Process optimization for enzymatic assisted extraction of anthocyanins from the mulberry wine residue

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Abstract. In order to improve the utilization value of the by-product of mulberry, the remaining residue of mulberry wine was used as raw materials to extract anthocyanins from the residue of mulberry wine by enzymatic method, and the response surface method was used to optimize the extraction process. Firstly, the single factor experiment was carried out by controlling four factors: liquid-solid ratio, extraction time, temperature and pH value. Then, Box-Behnken was used to design a three-factor, three-level experiment. Finally, the response surface method was used to optimize the extraction process. The results showed that the optimal extraction process of pectinase was the liquid-solid ratio of 1:20, the extraction time was 58 min, the pH was 5.90 and the extraction temperature was 45 °C, and the extraction effect was the best. The highest anthocyanin content in the extract was 6.040 mg/g.

1. Introduction
Mulberry is the fruit of mulberry, a perennial woody plant of the genus mulberry. The mature mulberry has rich nutritional value, rich in active protein, cellulose, amino acid, superoxide dismutase, resveratrol and anthocyanins, etc., which are loved by people [1]. Abundant mulberry resources in China have been listed by the ministry of health as one of the food raw materials "both food and medicine". Mulberries are not resistant to storage, and the ripe fruits are highly perishable after harvest. At present, they are mostly processed into fruit juice, wine and jam [2].

Mulberries are a new fruit wine with high nutritional value, rich in anthocyanins, flavonoids, resveratrol and other bioactive substances, which have been widely studied [3]. However, the utilization of peel residue in the brewing process of mulberry wine is seldom studied. Fan et al. [4] studied mulberry slag by macroporous resin purification methods of alpha amylase inhibitors in the process optimization. Kong et al. [5] processed the optimizing research on the extraction technology of total flavonoids in mulberry wine slag. Hu et al. [6] used the response surface method for mulberry anthocyanins in wine residue extraction process. All these studies for mulberry wine residue using provides a preliminary theoretical basis. Especially, the research on anthocyanin extraction has a high application prospect.
Anthocyanins as a natural pigment, security, non-toxic, rich in resources, and has clear free radicals, anti-inflammatory, anti-cancer, hinder the intestinal absorption of cholesterol and bile acid, can relieve liver dysfunction, inhibition of lipid peroxidation, and used in the treatment of diabetic retinopathy, resistance mutation, treatment of breast cyst, treating microcirculation diseases and to prevent atherosclerosis and other health care function, has been widely used in food, health care products, cosmetics, medical and other industries [7-11].

At present, there are many extraction methods for anthocyanins, including organic solvent extraction, ultrasonic extraction, ultrasound-assisted extraction, enzyme extraction, etc. [12]. High pressure liquid extraction, high pressure pulsed electric field assisted extraction, high pressure extraction and other technologies were also used to extract anthocyanins from food, all of which could be extracted efficiently and accurately in a short period of time and the extraction effect was also very good [13-14]. However, the enzymatic extraction method is easy to operate, easy to control and efficient, so it has become a common extraction method. In order to provide theoretical support for the utilization of the by-product of mulberry wine, this paper explored the enzymatic assisted extraction of anthocyanins from the residue of mulberry wine.

2. Materials and methods

2.1. Materials, instruments and equipments
Mulberries come from Qinglonghe fruit wholesale market, Chuxiong City, Yunnan Province. Pectinase was purchased from Guangzhou Feibo Biotechnology Co. LTD.

Details of instruments and equipment used in this study are listed in Table 1.

Table 1. Instruments and equipment

| Instrument & Equipment Name                        | Manufacturer                                |
|--------------------------------------------------|---------------------------------------------|
| UV-5500 ultraviolet and visible spectrophotometer | Shanghai Yuanxi instrument co., LTD         |
| PB-10 pH meter, Quintix224-1CN electronic scales | Satorius scientific instrument (Beijing) co., LTD |
| HWS-12 electric-heated thermostat water bath      | Shanghai Yiheng scientific instrument co., LTD |
| DHG-9070A electrothermal blowing dry box          | Shanghai Yiheng scientific instrument co., LTD |
| SHZ-III A circulating water pump                  | Gongyi Yuhua instrument co., LTD            |
| GPF-50 double-stage high efficiency fine pulverizer| Taizhou Guopinleji machine co., LTD         |
| SK8210HP ultrasonic cleaner                       | Shanghai Science Guide Ultrasonic Instrument co. LTD |

2.2. Preparation of mulberry wine residue samples.
Fruit wine is brewed in small containers [15], after fermentation, the artesian wine was removed, and the remaining wine residue was pressed to obtain the wine residue after brewing. It was dried by electric blast drying oven at 105°C. Then, the dried mulberry wine residue was crushed by a crusher and sifted through 60 mesh screen.

2.3. Method for determination of anthocyanin content.
The determination was made by pH differential method. Specifically, 1.0ml of the extract was placed in two test tubes, and then the sample was diluted 20 times with buffer pH1.00 and pH4.50, respectively. The sample was placed in the dark for 0.5h, and the absorbance values were measured with UV spectrophotometer at the wavelengths of 510 nm and 700 nm after reaching stability. The absorption value of the sample and the anthocyanin content in the extract were calculated by the following formula:
where: $A$ is light absorption value; $T_{Acy}$ is anthocyanin content (mg/g); $V$ is total volume of extract (mL); $M$ is molecular weight of cornflower-3-glucoside (449.2); $\varepsilon$ is extinction coefficient (26900); $m$ is mass (g); $N$ is dilution multiple.

2.4. Single factor experiment

2.4.1. The effect of liquid to material ratio (skin slag mass: water mass) on anthocyanin extraction. In the five conical bottles, 1.0000 g of derm powder was put in respectively, and the solid-liquid ratio was 1:10, 1:15, 1:20, 1:25, and 1:30. At a pH of 4.00 and a temperature of 40 °C, the powder was extracted for 40 min, filtered, and the content (anthocyanin) was calculated. After comparison, the best liquid-liquid ratio was selected.

2.4.2. Effect of pH on anthocyanin extraction. Five portions (1.0000g/portion) of mulberries peel powder were weighed in five conical bottles, the ratio of liquid to material was 1:20, and the pH was 3.00, 4.00, 5.00, 6.00 and 7.00, respectively.

At the temperature of 40 °C, the powder was treated for 40 min. After filtration, the anthocyanin content was calculated and the optimum pH was selected.

2.4.3. Effect of extraction temperature on anthocyanin extraction. Five portions (1.0000 g/ portion) of mulberries peel powder were weighed in five conical bottles.

Under the condition of 1:20 and pH6.00, the set temperature was 30 °C, 35 °C, 40 °C, 45 °C and 50°C, and the treatment was carried out for 40 min. After filtration, the anthocyanin content was calculated, and the optimal extraction temperature was selected.

2.4.4. Effect of extraction time on anthocyanin extraction. Weigh 5 (1.0000g/portion) of mulberry peel powder into 5 conical bottles, and treat at 1:20, pH6.00 and 45 °C for 30 min, 40 min, 50 min, 60 min and 70 min.

After filtration, the content (anthocyanin) was calculated and the optimal extraction time was selected.

2.5. Response surface analysis test

Based on the results of the four single-factor tests, Box-Behnken designed a three-factor, three-level test, and optimized the extraction conditions with the response surface method.

The number of milligrams (mg/g) of anthocyanin extracted from the mulberry-bark residue per gram was used as the research object. See table 2 for details.

| Coding level | $X_1$: Extraction time/min | $X_2$: pH value | $X_3$: Extraction temperature/°C |
|--------------|---------------------------|-----------------|-------------------------------|
| -1           | 55                        | 5.50            | 42.5                          |
| 0            | 60                        | 6.00            | 45                            |
| 1            | 65                        | 6.50            | 47.5                          |

2.6. Statistical analysis.

Use the software of design-expert 8.0 and Excel to process and analyze the test data.
3. Results and analysis

3.1. Effect of liquid to solid ratio on anthocyanin extraction

As can be seen from Fig. 1, with the change of the ratio of feed to liquid, the content of anthocyanin increased first and then decreased. When the liquid-solids ratio was 1:20, the content of anthocyanin reached the maximum value of 4.239 mg/g. This is because pectinase can break down the pectin on the cell wall to destroy the cell and release anthocyanins effectively [16]. After that, the extraction of anthocyanin did not continue to rise with the increase of the solid-liquid ratio, but decreased slowly. The reason is that the glycosidic bond of anthocyanin is hydrolyzed by pectinase and then broken, thus destroying the structure of anthocyanin and causing the content of anthocyanin to decrease instead of increase [17]. Therefore, the liquid to material ratio of 1:20 is determined to be the best.

![Figure 1. Effect of liquid-material ratio on anthocyanin content](image)

3.2. Effect of pH on anthocyanin extraction

The effect of pH on anthocyanin extraction is shown in Fig. 2. The change of anthocyanin showed a trend of first rising and then falling with the increasing of pH. In the range of pH 3.00 ~ 6.00, the anthocyanin content kept rising and reached its maximum at pH 6.00, the value is 5.036 mg/g. However, with the further increase of pH value, anthocyanin content showed a downward trend. This is because under neutral conditions, the enzymatic hydrolysis effect of pectinase is not obvious, and the decomposition ability of pectinase to cell wall is weakened, leading to the reduction of anthocyanin extraction amount [18]. Therefore, determine the optimal pH value to be 6.00.
Figure 2. Effect of pH value on anthocyanin content

3.3. Effect of extraction temperature on anthocyanin extraction.
As can be seen from Fig. 3, the content of anthocyanin increased first and then decreased within the range of experimental temperature. Within the range of 30°C to 45°C, the anthocyanin content in the extract increased with the increase of temperature, and reached the maximum value of 4.799 mg/g at 45°C. This is due to the fact that the increase of temperature will fully interact with the extract after the destruction of the cell wall of mulberry, increase the enzymatic hydrolysis rate, and gradually release anthocyanin into the extract, thus increasing the anthocyanin content [19].

However, the content of anthocyanin in the extract decreased when the temperature exceeded 45°C. This may be due to the fact that with the increase of temperature, the enzyme activity will decrease, leading to the decrease of enzymatic hydrolysis force [20]. Therefore, 45°C was determined as the optimal extraction temperature.

Figure 3. Effect of extraction temperature on anthocyanin content

3.4. Effect of extraction time on anthocyanin content.
The results are shown in Fig. 4, within the experimental time range, the content of anthocyanin increased first and then decreased with time. Between 30min and 60 min, the anthocyanin content increased with time and reached its maximum value of 5.351 mg/g at 60 min. After 60min, the
The anthocyanin content in the extract began to decrease. Therefore, the extraction time of 60 min was determined to be the best.

![Figure 4. Effect of extraction time on anthocyanin content](image)

### 3.5. Response surface design and results

The response surface test design results are shown in Table 3.

| Serial number | $X_1$ | $X_2$ | $X_3$ | $Y$: Extraction amount (mg/g) |
|---------------|-------|-------|-------|-----------------------------|
| 1             | -1    | -1    | 0     | 5.960±0.008                 |
| 2             | 0     | 1     | -1    | 5.006±0.010                 |
| 3             | 0     | 0     | 0     | 6.249±0.015                 |
| 4             | 1     | -1    | 0     | 5.838±0.014                 |
| 5             | 1     | 0     | 1     | 5.153±0.017                 |
| 6             | -1    | 0     | 1     | 5.785±0.015                 |
| 7             | 0     | -1    | 1     | 5.324±0.003                 |
| 8             | -1    | 0     | -1    | 5.738±0.006                 |
| 9             | 1     | 0     | -1    | 5.121±0.006                 |
| 10            | 0     | -1    | -1    | 5.449±0.007                 |
| 11            | 0     | 0     | 0     | 6.237±0.006                 |
| 12            | 0     | 0     | 0     | 6.253±0.006                 |
| 13            | 0     | 0     | 0     | 6.030±0.007                 |
| 14            | -1    | 1     | 0     | 5.121±0.016                 |
| 15            | 1     | 1     | 0     | 5.048±0.006                 |
| 16            | 0     | 1     | 1     | 5.398±0.010                 |
| 17            | 0     | 0     | 0     | 6.094±0.009                 |

Table 3 the quadratic polynomial regression equation between the extraction quantity ($Y$) and the variables $X_1$, $X_2$ and $X_3$ can be obtained by using the software of Design-Expert 8.0: $Y=6.21-0.18X_1-0.25X_2+0.043X_1^2+0.012X_1X_2-3.750*10^{-5}X_1X_3+0.13X_2X_3-0.28X_1^2-0.44X_2^2-0.48X_3^2$. In the above formula, $X_1$, $X_2$ and $X_3$ respectively represent the extraction time (min), pH value and extraction temperature (°C).

For this equation, the absolute value of each coefficient can be used to reflect the influence degree of each experimental factor on the response value, and the positive and negative coefficients of each term in this equation can be used to represent the direction of the response value [21]. Studies have
shown that using the response surface optimization of anthocyanins extracted by the quadratic polynomial equation, the quadratic term coefficient are negative, can through the equation of the quadratic term coefficient values, further determine the parabola opening direction of corresponding response figure, if the quadratic term coefficient are negative, can represent the response figure opening down, there is a maximum points, which can optimize the experimental conditions is analyzed [22]. Therefore, the study of this experiment conforms to this optimization range. The variance analysis results of this model are shown in Table 4.

| Source          | Sum of Squares | df | Mean Square | F Value | P-value |
|-----------------|----------------|----|-------------|---------|---------|
| Model           | 3.19           | 9  | 0.35        | 5.55    | 0.0171  |
| $X_1$           | 0.26           | 1  | 0.26        | 4.08    | 0.0832  |
| $X_2$           | 0.50           | 1  | 0.50        | 7.81    | 0.0267  |
| $X_3$           | 0.015          | 1  | 0.015       | 0.23    | 0.6432  |
| $X_1X_2$        | $6.002 \times 10^{-4}$ | 1  | $6.002 \times 10^{-4}$ | 9.395$\times 10^{-3}$ | 0.9255 |
| $X_1X_3$        | $5.625 \times 10^{-5}$ | 1  | $5.625 \times 10^{-5}$ | 8.804$\times 10^{-4}$ | 0.9772 |
| $X_2X_3$        | 0.067          | 1  | 0.067       | 1.05    | 0.3405  |
| $X_1^2$         | 0.34           | 1  | 0.34        | 5.28    | 0.0553  |
| $X_2^2$         | 0.81           | 1  | 0.81        | 12.64   | 0.0093  |
| $X_3^2$         | 0.97           | 1  | 0.97        | 15.21   | 0.0059  |
| Residual        | 0.45           | 7  | 0.064       |         |         |
| Lack of fit     | 0.34           | 3  | 0.11        | 4.32    | 0.0958  |
| Pure error      | 0.11           | 4  | 0.026       |         |         |
| Cor total       | 3.64           | 16 |             |         |         |

Note: the difference of $P<0.001$ was extremely significant. The difference was significant at $P<0.05$.

According to the analysis in table 4, the model ($P=0.0171$) is significant, while the loss of fit term ($P=0.0958$) is not significant, $R^2=0.8771$, and $R^2_{Adj}=0.7191$, indicating that the model has a certain significance and the experimental fitting effect is good, and the experimental loss of fit is good. Therefore, the quadratic polynomial equation can be used to replace the real point of the experiment for the analysis of the experimental results [22]. According to the $F$ value in the table, it can also be seen that each factor has an obvious effect on the extraction amount of anthocyanin, and its influence size is $X_2 > X_1 > X_3$, namely pH value $>$ extraction time $>$ extraction temperature.

3.6. The results of the interaction of factors on the response surface.
The influence of the interaction of various factors on anthocyanin was analyzed by design-expert 8.0 software, and the results were shown in Fig. 5.
Figure 5. The effect of an interaction between various factors on anthocyanins

As can be seen from figure 5, when the extraction time is unchanged, the extraction amount of anthocyanin changes significantly with the increase of pH. When pH is constant, the extraction amount increases first and then decreases with the increase of extraction time, and the effect is relatively obvious. Also can be seen from the contour map, contour extraction time and pH of direction more and more intensive, this phenomenon can explain both the extracting time and pH interaction on mulberry anthocyanins in wine slag skin effect is obvious, the extraction of contour in the shape of the ellipse, further showed that the extracting time and pH of interaction of significance. When the extraction time was unchanged, the extraction amount of anthocyanin increased first and then decreased with the increase of extraction temperature. When the extraction temperature is constant, the effect of the extraction amount is stable with the increase of time. From the analysis of contour map, it can be seen that the contour line along the extraction time and extraction temperature is denser with the extraction time, while the contour line along the extraction temperature is more sparse with the extraction temperature, indicating that the extraction time has a more significant effect on the extraction of anthocyanin relative to the extraction temperature. When the pH value is fixed, the extraction amount of anthocyanin increases with the increase of extraction temperature, and the change effect is obvious. When the extraction temperature is constant, the extraction amount of anthocyanin decreases with the change of pH value. As can be seen from the contour map, the contour line is elliptic, and the denser the contour line is along the pH value, the thinner the contour line is along the extraction temperature than the contour line along the pH value, which indicates that the mutual factor effect of the two is relatively obvious [23].
3.7. Experimental verification.
The response surface analysis results showed that when the liquid-solid ratio was 1:20, the extraction time was 58.37 min, the pH value was 5.86 and the extraction temperature was 45.02 °C, the pectinase-assisted extraction of anthocyanin reached the optimal technological conditions, and the anthocyanin content was 6.27816 mg/g. In order to improve the operability of the experiment, the extraction time, pH value and extraction temperature were adjusted to 58 min, 5.90 °C and 45 °C, respectively. Under this condition, the anthocyanin extraction verification experiment was further carried out, and the anthocyanin content in mulberry peel residue was 6.040 mg/g, which was close to the optimized value. Therefore, the response surface method was used to optimize the extraction process of anthocyanin from the mulberry peel residue by pectinase, and the optimal extraction conditions could be obtained.

4. Discussion
The research in this paper showed that under the conditions of liquid to material ratio of 1:20, extraction time of 58 min, pH value of 5.90 and extraction temperature of 45 °C, pectinase-assisted extraction of anthocyanin from mulberry residue was the best technological conditions, and under this condition, the maximum extraction amount was 6.040 mg/g. The anthocyanins extracted amount is far lower than using the ultrasonic method from planting mulberries, wild mulberry, black fruit mulberry anthocyanins content, but much higher than the ginkgo mulberry [24], but slightly higher than the research results by Cheng et al. [6] and Hu et al. [25], this may be due to extract raw materials, extraction methods and the differences in mulberry varieties. Different extraction methods vary in complexity and cost. As for the extraction method of mulberry anthocyanin, the enzyme-assisted extraction method adopted in this paper is simpler and cheaper than the ultrasound-assisted extraction method [6, 26, 27]. However, because the enzyme is susceptible to the influence of temperature and lead to decreased activity, resulting in decreased extraction efficiency. Although the ultrasonic wave is less affected by the outside world, the heat generated in the ultrasonic process will affect the extraction effect.

5. Conclusion
In this study, pectinase-assisted extraction was only studied, but other enzymes were not involved. In future studies, the author will further study the effect of snail enzyme and cellulase on extraction of anthocyanin from mulberry residue.

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