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Bioactive compounds of mulberry fruit and assessment of the effect of *Saccharomyces cerevisiae* strains on the quality of mulberry wine products

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Abstract

In the winemaking process, yeast strain plays a crucial role in the quality of the wine products. Therefore, this study aimed to screen the phytochemical compounds from mulberry fruits and assess the effect of three commercial yeast strains (*Saccharomyces cerevisiae* var. *burgundy*, *kyokai* and *montrachet*) on the quality of mulberry wines. The results showed that mulberry juice was a rich source of phytochemicals compounds (tannins, flavonoids, anthraquinones, steroids, terpenoids and alkaloids). Yeast strains had a significant effect on oenological parameters, total anthocyanins, phenolics and flavonoid contents and antioxidant activities (DPPH and FRAP assay) of mulberry wines ($p \leq 0.05$). The total soluble solids contents continually reduced while the percentage of alcohol contents consistently increased after 24 days of fermentation. The total soluble solids, pH values and titratable acidity of three mulberry wines were in the range of 5.80±0.06 – 6.00±0.06°Brix, 3.23±0.01 – 3.35±0.01 and 1.06±0.01 – 1.12±0.040% CAE, respectively. Mulberry wine fermented by *S. cerevisiae* var. *burgundy* (MWB) had the highest alcohol content (7.80±0.17% (v/v)) and acceptance scores (4.27±0.45 points). Moreover, MWB had the highest antioxidant activities due to the presence of the total anthocyanins, phenolics and flavonoid contents in mulberry wine. Cyanidin-3-O-glucoside (C3G) and cyanidin-3-O-rutinoside (C3R) were two major anthocyanins in mulberry wines. The C3G contents ranged from 0.14±0.01 – 0.38±0.01 mg/100 mL, while C3R ranged from 0.15±0.01 – 0.49±0.01 mg/100 mL. Therefore, this could be considered that *S. cerevisiae* var. *burgundy* had the potential yeast to produce quality mulberry wine as a healthy alternative product. Mulberry exhibited good antioxidant activity of anthocyanins and other phenolic compounds.

1. Introduction

Wine is a product made from fruit juice by the various yeast strains through the fermentation process. Yeast strains play a crucial function in determining the colour, flavour and sensory quality of wine products. *Saccharomyces cerevisiae* is the popular commercial yeast strain that is used for wine production. Because it makes the fermentation conditions safer and easier to control. The wine production through a mechanism of spontaneous fermentation is often unforeseeable but it can assist in reducing the undesirable nature of the wine product from bacterial contamination. Yeast performs the biotransformation of compounds in fruit juices (must) by converting sugars into ethanol and other substances during alcoholic fermentation (Maicas, 2020). Many fruits are currently used in winemaking such as grapes, dragon fruit, pomegranate, strawberry, blackberry and mulberry (Romero-Cascales *et al*., 2005; Mahmood *et al*., 2012; Ordoudi *et al*., 2014; Wang, Sun, Li *et al*., 2015; Caridi *et al*., 2017; Tao *et al*., 2017; Jiang *et al*., 2020; Klarić *et al*., 2020; Zhang *et al*., 2020).

Black mulberry (*Morus nigra* L.) is a member of the family *Moraceae*. It is grown wild or cultivated in various regions such as Asia, Europe, North and South America and Africa. In general, there are three types of mulberries, including white (*Morus alba* L.), black (*Morus nigra* L.) and red (*Morus rubra* L.). However, the colour of mulberry fruits cannot be used to identify the mulberry species (Thakur *et al*., 2016). Black mulberry fruit is an edible fruit which is currently consumed in the form of fresh fruit, juice, jam and wine products (Ramappa *et al*., 2020). Mulberry fruit is rich in many secondary metabolites, especially anthocyanins (Wang *et al*., 2022), phenolics and flavonoids that were

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recommended as having antioxidant (Wang *et al*., 2022), antimicrobial (Budiman and Aulifa, 2020), anti-inflammatory (Yu *et al*., 2021), anti-diabetic (Jiao *et al*., 2017) properties. According to a study by Tao *et al*. (2017), mulberry wine obtained by *S. cerevisiae* ySR 127 fermentations contained up to 9.12±0.69 and 88.34±4.99 mg/L of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, respectively. Cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside are classified as an anthocyanin (Zhang *et al*., 2020; Wang *et al*., 2022). Previous research has reported that some yeast not only promotes the degradation of anthocyanins and causes wine to lose its colour but also affects the bioactivities of wine (Caridi *et al*., 2017; Echeverrigaray *et al*., 2020). *Saccharomyces cerevisiae* used for alcoholic fermentation has a profound influence on colour and phenolic, aromatic compounds and amino acid profiles in wines (Echeverrigaray *et al*., 2020). It can grow in environments with high sugar and low pH. Yeast metabolism during fermentation greatly influences the final properties of wine appearance, taste and aroma. However, the influence of *S. cerevisiae* on the physico-chemical and sensory properties of mulberry wine is unclear. In the production of high-quality mulberry wines, it is important to examine the impact of yeast strains on various aspects of mulberry wine quality. Thus, this study aimed to study the phytochemical screenings and antioxidant properties of black mulberry juice before and after making mulberry wine by different commercial yeast strains and assessed the efficiency of inoculated starters on wine quality by analyzing physic-chemical, antioxidant and sensory properties of black mulberry wine products.

2. Materials and methods

2.1 Mulberry samples and juice extraction

Ripe fruits of Mulberry (*Morus nigra* L.) were collected from Sam Phran District, Nakhon Pathom Province, Thailand. The red–black and purple-black mulberry fruits (1–2 cm in size) were used in this study. Fresh mulberry fruits were stored in plastic bags and kept frozen for no more than 3 days. The collected fruits (without stems) were first cleaned carefully with tap water (2–3 times) and sterile distilled water (1 time) and allowed to dry, respectively. After that, the plant materials were milled with a blender and squeezed for the juice. The mulberry juice was filtered through a sterile filter cloth and a sterile filter paper (0.45 mm), respectively. Then, the juice was centrifuged at 8,000 × *g* for 10 min, stored at 4°C under dark conditions, and then analyzed. The final weight of the mulberry juice was weighed and calculated for the percentage yield. The fresh fruit extract was stored and kept frozen until used.

2.2 Phytochemical Screenings

The qualitative phytochemical compounds through chemical screening of mulberry (*Morus nigra* L.) fruit were performed by using the prescribed methods of Harborne (1998) with slight modifications. The present study was carried out to analyze the presence of saponins, flavonoids, tannins, alkaloids, terpenoids, steroids, anthraquinones and cardiac glycosides.

2.3 Total of anthocyanins content

The total anthocyanins content (TAC) of mulberry juice/wine was determined by the pH differential spectroscopic method (Lee *et al*., 2005). Briefly, 0.025 M KCl (pH 1.0) and 0.4 M CH_3COONa (pH 4.5) buffers were prepared. Five hundred microliter of mulberry juices or mulberry wines were homogenized and adjusted the volume with buffer to 1 mL or with the ratio of sample solution:buffer = 5:5. The mixture solutions were allowed to react in the dark at ambient temperature for 30 min. After that, the samples were read against the blank at the wavelengths of 530 and 700 nm in buffers at pH 1.0 and 4.5. TAC in the mulberry juice/wine was calculated and expressed as cyanidin 3-glucoside (C3G) equivalent according to the equation (Fan *et al*., 2008).

$$TAC\ (mg/100\ mL) = (A \times Mw \times DF \times 1000) / (e \times L)$$

Where A is $[(A_{530} - A_{700})_{pH\ 1.0} - (A_{530} - A_{700})_{pH\ 4.5}]$, Mw is molecular weight (449.2 g/mol), DF is dilution factor, E is molar absorptivity (26,900 l/mol cm) and L is the optical path length (usually 1 cm).

2.4 Total phenolic contents

The total phenolic contents (TPC) of mulberry juice/ wine were determined with Folin-Ciocalteu reagent (Mahmood *et al*., 2012). Approximately 200 mL of juice/ wine was added into different test tubes and thoroughly mixed with 1,500 mL of Folin-Ciocalteu reagent. After reacting for 5 min, 1,500 mL of 6% sodium carbonate (Na_2CO_3 ;w/v) was added and allowed to stand in darkness for 90 mins at room temperature. The absorbance was measured at 725 nm by using spectrophotometers. TPC was expressed as mg of gallic acid equivalent per gram of fresh weight (mg GAE/100 mL) using calibration curves of standard gallic acid (0.025–0.50 mg/mL).

2.5 Total flavonoid content

The total flavonoid content (TFC) of mulberry juice/wine was estimated by using the colorimetric or spectrophotometric assay with aluminium chloride (Stanković *et al*., 2015). Briefly, 200 µL of mulberry juice/wine containing 2.3 mL of 30% methanol was mixed with 100 µL f 0.3 M $AlCl_3$. The mixture solution was added with 330 µL of 1.0 M NaOH and

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kept in the dark for 5 mins. Finally, the absorbance was read at 506 nm using spectrophotometer against a blank sample. Total flavonoid content was determined by comparing it with a standard curve of rutin solution (0.25 – 5.0 mg/mL). TFC was expressed as mg of rutin equivalents (RE) per gram fresh weight (mg RE/100 mL).

2.6 Antioxidant activities

2.6.1 Determination of free radical scavenging by DPPH method

The free radical scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in mulberry juice/wine was evaluated which was based on the method proposed by Wang *et al*. (2015) with some modifications. Briefly, 1,800 mL of 0.1 mM DPPH in methanolic solution was mixed with 200 µL of sample solution under vigorous shaking. After 15 – 30 min of dark incubation, the reduction of the DPPH radical was measured at 517 nm against the control solution (mixing 1,800 mL of DPPH with 200 µL methanol). Percentage inhibition of DPPH scavenging activity was designed to evaluate the antioxidant activity of mulberry juice/wine. A plot of ascorbic acid concentration with DPPH radical scavenging activity was used as a standard curve. The DPPH values were expressed as milligrams of ascorbic acid equivalents (AAE) per gram (mg AAE/100 mL).

2.6.2 Determination of ferric reducing/antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was provided for measuring the reducing ability of the plant sample and modified from the method of Si *et al*. (2015). FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6). The freshly prepared FRAP reagent was warmed to 37°C in the water bath (Memmert, Germany) prior to use. An aliquot of 300 µL mulberry juice/wine was mixed with 2.7 mL of the FRAP reagent. The absorbance was measured at 596 nm using spectrophotometers after standing for 30 mins. The standard curve of ascorbic acid (0.01–0.20 mg/mL) was prepared using a similar procedure. The results were expressed as mg of ascorbic acid equivalents (AAE) per gram fresh weight (mg AAE/100 mL).

2.7 Yeast inoculum preparation

The three commercial yeast *Saccharomyces cerevisiae*; including *Burgundy* (BG), *Kyokai* (KK) and *Montrachet* (MC) were purchased from the Institute of Food Research and Product Development (IFRPD), Kasetsart University, Bangkok, Thailand. Yeasts were cultured and maintained in Yeast Peptone Dextrose (YPD) with a composition of 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose; pH 6.5±0.2) agar at 30°C for 48 hrs before inoculation. Pre-cultures of the three *Saccharomyces* yeasts were used to inoculate mulberry juice blends at a final concentration of 10^6 CFU/mL. The morphology and growth curve of yeast strains were evaluated before winemaking experiments.

2.8 Mulberry samples and winemaking

The mulberry wine fermentation process was performed according to the procedure of Wang, Sun, Li *et al*. (2015) with some modifications. The mulberry fruits in this study had 9°Brix and pH 3.47±0.11. After that, ripened fruits of mulberry were crushed to extract juice and centrifuged at 4,000 rpm for 20 mins. Final extracted juices in the ratio of 1:7 (juices: distilled water) were then adjusted to 22°Brix with sucrose (food grade) and pH 3.5 through the addition of critic acid. Afterwards, all juices were treated with potassium metabisulphite (KMS; 150 ppm) at 20°C for 24 hrs to inhibit the growth of unfavourable microorganisms. Finally, ameliorated juice (must) was inoculated the one of three yeast *Saccharomyces cerevisiae* (2%; BG, KK and MC strains; 10^6 CFU/mL) and fermented at 23±2°C for the period of 24 days under static conditions. Periodic sampling was performed at intervals every 4 days until the termination of the fermentation process to analyze the process parameters, biochemical contents and antioxidant activities after removing undesired solids by centrifugation. After 24 days of completed fermentation, the resulting wines were added 5% of bentonite powder to clarify the wines for 1 – 2 days. The clarified mulberry wines were transferred into sterile glass bottles and stored at 5°C in a refrigerator for further analysis.

2.9 Oenological parameters of mulberry juice and wine

The total soluble solids (TSS) content of the mulberry juice and wine was determined using a hand refractometer (Erma, Japan) in terms of °Brix. The pH of the samples was determined using a digital pH meter (Hanon, China). The alcohol content in wine was determined by using an ebulliometer (Laboratoires Dujardin-Salleron, France), with the results expressed as a percentage (% v/v). Titratable acidity was determined by titration with 0.1 N NaOH and expressed as percentage citric acid equivalents (% CAE). Reducing sugar was estimated by the Nelson-Somogyi assay (Somogyi, 1952). Turbidity was measured by nephelometric method and H V/C color determination with Munsell's book of color.

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2.10 Determination of anthocyanins by high-performance liquid chromatography analysis

Anthocyanin derivatives were determined following the method of Capanoglu *et al.* (2008) with slight modifications. High-performance liquid chromatography (HPLC) was performed on a Waters RP C18 column (250 × 4.6 mm, 5 µm C18, Waters Assoc., Milford, USA) using a Waters 2696 separation module equipped with a 996 photodiode array detector. The sample solutions were filtered through a 0.45 mm membrane filter before analysis. The mobile phase consisted of solvent A, Milli-Q water with 0.1% (v/v) TFA and solvent B, acetonitrile with 0.1% (v/v) TFA. A linear gradient was used as follows: at 0 min, 95% solvent A and 5% solvent B; at 45 mins, 65% solvent A and 35% solvent B; at 47 mins, 25% solvent A and 75% solvent B; and at 54 mins returning to initial conditions. The flow rate was 1.0 mL/mins. Detection was done at 520 nm. Identification was based on the retention times and characteristic UV spectra.

2.11 Sensory analysis

The quantitative descriptive analysis (QDA) technique was designed to evaluate the sensory properties of mulberry wines. A total of 30 trained panelists were served 20 mL of *mulberry* wines (10–12° C) at room temperature (22±3°C) to analyze rate of the visual appearance (clarity and color), aroma, taste and aftertaste and overall acceptability of the wine by 5-point hedonic scales ranging from dislike extremely (1) to like extremely (5). Finally, the average scores of each descriptor were calculated.

2.12 Statistical analysis

The results were carried out in triplicates and reported as means±deviations (SD). Statistical analysis for multiple comparisons was evaluated by one-way analysis of variance (ANOVA) to compare the means of different parameters of mulberry wines. The paired T-test at $p \leq 0.05$ of statistical significance was used to separate the mean differences.

3. Results and discussion

3.1 Preliminary phytochemical analysis

After centrifugation, the yield of mulberry juice was 25.5±0.06%. Phytochemicals in plants are the key to the value of each plant. These substances displayed different biological effects such as antioxidant and antimicrobial activities. The mulberry fruit juice was tested qualitatively to find out the presence of various bioactive substances using standard methods and phytochemical characteristics were summarized in Table 1. This study indicated that tannins, flavonoids, anthraquinones, steroids, terpenoids and alkaloids were presented in mulberry fruit juice, but none contained saponins and cardiac glycosides. Previously, Malik *et al*. (2012) found cardiac glycosides, saponins, alkaloids, phenolic compounds and flavonoids in *M. nigra* L. fruit. *Morus alba* L. fruit phytochemicals have led to reports of their richer phenolic- and volatile-compound content, as well as better antioxidant capacity than other berry species like blueberry, strawberry, blackberry and raspberry (Chen *et al*., 2021).

Table 1. Preliminary phytochemical analysis of Mulberry fruit juice.

Phytochemical Compounds		Distilled water
Saponins		–
Tannins		+
Flavonoids	Shinoda Test	+
	10% Lead (IV) acetate	++
Anthraquinones		+++
Steroids	Libermann Test	+
	Keller-Kiliani Test	++
Terpenoids		+++
Cardiac Glycosides	Keddy reagent	–
	Keller-kiliani Test + 10% $FeCl_3$	–
Alkaloids	28% NH_4OH	+
	Wagner' reagent	–
	Dragendoff's reagent	+

–: absent, +: trace, ++: moderately present, +++: highly present.

3.2 Basic wine compositional parameters

Generally, alcoholic fermentation significantly affects the phenolic composition, colour property and antioxidant capacity of mulberry juice. Mulberry juices were separately fermented by *S. cerevisiae* var. *burgundy*, *kyokai* and *montrachet* for 24 days. The basic oenological parameters of mulberry juice and mulberry wines are summarized in Table 2. The results showed that the yeast strains significantly affected various parameters of mulberry wines. The changes in pH and acidity in the mulberry wines during fermentation at 30° C depended on *S. cerevisiae* strains. The pH value of mulberry wine by *S. cerevisiae* var. *montrachet* (3.35±0.01) was slightly higher than mulberry wine by *S. cerevisiae* var. *kyokai* (3.25±0.01) and *burgundy* (3.23±0.01). The total titratable acidity (TTA) values ranged from 1.06±0.01–1.12±0.040% CAE. Mulberry wine by *S. cerevisiae* var. *burgundy* contained the highest TTA and the lowest TTA was recorded in that by *S. cerevisiae* var. *kyokai*.

Moreover, total reducing sugar contents in fermented wine *S. cerevisiae* strains rapidly increased at 4 days (773.864±3.090 – 811.440±2.268 mg/L), reached its maximum at 8 days (812.046±1.714 –

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Table 2. Physicochemical properties of mulberry must and mulberry wine during fermentation.

Parameters	Mulberry must (Day 0)	Mulberry wine (day 24)		
		S. cerevisiae var.	*S. cerevisiae* var.	*S. cerevisiae* var.
Oenological parameters				
Total soluble solids (°Brix)	22.00±0.02[a]	6.00±0.06[b]	6.00±0.06[b]	5.80±0.06[c]
Alcohol (% v/v)	ND	7.80±0.17[a]	7.30±0.14[b]	7.10±0.11[b]
pH	4.50±0.01[a]	3.23±0.01[c]	3.25±0.01[c]	3.35±0.01[b]
Titratable acidity (% CAE)	0.830±0.02[b]	1.12±0.040[a]	1.09±0.02[a]	1.06±0.01[a]
Total reducing sugar (mg/L)	650.83 ± 2.97[a]	190.23 ± 2.34[b]	179.32 ± 0.86[c]	177.50±2.27[d]
Phenolic family				
Total anthocyanin content (mg C3G/100 mL)	96.44±0.21[a]	43.65±0.10[b]	36.59±0.16[d]	38.62±0.22[c]
Total phenolic content (mg GAE/100 mL)	322.18±0.35[a]	157.67±0.38[b]	141.15±0.32[d]	153.69±0.34[c]
Total flavonoid content (mg RE/100 mL)	55.17±0.29[a]	23.65±0.03[b]	19.35±0.10[d]	21.30±0.05[c]
Antioxidant activities				
DPPH scavenging activity (mg AAE/100 mL)	340.83±0.21[a]	209.35±0.23[b]	187.66±0.47[d]	196.47±0.28[c]
FRAP assay (mg AAE/100 mL)	134.45±0.11[a]	85.45±0.19[b]	84.48±0.0.27[b]	84.61±0.16[b]

Values are presented as mean±SD. Values with different superscripts within the same row are statistically significantly different ($p \leq 0.05$). C3G: Cyanidin-3-O-glucoside, ND: Not Detectable

848.410±2.268 mg/L) and then continuously decreased until the end of fermentation on the 24th day (177.501±2.268 – 190.228±2.339 mg/L). In fermented mulberry juice with a large amount of reducing sugar, *S. cerevisiae* strains are also able to convert large amounts of reducing sugar into alcohol. This is consistent with the study by Gaharwar *et al*. (2018). The pH values must be reduced throughout the fermentation period in the winemaking process because yeast breaks down sugars into organic acids, and then the organic acids are converted to alcohol (Maicas, 2020). The alcohol content of mulberry wines also varied according to the yeast strains. The wines obtained from the inoculation of *S. cerevisiae* var. *burgundy* contained a maximum alcohol content of 7.80±0.17% followed by that of *S. cerevisiae* var. *kyokai* (7.30±0.14%) and *burgundy* (7.10±0.11%), respectively ($p \leq 0.05$). Moreover, the total soluble solids (°Brix) in mulberry must be continuously decreased throughout the 24-day fermentation period with different *S. cerevisiae* strains (Figure 1). At the end of the fermentation process, the total soluble solids of mulberry wines decreased below 6.00°Brix from 22.00°Brix, indicating the high fermentative capability of yeast strains under investigation. This is consistent with research by Saelim *et al*. (2018) which found that the total soluble solids of wine throughout the experiment. Besides, alcoholic fermentation of mulberry juice resulted in the increase of total acidity value and the decrease of pH value at the same time. Alcoholic fermentation had a profound influence on the organic acid profiles in mulberry as well as the formation of new organic acids (Ordoudi *et al*., 2014).

3.3 Total anthocyanins, phenolics and flavonoids content of mulberry wine

The total anthocyanins content (TAC) of mulberry juice (must) by the pH differential spectroscopic method

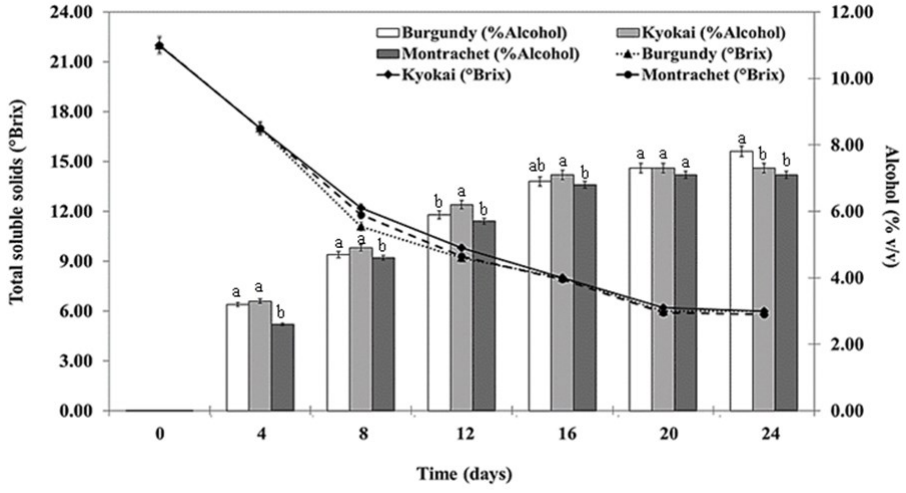

Figure 1. Changes in total soluble solids (°Brix) and alcohol (% v/v) of mulberry wines during lab scale fermentation. Bars with different notations are statistically significantly different ($p \leq 0.05$).

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at 0 and 24th days of fermentation were examined and presented in Table 2. Mulberry wine fermented by *S. cerevisiae* var. *burgundy* (MSB) showed the highest total anthocyanin contents of 43.65±0.10 mg C3G/100 mL (Figure 2) and followed by *S. cerevisiae* var. *montrachet* (38.62±0.22 mg C3G/100 mL) and *kyokai* (36.59±0.16 mg C3G/100 mL), respectively. There was a declining trend in TAC of mulberry wines which were significantly reduced with approximately 54.74%, 59.95% and 62.06% for wine fermentation by *S. cerevisiae* var. *burgundy*, *montrachet* and *kyokai* at the end of the 24th day experiment period, respectively ($p \leq 0.05$). During alcoholic fermentation, yeasts can interact with anthocyanins to eliminate polar compounds, resulting in the reduction of anthocyanin content (Medina *et al*., 2005; Tofalo *et al*., 2021). Meanwhile, the enzymes (β-glycosidase or anthocyanidase) produced by yeast can also negatively affect anthocyanin stability (Romero-Cascales *et al*., 2005; Tofalo *et al*., 2021). Thus, the low total anthocyanin content in samples fermented with *Saccharomyces cerevisiae,* especially *S. cerevisiae* var. *kyokai* may be due to the strong interaction between this yeast strain and anthocyanins and the high sensitivity of anthocyanins to enzymes.

The total phenolic contents (TPC) of mulberry juice (must) and mulberry wines by using the diluted Folin-Ciocalteu reagent are shown in Table 2. Generally, these compounds showed differences in their total phenolic contents depending on solvent polarities. The TPC is expressed as mg of GAE/100 mL of fresh weight. The result clearly showed that the mulberry juice had the TPC of 322.18±0.35 mg GAE/mL. There is a significant difference in the TPC in three mulberry wines at 24th days of fermentation, which can be ranked as *S. cerevisiae* var. *burgundy* (157.67±0.38 mg GAE/100 mL; 51.06% of reduction) > S. *cerevisiae* var. *montrachet* (153.69±0.34 mg GAE/100 mL; 52.30% of reduction) > *S. cerevisiae* var. *kyokai* (141.15±0.32 mg GAE/100 mL; 56.19% of reduction) (Figure 1). The total phenolic contents (141.15±0.32–157.67±0.38 mg GAE/100 mL) in three mulberry wines were higher than rose wine (an average of 914 mg GAE/L) (Li *et al*., 2009), gingko wine (456 mg GAE/L) (Wang, Xie, Zhuang *et al*., 2015b) and blueberry wine (an average of 360.27 mg GAE/L) (Cabanillas-Bojorquez *et al*., 2021), although they were still less than those of red wines (an average of 2,141–4,274 mg GAE/L) (Bajčan *et al*., 2015), pineapple wine (an average of 365.80 mg GAE/L), (Cendrowski *et al*., 2021), rose wine (an average of 4,730–9,580 GAE mg/L) (Cendrowski *et al*., 2021) and blackberry wines (2581 GAE mg/L; OBW 11) (Klarić *et al*., 2020).

The total flavonoid contents (TFC) in fruit juice of black mulberry (*Morus nigra*) were determined by using the spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of mg rutin equivalent/100 mL of fresh weight (Table 2). Total flavonoid content of mulberry juice was 55.17±0.29 mg RE/100 mL which had higher than the TFC of *Morus nigra* fruit from Serbia (1.508±0.015 mg RE/g) (Radojković *et al*., 2012). The TFC in three mulberry wines, which can be ranked as *S. cerevisiae* var. *burgundy* > *montrachet* > *kyokai* which ranged from 19.35±0.10 to 23.65±0.03 mg/100 mL after the end of the fermentation process. There were the downward trends about TFC which was significantly declined with approximately 57.13%, 61.39% and 64.92% by *S. cerevisiae* var. *burgundy*, *montrachet* and *kyokai* fermentation, respectively ($p \leq 0.05$). This is consistent with a previous study in which flavonoids concentration (TFC) significantly decreased during the fermentation period (Hu *et al*., 2021).

3.4 Antioxidant activities of mulberry wine

The antioxidant capacity of food and beverage products is dictated by the different mechanisms of action of their antioxidant constituents; therefore, this capacity could be evaluated by a variety of methods pertaining to the different mechanisms (Pérez-Jiménez *et al*., 2008). Consequently, DPPH and FRAP assays were used to evaluate the antioxidant activity of mulberry fruits and their products (wines) in this study. The antioxidant activities (FRAP and DPPH assays) of mulberry juice are reported in Table 2. In the DPPH assay, it averaged 340.83±0.21 AAE/100 mL. The antioxidant activity averaged 134.45±0.11 mg AAE/100 mL in the FRAP assay. Mulberry wine fermented by *S. cerevisiae* var. *burgundy* had the highest antioxidant activity with DPPH assay of 209.35±0.23 mg AAE/100 mL and FRAP assay of 85.45±0.19 mg AAE/100 mL (Figure 2). The high total phenolics content in mulberry wine fermented by *S. cerevisiae* var. *burgundy* contributed to its increased antioxidant capacity in comparison to other wines (Table 2). However, free anthocyanin is an important source of the red colour in young wines which is consistent with this study. The red colour in young wines are primarily derived from free anthocyanins. It can combine with small molecules such as pyruvic acid and acetaldehyde, which complicate the composition of anthocyanin (Ruta and Farcasanu, 2019). Moreover, the deep red/purple colour in mulberry juice (red purple colour; 5R, 2/4) is caused by anthocyanin compounds, when it was fermented by yeast to form mulberry wine (bright red purple colour; 5R, 2/8), the colour is lighter than its original anthocyanins in the

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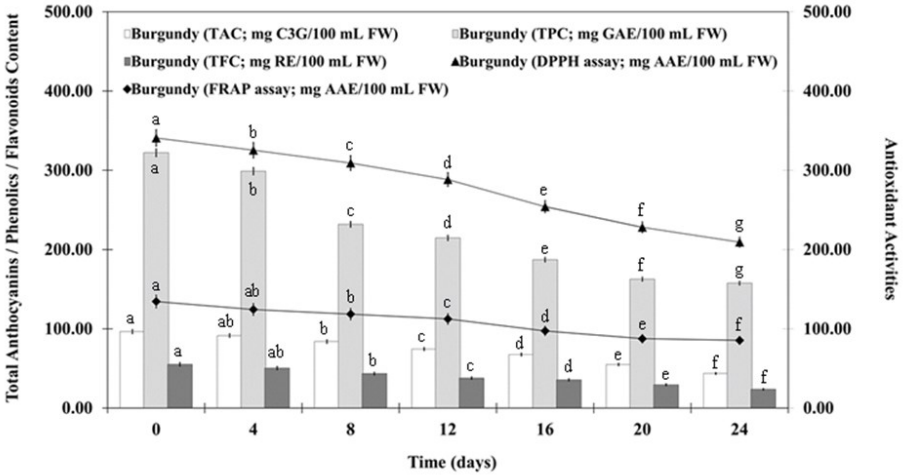

Figure 2. Changes in total anthocyanin, phenolic and flavonoid contents and antioxidant activities of mulberry wines during lab scale fermentation. Bars and lines with different notations are statistically significantly different ($p \leq 0.05$).

present study. There had been previous report that yeast cells of *S. cerevisiae* and anthocyanins had a competitive or interacting phenomenon, and yeast cells in wine could significantly decline the anthocyanin content, resulting in a loss of colour (Echeverrigaray *et al*., 2020).

3.5 Anthocyanin content of mulberry wine by HPLC-PAD

Anthocyanin plays an important role in the food industry and human health. The results of HPLC showed that there were mainly two types of monomer anthocyanins in mulberry juices and wines in this study (Figure 3). The peak time was consistent with the Cyanidin-3-O-rutinoside chloride (C3R) and Cyanidin-3-O-glucoside chloride (C3G) standard. Results agree with those obtained by the study of Chen *et al*. (2020) who reported that mulberry anthocyanins are mainly C3R and C3G. The content of monomeric anthocyanins in mulberry wine was significantly affected by different yeast strains ($p \leq 0.05$). The contents of C3R (0.49 mg/100 mL) and C3G (0.38 mg/100 mL) in mulberry wine by *S. cerevisiae* var. *burgundy* were significantly higher than in mulberry wines by *S. cerevisiae* var. *kyokai* and *Montrachet* ($p < 0.05$) . The lowest content of monomeric anthocyanins was detected in mulberry wine by *S. cerevisiae* var. *montrachet* which had 0.14±0.01 mg/100 mL (C3G content) and 0.15±0.01 mg/100 mL (C3R content). The C3R and C3G contents of *S. cerevisiae* var. *kyokai* were 0.15±0.01 mg/100 mL and 0.16±0.01 mg/100 mL, respectively. This is consistent with the study of Tao *et al*. (2017) who reported that the contents of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside in mulberry wines fermented with *S. cerevisiae* ySR 127 were higher than that in mulberry wines fermented with *S. cerevisiae* Y1 and YJM 681. The total anthocyanin content (expressed as C3G and C3R) ranged from 6.29±0.41 to 9.12±0.69 mg/L and

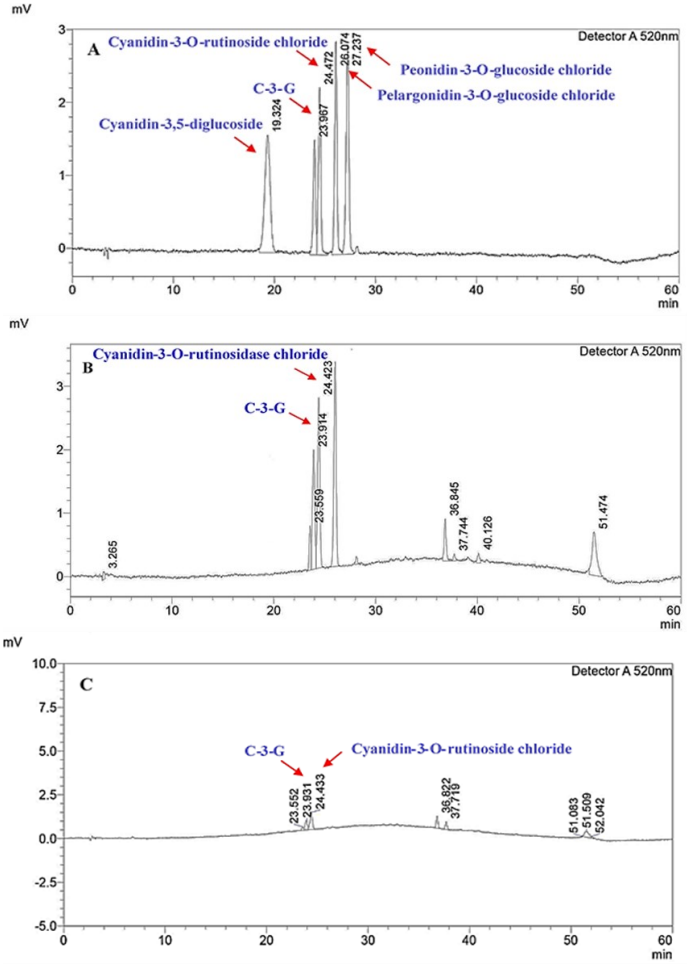

Figure 3. Changes in anthocyanin content in mulberry wines before and after fermentation by HPLC chromatograms with different *S. cerevisiae* var. *burgundy*: (A) anthocyanin derivatives standard, (B) mulberry must and (C) mulberry wine.

84.53±6.40 to 88.34±4.99 mg/L in mulberry wine. The type of yeast greatly affects the composition of the wine during fermentation. The structure and composition of the yeast cell walls can affect the monomeric anthocyanin content, and thereby affect the appearance quality of red wine. Differences in anthocyanin absorption capacity differ between individual species of *S. cerevisiae* (Echeverrigaray *et*

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al., 2020). Moreover, the C3R and C3G contents of three mulberry wines decreased from mulberry must (6.72±0.01 mg/100 mL of C3R and 5.46±0.01 mg/100 mL of C3G) in this study. Moreover, it was also found that C3R and C3G content were lost after 6 months and 9 months of mulberry wine (fermented with three strains of *S. cerevisiae*) storage under room temperature and at 5°C, respectively (data not shown). Mulberry wine production is accompanied by a reduction or disappearance of some anthocyanins. These changes may be due to the variety in anthocyanin derivatives at different immersion leaching rates which is part of the degradation or polymerization of anthocyanins (Morata *et al*., 2003) as well as anthocyanin adsorption capacity of yeast cell walls (Echeverrigaray *et al*., 2020).

Overall, mulberry wine fermented with *S. cerevisiae* var. *burgundy* was the suitable inoculum for mulberry wine fermentation. Mulberry wine made by this strain had the highest TAC, TPC, TFC and alcohol percentage. The contents of phenolic families, colour parameters and antioxidant capacity of mulberry wine were dependent on the yeast strains used for alcoholic fermentation. Moreover, it can be seen that the percentage alcohol of mulberry wines fermented with *S. cerevisiae* var. *burgundy* tends to increase, which varies with the total soluble solids content. While the mulberry wine is fermented with *S. cerevisiae* var. *kyokai* and *montrachet* showed stable initial alcohol percentages between the 21st–24th days of the fermentation process (Figure 1). Regarding antioxidant activities of the three mulberry wines, the mulberry fermented with *S. cerevisiae* var. *burgundy* showed significantly higher antioxidant activities (DPPH assay) than the mulberry fermented with other *Saccharomyces* strains ($p \leq 0.05$), while the reducing powers (FRAP assay) of mulberry wines fermented with *S. cerevisiae* var. *burgundy* were close to two yeasts (*S. cerevisiae* var. *kyokai* and *montrachet*).

3.6 Sensory evaluation

The sensory profiles of three mulberry wines were evaluated in terms of colour and clarity, aroma, taste and aftertaste and overall acceptability by 5-point hedonic scales. The sensory quality test in three mulberry wines was determined by their overall acceptance score, which can be ranked as *S. cerevisiae* var. *burgundy* (4.27±0.45 points; like moderately) > *S. cerevisiae* var. *kyokai* (4.200±0.40 points; like moderately) > S. *cerevisiae* var. *montrachet* (4.00±0.64 points; like moderately). The mean scores on colour and clarity, aroma, taste and aftertaste of mulberry wine fermented with *S. cerevisiae* var. *burgundy* were 4.10±0.66, 4.03±0.67, 3.87±0.73, 3.80±0.96 and 4.27±0.45 points, respectively. However, the quality in terms of appearance (colour and clarity), aroma and taste for all the mulberry wine samples were regarded to be equal to each other.

4. Conclusion

Mulberry juice could be a good source of phytochemical compounds (tannins, flavonoids, anthraquinones, steroids, terpenoids and alkaloids) which are natural antioxidants. The different yeast strains of fermented mulberry wine have significant differences in % alcohol, total anthocyanins, phenolics and flavonoids contents and antioxidant activities (DPPH and FRAP assay). The following tested parameters were significantly different among yeast strains. Yeast strain choice is an important to use in the winemaking process. *S. cerevisiae* var. *burgundy* was the suitable inoculated starter for mulberry wine fermentation when compared with *S. cerevisiae* var. *kyokai* and *montrachet* in this study. *Saccharomyces cerevisiae* var. *burgundy* produced the highest %alcohol content in mulberry wines. An increase in the alcohol content of mulberry wine with *S. cerevisiae* corresponded to a decrease in the total soluble solids content. The higher alcohol content had also a direct correspondence with the length of time it took to ferment. Moreover, total anthocyanins, phenolics, flavonoids contents and antioxidant capacity in fermented wine with *S. cerevisiae* var. *burgundy* were significantly higher than in fermented wine by *S. cerevisiae* var. *kyokai* and *montrachet*. Two major anthocyanin derivatives (C3G and C3R) were identified in mulberry wines that both compounds tend to decrease after 24 days of fermentation by HPLC. The results showed that mulberry juice could be a good source of these natural antioxidants. *S. cerevisiae* var. *burgundy* was suitable as a starter culture that had the potential to be utilized for obtaining quality mulberry wines. Mulberry wine contains many important compounds that are beneficial to the health of the body, especially antioxidant substances. Mulberry wine is therefore an attractive alternative beverage product for health-conscious consumers.

Conflict of interest

The authors declare no conflict of interest.

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