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Box–Behnken design to determine optimal fermentation conditions for apple-fortified mulberry wine using *Saccharomyces bayanus*

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Abstract

Wine is a fermented product of fruit juice. Mulberry and apple juice can also be used to produce wine. Several factors influence the alcoholic fermentation of the must; therefore, the optimization of the mulberry wine fermentation conditions with the dry yeast strain *Saccharomyces bayanus* (concentration varied from 0.15 to 0.25 g/L), fermentation conditions as pH value (from 3.5 to 4.5), and total soluble solid (TSS) content (24–28°Brix) were performed in this study. To evaluate the influence of these factors, a Box–Behnken design was used to minimize the number of factor combinations required, which allows the determination of optimal fermentation conditions (pH, TSS content, and dry yeast concentration) to produce wines with high alcohol and bioactive compounds (total anthocyanin (TAC) and polyphenol content (TPC)). Based on the analysis of experimental data, the second-order response surface models were developed to describe the relationship between initial pH value, TSS, and dry yeast concentration on wine quality acquisition (ethanol content and bioactive compounds). The results of the analysis of variance (ANOVA) showed that the model setup by response surface quadratic regression was suitable for predicting the wine quality. It was observed that the quality of the apple-fortified mulberry wine was significantly affected by all variables. The optimal contents of ethanol, total anthocyanin, and total polyphenol were achieved at 12.97% (v/v), 171.85 mg/L, and 87.17 mgGAE/L, respectively, when fermented in juice medium with optimal values of pH, TSS, and yeast concentration of 3.9, 26°Brix, and 0.22 g/L, respectively.

Keywords: apple-fortified mulberry wine; S. bayanus; ethanol; bioactive compounds; optimization.

Practical Application: The yeast strain *S. bayanus* proved to be effective in the production of mulberry wine with the addition of apples. The product has high contents of alcohol and bioactive compounds, has good sensory value, and is well received by a large number of consumers.

1 INTRODUCTION

Vietnam has an abundant and rich source of fresh fruits, but with the tropical climate, it is very difficult to preserve fresh fruits which are easily damaged during harvesting and transportation by reducing their quality. Among the fruits with attractive colors and high contents of nutrients and bioactive compounds, mulberry is most popular one. Mulberry (*Morus* spp.) is a fast-growing, deciduous tree belonging to the Moraceae family. It is long-lived and grows well in provinces such as Lam Dong, Kien Giang, and An Giang in Vietnam. Mulberry fruits can be grown in different forms; their foliage has many uses and positive impacts on environmental safety, including ecological restoration of degraded land, bioremediation of contaminated areas, water conservation, and prevention of soil erosion, and it improves air quality by sequestering carbon (Rohela et al., 2020). In most localities in our country where mulberry is grown, the plant is being effectively exploited for silkworm rearing and food processing such as candy, jam, and juice. Mulberry fruit contains high levels of vitamins, minerals, fiber, amino acids, polysaccharides, a variety of polyphenols, flavonols, phenolic acids, and anthocyanins (You et al., 2018; Yuan and Zhao, 2017). With high anthocyanin content, the fruit has pharmacological, antioxidative, anti-diabetic, anti-atherosclerotic, and anti-obesity effects (Chan et al., 2016; Peng et al., 2011; Thuy et al., 2022a), which reduces excess fat, lowers cholesterol, improves the ratio of LDL (bad) to HDL (good) cholesterol, and potentially helps prevent fatty liver disease (Liu et al., 2009; Wu et al., 2011). Therefore, efficient use of fruit for different processing techniques is also important to increase sustainability, increase crop value, and meet the diverse food needs of consumers.

Besides, apples are widely consumed in all countries of the world, which are very popular because of their taste, juiciness, and nutritional content. In addition, they are available year-round on the market, relatively low in price, and considered healthy foods. A total of 20 polyphenolic compounds were identified in all studied apples (Kschonsek et al., 2018). Due to the antioxidant capacity of polyphenols, combining mulberry

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and apple for wine fermentation is a matter of interest in our research, positively supporting fermentation, protecting anthocyanins of mulberry, and improving fermentation and health support of the user. In addition, natural anthocyanin pigments and co-pigments (e.g., polymers, phenolic compounds, and carbohydrates) form non-covalent complexes that stabilize and regulate color in a wide variety of plants, fruits, and foods derived from them, such as wine, jam, juice, and syrup products (Gençdağ et al., 2022; Tai & Thanh, 2020).

Fermentation of apple-fortified mulberry wine can be considered a relatively efficient preservation process, using less energy, increasing shelf life, and reducing the need for cold storage systems. This technique is very suitable for use in developing countries where access to modern equipment is limited. Fermentation is a viable technique in developing new products with altered organoleptic and physicochemical properties, especially in terms of taste and nutritional composition. Currently, the wine market is also moving toward diversification by evolving from different valuable ingredients through more traditional or modern techniques, along with alternative fruit or vegetable ingredients. The wine industry is popular in different regions of the world (Maicas & Mateo, 2020). To date, many wine production studies have used *Saccharomyces cerevisiae*, a yeast isolated or commercially available. The study on the change and correlation between phenol and product quality of mulberry wine fermented with *Lactiplantibacillus plantarum* in combination with *S. cerevisiae* was performed by Hu et al. (2021). A study on chemical composition and sensory value of mulberry wine fermented with yeast strain *S. cerevisiae* was carried out by Tao et al. (2017). However, very few studies have used the yeast strain *Saccharomyces bayanus*. *S. bayanus* is now accepted as the result of multiple hybridization events between three purebred species, namely, *Saccharomyces uvarum*, *S. cerevisiae,* and *Saccharomyces eubayanus* (Libkind et al., 2011). The hybrid strains seem to be well adapted to the stressful conditions (low pH, high sugar concentration, and ethanol content) that occur during wine fermentation (Belloch et al., 2008). In addition, their enology has confirmed their interesting properties in wine making (González et al., 2007). *S. bayanus* is also the only species of the genus that can grow in vitamin-deficient conditions (Batt and Tortorello, 2014). The yeast *S. bayanus* has a distinctive organoleptic flavor that it imparts to wines (Muñoz-Bernal et al., 2016). Eglinton et al. (2000) stated that strains of *S. bayanus* could induce a different organoleptic characteristic in wine when compared with a widely used yeast *S. cerevisiae*. Januszek et al. (2020) suggested that *S. bayanus* uses up its carbon source to produce more potent volatile compounds than *S. cerevisiae*. *S. bayanus* produces large amounts of 2-phenylethanol, ethyl lactate, 2-phenylethyl acetate, and other acetate esters (Gamero et al., 2014; Tosi et al., 2009). The objective of this study is to effectively utilize ripened mulberry fruit grown in An Giang Province, Vietnam and to establish an optimal fermentation condition for apple-supplemented mulberry wine by varying pH value, total soluble solid (TSS) content (Brix degree), and *S. bayanus* yeast concentration using a Box–Behnken design.

2 MATERIALS AND METHODS

2.1 Sample preparation

Ripe mulberries (*Morus nigra*) were collected from fruit orchards in Chau Phu district, An Giang Province. After collection, raw materials were selected; only intact fruits were selected, while the damaged and crushed fruits were removed. The fruits were washed with clean water, drained, quantified, put in PA bags, and stored in the freezer at ≤-10±2°C for study. For wine making, mulberry fruit was removed from the freezer and soaked in warm water (40–50°C) with a fruit:water ratio of 1:1. Then, the filtration was carried out to obtain mulberry juice.

Apples (Fuji) were purchased from supermarkets. Riped, attractive bright colored, and shiny skinned apples were selected for this study. After collection, they were washed, dried, and stored in a refrigerator (3–5°C) for the research. For wine making, apples were finely chopped (both the flesh and the skin). Water was added at the ratio of 1:1 (fruit:water), followed by the addition of pectinase with a concentration of 0.2% (according to the total weight of the fruit and water added) and the mixture was incubated for 2 h (Thuy *et al*., 2011), filtered, and recovered to obtain the apple juice.

2.2 Yeast strain

The dried yeast *S. bayanus* emulsifier, sorbitan monostearate (E491) (France), was used to ferment mulberry fruit juice at ambient temperature (28±2°C). Yeast was activated in 5% glucose solution at 35–38°C (according to the manufacturer's instructions). The solution was stirred well to avoid lumps and allowed to stand for 15 min.

2.3 Effect of combined apple juice on the quality of apple-fortified mulberry wine

To prepare for alcoholic fermentation, apple juice was added to the mulberry juice in concentrations ranging from 100 to 350 mL/L. Then, water was added to the mixed juice with the ratio of 3:1. Sugar and pH were adjusted to 26°Brix (by refined sugar) and pH 4 (by $NaHCO_3$). Notably, 100 ppm of sodium metabisulfite ($Na_2S_2O_5$) was added to the mixed juice for about 2 h in order to inhibit microorganisms that are not conducive to alcohol fermentation. The fixed dry yeast concentration of 0.15% was added after activation (as mentioned above). The product was analyzed for quality after 12 days of fermentation, as well as for alcohol and bioactive compounds. Sensory evaluation of the product was also performed concurrently. The product chosen from this experiment (with a proper combination of mulberry and apple juice) was further studied to optimize the conditions affecting fermentation, such as pH, TSS content, and concentration of yeast *S. bayanus* used.

2.4 Optimization of fermentation conditions (pH value, TSS content, and dry yeast concentration)

The factors investigated in this experiment included pH value (X_1), TSS content (X_2), and the percentage of yeast *S. bayanus* (X_3) (Table 1). The apple-fortified mulberry wine fermentation

optimization was experimentally designed using a Box–Behnken model with 18 experimental units and 6 central points. Each experiment was repeated three times.

2.4.1 Fruit wine fermentation

To prepare alcoholic fermentation, apple juice was added at a selected ratio from the previous study. The mixed juice was adjusted for Brix (with fine granulated sugar) and pH (not adjusted if required pH was reached or adjusted with $NaHCO_3$ to increase pH) according to the experimental arrangements. Notably, 100 ppm of sodium metabisulfite ($Na_2S_2O_5$) was added for approximately 2 h and the dry yeast *S. bayanus* was supplemented with different concentrations following the experimental setup. The mixture was stirred slowly until the sugar dissolved completely. The activated yeast was added to the fermenter slowly so that it was gradually acclimatized to the fermentation environment. Apple-fortified mulberry wine fermentation in 30 L fermenter tank with stopper and spout and white polypropylene as synthetic material is specialized for food (Enolandia, Italy). Each treatment was repeated three times. The analytical parameters during fermentation were ethanol (% v/v) and bioactive substances such as total anthocyanins (mg/L) and total polyphenols (mgGAE/L).

2.4.2 Wine quality analysis

Samples were taken after 12 days of fermentation. A distillation system was used to separate volatile and non-volatile components in alcohol samples. The alcohol content of the resulting distillate was then readily measured by hydrometry using an alcohol hydrometer of appropriate proportions. The measurement was influenced by temperature; therefore, the temperature was measured and the observed reading was corrected using the published tables. The samples are usually measured at 25°C for alcohol content.

Total anthocyanin content (TAC) was determined by pH differential method by measuring absorbance at pH 1.0 and 4.5 using a UV-vis spectrophotometer (Cary 60 UV-Vis spectrophotometer, USA) (Thuy et al., 2022b). Total polyphenol content (TPC) was determined by the Folin-Ciocalteu method (Hossain et al., 2013). Chromatometer CR-400 (USA) was used to observe the color of wine products after fermentation.

2.5 Multiple regression analysis

Multiple regression was applied on the entire data set to derive a relationship between response/dependency (ethanol/total anthocyanin/total polyphenol) and multiple independent variables (pH value, TSS, and dry yeast concentration). Multiple regression was applied on the entire data set to derive a relationship between a response/dependency and multiple independent variables. The following quadratic polynomial equation (Equation 1) is used in this case:

$$Y = \beta_o + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij} X_i X_j \quad (1)$$

where:

Y: a dependent variable;

X: an independent variable;

β_o, β_i, β_{ii}, and β_{ij}: the regression coefficients.

3 RESULTS AND DISCUSSION

3.1 Effect of apple juice supplement on some physicochemical and sensory properties of combined wine products

Adding apple juice to mulberry juice during fermentation has positively changed some quality parameters of wine products. Product quality was analyzed after 12 days of fermentation and is presented in Table 2.

3.1.1 Ethanol

With the addition of different percentages of apple juice, ethanol content tended to increase slightly, probably because apples contain many nutrients that are good nutritional support

Table 1. Factor levels used for optimization.

Factors	Levels			
	Coded	-1	0	1
pH	X_1	3.5	4	4.5
TSS content (%)	X_2	24	26	28
Dry yeast concentration (g/L)	X_3	0.15	0.2	0.25

Table 2. Some physicochemical parameters of mulberry wine with different ratios of apple juice added.*

Ratio of apple juice added (mL/L)	Ethanol (% v/v)	Total anthocyanin (mg/L)	Total polyphenol (mgGAE/L)	Color (L value)
0	12.25[a]	171.89[a]	83.01[a]	18.53[a]
100	12.30[a]	172.05[a]	84.88[b]	18.25[a]
200	12.60[ab]	172.21[a]	88.74[c]	19.24[b]
300	13.10[b]	175.37[b]	89.61[d]	18.98[b]
400	13.15[b]	175.03[b]	102.40[e]	19.38[b]

*Means with the same letters represent no significant difference (P>0.05).

for yeast growth. With the addition of 30 mL/L of apple juice, the ethanol content in the product was high (13.10% v/v) and significantly different from the control sample (12.25% v/v); however, no significant difference was noted in the ratio of 300 and 400 mL/L apple juice supplementation (Table 2). Apple juice contains many forms of sugars such as fructose, glucose, and sucrose. Given the high fructose content present in apple juice, the selection of a fructose-favoring yeast strain could have important implications for the cider industry (Wang et al., 2004). Some studies also show that cider is a fermented product from apples and it uses a popular strain of yeast *S. bayanus* (Hoff, 2012; Magalhães et al., 2017).

3.1.2 Bioactive compounds

The content of bioactive compounds (TPC and TAC) increased with increasing apple juice intake, especially for TPC. TPC was found to increase gradually from 2.25 to 23.36% when apple juice was added from 10 to 40% and was significantly different (P£0.05) from the control sample (without apple juice). This difference is due to the fact that apples contain high polyphenol content (skin: 3.25 mg/g and fruit pulp: 1.68 mg/g), so when the ratio of apple juice is increased, this content in wine also increases (although about 30–35% of polyphenols are lost during fermentation). However, there was no significant difference in TAC with increasing apple juice ratio. After fermentation, the TAC content varied from 0.09 to 1.83% while increasing the percentage of apple juice added ranging from 10 to 40%. This result is probably due to the fact that the apple juice is taken with both the skin and the flesh, the peel contains 125.15 µg/g TAC, and the pulp contains about 52.80 µg/g TAC, so it did not reduce this content in the finished product.

3.1.3 Color

The color of the wine is slightly brighter as the percentage of apple juice increases. However, as the color of mulberry juice is very dark, it is not possible to see a significant difference by increasing the amount of apple juice, and only when the percentage of about 20–40% of apple juice is added, the brighter color is noted, but there is no significant difference between these three ratios (as indicated in Table 2 and Figure 1).

3.2 Optimization of fermentation conditions on mulberry wine quality

3.2.1 Ethanol content

Optimization was performed according to the response surface method (RSM). The factors of pH (X_1), degree of Brix (X_2), and dry yeast concentration (X_3) affect the ethanol content. The statistical results showed that the P-values of both the independent and interacting variables are less than 0.05, showing a high level of significance of these components shown in the equation (Table 3). However, it is also easy to see that the X_1X_3 interaction does not affect the ethanol content when the expressed P-value is greater than 0.05 (P=0.356).

The model fitting was also evaluated through the P-value of lack-of-fit. A good correlation model needs a goodness-of-fit between the actual data and the model's predictions, so a model built with the lack-of-fit test that has no statistical significance is desirable (Van Tai et al., 2021; Thuy et al., 2022a; Thuy et al., 2022b). From the data analysis, it is also showed that lack-of-fit did not show statistical significance (0.1184), so the model fitting

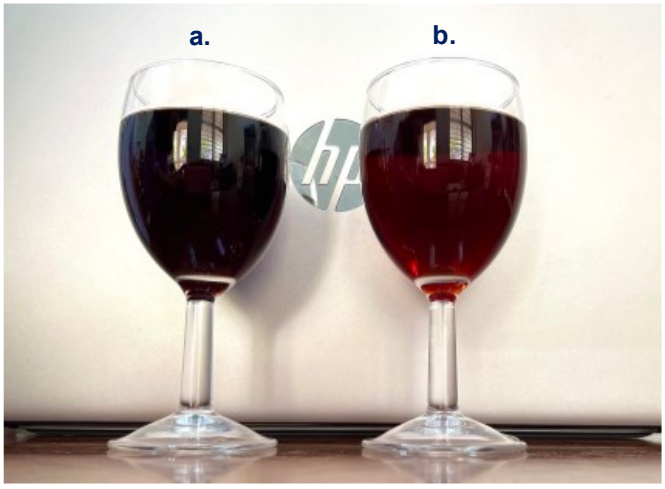


Figure 1. Mulberry wine: (A) control samples (without apple juice) and (B) sample supplemented with 30% apple juice.

Table 3. Analysis of variance (ANOVA) for the regression response surface model for ethanol content

Sources	Sum of Square	dF	Mean Square	F value	P-values	Significant
X_1	57.1651	1	57.1651	219.88	0.0000	**
X_2	1.51504	1	1.51504	5.83	0.0203	*
X_3	7.1177	1	7.1177	27.38	0.0000	**
X_1X_1	30.9792	1	30.9792	119.16	0.0000	**
X_1X_2	1.62067	1	1.62067	6.23	0.0166	*
X_1X_3	0.226875	1	0.226875	0.87	0.3557	Not significant
X_2X_2	5.94983	1	5.94983	22.89	0.0000	**
X_2X_3	1.74803	1	1.74803	6.72	0.0131	*
X_3X_3	7.61114	1	7.61114	29.28	0.0000	**
Lack-of-fit	1.61829	3	0.539431	2.07	0.1184	Not significant
Pure error	10.6593	41	0.259982			
R^2 = 90.86%			R^2 (adjusted for d.f.) = 88.99%		Standard Error of Estimation = 0.51	

X_1: pH value; X_2: TSS (°Brix); X_3: dry yeast concentration (%); *significant difference and **extremely significant difference at P<0.05.

was high, with the correlation coefficient R^2=90.86% and the adjusted (Adj.) R^2=88.99% with a low standard error (SE=0.54), showing a high degree of compatibility between experimental data and theoretical data. When eliminating the insignificant interaction (X_1X_3), the final regression equation shows the relationship between ethanol content and three variables according to the quadratic model of the Box–Behnken design for the fermentation (Equation 2) (R^2 = 90.69%; $R^2_{Adj.}$ = 89.03%; standard error of the estimate (SEE)=0.51).

$$Ethanol\left(\%\frac{v}{v}\right) = -204.36 + 42.76X_1 + 7.93X_2 + 232.13X_3 - 6.15X_1^2 + 0.37X_1X_2 - 0.17X_2^2 - 3.82X_2X_3 - 305X_3^2 \quad (2)$$

The response surface models presented in Figure 2 showed that the factors pH, Brix, and yeast percentage affect the ethanol content. When the pH values, Brix, and yeast percentage increase, the ethanol content increases, but when the pH is raised too high, the sugar content in the fermentation broth is also too high, and the number of yeasts is large, the ethanol content tends to reduce. In this study, when the pH was increased from 3.5 to 4, the alcohol content tended to increase markedly but then did not seem to increase anymore at higher pH. In general, a low initial pH prolongs yeast lagging, affects cumulative mass loss, changes the rate of total sugar consumption, increases final acetic acid and glycerol content, and reduces the final content of ethanol (Liu et al., 2015). At high pH, the harmful bacteria can be grown, causing competition and inhibiting the yeast growth and development. Fruit juice with suitable pH can improve alcohol stability, inhibit bacterial growth, and also facilitate sugar fermentation (Ribéreau-Gayon et al., 2006). Normally, yeast grows at pH 2–8, but most of the yeasts grow very well between pH 4.5 and 6.5 (Yalcin & Ozbas, 2008). However, previous studies have also suggested that the pH ranging from 2.75 to 4.25 is also considered an important factor for the survival and growth of yeast (Fleet and Heard, 1993). The suitable pH for the growth and yeasts depends on many factors such as yeast strain, environmental composition, and fermentation conditions (time and temperature). Reddy and Reddy (2011) studied alcoholic fermentation from mango and showed that ethanol changed with pH change of mango juice. The results of this study showed the lowest concentration (5% v/v) of ethanol when produced at pH 3.0 and the highest (7.8% v/v) at pH 5.0.

Sugar is an essential substrate for fermentation, so it greatly affects the ethanol content. Yeast has the ability to ferment sugar into alcohol, so the alcohol level is high or low depending on the sugar content used in the fermentation solution. The higher the sugar concentration, the more the alcohol production (Attri, 2009). However, high sugar content will increase osmotic pressure and unbalance the physiological state of yeast, adversely affecting the fermentation process. If the sugar content is too low, there will not be enough substrate for the yeast to work, which will reduce the fermentation efficiency. Sugar concentrations from 200 g/L to 300 g/L reduced the growth rate of *S. cerevisiae*,

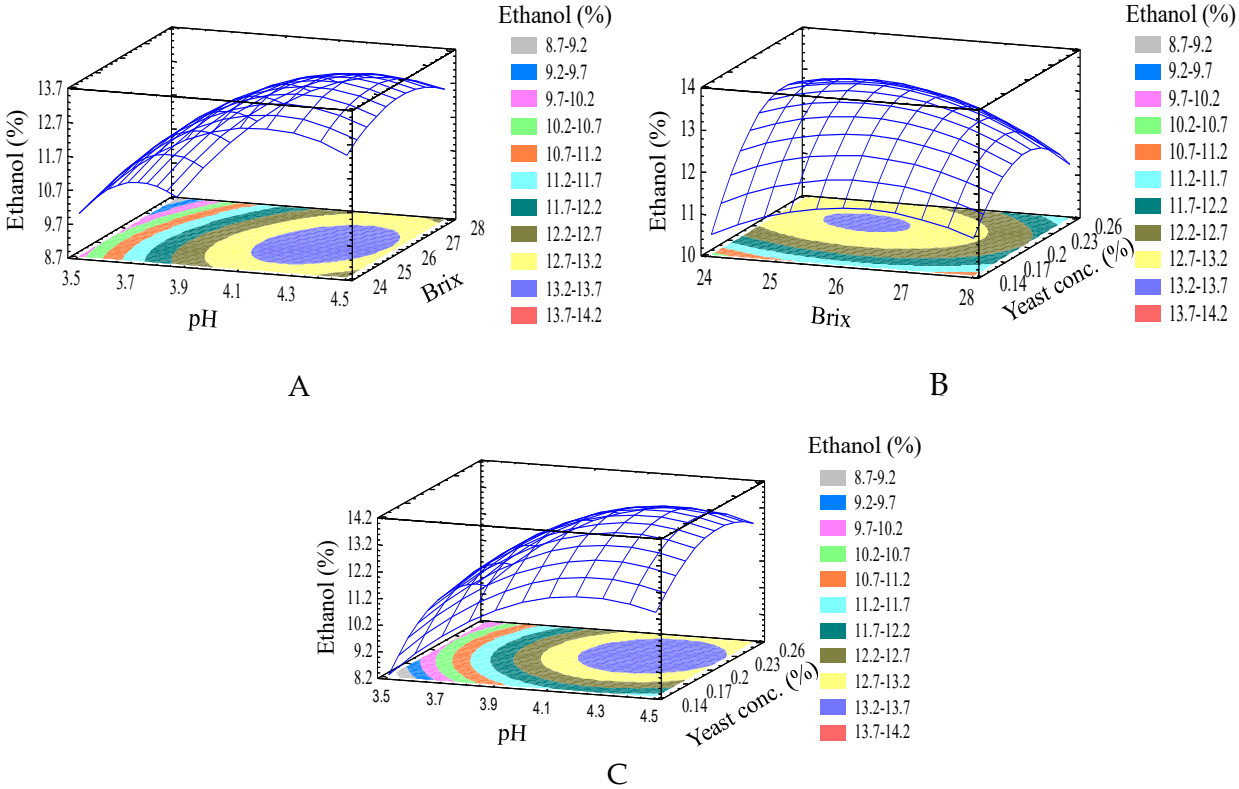


Figure 2. Correlation between variables: (A) Ethanol content (% v/v) with pH and TSS (%) (With a fixed yeast concentration of 0.2 g/L); (B) Ethanol content (% v/v) with TSS (%) and dry yeast concentration (at a fixed pH of 4); (C) Ethanol content (% v/v) with pH and dry yeast concentration (TSS is fixed at 26%).

as reported by D'Amato et al. (2006), who found that the growth rate was lowest at higher glucose concentrations.

As the concentration of yeast (g/L) increases, the fermentation rate also increased until the optimum level was reached. At low yeast concentrations, the ability to continue budding and the time to reach the norm is very long, which will affect the activity and metabolism of yeast. However, when the amount of yeast is high, they can compete for nutrients, affecting the fermentation process. Therefore, when the yeast ratio is between 0.15 and 0.25 g/L, the ethanol content is not higher than the 0.2 g/L of dry yeast used.

Pareto charts (Figure 3) was used to determine the magnitude and importance of effects. On this chart, the bars representing the factors X_1, X_2, and X_3 and their interactions cross the reference line at 1.99, so these factors and interactions have statistical significance at the 0.05 level with the current model conditions, except for the X_1X_3 interaction. The standardized Pareto chart for ethanol also showed that pH has the greatest influence on the alcoholic fermentation of *S. bayanus* according to the linear equation (X_1) and quadratic equation (X_1X_1). The other two variables, namely, sucrose and yeast also have a similar but relatively less significant effect. The interaction between pH and yeast content (X_1X_3) was not significant for that response. The optimal ethanol content found from the model was 13.62% (v/v) corresponding to pH, Brix, and yeast concentration of 4.24, 25.7%, and 0.22 g/L, respectively. By substituting the empirical values of the variables into Equation 2, the ethanol content can be predicted. It can be seen that a high compatibility has been achieved between the experimental data and the calculated results from the model.

3.2.2 Total anthocyanin content

The influence of factors on the TAC of wine after fermentation was also examined. The ANOVA panel statistical significance of each effect was checked by comparing the mean squared with an estimate of experimental error. In this case, eight effects have P-values less than 0.05 (from ANOVA for TAC was performed), showing that they are significantly different at the 95% confidence level. However, the X_1X_3 interaction also shows no significance when the P-value is greater than 0.05 (P=0.402). Since the P-value of lack-of-fit is 1.0 in the ANOVA table, which is larger than 0.05, the model appears to be adequate for the observed data at the 95% confidence level. R-squared and adjusted R-squared with values of 98.78% and 98.53% showed that the fit model explained more than 98% of the variation in TAC with the SEE, showing that the standard deviation of the residuals is 0.583. The second-order polynomial model equations for TAC with pH (X_1), TSS (X_2), and dry yeast concentration (X_3) are shown in Equation 3 (R^2=98.76%; $R^2_{Adj.}$ = 98.54%; and SEE=0.58).

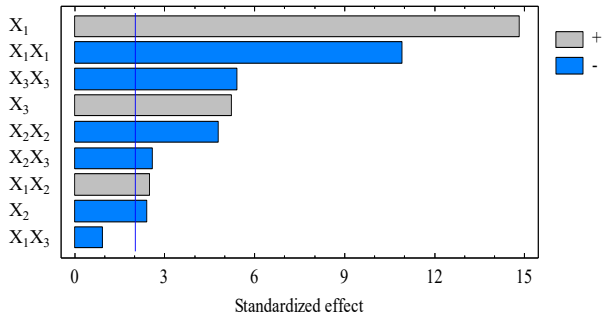
$$TAC\ (mg/L) = -1049.24 + 18.71X_1 + 88.21X_2 + 371.99X_3 - 4.72X_1^2 + 0.45X_1X_2 - 1.69X_2^2 - 5.28X_2X_3 - 539.78X_3^2 \quad (3)$$

The Pareto plot (Figure 4) shows that the bars representing the variables X_1 (pH), X_2 (°Brix), and X_3 (dry yeast concentration) and their interactions cross the reference line at 2.006, so all the three factors and their interactions are statistically significant at the 0.05 level, except for the X_1X_3 interaction. It was observed that sugar (as TSS) had the greatest influence on TAC in wine when fermenting with yeast strain *S. bayanus*, then pH and least affected is yeast. In this study, according to statistical analysis, the sugar (sucrose) concentration of 26% had a protective effect on anthocyanins, but at higher concentrations this effect was reduced. Sucrose protects anthocyanins from degradation during fermentation, preventing browning, and the formation of polymeric pigments, which may be due to inhibition of enzymatic reactions or interference with other condensation reactions of sucrose (Wrolstad et al., 1990). Therefore, it is necessary to use sugar effectively for the fermentation process to create high alcohol content and maintain the anthocyanin content of the product.

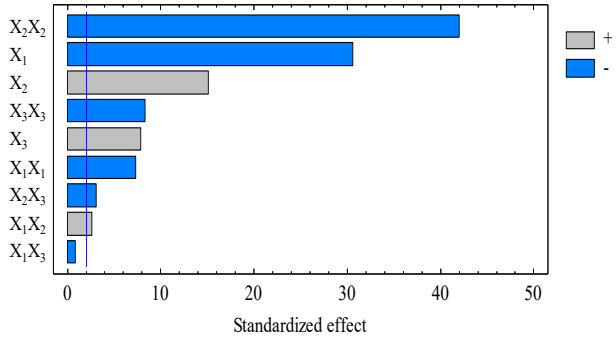
The higher the pH of the initial fermentation broth, the lower the anthocyanin content in the strawberry juice. Most anthocyanin pigments are highly stable under acidic versus basic conditions and degradation occurs at higher pH (Khoo et al., 2017). Sui et al. (2014) also suggested that anthocyanins are pH dependent. Using Optimize Response, the highest (optimal) anthocyanin content value was found from the model, which was 173.82 mg/L when the initial fermentation broth had pH of 3.5, Brix degree of 26.17, and dry yeast content of 0.22%.

3.2.3 Total polyphenols

The ANOVA table was also performed in the case of TPC (full data not provided here). This analysis tested the statistical



X_1: pH; X_2: Brix; X_3: dry yeast concentration.
Figure 3. Standardized Pareto chart for ethanol.



X_1: pH; X_2: Brix; X_3: dry yeast concentration.
Figure 4. Standardized Pareto chart for anthocyanin.

significance of each effect by comparing the mean square with an estimate of experimental error. In the case of analysis for TPC, 8 effects had P-values less than 0.05, indicating that they were significantly different from 0 at 95% confidence. There is only one effect (X_1X_3) with a P-value greater than 0.05 (0.4022). From the ANOVA analysis, the P-value of lack-of-fit is greater than 0.05 (P-value of lack-of-fit=1), so this model seems to agree with the observed data at 5% significance level (95% confidence level). The R-squared statistics showed that the model was well established and explained 98.78% of the variation in the TPC. The adjusted R-squared statistics with a value of 98.53% is more suitable when comparing models with different numbers of independent variables. The SEE showed that the standard deviation of the residual is 0.583. After removing the insignificant interaction (X_1X_3), the quadratic polynomial model equation for TPC in terms of pH (X_1), TSS (X_2), and dry yeast concentration (X_3) is given in Equation 4 (R^2 = 98.76%; $R^2_{Adj.}$ = 98.54% ; and SEE = 0.583).

$$TPC = -1133.92 + 18.71X_1 + 88.21X_2 + 371.99X_3 - 4.72X_1^2 + 0.45X_1X_2 - 1.69X_2^2 - 5.28X_2X_3 - 539.78X_3^2 \quad (4)$$

The Pareto plot shows the bars representing the variables X_1 (pH), X_2 (°Brix), and X_3 (dry yeast concentration) and their interactions cross the reference line at 1.929, and all three factors and their interactions are statistically significant at the 0.05 level, except for the X_1X_3 interaction (Figure 5). Sugars and acids show the greatest influence on TPC in wine and the least effect on yeast. The optimal TPC value obtained from the model (Equation 4) is 89.14 mgGAE/L when the pH of the initial fermentation is 3.5, the Brix is 26.17, and the dry yeast content is 0.216% using Optimize Response from Statgraphic.

3.2.4 Optimization and Validation of Process

The fitted model for all three responses was reliable within the region of the experiment based on the results of ANOVA. So, concurrent optimization for all responses can also be performed using overlapping histograms (Figure 6). It was observed that the optimal conditions of pH, TSS, and yeast concentration are 3.93, 26°Brix, and 0.22 g/L, respectively, giving the maximum estimated value for ethanol, TAC, and TPC of 12.97% (v/v), 171.85 mg/L, and 87.17 mgGAE/L, respectively.

Validation testing was performed to determine the accuracy and reliability of the built prediction models. In addition, the difference between the experimental value and the estimated value from the optimal conditions is also considered.

The results presented in Table 4 show that the experimental ethanol and TAC concentrations were slightly higher than the predicted values, 1.77% and 0.76% respectively, but still within

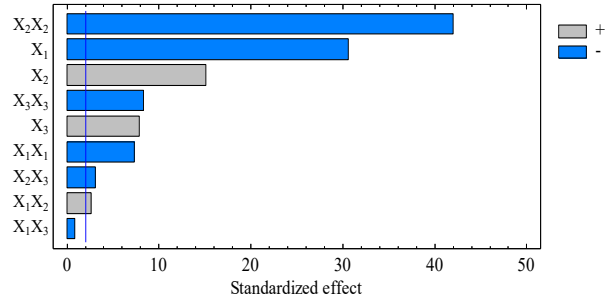


Figure 5. Standardized Pareto chart for TPC.

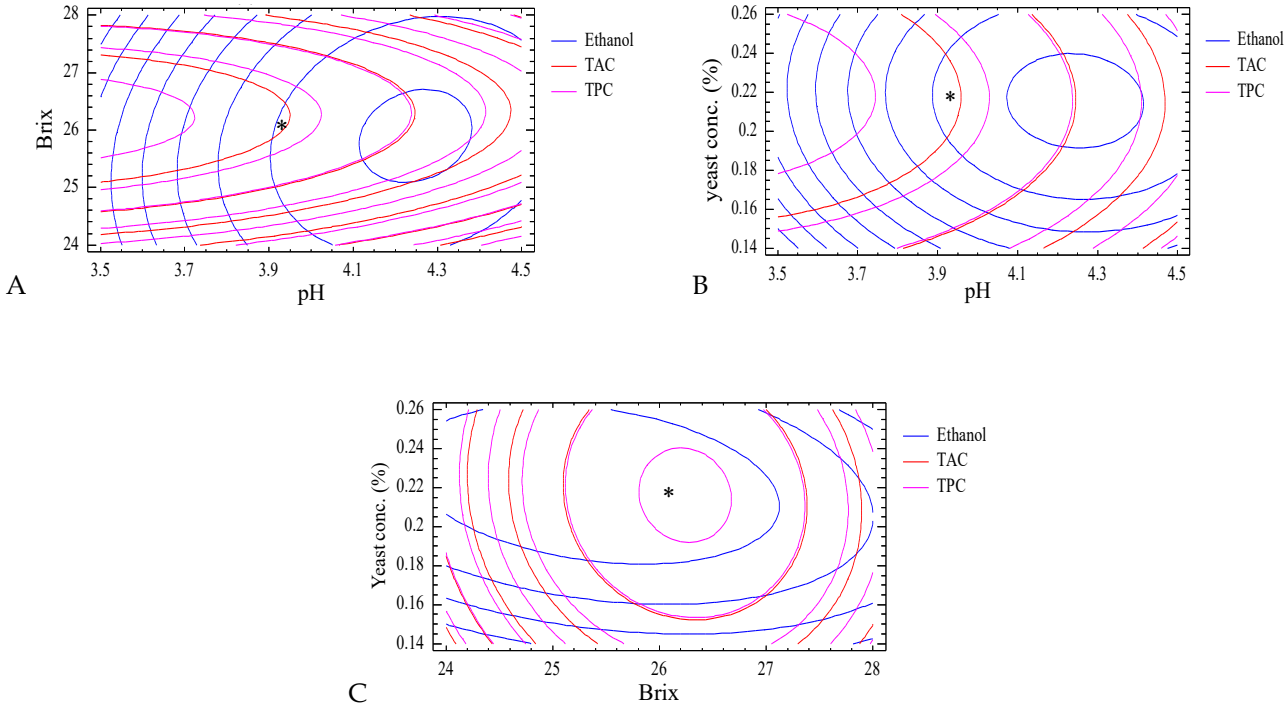


Figure 6. Overlay plot for optimal area: (A) dry yeast concentration 0.2 g/L; (B) TSS content 26%; (C) pH 4.

Table 4. The criteria used to optimize with the predicted value and the empirical value of the responses*.

Constraints	Goal	Lower limit	Upper limit	Predicted values	Empirical values
pH	In range	3.5	4.5	3.93	-
TSS (°Brix)	In range	24	28	26.08	-
Dry yeast concentration (g/L)	In range	0.15	0.25	0.218	-
Ethanol (% v/v)	Maximize	8.22	13.93	12.97	13.2±0.5
TAC (mg/L)	Maximize	156.28	173.33	171.85	173.15±8.5
TPC (mgGAE/L)	Maximize	71.6	88.65	87.17	86.33±3.8

*Mean±SD.

the allowable limits. While TPC was found to be slightly below the predicted value with the calculated data approximately to 1%, it is likely that this content is susceptible to oxidation during alcohol preparation and fermentation.

4 CONCLUSION

Mulberry wine with apple addition showed higher concentrations of ethanol and bioactive compounds than fermentation with mulberry juice alone. RSM has been used successfully in optimizing fermentation conditions. RSM's Box–Behnken design has been shown to be effective in determining the optimal region in the test region. Optimization conditions of various input variables such as pH, TSS, and dry yeast concentration were found, which, upon validation, showed a high content of alcohol and bioactive compounds. The results obtained may contribute to enhance wine development from locally abundant fruit ingredients, containing high concentrations of phytochemical compounds with attractive organoleptic properties.

REFERENCES

Attri, B. L. (2009). Effect of initial sugar concentration on phisyco-chemical characteristics and sensory quality of cashew apple wine. *Natural Product Radiance*, *8*(4), 374-379.

Batt, C. A., & Tortorello, M. L. (2014). *Encyclopedia of Food Microbiology*. Elsevier.

Belloch, C., Orlic, S., Barrio, E., & Querol, A. (2008). Fermentative stress adaptation of hybrids within the Saccharomyces sensu stricto complex. *International Journal of Food Microbiology*, *122*(1-2), 188-195. https://doi.org/10.1016/j.ijfoodmicro.2007.11.083

Chan, E. W. C., Phui-Yan, L. Y. E., & Siu-Kuin, W. O. N. G. (2016). Phytochemistry, pharmacology, and clinical trials of Morus alba. *Chinese Journal of Natural Medicines*, *14*(1), 17-30. https://doi.org/10.3724/sp.j.1009.2016.00017

D'Amato, D., Corbo, M. R., Nobile, M. A. D., & Sinigaglia, M. (2006). Effects of temperature, ammonium and glucose concentrations on yeast growth in a model wine system. *International Journal of Food Science & Technology*, *41*(10), 1152-1157. https://doi.org/10.1111/j.1365-2621.2005.01128.x

Eglinton, J. M., McWilliam, S. J., Fogarty, M. W., Francis, I. L., Kwiatkowski, M. J., Hoj, P. B., & Henschke, P. A. (2000). The effect of Saccharomyces bayanus-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Australian Journal of Grape and Wine Research*, *6*(3), 190-196. https://doi.org/10.1111/j.1755-0238.2000.tb00178.x

Fleet, G. H. & Heard, U. (1993). The microbiology of alcoholic beverages. In B. B. B. Wood (ed.). *Microbiology of Fermented Foods*. Blackie Academic and Professional.

Gamero, A., Belloch, C., Ibanez, C., & Querol, A. (2014). Molecular analysis of the genes involved in aroma synthesis in the species S. cerevisiae, S. kudriavzevii and S. bayanus var. uvarum in winemaking conditions. *PLoS One*, *9*(5), e97626. https://doi.org/10.1371/journal.pone.0097626

Gençdağ, E., Özdemir, E. E., Demirci, K., Görgüç, A., & Yılmaz, F. M. (2022). Copigmentation and stabilization of anthocyanins using organic molecules and encapsulation techniques. *Current Plant Biology*, *29*, 100238. https://doi.org/10.1016/j.cpb.2022.100238

González, S. S., Gallo, L., Climent, M. D., Barrio, E., & Querol, A. (2007). Enological characterization of natural hybrids from *Saccharomyces cerevisiae* and S. kudriavzevii. *International Journal of Food Microbiology*, *116*(1), 11-18. https://doi.org/10.1016/j.ijfoodmicro.2006.10.047

Hoff, J. W. (2012). *Molecular typing of wine yeasts: Evaluation of typing techniques and establishment of a database* (Doctoral dissertation). Stellenbosch University.

Hossain, M. A., AL-Raqmi, K. A. S., Al-Mijizy, Z. H., Weli, A. M., & Al-Riyami, Q. (2013). Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown Thymus vulgaris. *Asian Pacific Journal of Tropical Biomedicine*, *3*(9), 705-710. https://doi.org/10.1016/s2221-1691(13)60142-2

Hu, J., Vinothkanna, A., Wu, M., Ekumah, J. N., Akpabli-Tsigbe, N. D. K., & Ma, Y. (2021). Tracking the dynamic changes of a flavor, phenolic profile, and antioxidant properties of Lactiplantibacillus plantarum-and Saccharomyces cerevisiae-fermented mulberry wine. *Food Science & Nutrition*, *9*(11), 6294-6306. https://doi.org/10.1002/fsn3.2590

Januszek, M., Satora, P., Wajda, Ł., & Tarko, T. (2020). Saccharomyces bayanus enhances volatile profile of apple brandies. *Molecules*, *25*(14), 3127. https://doi.org/10.3390/molecules25143127

Khoo, H. E., Azlan, A., Tang, S. T., & Lim, S. M. (2017). Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food & Nutrition Research*, *61*(1), 1361779. https://doi.org/10.1080%2F16546628.2017.1361779

Kschonsek, J., Wolfram, T., Stöckl, A., & Böhm, V. (2018). Polyphenolic compounds analysis of old and new apple cultivars and contribution of polyphenolic profile to the in vitro antioxidant capacity. *Antioxidants*, *7*(1), 20. https://doi.org/10.3390%2Fantiox7010020

Libkind, D., Hittinger, C. T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P., & Sampaio, J. P. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proceedings of the National Academy of Sciences*, *108*(35), 14539-14544. https://doi.org/10.1073/pnas.1105430108

Liu, L. K., Chou, F. P., Chen, Y. C., Chyau, C. C., Ho, H. H., & Wang, C. J. (2009). Effects of mulberry (Morus alba L.) extracts on lipid homeostasis in vitro and in vivo. *Journal of Agricultural and Food Chemistry*, *57*(16), 7605-7611. https://doi.org/10.1021/jf9014697

Liu, X., Jia, B., Sun, X., Ai, J., Wang, L., Wang, C., Zhao, F., Zhan, J., & Huang, W. (2015). Effect of initial pH on growth characteristics and fermentation properties of Saccharomyces cerevisiae. *Journal of Food Science*, *80*(4), M800-M808. https://doi.org/10.1111/1750-3841.12813

Magalhães, F., Krogerus, K., Vidgren, V., Sandell, M., & Gibson, B. (2017). Improved cider fermentation performance and quality with newly generated Saccharomyces cerevisiae× Saccharomyces eubayanus hybrids. *Journal of Industrial Microbiology and Biotechnology*, *44*(8), 1203-1213. https://doi.org/10.1007/s10295-017-1947-7

Maicas, S., & Mateo, J. J. (2020). Sustainability of wine production. *Sustainability*, *12*(2), 559. https://doi.org/10.3390/su12020559

Muñoz-Bernal, E., Deery, M. J., Rodríguez, M. E., Cantoral, J. M., Howard, J., Feret, R., Natera, R., Lilley, K. S., & Fernández-Acero, F. J. (2016). Analysis of temperature-mediated changes in the wine yeast Saccharomyces bayanus var uvarum. An oenological study of how the protein content influences wine quality. *Proteomics*, *16*(4), 576-592. https://doi.org/10.1002/pmic.201500137

Peng, C. H., Liu, L. K., Chuang, C. M., Chyau, C. C., Huang, C. N., & Wang, C. J. (2011). Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. *Journal of Agricultural and Food Chemistry*, *59*(6), 2663-2671. https://doi.org/10.1021/jf1043508

Reddy, L. V. A., & Reddy, O. V. S. (2011). Effect of fermentation conditions on yeast growth and volatile composition of wine produced from mango (Mangifera indica L.) fruit juice. *Food and Bioproducts Processing*, *89*(4), 487-491. https://doi.org/10.1016/j.fbp.2010.11.007

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (Eds.). (2006). *Handbook of enology* (V. 1). John Wiley & Sons. https://doi.org/10.1002/0470010363

Rohela, G. K., Shukla, P., Kumar, R., & Chowdhury, S. R. (2020). Mulberry (Morus spp.): An ideal plant for sustainable development. *Trees, Forests and People*, *2*, 100011. https://doi.org/10.1016/j.tfp.2020.100011

Sui, X., Dong, X., & Zhou, W. (2014). Combined effect of pH and high temperature on the stability and antioxidant capacity of two anthocyanins in aqueous solution. *Food Chemistry*, *163*, 163-170. https://doi.org/10.1016/j.foodchem.2014.04.075

Tai, N. V., & Thanh, N. V. (2020). Identification and characterization of yeast isolates from maprang (Bouea macrophylla Griffith) growing in Vietnam. *Malaysian Applied Biology*, *49*(3), 23-30. https://doi.org/10.55230/mabjournal.v49i3.1537

Tao, Y., Wang, Y., Yang, J., Wang, Q., Jiang, N., Chu, D. T., ... & Zhou, J. (2017). Chemical composition and sensory profiles of mulberry wines as fermented with different Saccharomyces cerevisiae strains. *International Journal of Food Properties*, *20*(Suppl. 2), 2006-2021. https://doi.org/10.1080/10942912.2017.1361970

Thuy, N. M., Cuong, N. P., Tuyen N. T. M., Phuoc, N. H. (2011). Clarification and stabilization of pineapple wine. *Scientific Journal of Can Tho University*, 18b, 73-82.

Thuy, N. M., Nhu, P. H., Tai, N. V., & Minh, V. Q. (2022a). Extraction Optimization of Crocin from Gardenia (Gardenia jasminoides Ellis) Fruits Using Response Surface Methodology and Quality Evaluation of Foam-Mat Dried Powder. *Horticulturae*, *8*(12), 1199. https://doi.org/10.3390/horticulturae8121199

Thuy, N. M., Tien, V. Q., Tuyen, N. N., Giau, T. N., Minh, V. Q., & Tai, N. V. (2022b). Optimization of Mulberry Extract Foam-Mat Drying Process Parameters. *Molecules*, *27*(23), 8570. https://doi.org/10.3390/molecules27238570

Tosi, E., Azzolini, M., Guzzo, F., & Zapparoli, G. (2009). Evidence of different fermentation behaviours of two indigenous strains of Saccharomyces cerevisiae and Saccharomyces uvarum isolated from Amarone wine. *Journal of Applied Microbiology*, *107*(1), 210-218. https://doi.org/10.1111/j.1365-2672.2009.04196.x

Van Tai, N., Linh, M. N., & Thuy, N. M. (2021). Optimization of extraction conditions of phytochemical compounds in "Xiem" banana peel powder using response surface methodology. *Journal of Applied Biology and Biotechnology*, *9*(6), 56-62. https://doi.org/10.7324/JABB.2021.9607

Wang, D., Xu, Y., Hu, J., & Zhao, G. (2004). Fermentation kinetics of different sugars by apple wine yeast Saccharomyces cerevisiae. *Journal of the Institute of Brewing*, *110*(4), 340-346. https://doi.org/10.1002/j.2050-0416.2004.tb00630.x

Wrolstad, R. E., Skrede, G., Lea, P. E. R., & Enersen, G. (1990). Influence of sugar on anthocyanin pigment stability in frozen strawberries. *Journal of Food Science*, *55*(4), 1064-1065. https://doi.org/10.1111/j.1365-2621.1990.tb01598.x

Wu, C. H., Lin, M. C., Wang, H. C., Yang, M. Y., Jou, M. J., & Wang, C. J. (2011). Rutin inhibits oleic acid induced lipid accumulation via reducing lipogenesis and oxidative stress in hepatocarcinoma cells. *Journal of Food Science*, *76*(2), T65-T72. https://doi.org/10.1111/j.1750-3841.2010.02033.x

Yalcin, S. K., & Ozbas, Z. Y. (2008). Effects of pH and temperature on growth and glycerol production kinetics of two indigenous wine strains of Saccharomyces cerevisiae from Turkey. *Brazilian Journal of Microbiology*, *39*(2), 325-332. https://doi.org/10.1590%2FS1517-838220080002000024

You, Y. L., Li, N., Han, X., Guo, J. L., Zhao, Y., Liu, G. J., Huang, W. D., & Zhan, J. C. (2018). Influence of different sterilization treatments on the color and anthocyanin contents of mulberry juice during refrigerated storage. *Innovative Food Science & Emerging Technologies*, 48, 1-10. https://doi.org/10.1016/j.ifset.2018.05.007

Yuan, Q. & Zhao, L. (2017). The mulberry (*Morus alba L.*) fruit-A review of characteristic components and health benefits. *Journal of Agricultural and Food Chemistry*, *65*(48), 10383-10394. https://doi.org/10.1021/acs.jafc.7b03614