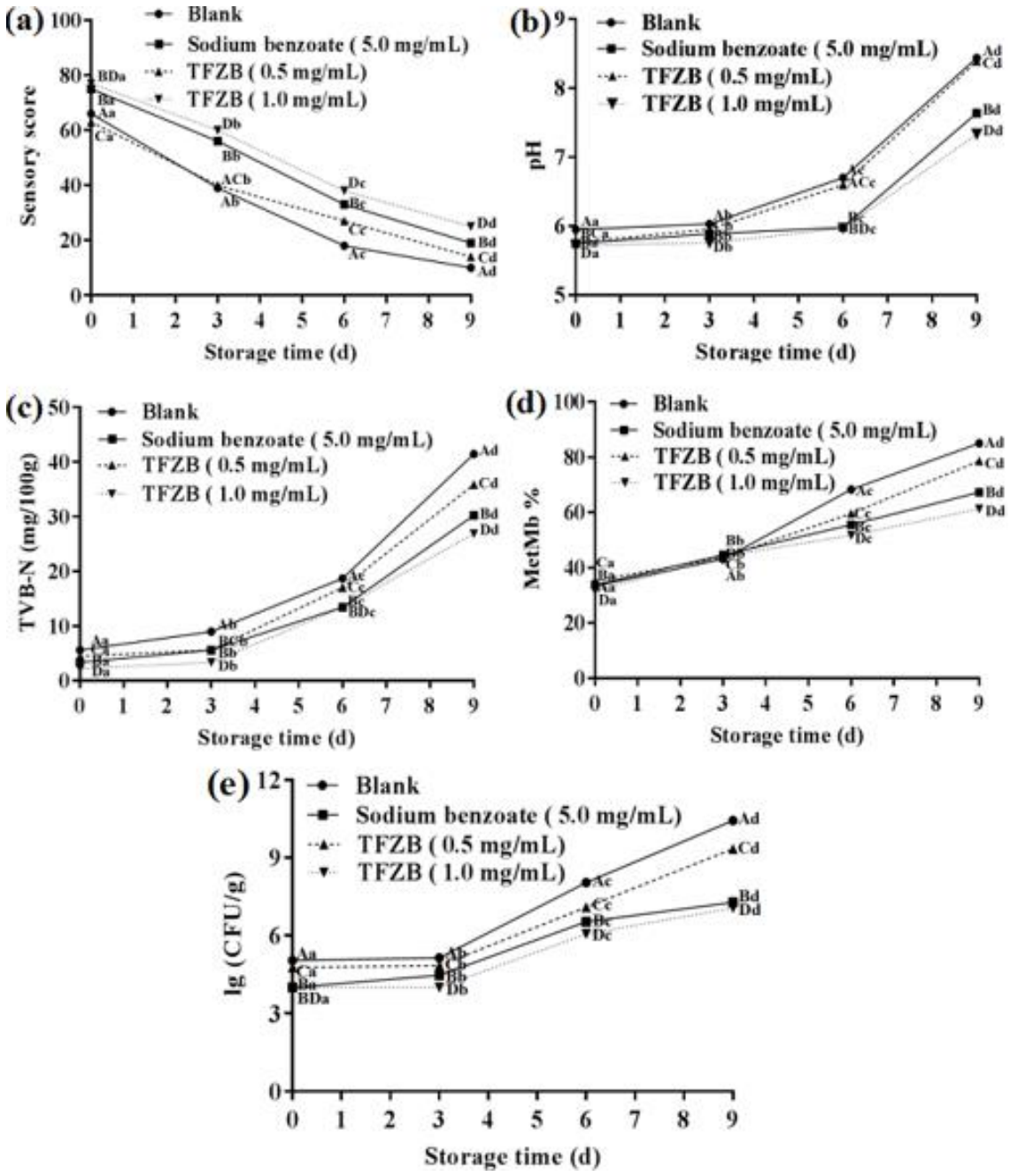

Fig. 7: Changes in index values of chilled pork with different treatment groups during storage. (a) Sensory score; (b) pH; (c) TVB-N; (d) MetMb %. A, B, C, and D indicate the same letters are not significantly different (P>0.05) within the same storage time, and different letters imply significant differences (P<0.05). a, b, c, and d indicate the same letters are not significantly different (P>0.05) in the same group, and different letters imply significant difference (P<0.05)

pH Measurement

As the storage time of chilled meat was extended, the proteins in the meat were decomposed by bacteria and enzymes to ammonia, amine, and other alkaline substances, making the meat alkaline. The pH >6.7 indicates that the pork has been already rotten and bad (Lu *et al*., 2013). The effects of TFZB on the pH of chilled pork were shown in Fig. 7b. The pH of all processed chilled pork samples rose with the prolonging of storage time (Fig. 7b). The pH of the blank group was higher than those of other groups since the start of storage, turned >6.7 on day 6, and then rapidly rose to 8.4 on day 9. The pH values of test group 2 and the control group were consistent on day 6, but the pH of test group 2 on

day 9 was lower than that of the control group and changed very slowly during the storage period.

TVB-N Detection

TVB-N refers to the alkaline nitrogen-containing substances produced from deamination after proteins in meat are decomposed by some microbes, and TVB-N concentration is an indicator of meat freshness (Lu *et al*., 2013). The effects of TVB-N concentration of chilled pork during the storage period were shown in Fig. 7c. The TVB-N concentrations in all groups increased with the prolonging of storage time (Fig. 7c). The TVB-N concentrations in the blank group and test group 1 reached 18.68 and 17.00 mg/100 g on day 6, respectively, which were both higher than that of the national limit standard of China (15.00 mg/100 g). Moreover, the TVB-N concentrations of test group 2 and the control group on day 6 were both lower than that of the national limit standard. On day 9, the TVB-N concentrations of test group 2 and the control group reached 26.88 and 30.24 mg/100 g, respectively, which surpassed the national limit standard.

MetMb % Measurement

During the storage of chilled meat, the oxidation of myoglobin to brown metmyoglobin makes the meat dark and of lower quality. The color of meat is an important indicator of the freshness and quality of meat and meat products (XiaoRan *et al*., 2019). On the 3rd day of storage, the MetMb % was nearly equal among all groups, but significantly changed after the 3rd day (Fig. 7d). The MetMb % of the blank group on day 6 was close to 70%, indicating that the chilled meat had begun to rot and deteriorate. On the same day, MetMb % of test group 2 was the lowest (51.67%). On day 9, the MetMb % was less than 70% in both the control group and test group 2, but the MetMb % exceeded 70% in both the blank group and test group 1, indicating that the chilled pork had already deteriorated.

Total Number of Bacterial Colonies Determination

The total count of bacterial colonies during the storage period directly reflects the quality variation of chilled pork (Shi *et al*., 2013). During the 9-day storage, the total count of bacterial colonies among different time points ranked as blank group > test group 1 > control group > test group 2, and the total counts of bacterial colonies in the former three groups on day 6 all exceeded 6 lg CFU/g (Fig. 7e). These results indicated that the chilled pork had already deteriorated, but the pork in the test group 2 begun to deteriorate until the 6th day.

Discussion

In the present study, the single-factor experiments for the UAEE of TFZB were first implemented for acquiring the value range of independent variables of RSM optimization. From Fig. 2, we can observe the effects of enzyme dosage, enzymolysis time, ultrasonic power, and ultrasonic time on the extraction yields of TFZB. The following reasons could be uncovered to explain these results. (1) Within a certain enzymolysis time, as the enzyme dosage rose, the enzymolysis was accelerated, so the cellulase can react with more substrate, and facilitated the subsequent extraction. However, excessive enzymes, because of their adhesiveness, will block the channel of flavonoid dissolution, so the increase in enzyme dosage will induce competition between enzymes and even inhibit enzyme activity (Liu *et al*., 2015). (2) Further, with prolonging enzymolysis time, the enzyme and substrate reaction were more sufficient, and the bract cell walls were structurally degraded under the enzymatic action, which facilitated the dissolution and diffusion of flavonoids in the medium. When the enzymolysis time was further prolonged to 70 min, the TFZB yield significantly dropped, which was probably associated with the fact that a part of flavonoids had been oxidized due to too long a heating time (Patindol *et al*., 2007). (3) Moreover, when the ultrasonic power was weak, the cavitation effect of ultrasonic waves slowed down the dissolution of flavonoids, but as the ultrasonic power rose, the molecular kinetic energy of the extracts was improved and the cavitation effect of ultrasonic waves was enhanced, which accelerated the dissolution of flavonoids. But when the ultrasonic power was too high, a molecular motion was fierce, so the reaction between flavonoids and other components was intensified, leading to the destruction of flavonoids and a decrease in extraction yield (Ko *et al*., 2013). (4) Finally, the effects of ultrasonic time on TFZB yield mainly indicated that excessive ultrasonic treatment can destroy the flavonoids.

Then, a four-factor-three-level RSM was designed to further optimize the UAEE for TFZB extraction, and the results indicated that the model is accurate to predict the UAEE of TFZB and interpreting the results. Fig. 4 illustrated the importance of introducing enzymatic hydrolysis and ultrasonic technology to TFZB extraction, which was consistent with the results of Table 5 that enzymatic hydrolysis time, ultrasonic time, and their interaction have significant effects on TFZB extraction, especially ultrasonic power has a highly significant impact. The SEM observation (Fig. 5) also further explained the reason why the high extraction rate of TFZB was achieved when cellulase treatment was combined with ultrasonic irradiation, where the bracts cytoskeletons had been severely damaged, and a uniform large aperture appeared on the skeleton (Fig. 5d).

The FT-IR spectrum of TFZB showed vibration peaks at 3421.10, 1635.34, and 1058.87 cm^{-1} (Fig. 6), indicating TFZB has the typical characteristic peaks of flavonoids. The TFZB extracted with optimized UAEE has antibacterial properties against *E. coli* and *S. aureus*, suggesting a potential for the fresh-keeping of meat. Therefore, TFZB was applied for the fresh-keeping of chilled pork, and the fresh-keeping effects were investigated through sensory, chemical, and microbial indicators. Based on the changes in index values of chilled pork with different treatment groups during storage (Fig. 7), indicate that TFZB can moderately inhibit the microbes that cause the pork to rot, thereby suppressing the rise of pH. Besides, the use of TFZB at 1.0 mg/mL has a higher antibacterial effect than that of sodium benzoate solution at 5.0 mg/mL during the preservation of chilled pork, and these results were in line with those of our previous findings that TFZB can prolong the shelf life of strawberry (Jiang *et al*., 2016).

In short, the use of TFZB extracted with optimized UAEE can extend the shelf life of chilled pork.

Conclusion

In this study, based on preliminary research (Jiang *et al*., 2016) on the cellulase-assisted extraction of TFZB, a UAEE method was further used to extract TFZB. After single-factor experiments and RSM optimization, the optimal process conditions were: Cellulase dosage of 0.7%, enzymolysis time of 60 min, ultrasonic power of 704 W, and ultrasonic time of 47 min. Under these conditions, the TFZB extraction rate was up to 1.922%, which was 3.92-folds higher than that of the previous cellulase-assisted method (0.49%) (Jiang *et al*., 2016). The UAEE method can more severely destroy the cell walls of *Z. latifolia* bracts, and make the flavonoids in the cells more dissoluble and diffuse to the ethanol solution, largely improving the TFZB extraction rate.

The counts of *E. coli* and *S. aureus* are important indicators of measuring food safety. The antibacterial experiments showed that the MICs of TFZB against *E. coli* and *S. aureus* were 2.5 and 1.25 mg/mL, respectively, indicating that TFZB could be a candidate resource to produce environmental disinfection and insecticidal products.

Finally, sterile water, 5.0 mg/mL of sodium benzoate, and 0.5 and 1.0 mg/mL of TFZB served as the blank, positive control, test 1, and 2 groups, respectively. The four solutions were separately sprayed to the surface of fresh pork, and the fresh-preserving effects of these four solutions during the cold storage were investigated using five indices, including sensory score, pH, TVB-N, MetMb %, and total count of bacterial colonies. The quality indices of pork sprayed with TFZB solution at 1.0 mg/mL were all higher than those of sodium benzoate at 5.0 mg/mL during the storage. The results again convincingly verified the antibacterial effects of TFZB and implied that the TFZB extracted with optimized UAEE could be a natural flavonoid resource to produce chilled pork preserving agents. Lee *et al*. (2020) separated tricin and five derivatives of tricin from the leaves of *Z. latifolia* and found that they all possessed certain anti-allergic activity (Lee *et al*., 2020). Yan *et al*. (2018) isolated and identified 9 derivatives of tricin from the leaves of *Z. latifolia* and found that they have anti-fatigue, anti-inflammatory, and anti-allergic effects (Yan *et al*., 2018). Hence, we courageously speculated that the antibacterial and chilled pork-preserving effects of TFZB might be mainly attributed to the active ingredients dominated by tricin-type flavonoids (Lee *et al*., 2020), but the identification of other types of flavonoids needs further research. In the future, we will further clarify the underlying mechanisms that TFZB exhibits. This study theoretically underlies the high-value utilization of *Z. latifolia* bracts and other similar agricultural wastesof TFZB crude extract (g).M is the maeeow Bcrude.

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Author's Contributions

Chen Pan and Shuyi Zhu: Performed the experiments extraction and antibacterial experiments and FT-IR analysis.

Na Niu: Carried out the scanning experiment.

Yuzhu Zhang and Kai Gao: Carried out the chilled pork preservation experiments.

Yang Zhang: Designed the experiments, revised and polish the manuscript.

Lixue Zheng: Designed the experiments, wrote, and polish the manuscript.

Ethics

This study is original and contains unpublished data. All authors state that there are no ethical issues involved that may arise after the publication of this manuscript.

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