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Optimisation of clarification process of glutinous rice tea wine, and its antioxidant activity

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Abstract

Glutinous rice tea wine is an alcoholic beverage with antioxidant function which has good market value. However, it is prone to turbidity and precipitation during storage. To improve the unstable quality of glutinous rice tea wine, pectinase is used to clarify it. Based on single factor test and response surface test, the optimal clarification process parameters of pectinase, including pectinase addition, enzymolysis temperature, and enzymolysis time were optimised. Then, the antioxidant activity of glutinous rice tea wine was determined. Results showed that the effect of three clarification treatments on the clarification of glutinous rice tea wine was in the order of pectinase addition > enzymolysis temperature > enzymolysis time, and the optimal clarification conditions were: pectinase addition was 0.4 mL/L, enzymolysis temperature was 40.3°C, and enzymolysis time was 120.2 min. Under the optimal condition, the transmittance of glutinous rice tea wine was 92.3%, while the half maximal inhibitory concentration (IC_{50}) of glutinous rice tea wine on the scavenging rates of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·), hydroxyl radical (·OH), and superoxide anion radical (O_2^{-}·) were 53.64, 41.18, and 66.56 mg/mL (ethanol content), respectively. Therefore, glutinous rice tea wine had certain antioxidant capacity and health promotion effect which provided a theoretical basis for improving the added value of medium- and low-grade teas.

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Introduction

Tea is one of the most important economic crops in the world, and has abundant production (Komes *et al.*, 2010). However, most of the medium- and low-grade teas, especially summer and autumn teas, are generally low in prices, and often unsaleable (Li and Tang, 2012). In order to increase their economic added value, it is necessary to use scientific and technological means to process the tea (Lu *et al.*, 2010; Fang *et al.*, 2017). It is found that some companies use flavonoids in medium- and low-grade teas through extraction and microbial fermentation to obtain higher economic value (Bajerska *et al.*, 2011; Saini *et al.*, 2020). Some companies use medium- and low-grade teas to produce fermented tea wine, and have obtained good achievements (Sandri *et al.*, 2011; Li *et al.*, 2020). However, most fermented tea wines have uncoordinated taste (Joshi and Kumar, 2017). To mitigate this, it is, therefore, urgent to improve the production process. Glutinous rice wine is a traditional alcoholic beverage in Asia, and loved by consumers not only because of its pleasant aroma and taste (Ren *et al.*, 2021), but also because of its richer nutrients and therapeutic effects (Zhu *et al.*, 2016). Therefore, its production technology can be used to improve the quality of fermented tea wine.

By comparing the similarity of aroma characteristics between six kinds of tea and glutinous rice wines, it was found that the similarity of aroma characteristics between black tea and glutinous rice wine was the highest (Yoto *et al.*, 2018; Ren *et al.*, 2021; Chen *et al.*, 2022). So, we used the production technology of glutinous rice wine to produce a rice-flavour glutinous rice tea wine with harmonious aroma and refreshing taste (Wang *et al.*, 2020).

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However, the glutinous rice tea wine was prone to turbidity and precipitation during storage, which affected the quality of the product.

The formation of turbidity and precipitation is related to the microorganisms, macromolecular substances, and metal ions in the tea wine (Held *et al.*, 2015; Pal *et al.*, 2018). Meanwhile, the formation mechanism of turbidity and precipitation is more complicated; choosing an appropriate clarification method based on the characteristics of the wine body is a current way to eliminate precipitation (Kashyap *et al.*, 2001; Manavalan *et al.*, 2015). At present, there are many clarification methods for tea wine including physical, chemical, and biological (Sandri *et al.*, 2011; Ghosh *et al.*, 2017). Physical clarification methods use adsorbents to adsorb the substances which produce precipitation in tea wine (Manavalan *et al.*, 2015). Chemical clarification methods use clarifying agents such as gelatine, bentonite, chitosan, and plant-based clarifying agents (Rocha *et al.*, 2015). Biological clarification methods use hydrolysing pectin and other substances with enzyme preparations (Vaillant *et al.*, 1999; Lakhanpal *et al.*, 2016). Studies have shown that the use of pectinase clarification technology can achieve a better clarification effect on fruit juices and wines, and this method had been proved to be safe and effective (Kashyap *et al.*, 2001; Ma *et al.*, 2018). However, few studies to date have sought to clarify the tea wine by using pectinase. Therefore, in the present work, pectinase was used to clarify the glutinous rice tea wine. Meanwhile, based on the single factor test, the clarification process was optimised by response surface methodology, and the scavenging rates of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·), hydroxyl radical (·OH), and superoxide anion radical ($O_2^-\cdot$) were analysed to improve quality characteristics, and provide a theoretical basis for further processing and utilisation of fermented tea wine with antioxidant function, and to improve the added value of medium- and low-grade teas.

Materials and methods

Materials, chemicals, and reagents

Tea wine was self-brewed in this experiment; black tea (the raw material of tea was a small-leaf population variety, and the picking standard was one bud and four leaves) was procured from Chuanhong Group (Yibin, Sichuan, China; 104.56°E, 28.71°N; July 2019); orange and glutinous rice were procured from the local market; distiller's yeast was procured from Grams of Biological Technology Co., Ltd (Lishui, Zhejiang, China); pectinase (enzyme activity was 20,000 U/g) was manufactured by Lanji Technology Development Co., Ltd. (Shanghai, China); DPPH·, ·OH, and $O_2^-\cdot$ kits were purchased from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China); vitamin C (VC) was procured from Kelong Chemical Reagent Factory (Chengdu, Sichuan, China); and the other reagents were of analytical grade (AR).

Test flow

Test flow is shown in Figure 1. The operation points were as follows: removing impurities and stems from black tea, and black tea was passed through 60 mesh sieves after grinding. After sorting and impurity removal, the glutinous rice was washed and steamed for 40 min, and then added to the fermentation tank with 2% black tea powder and equal amount of orange juice. After cooling to room temperature, 2% distiller's yeast was added. Then, the glutinous rice tea wine was statically fermented for 14 d at 30°C. After the fermentation, the tea wine was filtered and boiled at 80°C for 20 min to obtain the final product.

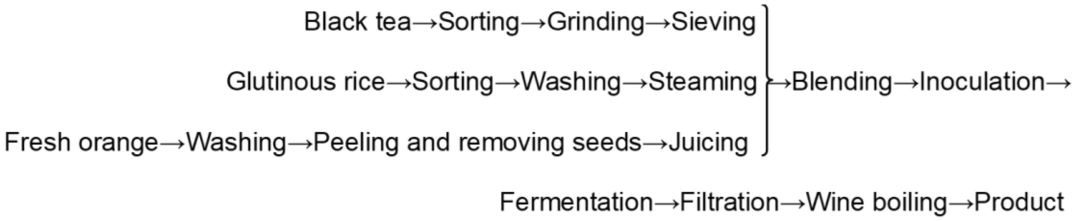

Figure 1. Test flow of the present work.

Clarity determination of glutinous rice tea wine

The clarity of glutinous rice tea wine was measured by spectrophotometry: the glutinous rice tea wine treated with pectinase was centrifuged at 1,504 *g* and 4°C for 10 min. And the light transmittance (%) of the supernatant was measured at 550 nm. The clarity of the glutinous rice tea wine was expressed by transmittance (distilled water as blank).

Optimisation of production technology of glutinous rice tea wine by single factor test

Under the basic conditions (factor) of pectinase addition, enzymolysis temperature, enzymolysis time of 0.3%, 40°C, and 100 min, respectively, each single factor was kept constant, and the others were changed. The variables were: pectinase addition (0.1, 0.2, 0.3, 0.4, and 0.5%), enzymolysis temperature (30, 35, 40, 45, and 50°C), and enzymolysis time (60, 80, 100, 120, and 140 min). Using transmittance as an indicator, each group of experiments was repeated three times for single factor experiments.

Optimisation of production technology of glutinous rice tea wine by response surface methodology

Based on single factor test, response surface test was conducted with pectinase addition (*A*), enzymolysis temperature (*B*), and enzymolysis time (*C*). Each factor was divided into three levels of change as presented in Table 1.

Table 1. Design and results of response surface methodology for clarification condition optimisation of glutinous rice tea wine.

Experimental level	*A:* Pectinase addition (%)	*B:* Enzymolysis temperature (°C)	*C:* Enzymolysis time (min)	The transmittance (%)
1	-1 (0.3)	-1 (35)	0 (120)	82.11
2	1 (0.5)	-1	0	81.47
3	-1	1 (45)	0	83.57
4	1	1	0	82.84
5	-1	0 (40)	-1 (100)	82.23
6	1	0	-1	81.46
7	-1	0	1 (140)	83.54
8	1	0	1	80.73
9	0 (0.4)	-1	-1	81.43
10	0	1	-1	84.42
11	0	-1	1	84.24
12	0	1	1	82.13
13	0	0	0	89.96
14	0	0	0	90.64
15	0	0	0	89.98
16	0	0	0	89.86
17	0	0	0	90.56

Determination of physicochemical indexes and sensory evaluation of glutinous rice tea wine

The determination of alcohol contents, total sugar (calculated as glucose), total acid, amino acid nitrogen, non-sugar solids, pH, and the sensory evaluation were performed according to Chinese standard (GB/T 13662-2018). The sensory evaluation was taken by a sensory evaluation panel consisting of 11 tea wine-tasters (5 men and 6 women), with the tea wine-tasters having > three years of sensory analysis experience in tea wine, and aged 20 to 30 years old. Furthermore, four sensory indexes (Table 2) including appearance (0 - 10 score), aroma (0 - 30 score), taste (0 - 40 score), and typicalness (0 - 20 score) were considered, as described previously (Wang *et al.*, 2020). The sensory score of the glutinous rice tea wine was an average result of 11 tea wine-tasters.

Table 2. Sensory score standards of glutinous rice tea wine.

Sensory index	Weightage of sensory index	Grading standard
Appearance	0.1	Hull of glossy, no obvious suspension
Aroma	0.3	Harmonious of wine and tea, full-bodied
Taste	0.4	Harmonious, delicate, mellow, and full-bodied
Typicalness	0.2	Unique style of tea wine and elegant quality

Highly bad: 0 - 19 score; moderately bad: 20 - 39 score; bad: 40 - 59 score; good: 60 - 79 score; moderately good: 80 - 89 score; and highly good: 90 - 100 score.

Antioxidant activity determination

The DPPH·, ·OH, and O_2^{-}· scavenging activities of the glutinous rice tea wine were determined following the manufacturer's instruction. VC solution served as positive control.

To measure DPPH· scavenging activity, 400 µL of glutinous rice tea wine (ethanol content of tea wine was 50 - 100 mg/mL) was mixed with DPPH solution (600 µL) or ethanol (600 µL) to produce test (T) and control solutions (CS), respectively. The blank solution (BS) contained a mixture of DPPH solution (600 µL) and ethanol (400 µL). These solutions were held for 30 min in the dark at room temperature, and centrifuged for 5 min at 13,000 *g*. The absorbance at 517 nm of the supernatant was then measured. The activity was calculated using Eq. 1:

DPPH· scavenging activity (%) =
$$[(1 - (A_T - A_{CS}) / A_{BS}) \times 100] \quad \text{(Eq. 1)}$$

For analysis of ·OH scavenging activity, glutinous rice tea wine (125 µL, ethanol content of tea wine was 40 - 100 mg/mL) was mixed with ddH_2O (500 µL), solution I (125 µL), solution II (125 µL), and solution III (125 µL) to produce the test solution (T). The control solution (CS) contained a mixture of glutinous rice tea wine (125 µL), ddH_2O (625 µL), solution I (125 µL), and solution II (125 µL). The blank solution (BS) contained a mixture of ddH_2O (625 µL), solution I (125 µL), solution II (125 µL), and solution III (125 µL). These mixtures were held for 20 min at 37°C, and their absorbances were measured at 510 nm. The activity was calculated using Eq. 2:

·OH scavenging activity (%) =
$$[A_{BS} - (A_T - A_{CS})]/A_{BS}) \times 100] \quad \text{(Eq. 2)}$$

To measure O_2^{-}· scavenging activity, solution I (260 µL), solution II (320 µL), solution III (40 µL), and solution IV (60 µL) were mixed with glutinous rice tea wine (40 µL, ethanol content of tea wine was 50 - 100 mg/mL) or ddH_2O (40 µL) to produce test (T) and control solutions (CS), respectively. The blank solution (BS) contained a mixture of ddH_2O (80 µL), solution I (260 µL), solution II (320 µL), and solution IV (60 µL). The mixtures were held for 10 min at 37°C, and their absorbances were measured at 570 nm. The activity was calculated using Eq. 3:

O_2^{-}· scavenging activity (%) =
$$(A_{CS} - A_T)/(A_{CS} - A_{BS}) \times 100 \quad \text{(Eq. 3)}$$

Statistical analysis

OriginPro 12.1 software was used for data mapping. Analysis of variance was performed by SPSS 20.0 software, and significant differences between means were identified using Duncan's multiple range test ($p < 0.05$). Response surface design and result analysis were performed by Design Expert 10.1 software.

Results and discussion

Effect of pectinase addition on the clarity of glutinous rice tea wine

As seen in Figure 2A, with increasing amount of pectinase addition, the transmittance of glutinous rice tea wine also increased. In particular, when the amount of pectinase addition increased from 0.1 to 0.4 mL/L, the transmittance of glutinous rice tea wine increased significantly ($p < 0.05$) from 78.80 ± 0.46 to 89.37 ± 0.25%. Due to the fact that pectinase can effectively interact with pectin and other substances, it was easy to precipitate in the beverage (Robles *et al.*, 2019); as the amount of pectinase addition increased, the rate of enzymolysis reaction increased, and the transmittance of glutinous rice tea wine also increased. However, when the amount of pectinase addition continued to increase, the effect of improving the clarity of glutinous rice tea wine would no longer be significant ($p > 0.05$) due to the exhaustion of substrate. In addition, considering the food safety, 0.4 mL/L pectinase addition was selected for subsequent experiments.

Effect of enzymolysis temperature on the clarity of glutinous rice tea wine

The enzymolysis temperature increased from 30 to 40°C, and the enzyme activity increased with the increase in temperature, which in turn significantly improved the transmittance of glutinous rice tea wine ($p < 0.05$). But when the enzymolysis temperature further increased, its transmittance decreased significantly ($p < 0.05$, Figure 2B). Since the enzyme is a protein, as the temperature further increases, the enzyme denatures, thus resulting in a decrease in activity (Reynolds *et al.*, 2018). Therefore, the optimum enzymolysis temperature of pectinase used in the experiment was 40°C. At this temperature, the transmittance of the glutinous rice tea wine reached 89.37 ± 0.25% after enzymolysis. Therefore, the enzymolysis temperature of 40°C was used for subsequent experiments.

Effect of enzymolysis time on the clarity of glutinous rice tea wine

As seen in Figure 2C, the transmittance of glutinous rice tea wine increased with the prolongation of the enzymolysis time, reaching a maximum of 90.50 ± 0.36%. When the enzymolysis time increased from 60 to 120 min, the transmittance of glutinous rice tea wine increased significantly ($p < 0.05$). When the enzymolysis time increased to 140 min, the substrate of pectinase was decomposed, and the enzymolysis reaction ended. Although the transmittance of glutinous rice tea wine was improved, it was not significant ($p > 0.05$). Furthermore, as the enzymolysis time increased, the nutrients in the product would also be lost (Shah *et al.*, 2015). Therefore, the enzymolysis time of 120 min was used for subsequent experiments.

Response surface test of glutinous rice tea wine clarity

Through the single factor test, the Box-Behnken central combination design was used to conduct the response surface test for the pectinase addition (A), enzymolysis temperature (B), enzymolysis time (C), and the transmittance was the response value. The results are shown in Table 1.

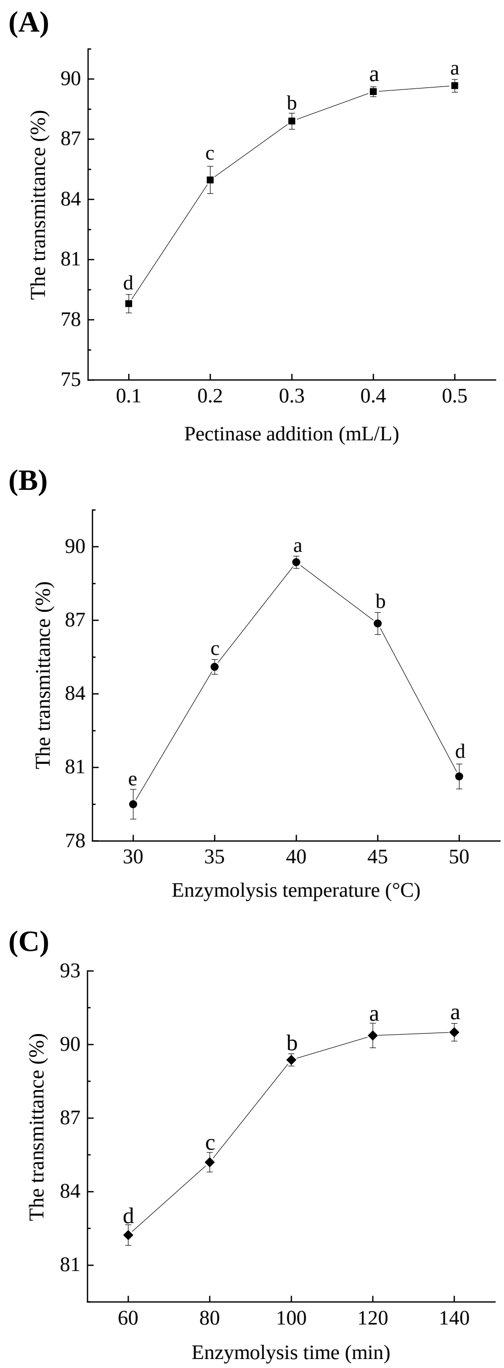

Figure 2. Effects of different pectinase additions on transmittance of glutinous rice tea wine **(A)**, effects of different enzymolysis temperatures on transmittance of glutinous rice tea wine **(B)**, and effects of different enzymolysis times on transmittance of glutinous rice tea wine **(C)**. Different lowercase letters indicate significant difference at $p < 0.05$.

Analysis of variance (ANOVA) was used to analyse the experimental data; the results are represented in Table 3. After regression fitting, Eq. 4 was obtained:

Table 3. Variance analysis results of response surface methodology for clarification condition optimisation of glutinous rice tea wine.

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*p*-value	Significant
Model	223.24	9	24.8	106.45	< 0.0001	**
A	3.06	1	3.06	13.14	0.0084	**
B	1.72	1	1.72	7.38	0.0299	*
C	0.15	1	0.15	0.65	0.4469	
AB	< 0.01	1	< 0.01	< 0.01	0.9283	
AC	1.04	1	1.04	4.47	0.0725	
BC	6.50	1	6.50	27.91	0.0011	**
A^2	80.91	1	80.91	347.26	< 0.0001	**
B^2	46.38	1	46.38	199.03	< 0.0001	**
C^2	61.64	1	61.64	264.55	< 0.0001	**
Residual	1.63	7	0.23			
Lack of fit	1.09	3	0.36	2.66	0.1842	
Pure error	0.54	4	0.14			
Total dispersion	224.87	16				

R^2 = 0.9927, R^2_{Adj} = 0.9834. *significant at $p < 0.05$, **highly significant at $p < 0.01$.

$$\text{Transmittance (\%)} = 90.20 + 0.62A + 0.46B + 0.14C - 0.02AB - 0.51AC - 1.27BC - 4.38A^2 - 3.32B^2 - 3.83C^2 \quad \text{(Eq. 4)}$$

Based on ANOVA, the correlation coefficient of the corresponding regression equation model was R^2 = 0.9927 and R^2_{Adj} = 0.9834, which indicated that the accuracy of the model was very good (Wang *et al.*, 2022). The influence of three factors on the clarity of glutinous rice tea wine showed the following order: $A > B > C$, that is, pectinase addition had the most significant effect on the clarity of glutinous rice tea wine, followed by enzymolysis temperature and enzymolysis time. The linear term of *A*, the interaction term of *BC*, the quadric terms of A^2, B^2, C^2 had a very significant effect ($p < 0.01$) on the transmittance of the glutinous rice tea wine, and the linear term of *B* had a significant effect ($p < 0.05$).

The response surface graph formed by the response value to each test factor (Singh *et al.*, 2004) are presented in Figure 3. From the response surface graph, the best parameters and the interaction between the parameters can be seen clearly (Bayraktar, 2001; Darwish *et al*., 2021). Among the interaction terms of three factors, the slope of the response surface formed by factor *B* and factor *C* was the steepest, which fitted with the ANOVA result that the interaction terms of *BC* had a very significant effect ($p < 0.01$) on the transmittance of the glutinous rice tea wine (Table 3).

Based on the analysis of Design Expert 10.1, with the pectinase addition of 0.39 mL/L, enzymolysis temperature of 40.34°C, and enzymolysis time of 120.23 min, glutinous rice tea wine could obtain the highest theoretical value of 90.7386% in transmittance. For the operability of the experiment, the optimal clarification conditions obtained by the software were appropriately adjusted to the pectinase addition of 0.4 mL/L, enzymolysis temperature of 40.3°C, and enzymolysis time of 120.2 min. After three parallel experiments, the transmittance of glutinous rice tea wine was 90.78 ± 0.36%.

Analysis of physicochemical indexes of glutinous rice tea wine and sensory evaluation

The physicochemical indexes of glutinous rice tea wine optimised by response surface methodology were determined, with wine samples without pectinase serving as control. The results are shown in Table 4. As can be seen from Table 4, the physicochemical indexes of glutinous rice tea wine were not significantly different from those of the control wine ($p > 0.05$), except for the solid content of sugar. The physicochemical indexes were both in line with the Chinese standard (GB/T 13662-2018).

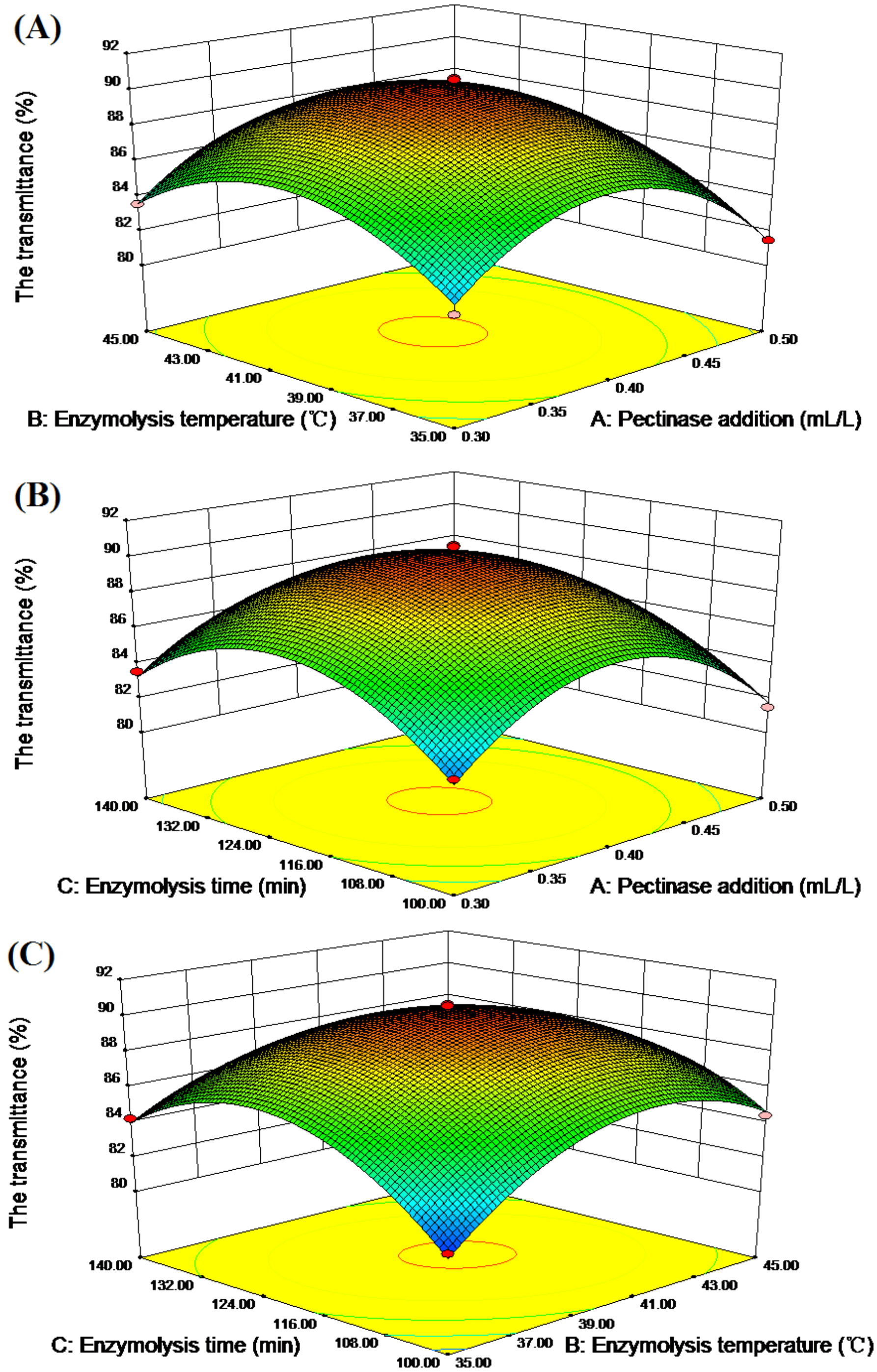

Figure 3. Response surface graph of interactive of pectinase addition and enzymolysis temperature on transmittance of glutinous rice tea wine **(A)**, response surface plot of interactive of pectinase addition and enzymolysis time on transmittance of glutinous rice tea wine **(B)**, and response surface plot of interactive of enzymolysis time and enzymolysis temperature on transmittance of glutinous rice tea wine **(C)**.

Table 4. Physicochemical indexes and sensory scores of glutinous rice tea wine and control sample.

Item	Control sample	Glutinous rice tea wine
Total sugar (g/L)	7.62 ± 0.16	7.68 ± 0.14
Total acid (g/L)	6.14 ± 0.10	6.08 ± 0.09
Non-sugar solid (g/L)	13.37 ± 0.08[a]	11.15 ± 0.12[b]
Amino acid nitrogen (g/L)	0.29 ± 0.01	0.30 ± 0.01
pH	4.92 ± 0.06	4.91 ± 0.04
Alcohol content (g/L)	12.36 ± 0.05	12.33 ± 0.07
Sensory score	91.87 ± 1.24[b]	95.93 ± 0.76[a]

Means followed by different lowercase superscripts in a row indicate significant differences at $p < 0.05$.

Therefore, it is feasible to use pectinase to clarify glutinous rice tea wine.

When compared with the glutinous rice tea wine without enzyme treatment, the sensory score of the enzyme-treated glutinous rice tea wine (95.93 ± 1.24 score) increased significantly ($p < 0.05$), which was mainly reflected in the improvement of clarity and reduction of bitterness and astringency of the glutinous rice tea wine after enzyme treatment. The enzyme-treated glutinous rice tea wine had bright yellow transparent, typical harmonious tea aroma of the wine, mellow taste, and elegant quality. Pectinase can improve the sensory characteristics of products (Adelakun *et al.*, 2020; Gani *et al.*, 2021); the reason being pectinase can remove substances with bitterness and astringency such as tannins and polyphenols (Liu *et al.*, 2021) in the process of pectin flocculation (Yamasaki *et al.*, 1964; Vijayanand *et al.*, 2010), which improved the sensory characteristics of glutinous rice tea wine.

Antioxidant activity of glutinous rice tea wine

The results of the antioxidant activity of glutinous rice tea wine are shown in Figure 4. The DPPH·, ·OH, and O_2^-· scavenging rate of glutinous rice tea wine all increased with the increase in their concentration. When the ethanol content of glutinous rice tea wine was 100 mg/mL, the scavenging rate reached the highest, and the scavenging rates of DPPH·, ·OH, and O_2^-· scavenging activity were 76.62 ± 1.56, 90.53 ± 0.53, and 68.61 ± 0.77%, respectively. But the scavenging rates were both lower than the positive control of VC. The inhibitory concentration 50% (IC_{50}) of glutinous rice tea wine on DPPH·, ·OH, and O_2^-· were 53.64, 41.18, and 66.56 mg/mL, respectively, which were all higher than the IC_{50} of VC (0.0308, 0.1211, and 0.1377 mg/mL). Due to tea leaves containing catechins and flavonoids which

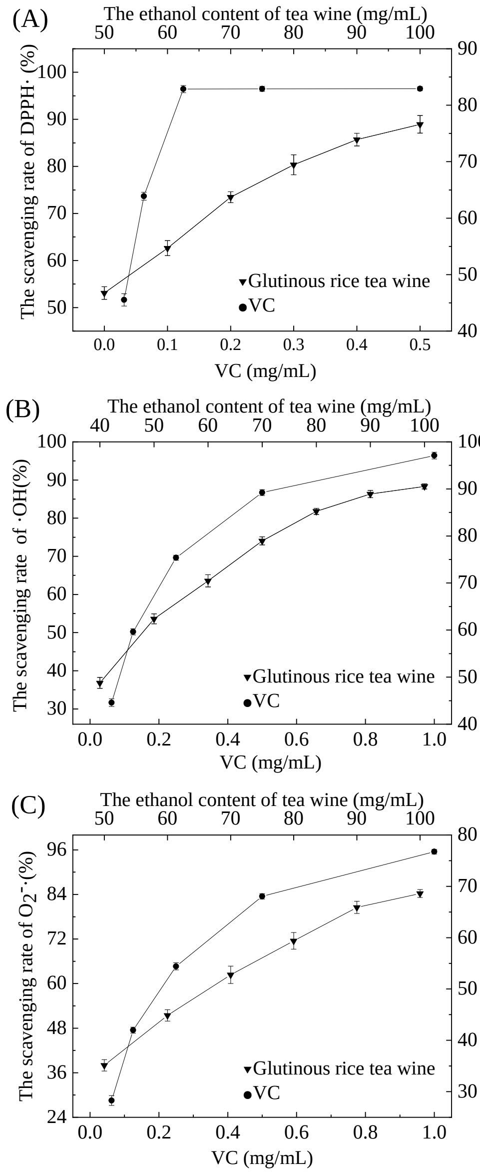

Figure 4. Analysis of antioxidant activity of glutinous rice tea wine. The scavenging rates of DPPH· of glutinous rice tea wine **(A)**, the scavenging rates of ·OH of glutinous rice tea wine **(B)**, and the scavenging rates of O_2^-· of glutinous rice tea wine **(C)**.

have certain antioxidant activity (Peng *et al.*, 2015; Rameshrad *et al.*, 2017; Jakubczyk *et al.*, 2020), and these active substances might be well retained in the fermentation process of glutinous rice tea wine, therefore, the glutinous rice tea wine had antioxidant activity.

Conclusion

Pectinase was used to study the clarification effect on glutinous rice tea wine. The optimum conditions for the clarification process were pectinase addition of 0.4 mL/L, enzymolysis temperature of 40.3°C, and enzymolysis time of 120.2 min through single factor test and response surface test. Under these conditions, the clarification effect of pectinase was significant ($p < 0.05$), and transmittance of glutinous rice tea wine reached 90.78 ± 0.36%. Meanwhile, antioxidant activity test results showed that the IC_{50} of glutinous rice tea wine for DPPH·, ·OH, and O_2^{-}· were 53.64, 41.18, and 66.56 mg/mL (ethanol content), respectively. It was concluded that glutinous rice tea wine had good antioxidant capacity and health promotion effect, and the present work provided a theoretical basis for increasing the added value of medium- and low-grade teas.

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