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STUDY ON WINES PRODUCED FROM MONOSTROMA NITIDUM HYDROLYSATE SOLUTION

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STUDY ON WINES PRODUCED FROM *MONOSTROMA NITIDUM* HYDROLYSATE SOLUTION

Shao-Chi Wu*, Fu-Jin Wang**, Jiun-Ru Chen***, and Chorng-Liang Pan***

Key words: agarase, cellulase, *Monostroma nitidum*, wine.

ABSTRACT

Two yeast groups, S5: *Saccharomyces* (*S*.) *cerevisiae* BCRC21686 and *S. cerevisiae* BCRC21962, and S6: *S. cerevisiae* BCRC21824 and *S. cerevisiae* BCRC21962, were used to ferment *Monostroma* (*M*.) *nitidum* hydrolysate (MNH) solution under various conditions, such as (1) *M. nitidum* powder; (2) carbon source; (3) nitrogen source; and (4) yeast group inoculation concentrations, respectively, at 25°C in a 10-day *M. nitidum* wine study. First, the 2.5% *M. nitidum* powder particles in the MNH solution elicited better alcohol content than the other concentrations did. Then, the 15% sucrose and 0.500% proline in the MNH solution produced good alcohol content in a carbon source and a nitrogen source, respectively. The S5 and S6 groups also showed improved alcohol content in the yeast group inoculation concentration of 4%. Therefore, we combined these conditions to out carry *M. nitidum* wine fermentation on days 0, 5, 7, 10, 14, 18, or 21. The *M. nitidum* wines with 10%, 15%, or 20% sucrose ferment showed good alcohol and compositional changes after 7, 10, or 14 days, respectively. During the 3 months of aging, the influence on the alcohol content, pH value, titratable acidity, reducing sugar content and residual sugar content caused by storage temperatures at 15°C and 25°C on the components of *M. nitidum* wines were not observed. The *M. nitidum* wines aged at 25°C showed lower Hunter L, a, and b values than did the *M. nitidum* wines aged at 15°C. Overall, sensory evaluations showed the best *M. nitidum* wines were fermented with 20% sucrose added to the *M. nitidum* wine substrate at 25°C and then aged at 15°C for 3 months. *M. nitidum* wine can be further studied in the field of *M. nitidum* wine manufacturing.

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I. INTRODUCTION

The green alga, *M. nitidum*, has thalli that are foliaceous, soft and gelatinous. It is yellowish green in color, about 2-4.5 cm tall, 18-33 μ thick, and is found in Hong Kong, Taiwan, the China Sea, Ryukyu, and Japan. The main component of *M. nitidum* is mucilage, which contains xylose (1.98%), galactose (19.25%), galacturonic acid (23.17%) and rhamnose (52.95%) [26].

Sulfated polysaccharides from *Monostromaceae* exhibit many biological activities; they are anticoagulant, antiviral, antiherpetic and antioxidant [48]. Maeda *et al*. [25] studied heparinoid-active sulfated polysaccharides from *M. nitridum* and found that they were 6-fold more antithrombin-active than the heparin standard. Harada and Maeda [17] analyzed the chemical structure and found it was rhamnan sulfate. The study by Lee *et al*. [23] of sulfated polysaccharides from *M. nitidum* showed potent anti-Herpes simplex virus type 1 (HSV-1) activity. An *M. nitidum* diet fed to 30 Sprague-Dawley rats demonstrated the potential of seaweed as a natural source of sulfated polysaccharide substances with potential for use in chemoprevention medicine [8]. A notable reducing effect of plasma cholesterol in rats was found in the basic fraction of water-extractives; it was able isolate arginine, glycine betaine and β-homonetaine [1]. Of the two betaines, only β-homobetaine was able to reduce plasma cholesterol [2]. The water-soluble mucilage of *M. nitidium* showed skin hydration effects which could be useful in the production of cosmetics [9]. Hot water polysaccharide extract and oligosaccharide-lysates prepared by two bacterial agarases hydrolyzed, which derived from *M. nitidium*, showed anti-oxidative properties in our previous study [43, 46]. Wu *et al*. [44] observed that lactic acid bacteria fermented hot water polysaccharide extract and oligosaccharide-lysates prepared by two bacterial agarases which were stepwise hydrolyzed and derived from *M. nitidium*.

During fermentation in alcoholic beverage production, volatile compounds are produced at various concentrations. These compounds play an important role in the flavour and sensory properties of the alcoholic beverage [37]. Higher alcohols can be classified as aliphatic [n-propanol, isobutanol, 2-methylbutanol (or active amyl alcohol), 3-methyl butanol (or isoamyl alcohol)] or aromatic (2-phenylethanol, tyrosol

and tryptophol). Aliphatic higher alcohols contribute to the 'alcoholic' or 'solvent' aroma of wine and produce a warm sensation in the mouth. The aromatic alcohol 2-phenylethanol has a sweet aroma, which makes the wine desirable, whereas the aromas of tyrosol and tryptophol are undesirable. Higher alcohols are synthesized by yeast during fermentation via the catabolic (Ehrlich) and anabolic pathways (amino acid metabolism) [42].

S. cerevisiae is widely used worldwide in many industries and it is one of the most extensively studied microorganisms [21]. The quality of the wine produced greatly depends on the yeast strain [32]. Conditions which promote yeast cell growth, such as high levels of nutrients (amino acids, oxygen, lipids, zinc), increased temperature and agitation, stimulate the production of higher alcohols. The synthesis of aromatic alcohols is especially sensitive to temperature changes. On the other hand, conditions which restrict yeast growth, such as lower temperature and higher (CO_2) pressure, can reduce the extent of higher alcohol production. The amino acid composition has a major effect on higher alcohol formation: growth medium supplemented with valine, isoleucine and leucine induced the formation of isobutanol, amyl alcohol and isoamyl alcohol, respectively [42].

Many researchers have studied alcoholic beverages employing yeast from different raw materials: sugar beets, wheat, maize, potatoes, sugar cane, and sweet sorghum are rich in single sugars or polymers which are easy to hydrolyze [33]. This work deals with wine prepared from *M. nitidium* and the compositional changes during fermentation and aging.

II. MATERIALS AND METHODS

1. Strains

1) Agarases-Producing Strains

Agarases-producing strains, *Aeromonas* (*A.*) *salmonicida* MAEF108 with agar-softening isolated from the seawater off the coast at Keelung in Taiwan [43-46].

2) Wine-Producing Strains

S. cerevisiae BCRC21686, BCRC21824, and BCRC21962, obtained from the Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan, were tested for alcohol fermentation using *M. nitidum*.

2. *Monostroma nitidum* Powder Particles

M. nitidum was purchased from a traditional market in Penghu, Taiwan. The proximate composition of ash, carbohydrate, crude fiber, crude lipid, crude protein, and moisture were prepared according to the methods described in AOAC [4]. The dried *M. nitidum* alga was crushed, and then screened by means of a standard screening sieve (Tokyo Garasu KiKai Co. Ltd, Tokyo, Japan), so the *M. nitidum* powder particles were smaller than 0.42 mm [43, 46].

3. Preparation of *M. nitidum* Hydrolysate Solution

1) Types of Digestive Enzyme

The commercial enzyme cellulase was purchased from Challenge Bioproducts Co. Ltd, Yunlin, Taiwan. The preparation of crude enzyme solution of MAEF108-agarases was prepared according to the method of previous studies [43, 46], and the assay of agarase activity was carried out in accordance with the same references.

2) M. nitidum Hydrolysate Solution

The hydrolysate solution of *M. nitidum* was obtained by a modified method described in previous studies [43, 46]. First, *M. nitidum* powder particles of 2.5% *M. nitidum* were suspended in 300 mL of deionized water and heated at 90°C for 10 min. The *M. nitidum* solution cooled to 40°C with the pH value adjusted to 5.5 by lactic acid (Panreac, Monteada i Reixac, Barcelona, Spain) is referred to as the MN (*M. nitidum*) solution. Then, 10 unit/mL MAEF108-agarases and 10 unit/mL cellulase were added to the MN solution which was placed at 40°C. Then, enzyme digestion was carried out for 6 h. After digestion, the MN solution containing 2.5% *M. nitidum* powder particles digested by 10 units/mL MAEF-108 agarases and 10 units/mL cellulase is referred to as MNH (*M. nitidum* hydrolysate) solution.

4. Fermentation Conditions of *M. nitidum* Wine

1) M. nitidum Wine-Producing Starter Groups

Our pervious study showed that the *M. nitidum* wine-producing starter groups that obtained the highest alcohol content: S5: BCRC21686 + BCRC21962 and S6: BCRC21824 + BCRC21962, were used as *M. nitidum* wine-producing starters in this study. The duration of the fermentation time was 10 days at 25°C, and the microbiological composition of the *M. nitidum* wine were determined as describe in exp. 6.

2) Carbon Source

Three hundred mL of MNH solution had 15% glucose, maltose or sucrose added in order to choose the best carbon source. The duration of the fermentation time and the microbiological composition of the *M. nitidum* wine were determined in the same way as in exp. 4-1.

3) Nitrogen Source

Three hundred mL of MNH solution containing 15% sucrose was added to 0.125%-1.000% NH_4Cl, $NH_4H_2PO_4$, or proline to choose the best nitrogen source. The duration of the fermentation time and the microbiological composition of the *M. nitidum* wine were determined in the same way as in exp. 4-1.

4) Inoculated Concentration of M. nitidum Wine-Producing Starter Groups

M. nitidum wine-producing starter groups were added in amounts of 2%, 4%, 6%, 8%, or 10% to choose the best in-

oculated concentration. The duration of the fermentation time and the microbiological composition of the *M. nitidum* wine were determined in the same way as in exp. 4-1.

5) Fermentation Time of M. nitidum Wine Producing Starter Groups

According to the results of Experiments 4-2, 4-3, and 4-4, the carbon source, nitrogen source, and inoculated concentration which exhibited the highest alcohol content were selected. The fermentation times of the *M. nitidum* wine-producing starter groups were 0, 5, 7, 10, 14, 18, or 21 days. The duration of the fermentation time and the microbiological and composition of the *M. nitidum* wine were determined in the same way as in exp. 4-1.

5. Aging of *M. nitidum* Wine

The aging of *M. nitidum* wine prepared from the MNH solution containing 2.5% *M. nitidum* powder particles, 0.5% proline, and 10%-20% sucrose was fermented by inoculation with 4% yeast groups S5: BCRC21686 + BCRC21962 and S6: BCRC21824 + BCRC21962 at 15 or 25°C, respectively. The duration of the aging was 3 months and the microbiological and chemical composition of the *M. nitidum* wine was determined in the same way as in exp. 4-1 every 2 weeks.

6. Microbiological and Chemical Analyses

1) Determination of Acidity

The pH levels of the *M. nitidum* wine samples were measured at room temperature (23 to 25°C) using a pH meter (MP220, Mettler Toledo, Schwerzenbach, Switzerland) (triplicatelly) after calibration with fresh pH 4.0 and 7.0 standard buffers (Panreac) [44, 45].

2) Determination of Titratable Acidity (TA)

TA was determined by the AOAC method and expressed as % tartaric acid [4].

3) Determination of Alcohol Content

Fifty milliliter *M. nitidum* wine samples were placed in 250 mL distilling flasks and then loaded into a Soxtec manual extraction unit (Model 2058, Foss Tecator, Sweden) for distillated alcohol. After the distillated alcohol reached 45 mL, 50 mL of deionized water was added and mixed well. Finally, an alcohol hydrometer measured the alcohol content expressed as % (v/v) [35].

4) Determination of Reducing Sugar Content

The dinitro salicylic acid method of Miller *et al.* [28] was used to estimate reducing sugar. One milliliter of the *M. nitidum* wine was reacted with an alkaline solution of 3,5-dinitrosalicylate reagent to give the brown-colored 3-amino-5-nitrosalicylic acid solution. Extinction was measured at 540 nm. The quantity of reducing sugar was extrapolated from a calibration curve prepared with D-glucose.

5) Determination of Total Sugar Content

Total sugar content was measured by the phenol-sulphuric-acid method using glucose as the standard [13].

6) Determination of Residual Sugar Content

The residual sugar content was determined following the method of Amerine and Ough [3].

7) Determination of S. cerevisiae Count

YM agar was used for the enumeration of *M. nitidum* alcohol-producing starters. One milliliter of each sample was diluted with 9 mL of sterilized 0.85% (w/v) NaCl (Panreac) solution and vortex thoroughly. Subsequent serial dilutions were prepared, and viable numbers were enumerated using the spread-plated technique onto the YM agar. After 48 h of incubation at 25°C, the colonies appearing on the plates were counted and the CFU/mL was calculated [15].

8) Determination of Hunter L, a, and b values

Samples of *M. nitidum* wine were centrifuged at 5,000 × g at 4°C for 10 min, and the supernatant was collected. Visual color was measured using a Hunter color meter (Model TC-10 Analyzer, Denshoku Co., Tokyo, Japan) and was expressed as L, a, or b (brightness, redness and yellowness, respectively) [7].

7. Sensory Evaluation for *M. nitidum* Wines

The sensory evaluation and aging of all *M. nitidum* wines were initially studied on the basis of a 9-point hedonic scale test (1 = dislike extremely; 3 = dislike; 5 = neither like nor dislike; 7 = like; 9 = like extremely), and used to evaluate sensory attributes for color, flavor, taste, or overall preference.

8. Statistical Analysis

All results from testing the acidity, TA, alcohol content, reducing sugar content, total sugar content, residual sugar content, *S. cerevisiae* count, and Hunter L, a, b values of the *M. nitidum* wine are expressed as mean ± SD (n = 3). Results from the sensory evaluation of *M. nitidum* wines are expressed as mean ± SD (n = 16). Results from the sensory evaluation aging of *M. nitidum* wines are expressed as mean ± SD (n = 20). Data were analyzed by a one-way analysis of variance (ANOVA). When the ANOVA identified differences between the groups, multiple comparisons of the means were made using Duncan's new multiple range tests. Statistical significance was determined by setting the aggregate type I error at 5% ($p < 0.05$) for each set of comparisons, using the Statistical Analysis System software package [36].

III. RESULTS AND DISCUSSION

1. Concentrations of *M. nitidum* Powder Particles in MNH Solution

Table 1 shows the analyses of yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar con-

Table 1. The analyses of the yeast count, alcohol content, pH value, titratable acidity, reducing sugar content, and residual sugar content of 0.5%-3.0% *M. nitidum* hydrolysate solution with 15% sucrose added that fermented at 25°C for 10 days.

Groups[1]	*M. nitidum* powder (%)	Yeast count (Log CFU/mL)	Alcohol content (%, v/v)	pH value	Titratable acidity (%)	Reducing sugar (%)	Residual sugar (°Brix)
S5	0.5	7.10 ± 0.10^{c}[2]	5.45 ± 0.10^{d}	3.79 ± 0.27^{f}	0.06 ± 0.01^{d}	2.84 ± 0.03^{a}	3.63 ± 0.06^{e}
	1.0	7.42 ± 0.06^{a}	6.88 ± 0.17^{ab}	3.86 ± 0.23^{e}	0.08 ± 0.00^{c}	0.42 ± 0.02^{b}	4.06 ± 0.03^{d}
	1.5	7.40 ± 0.05^{a}	6.71 ± 0.20^{bc}	3.92 ± 0.00^{d}	0.09 ± 0.00^{c}	0.37 ± 0.00^{c}	4.20 ± 0.00^{c}
	2.0	7.35 ± 0.04^{ab}	6.85 ± 0.34^{ab}	3.95 ± 0.35^{c}	0.11 ± 0.01^{b}	0.39 ± 0.01^{bc}	4.30 ± 0.00^{c}
	2.5	7.37 ± 0.00^{ab}	7.25 ± 0.12^{a}	4.03 ± 0.21^{b}	0.12 ± 0.00^{b}	0.35 ± 0.04^{c}	4.60 ± 0.10^{b}
	3.0	7.01 ± 0.12^{c}	6.68 ± 0.56^{c}	4.10 ± 0.35^{a}	0.16 ± 0.01^{a}	0.37 ± 0.01^{c}	4.86 ± 0.03^{a}
S6	0.5	6.97 ± 0.20^{a}	4.94 ± 0.30^{e}	3.73 ± 0.01^{e}	0.05 ± 0.00^{d}	3.25 ± 0.02^{a}	6.17 ± 0.06^{a}
	1.0	6.96 ± 0.01^{a}	6.84 ± 0.09^{bc}	3.81 ± 0.01^{d}	0.06 ± 0.01^{d}	0.51 ± 0.00^{c}	4.20 ± 0.03^{e}
	1.5	6.89 ± 0.15^{a}	6.90 ± 0.21^{ab}	3.83 ± 0.02^{d}	0.08 ± 0.01^{c}	0.41 ± 0.04^{d}	4.30 ± 0.00^{d}
	2.0	6.81 ± 0.06^{a}	6.62 ± 0.10^{c}	3.87 ± 0.02^{c}	0.09 ± 0.01^{c}	0.36 ± 0.01^{e}	4.33 ± 0.06^{d}
	2.5	6.73 ± 0.10^{a}	7.13 ± 0.26^{a}	3.95 ± 0.00^{b}	0.11 ± 0.00^{b}	0.45 ± 0.00^{d}	4.50 ± 0.00^{c}
	3.0	6.14 ± 0.25^{b}	6.80 ± 0.20^{bc}	4.02 ± 0.01^{a}	0.14 ± 0.00^{a}	0.90 ± 0.05^{b}	4.76 ± 0.03^{b}

[1]: S5: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21686 (2.5%) and BCRC21962 (2.5%); S6: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21824 (2.5%) and BCRC21962 (2.5%).

[2]: Each value is the means ± standard deviation (n = 3) and the different superscript letters in the same column are significantly different ($p < 0.05$).

tent of 0.5%-3.0% *M. nitidum* hydrolysate solution, with 15% sucrose added, fermented at 25°C for 10 days. The yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content were 7.01-7.42 log CFU/mL, 5.45-7.25%, 3.79-4.10, 0.06-0.16%, 0.35-2.84%, and 3.63-4.86 °Brix, respectively, for the S5 yeast group fermented with different *M. nitidum* powder particles concentrations; and 6.14-6.97 log CFU/mL, 4.94-7.13%, 3.73-4.02, 0.05-0.14%, 0.36-3.25%, and 4.20-6.17 °Brix, respectively, for the S6 yeast group fermented with different *M. nitidum* powder particle concentrations. The highest alcohol contents of the S5 and S6 yeast groups were 7.25% and 7.13%, respectively, and both occurred with 2.5% *M. nitidum* powder particles in the MNH solution. The increase in alcohol content with the concentration in MNH solution could be due to the formation of simple sugar by the enzyme hydrolyzed polysaccharide in *M. nitidum*. In our previous study, the reducing sugar content increased with *M. nitidum* powder particle concentrations from 0.5 to 3.0% in MNH solution, attesting to this phenomenon.

In many types of cells, osmotic stress interferes with cell volume and intracellular inorganic ion homeostasis. Most cells shrink when exposed to hyperosmotic medium and swell in hypo-osmotic medium as a result of osmosis. The resulting change in cell volume is accompanied by an alteration in the intracellular density of macromolecules. Osmotic stress may damage cellular macromolecules and impair cell function until compensatory adaptations counteract the stress. Damage to DNA and proteins leads to impairment of cell function and to the induction of repair processes and protection systems [22]. Moreover, high osmotic pressure, the formation of elevated levels of toxic fermentation by-products (such as ethanol), and limiting yeast nutritional factors have been implicated as key factors contributing to the deleterious results in yeast fermentation [20]. The fermentation of high sugar grape musts can occur in winemaking for wine production from dried, botrytized or late-harvest grapes, for ice-wine production or in processing industries that use grape juice concentrate. When using musts with high sugar concentrations, stuck or sluggish fermentations are probable because of the high osmotic pressure and ethanol toxicity of yeast cells [27]. Therefore, in accordance with the research results of Chiang *et al*. [10] on the conversion of D-xylulose to ethanol in the presence of D-xylose, the rate of ethanol production increased with an increase in yeast cell density. The rate of D-xylulose fermentation decreased when the production of ethanol yielded concentrations of 4% or more. The slow conversion rate of D-xylulose to ethanol was increased by increasing the yeast cell density. In the study by D'Amore *et al*. [12] on the intracellular accumulation of ethanol in *S. cerevisiae*, fermentation was observed after 3 h. After 12 h of fermentation, the intracellular and extracellular ethanol concentrations were similar. Increasing the osmotic pressure of the medium caused an increase in the ratio of intracellular to extracellular ethanol concentrations at 3 h of fermentation. Increasing the osmotic pressure caused a decrease in yeast cell growth and fermentation activities. D'Amore *et al*. [12] suggested that nutrient limitation was a major factor responsible for the decreased growth and fermentation activities observed in yeast cells at higher osmotic pressures.

2. Carbon Source

The most efficient sugar-fermenting yeast, *S. cerevisiae* (fermentable substrates such as glucose or fructose that convert into alcohol and CO_2) are metabolized more easily than

Table 2. The analyses of the yeast count, alcohol content, pH value, titratable acidity, reducing sugar content, and residual sugar content of 2.5% *M. nitidum* hydrolysate solution with 15% different types of sugars added that fermented at 25°C for 10 days.

Groups[1]	Sugars	Yeast count (Log CFU/mL)	Alcohol content (%, v/v)	pH value	Titratable acidity (%)	Reducing sugar (%)	Residual sugar (°Brix)
S5	Glucose	6.88 ± 0.00^{cd2}	6.10 ± 0.21^{b}	4.02 ± 0.04^{c}	0.17 ± 0.02^{a}	0.16 ± 0.02^{e}	4.03 ± 0.06^{f}
	Maltose	7.33 ± 0.15^{b}	3.71 ± 0.35^{c}	4.07 ± 0.01^{a}	0.18 ± 0.01^{a}	8.91 ± 0.01^{b}	9.80 ± 0.00^{b}
	Sucrose	7.86 ± 0.10^{a}	7.17 ± 0.18^{a}	4.08 ± 0.09^{bc}	0.12 ± 0.01^{b}	0.39 ± 0.07^{d}	4.10 ± 0.00^{e}
S6	Glucose	6.91 ± 0.06^{c}	3.71 ± 0.31^{c}	4.06 ± 0.01^{ab}	0.18 ± 0.00^{a}	0.17 ± 0.01^{e}	4.66 ± 0.03^{c}
	Maltose	6.75 ± 0.12^{d}	1.91 ± 0.11^{d}	4.05 ± 0.00^{abc}	0.19 ± 0.01^{a}	11.05 ± 0.03^{a}	10.70 ± 0.00^{a}
	Sucrose	7.23 ± 0.15^{b}	7.10 ± 0.16^{a}	3.99 ± 0.02^{d}	0.11 ± 0.01^{b}	0.48 ± 0.05^{c}	4.56 ± 0.03^{d}

[1]: S5: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21686 (2.5%) and BCRC21962 (2.5%); S6: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21824 (2.5%) and BCRC21962 (2.5%).

[2]: Each value is the means ± standard deviation (n = 3) and the different superscript letters in the same column are significantly different ($p < 0.05$).

alternative sugars like maltose, galactose, sucrose etc. [6]. This type of yeast requires the addition of carbon sources for *S. cerevisiae* fermented wines to reach ideal alcohol content when reducing sugar is lacking in raw materials. Therefore, in the *M. nitidum* wine from the MNH solution that was fermented by *S. cerevisiae* a carbon source, glucose, maltose, or sucrose were used as the energy sources for the test. The analyses of the yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content of the 2.5% MNH solution, with 15% of different types of sugars added and fermentation at 25°C for 10 days, are shown in Table 2. The yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content were 6.88-7.86 log CFU/mL, 3.71-7.17%, 4.02-4.08, 0.12-0.18%, 0.16-8.91%, and 4.10-9.80 °Brix, respectively, for the S5 yeast group with different sugar types; and 6.75-7.23 log CFU/mL, 1.91-7.10%, 3.99-4.06, 0.11-0.19%, 0.17-11.05%, and 4.56-10.70 °Brix, respectively, for the S6 yeast group with different sugar types. The highest alcohol contents of the S5 or S6 yeast groups were 7.17% and 7.10%, respectively, and both occurred in 2.5% *M. nitidum* powder particles in MNH solution with 15% sucrose. The trend of the carbohydrates used by yeast in *M. nitidum* wines was sucrose > glucose > maltose. In compliance with the results of *S. cerevisiae,* sucrose was used as the carbon source for producing alcohol rather than the other sugars in later *M. nitidum* wine fermentation experiments.

Using sucrose as the carbon source, *S. cerevisiae* produced higher ethanol content than when using glucose or maltose. During wine fermentation, yeasts convert most of the glucose and fructose present into alcohol and CO_2 [16]. Depending on the type of wine being produced, all the sugar may be fermented into alcohol to produce a dry wine [11]. The disaccharides used in this study can easily be accepted as nutrients, by breaking maltose down into two glucoses, and sucrose hydrolyzed by invertase to form glucose and fructose. Grape musts contain equal amounts of glucose and fructose, and the *S. cerevisiae* is glucophilic yeast which prefers glucose to fructose. During the fermentation period, glucose is fermented at a higher rate than fructose, and the proportion of fructose therefore increases as fermentation progresses. Consequently, fructose becomes the main sugar present during the late stages of alcoholic fermentation, and wine yeasts have to ferment this nonpreferred sugar after long periods of starvation and in the presence of large amounts of ethanol [19]. Phowchinda and Strehaiano [33] observed that the hydrolysis of sucrose by *S. cerevisiae* takes place very early during fermentation, and the glucose level is lower than the fructose level, confirming the faster use of glucose. In fact, sucrose is hydrolyzed faster when the glucose is exhausted. Finally, by the end of fermentation, it seems that a stationary phase was reached. The sugar consumption rate equaled the hydrolysis rate and no glucose or fructose accumulation was noticed during this phase.

Houghton-Larsen and Brandt [18] indicated that maltose metabolism in *S. cerevisiae* was negatively regulated by glucose at both the transcriptional level and at the enzyme activity level. Despite this multilayered regulation of maltose metabolism, *S. cerevisiae* cells can have difficulties in coping with sudden changes in extracellular maltose concentration. Exposure of aerobic, maltose-limited chemostat cultures, to excess maltose has even reportedly resulted in maltose-accelerated death. This loss of viability, which was accompanied by the release of glucose in the medium, was interpreted to be a result of non-restricted maltose uptake and hydrolysis, with the resulting accumulation of glucose and protons in the cells leading to cell death and lysis. Release of glucose upon exposure to excess maltose was also observed in *S. cerevisiae* mutants that were defective in glucose catabolite repression [19]. Therefore, the alcohol content of sucrose in the MNH solution fermented by *S. cerevisiae* was higher than that with maltose as the carbon source.

3. Nitrogen Source

During ethanol fermentation, yeast must adapt rapidly to the rich environment using the available nitrogen for the

Table 3. The analyses of the yeast count, alcohol content, pH value, titratable acidity, reducing sugar content, and residual sugar content of 2.5% *M. nitidum* hydrolysate solution with various contents of ammonium dihydrogen phosphate ($NH_4H_2PO_4$) added that fermented at 25°C for 10 days.

Groups[1]	$NH_4H_2PO_4$ (%)	Yeast count (Log CFU/mL)	Alcohol content (%, v/v)	pH value	Titratable acidity (%)	Reducing sugar (%)	Residual sugar (°Brix)
S5	0.000	7.30 ± 0.12^{a2}	7.22 ± 0.12^{bc}	4.03 ± 0.02^{bcd}	0.12 ± 0.00^{d}	0.35 ± 0.04^{c}	4.60 ± 0.00^{bc}
	0.125	7.24 ± 0.21^{a}	7.26 ± 0.25^{bc}	4.09 ± 0.01^{abc}	0.15 ± 0.01^{c}	0.30 ± 0.01^{d}	4.76 ± 0.03^{ab}
	0.250	7.15 ± 0.11^{a}	7.29 ± 0.13^{bc}	4.15 ± 0.02^{ab}	0.15 ± 0.01^{c}	0.27 ± 0.02^{e}	4.80 ± 0.00^{a}
	0.500	7.11 ± 0.05^{ab}	7.43 ± 0.18^{ab}	4.18 ± 0.00^{a}	0.19 ± 0.02^{a}	0.31 ± 0.01^{d}	4.80 ± 0.00^{a}
	1.000	6.94 ± 0.20^{b}	6.92 ± 0.19^{d}	3.90 ± 0.02^{ef}	0.17 ± 0.00^{b}	0.33 ± 0.00^{cd}	4.70 ± 0.00^{ab}
S6	0.000	6.72 ± 0.06^{c}	7.13 ± 0.17^{c}	3.95 ± 0.00^{def}	0.11 ± 0.00^{d}	0.45 ± 0.00^{a}	4.53 ± 0.06^{c}
	0.125	6.70 ± 0.10^{c}	7.24 ± 0.12^{bc}	4.08 ± 0.01^{abc}	0.14 ± 0.01^{c}	0.39 ± 0.02^{b}	4.40 ± 0.00^{d}
	0.250	6.63 ± 0.05^{c}	7.50 ± 0.26^{a}	4.06 ± 0.02^{abcd}	0.17 ± 0.00^{b}	0.43 ± 0.00^{a}	4.56 ± 0.03^{c}
	0.500	6.62 ± 0.06^{c}	7.16 ± 0.20^{c}	4.02 ± 0.00^{cde}	0.18 ± 0.00^{ab}	0.45 ± 0.02^{a}	4.40 ± 0.00^{bc}
	1.000	6.24 ± 0.13^{d}	6.89 ± 0.21^{d}	3.84 ± 0.01^{f}	0.17 ± 0.01^{b}	0.45 ± 0.01^{a}	4.50 ± 0.00^{ab}

[1]: S5: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21686 (2.5%) and BCRC21962 (2.5%); S6: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21824 (2.5%) and BCRC21962 (2.5%).

[2]: Each value is the means ± standard deviation (n = 3) and the different superscript letters in the same column are significantly different ($p < 0.05$).

synthesis of cellular proteins and other cell compounds [24]. Tables 3 to 5 show the results of the addition of different nitrogen sources to MNH solution fermented by *S. cerevisiae*. Table 3 shows the analyses of the yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content of the 2.5% MNH solution with various concentrations of ammonium dihydrogen phosphate ($NH_4H_2PO_4$) added with fermentation at 25°C for 10 days. The yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content were 6.94-7.30 log CFU/mL, 6.92-7.43%, 3.90-4.18, 0.12-0.19%, 0.27-0.35%, 4.6-4.8 °Brix, respectively, for the S5 yeast group with different $NH_4H_2PO_4$ concentrations; and 6.24-6.72 log CFU/mL, 6.89-7.50%, 3.84-48, 0.11-0.18%, 0.39-0.45%, and 4.40-4.56 Brix, respectively, for the S6 yeast group with different $NH_4H_2PO_4$ concentrations. The highest alcohol contents of the S5 or the S6 yeast group were 7.43% or 7.50%, respectively, occurring with 2.5% *M. nitidum* powder particles in MNH solution with 0.500% or 0.250% $NH_4H_2PO_4$.

The analyses of the yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content of the 2.5% MNH solution with various amounts of ammonium chloride (NH_4Cl) added with fermentation at 25°C for 10 days are shown in Table 4. The yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content were 7.15-7.43 log CFU/mL, 7.01-7.50%, 3.61-4.03, 0.12-0.18%, 0.32-0.41%, and 4.50-4.60 Brix, respectively, for the S5 yeast group with different NH_4Cl concentrations; and 6.58-6.88 log CFU/mL, 7.13-7.40%, 3.57-3.95, 0.11-0.18%, 0.40-0.45%, and 4.40-4.50 Brix, respectively, for the S6 yeast group with different NH_4Cl concentrations. The highest alcohol contents of the S5 or the S6 yeast group were 7.50% or 7.40%, respectively, and both occurred in 2.5% *M. nitidum* powder particles in an MNH solution with 0.250% NH_4Cl.

Table 5 shows the analyses of the yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content of 2.5% MNH solution with various amounts of proline added with fermentation at 25°C for 10 days. The yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content were 7.12-7.58 log CFU/mL, 7.17-7.73%, 4.03-4.13, 0.12-0.16%, 0.32-0.41%, and 4.60-4.83 °Brix, respectively, for the S5 yeast group with different proline concentrations; and 6.60-7.10 log CFU/mL, 7.13-7.86%, 3.92-3.99, 0.11-0.16%, 0.40-0.45%, and 4.50-4.67 °Brix, respectively, for the S6 yeast group with different proline concentrations. The highest alcohol contents of the S5 or the S6 combination starter were 7.73% or 7.86%, respectively, and both occurred in 2.5% *M. nitidum* powder particles in MNH solution with 0.500% proline.

In the *M. nitidum* wine-producing starter groups, S5 and S6, the organic nitrogen source proline resulted in higher alcohol content than with the mineral nitrogen sources of ammonium chloride or dihydrogen phosphate. At only 7.53%, the crude protein of *M. nitidum* powder may not have been enough for *S. cerevisiae* growth under alcohol fermentation. In the berry and the must, nitrogen can be found under mineral (NH_4^+, NO_3^-, and NO_2^-) and organic (free amino acids, proteins and other nitrogenated organic compounds such as urea, ethyl carbamate and nucleic acids) forms. This nitrogen, called fermentable nitrogen, is used by yeast to carry on the normal alcoholic fermentation of the must [11]. Takagi *et al.* [38] indicated that, during sake fermentation, yeast cells are exposed to various stresses under anaerobic conditions, including high concentrations of ethanol (~20% [vol/vol]). These stresses make it toxic for *S. cerevisiae*, damaging the cell membrane and functional proteins, and gradually reducing cell viability and leading to cell death during fermentation. L-proline is an osmoprotectant and a sweet amino acid that

Table 4. The analyses of the yeast count, alcohol content, pH value, titratable acidity, reducing sugar content, and residual sugar content of 2.5% *M. nitidum* hydrolysate solution with various contents of ammonium chloride (NH_4Cl) added that fermented at 25°C for 10 days.

Groups[1]	NH_4Cl (%)	Yeast count (Log CFU/mL)	Alcohol content (%, v/v)	pH value	Titratable acidity (%)	Reducing sugar (%)	Residual sugar (°Brix)
S5	0.000	7.30 ± 0.12^{a2}	7.22 ± 0.28^{abc}	4.03 ± 0.02^{a}	0.12 ± 0.00^{de}	0.35 ± 0.04^{bc}	4.60 ± 0.00^{a}
	0.125	7.38 ± 0.31^{a}	7.37 ± 0.23^{ab}	3.97 ± 0.01^{ab}	0.14 ± 0.02^{cd}	0.32 ± 0.12^{c}	$4.50 \pm 0.10a^{b}$
	0.250	7.43 ± 0.15^{a}	7.50 ± 0.25^{a}	3.88 ± 0.00^{bc}	0.15 ± 0.00^{bc}	0.38 ± 0.02^{abc}	4.60 ± 0.00^{a}
	0.500	7.33 ± 0.12^{a}	7.34 ± 0.13^{ab}	3.79 ± 0.01^{c}	0.18 ± 0.02^{a}	0.36 ± 0.03^{abc}	4.57 ± 0.03^{a}
	1.000	7.15 ± 0.04^{ab}	7.01 ± 0.11^{c}	3.61 ± 0.01^{d}	0.17 ± 0.02^{ab}	0.41 ± 0.00^{abc}	4.57 ± 0.03^{a}
S6	0.000	6.73 ± 0.06^{c}	7.13 ± 0.08^{bc}	3.95 ± 0.00^{ab}	0.11 ± 0.00^{e}	0.45 ± 0.00^{a}	4.50 ± 0.06^{ab}
	0.125	6.88 ± 0.03^{bc}	7.21 ± 0.16^{abc}	3.86 ± 0.00^{bc}	0.13 ± 0.01^{cde}	0.40 ± 0.06^{ab}	4.50 ± 0.00^{ab}
	0.250	6.80 ± 0.26^{c}	7.40 ± 0.19^{ab}	3.78 ± 0.01^{c}	0.15 ± 0.00^{bc}	0.41 ± 0.03^{abc}	4.43 ± 0.06^{b}
	0.500	6.70 ± 0.30^{c}	7.36 ± 0.16^{ab}	3.81 ± 0.02^{c}	0.18 ± 0.01^{a}	0.44 ± 0.04^{ab}	4.50 ± 0.10^{ab}
	1.000	6.58 ± 0.22^{c}	7.16 ± 0.31^{bc}	3.57 ± 0.00^{d}	0.17 ± 0.00^{ab}	0.45 ± 0.02^{a}	4.40 ± 0.00^{b}

[1]: S5: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21686 (2.5%) and BCRC21962 (2.5%); S6: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21824 (2.5%) and BCRC21962 (2.5%).

[2]: Each value is the means ± standard deviation (n = 3) and the different superscript letters in the same column are significantly different ($p < 0.05$).

Table 5. The analyses of the yeast count, alcohol content, pH value, titratable acidity, reducing sugar content, an residual sugar content of 2.5% *M. nitidum* hydrolysate solution with various contents of proline added that fermented at 25°C for 10 days.

Groups[1]	Proline (%)	Yeast count (Log CFU/mL)	Alcohol content (%, v/v)	pH value	Titratable acidity (%)	Reducing sugar (%)	Residual sugar (°Brix)
S5	0.000	7.30 ± 0.13^{ab2}	7.22 ± 0.10^{cd}	4.03 ± 0.02^{abc}	0.12 ± 0.00^{cd}	0.35 ± 0.04^{de}	4.63 ± 0.06^{bc}
	0.125	7.42 ± 0.28^{a}	7.32 ± 0.09^{c}	4.08 ± 0.02^{ab}	0.13 ± 0.01^{bcd}	0.36 ± 0.03^{cde}	4.70 ± 0.00^{b}
	0.250	7.51 ± 0.22^{a}	7.51 ± 0.07^{b}	4.11 ± 0.04^{ab}	0.14 ± 0.01^{abc}	0.38 ± 0.02^{bcde}	4.70 ± 0.00^{b}
	0.500	7.58 ± 0.06^{a}	7.73 ± 0.12^{a}	4.13 ± 0.01^{a}	0.15 ± 0.01^{ab}	0.41 ± 0.06^{abcd}	4.83 ± 0.06^{a}
	1.000	7.12 ± 0.18^{bc}	7.17 ± 0.15^{cd}	4.09 ± 0.00^{ab}	0.16 ± 0.00^{a}	0.32 ± 0.01^{e}	4.60 ± 0.00^{cd}
S6	0.000	6.73 ± 0.06^{de}	7.13 ± 0.13^{d}	3.95 ± 0.00^{c}	0.11 ± 0.00^{d}	0.45 ± 0.00^{a}	4.53 ± 0.06^{de}
	0.125	6.78 ± 0.12^{de}	7.34 ± 0.05^{c}	3.92 ± 0.03^{c}	0.12 ± 0.02^{cd}	0.42 ± 0.02^{abc}	4.60 ± 0.00^{cd}
	0.250	6.89 ± 0.15^{cd}	7.56 ± 0.06^{b}	$3.99 \pm 0.01b^{c}$	0.13 ± 0.02^{bcd}	0.43 ± 0.06^{ab}	4.50 ± 0.00^{e}
	0.500	7.10 ± 0.17^{bc}	7.86 ± 0.14^{a}	3.92 ± 0.02^{c}	0.14 ± 0.00^{abc}	0.44 ± 0.01^{ab}	4.67 ± 0.03^{bc}
	1.000	6.60 ± 0.09^{e}	7.19 ± 0.05^{cd}	3.93 ± 0.01^{c}	0.16 ± 0.01^{a}	0.40 ± 0.02^{abcd}	4.60 ± 0.00^{cd}

[1]: S5: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21686 (2.5%) and BCRC21962 (2.5%); S6: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21824 (2.5%) and BCRC21962 (2.5%).

[2]: Each value is the means ± standard deviation (n = 3). The different superscript letters in the same column are significantly different ($p < 0.05$).

helps protect yeast cells from damage by freezing, desiccation, or oxidative stress. L-proline enhances the stability of proteins and membranes in environments with low water activity and inhibits aggregation during protein refolding. This suggests that L-proline could play a crucial role in reducing ethanol stress by preventing protein denaturation and membrane disorder during sake fermentation [31, 38]. Hence, it was not unexpected when proline revealed a higher ethanol content than ammonium chloride or dihydrogen phosphate. However, the results showed that the nitrogen sources of ammonium chloride, dihydrogen phosphate, or proline were greater (from 0.500% raised to 1.000), causing less ethanol; perhaps, great osmotic pressure led to this phenomenon.

4. Inoculation Concentration of *M. nitidum* Wine-Producing Starter Groups

The analyses of the yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content of 2.5% MNH solution with different starter concentrations added and with fermentation at 25°C for 10 days are shown in Table 6. The yeast count, alcohol content, pH value, TA, reducing sugar content, and residual sugar content were 7.14-7.75 log CFU/mL, 7.21-7.87%, 4.03-4.14, 0.12-0.15%, 0.39-0.45%, and 4.50-4.90 Brix, respectively, for the S5 yeast

Table 6. The analyses of the yeast count, alcohol content, pH value, titratable acidity, reducing sugar content, and residual sugar content of 2.5% *M. nitidum* hydrolysate solution with different starter contents added that fermented at 25°C for 10 days.

Groups[1]	Starter contents (%)	Yeast count (Log CFU/mL)	Alcohol content (%, v/v)	pH value	Titratable acidity (%)	Reducing sugar (%)	Residual sugar (°Brix)
S5	2	7.28 ± 0.12^{c2}	7.51 ± 0.11^{de}	4.03 ± 0.21^{bcd}	0.13 ± 0.00^{bc}	0.40 ± 0.04^{abc}	4.73 ± 0.12^{bcd}
	4	7.68 ± 0.14^{ab}	7.87 ± 0.05^{b}	4.11 ± 0.01^{abc}	0.15 ± 0.01^{a}	0.45 ± 0.04^{a}	4.90 ± 0.10^{a}
	6	7.75 ± 0.12^{a}	7.66 ± 0.03^{cd}	4.14 ± 0.02^{ab}	0.14 ± 0.00^{ab}	0.43 ± 0.05^{abc}	4.73 ± 0.06^{de}
	8	7.23 ± 0.05^{cd}	7.39 ± 0.06^{e}	4.11 ± 0.01^{abc}	0.12 ± 0.01^{c}	0.44 ± 0.02^{ab}	4.60 ± 0.00^{ef}
	10	7.14 ± 0.09^{cde}	7.21 ± 0.13^{f}	4.05 ± 0.01^{bcd}	0.13 ± 0.01^{bc}	0.39 ± 0.08^{bc}	4.50 ± 0.00^{f}
S6	2	7.00 ± 0.07^{e}	7.82 ± 0.08^{b}	3.87 ± 0.00^{e}	0.14 ± 0.00^{ab}	0.41 ± 0.01^{abc}	4.80 ± 0.10^{ab}
	4	7.25 ± 0.16^{c}	8.07 ± 0.09^{a}	3.93 ± 0.00^{de}	0.13 ± 0.01^{bc}	0.38 ± 0.11^{c}	4.77 ± 0.06^{bc}
	6	7.52 ± 0.19^{b}	7.73 ± 0.03^{bc}	3.99 ± 0.01^{cd}	0.13 ± 0.02^{bc}	0.45 ± 0.02^{a}	4.80 ± 0.00^{ab}
	8	7.20 ± 0.10^{cde}	7.53 ± 0.21^{de}	4.07 ± 0.01^{abc}	0.14 ± 0.01^{ab}	0.24 ± 0.01^{d}	4.67 ± 0.06^{cde}
	10	7.01 ± 0.18^{de}	7.40 ± 0.11^{e}	4.18 ± 0.00^{a}	0.15 ± 0.00^{a}	0.20 ± 0.01^{d}	4.77 ± 0.06^{bc}

[1]: S5: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21686 (2.5%) and BCRC21962 (2.5%); S6: *M. nitidum* hydrolysate solution + 15% sucrose fermented by *S. cerevisiae* BCRC21824 (2.5%) and BCRC21962 (2.5%).

[2]: Each value is the means ± standard deviation (n = 3). The different superscript letters in the same column are significantly different ($p < 0.05$).

group with different inoculation concentrations; and 7.00-7.52 log CFU/mL, 7.40-8.07%, 3.87-4.18, 0.13-0.15%, 0.20-0.45%, and 4.67-4.80 °Brix, respectively, for the S6 yeast group with different inoculation concentrations. The highest alcohol contents of the S5 or the S6 yeast group were 7.87% or 8.07%, respectively, and both occurred in 2.5% *M. nitidum* powder particles in MNH solution with a 4% yeast concentration. In this study, when the *S. cerevisiae* inoculation concentration was higher, the *S. cerevisiae* content formed more rapidly, and higher alcohol content was produced in *M. nitidum* wine. Vriesekoop and Pamment [41] indicated that during industrial fermentations, microorganisms may be exposed to a range of unfavorable conditions that can impede growth and fermentation. The other reason is that more inoculation concentration may cause growth of *S. cerevisiae* that stops ethanol production. On the basis of the wine cost of fermented *M. nitidum*, the *S. cerevisiae* inoculation concentration was 4% for the S5 or S6 starter combinations.

In brief, a higher alcohol content results in more TA, less reducing sugar, less residual sugar, and lower pH in tests of the concentrations of *M. nitidum* powder particles, carbon sources, nitrogen sources, and wine-producing starter groups.

5. Fermentation Time of *M. nitidum* Wines

To determine the best fermentation day and the superior chemical composition of *M. nitidum* wines, this study integrated the above powder particle concentrations of *M. nitidum*, carbon sources, nitrogen sources, and starter groups concentration results under the following fermentation conditions: 2.5% *M. nitidum* powder particles, 10%-20% sucrose, 0.5% proline, and 4% yeast group inoculated concentrations (2% for each) at 0, 5, 7, 10, 14, 18, or 21 day. The *M. nitidum* wine experiments were carried out under these conditions, as shown in Fig. 1.

The changes in yeast counts of 2.5% MNH solutions with 10%-20% sucrose fermented by the 4% S5 or S6 yeast groups at 25°C over 21 days are displayed in Fig. 1(a). The yeast counts increased sharply from the initial fermentation stage (6.25-6.35 log CFU/mL) to the 5th day (7.39-7.89 CFU/mL), and slowly decreased with *M. nitidum* wine fermentation from the 5th to 21st day. Fig. 1(b) shows the changes in alcohol content of 2.5% MNH solution with 10%-20% sucrose fermented by the 4% S5 and S6 yeast groups at 25°C for 21 days. The alcohol contents of *M. nitidum* wines containing 10%, 15%, and 20% sucrose were 5.22-5.32%, 7.89-8.11% and 10.09-10.24%, respectively; their alcohol transform ratios were 81.7-83.3%, 82.3-84.6%, and 78.7-80.2% after 7, 10, or 14 days, respectively. They reached a stationary phase without undergoing any changes. The changes in pH values of 2.5% MNH solution with 10%-20% sucrose fermented by the 4% S5 and S6 yeast groups at 25°C for 21 days are shown in Fig. 1(c). The pH values of all *M. nitidum* wines ranged from 5.56-5.64 at the onset stage, then sharply decreased to 3.51-4.01 after 5 days of fermentation, rose again to 3.64-4.21 during 7-10 days, and finally maintained at 3.79-4.19 until the 21st day. Fig. 1(d) shows the changes in TA of 2.5% MNH solution with 10%-20% sucrose fermented by the 4% S5 and S6 yeast groups at 25°C for 21 days. The TA content of *M. nitidum* wines containing 10%, 15%, or 20% sharply increased to 0.11-0.12%, 0.13-0.14%, or 0.16-0.17% after 7, 10, or 14 days, respectively, then stayed at 0.09-0.11%, 0.13-0.15%, or 0.16-0.17% , respectively, until the 21st day. The changes in the reducing sugar content of 2.5% MNH solution with 10%-20% sucrose fermented by the 4% S5 and S6 yeast groups at 25°C for 21 days are shown in Fig. 1(e). The reduced sugar content of *M. nitidum* wines containing 10%, 15%, or 20% sucrose sharply increased by fermentation day 5, and took 0.23-0.26%, 0.33-0.44%, or 0.53-0.58% at the 10, 14, or

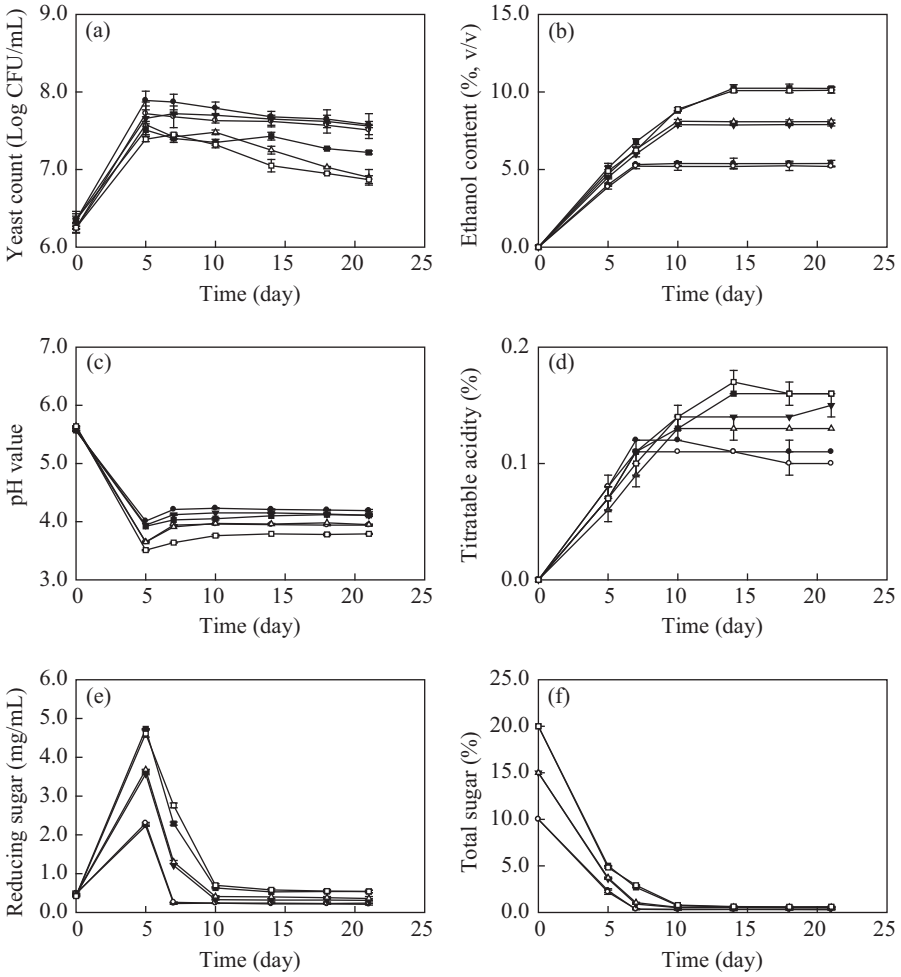

Fig. 1. The changes in (a) yeast count; (b) alcohol content; (c) pH value; (d) titratable acidity; (e) reducing sugar content; and (f) total sugar content of 2.5% *M. nitidum* hydrolysate solution containing 10%-20% sucrose with fermentation by 4% S5 and S6 combination starters at 25°C over 21 days. ●: S5-10% sucrose; ○: S6-10% sucrose; ▼: S5-15% sucrose; ▽: S6-15% sucrose; ■: S5-20% sucrose; □: S6-20% sucrose.

21 day, respectively, reaching a stationary phase without undergoing any changes. Fig. 1(f) shows the changes in total sugar content of 2.5% MNH solution with 10%-20% sucrose fermented by the 4% S5 and S6 yeast groups at 25°C for 21 days. The total sugar content of *M. nitidum* wines containing 10%, 15%, or 20% sucrose was 2.17-2.34%, 3.61-3.72%, or 4.82-5.01% after 5 days, respectively, and decreased to 0.33-0.35%, 0.51-0.54%, or 0.59-0.63% after 10, 14, or 21 days, respectively, reaching a stationary phase without undergoing any changes.

Many studies have been done on wines fermented with different raw materials with *S. cerevisiae*. Reddy and Reddy [34] used *S. cerevisiae* inoculated into a high (30-40%, w/v) sugar-containing medium with and without supplementation of horse gram flour. The fermentation rate increased in 3 to 5 days with increased viable cell counts, and decreased with the fermentation time in the end. The residual sugar content was also reduced by an increase in alcohol content. Malacrinò *et al*. [27] carried out alcoholic fermentation using four commercial strains of fermented grape musts with a sugar concentration of 35 °Brix. Ethanol production started quickly in trials of inoculated strains, reaching a maximum production rate after 12 day, with a drastic reduction of ethanol production observed after 30 days. There is a strong correlation between the amount of sugar and the assimilable nitrogen consumption rate, with most of the TAN (total assimilable nitrogen) present in the must being utilized at the beginning of alcoholic fermentation in correspondence with the highest rate of sugar consumption. Therefore, 10%, 15%, or 20% sucrose concentrations were added to 25°C *M. nitidum* wines fermented from 2.5% *M. nitidum* powder particles, 0.5% proline, and 4% starter groups after 7, 10, or 14 days, respectively.

6. Composition Changes of *M. nitidum* Wine Aging

The changes in alcohol content on the aging period of *M. nitidum* wines fermented by S5 and S6 yeast groups are shown in Fig. 2(a) and Fig. 3(a). Whether at 15 or 25°C, the alcohol contents in *M. nitidum* wines containing 10%, 15%, or 20%

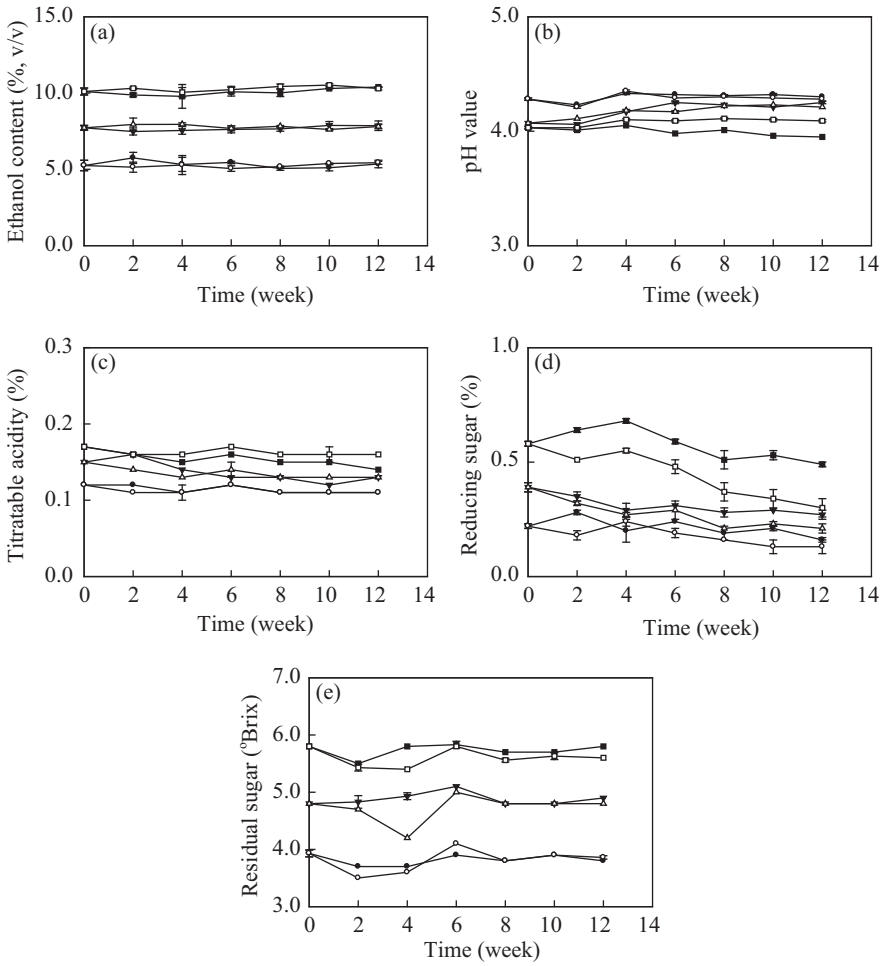

Fig. 2. The changes of (a) alcohol content; (b) pH value; (c) titratable acidity; (d) reducing sugar content; and (e) total sugar content on the aging period of *M. nitidum* wines fermented by the S5 yeast group. ●: 10% sucrose-15°C; ○: 10% sucrose-25°C; ▼: 15% sucrose-15°C; ▽: 15% sucrose-25°C; ■: 20% sucrose-15°C; □: 20% sucrose-25°C.

sucrose fermented by the S5 and the S6 yeast groups were 5.2-5.5%, 7.7-8.4%, and 10.1-10.3%, respectively. The alcohol contents of *M. nitidum* wines which did not undergo distinct changes, might have resulted because the *M. nitidum* wines were sealed up in bottles during the aging period, with very little alcohol evaporating. The changes in pH value and TA in the aging period of *M. nitidum* wines fermented by S5 and S6 yeast groups are shown in Fig. 2(b, c) and Fig. 3(b, c): the pH values increased slightly from 3.85%-4.27% to 3.93%-4.29%, while the TA showed a small decrease from 0.12%-0.17% to 0.11%-0.16% after three months. The changes of the aging period of *M. nitidum* wines fermented by S5 and S6 yeast groups by reducing sugar along with decreased aging time are shown in Fig. 2(d) and Fig. 3(d). After the *M. nitidum* wines had aged, reducing sugar decreased from 0.22%-0.58% to 0.13%-0.39% (25°C) or 0.16%-0.49% (15°C). Fig. 2(e) and Fig. 3(e) show the changes in residual sugar content in the aging period of *M. nitidum* wines fermented by the S5 and S6 yeast groups with no distinct changes in residual sugar after three months, and their contents ranged from 3.5-3.8, 4.7-4.9, or 5.5-5.8 Brix for *M. nitidum* wines containing 10%, 15%, or 20% sucrose. Moreno and Azpilicueta [30] studied wines that had been aged in oak barrels at the half-way stage of storage (243 days) and at the end of aging (540 days). The pH values, total acidity, and volatile acidity increased at 243 and 540 days for young wine. The alcohol content and free SO_2 content decreased at 243 and 540 days for young wine.

Fig. 4 shows the changes in the Hunter L, a, and b values in the aging period of *M. nitidum* wines fermented by the (a) S5 and (b) S6 yeast groups. Before the aging of *M. nitidum* wines fermented by the S5 and S6 yeast groups, the Hunter L values were 15.53-15.80 and 15.10-15.86, respectively. All the values decreased at 15°C (S5: 14.21-14.86; S6: 14.58-14.88) and 25°C (S5: 14.19-14.32; S6: 13.62-14.17) after the aging period. Before the aging of *M. nitidum* wines fermented by the S5 and S6 yeast groups, the Hunter a values were -1.99~-1.88 and -1.91~-1.87, respectively, and all increased at 15°C (S5: -1.89~-1.67; S6: -1.69~-1.60) and 25°C (S5: -1.84~-1.62; S6: -1.54~-1.50) after the aging period. Before the aging of *M. nitidum* wines fermented by the S5 and S6 yeast groups, the

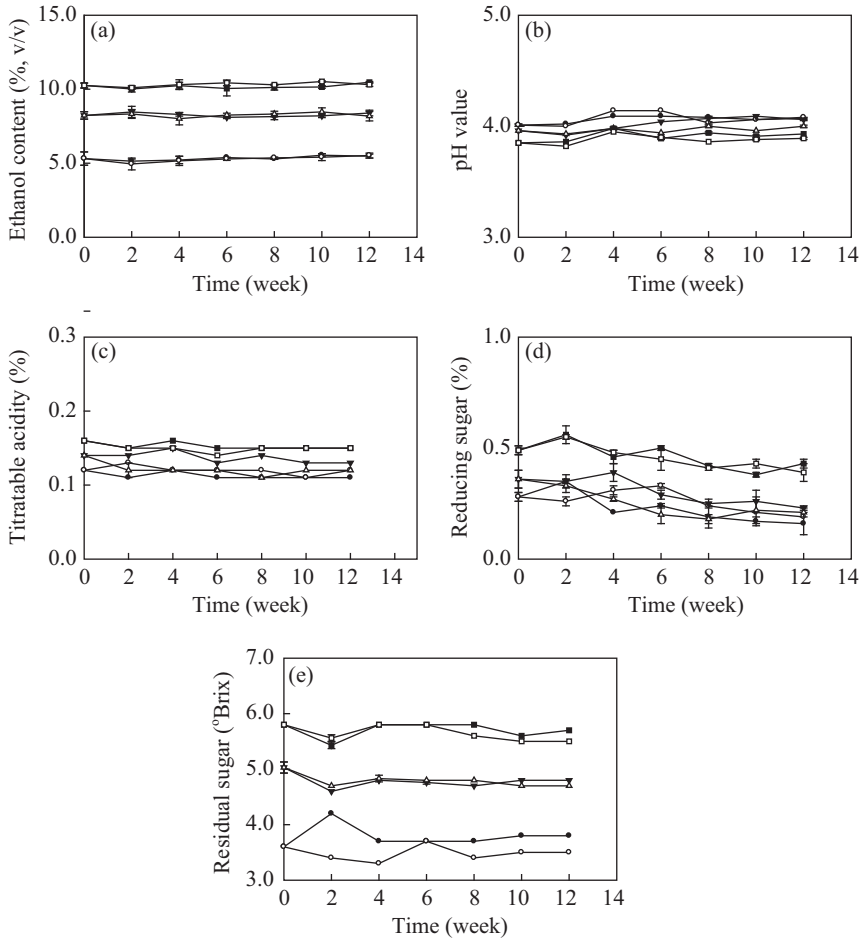

Fig. 3. The changes of (a) alcohol content; (b) pH value; (c) titratable acidity; (d) reducing sugar content; and (e) total sugar content on aging period of *M. nitidum* wines fermented by S6 yeast group. ●: 10% sucrose-15°C; ○: 10% sucrose-25°C; ▼: 15% sucrose-15°C; ▽: 15% sucrose-25°C; ■: 20% sucrose-15°C; □: 20% sucrose-25°C.

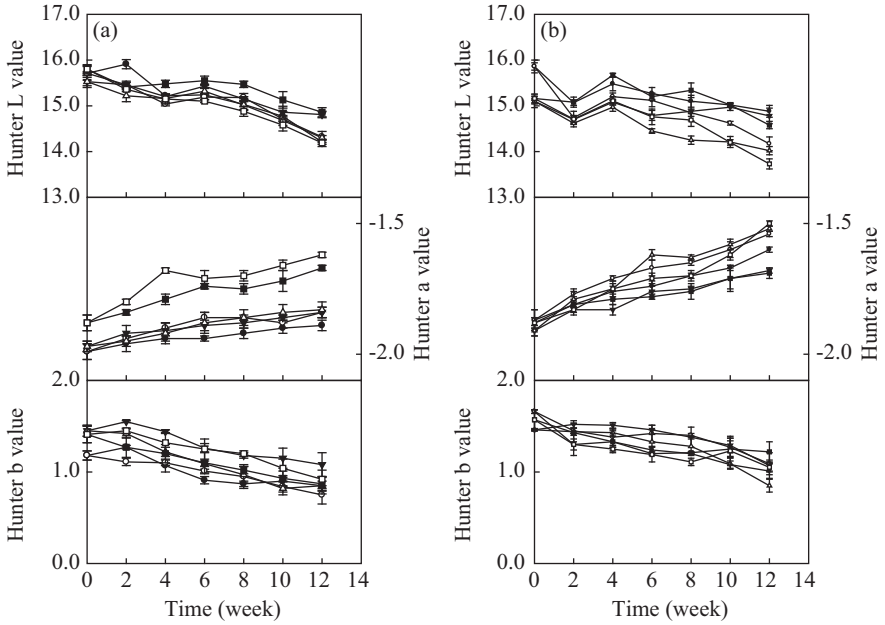

Fig. 4. The changes of Hunter L, a, and b value on the aging period of *M. nitidum* wines fermented by (a) S5 and (b) S6 yeast groups. ●: 10% sucrose-15°C; ○: 10% sucrose-25°C; ▼: 15% sucrose-15°C; ▽: 15% sucrose-25°C; ■: 20% sucrose-15°C; □: 20% sucrose-25°C.

Table 7. Results of the sensory evaluation for *M. nitidum* wines with 10%-20% sucrose.

Groups[1]	Sucrose content (%)	Color[2]	Flavor	Taste	Preference
Mn-W-S5	10	6.13 ± 2.94^{a}[3]	5.10 ± 2.75^{c}	5.35 ± 1.88^{b}	5.20 ± 1.76^{c}
	15	6.05 ± 1.58^{a}	5.28 ± 1.72^{bc}	5.60 ± 2.02^{ab}	5.45 ± 1.25^{bc}
	20	6.08 ± 1.85^{a}	5.82 ± 1.64^{ab}	5.95 ± 1.82^{a}	5.97 ± 2.04^{ab}
Mn-W-S6	10	6.10 ± 1.43^{a}	5.27 ± 1.83^{bc}	5.15 ± 2.15^{b}	5.33 ± 2.12^{c}
	15	6.07 ± 1.32^{a}	5.36 ± 1.89^{bc}	5.38 ± 1.91^{b}	5.43 ± 1.20^{bc}
	20	6.12 ± 1.38^{a}	5.94 ± 2.09^{a}	6.04 ± 2.22^{a}	6.12 ± 1.28^{a}

[1]: Mn-W: *M. nitidum* wines; S5: *M. nitidum* hydrolysate solution fermented by *S. cerevisiae* BCRC21686 (2.0%) and BCRC21962 (2.0%); S6: *M. nitidum* hydrolysate solution fermented by *S. cerevisiae* BCRC21824 (2.0%) and BCRC21962 (2.0%); 10%-20% sucrose: the fermented wines of the *M. nitidum* hydrolysate solution with 10%-20% sucrose added.
[2]: Grade: 1 = dislike extremely; 3 = dislike; 5 = neither like nor dislike; 7 = like; 9 = like extremely.
[3]: Each value is the means ± standard deviation (n = 16). The different superscript letters in the same column are significantly different ($p < 0.05$).

Table 8. Results of the sensory evaluation of *M. nitidum* wines treated at different aging temperatures.

Groups[1]	Aging temperature	Color[2]	Flavor	Taste	Preference
Mn-W-S5-20s	15°C aging	6.21 ± 1.43^{a}[3]	6.49 ± 1.95^{a}	6.43 ± 0.87^{a}	6.52 ± 1.85^{a}
Mn-W-S6-20s		6.13 ± 2.26^{a}	6.57 ± 2.13^{a}	6.54 ± 1.14^{a}	6.70 ± 1.73^{a}
Mn-W-S5-20s	25°C aging	5.92 ± 2.01^{a}	5.77 ± 1.68^{b}	5.97 ± 1.56^{a}	5.91 ± 1.09^{b}
Mn-W-S6-20s		6.05 ± 1.82^{a}	5.69 ± 2.25^{b}	6.12 ± 1.32^{a}	5.97 ± 1.96^{b}

[1]: Mn-W: *M. nitidum* wines; S5: *M. nitidum* hydrolysate solution fermented by *S. cerevisiae* BCRC21686 (2.0%) and BCRC21962 (2.0%); S6: *M. nitidum* hydrolysate solution fermented by *S. cerevisiae* BCRC21824 (2.0%) and BCRC21962 (2.0%); 20s: the fermented wines of the *M. nitidum* hydrolysate solution with 20% sucrose added; 15°C aging: aging at 15°C for 3 month; 25°C aging: aging at 25°C for 3 month.
[2]: Grade: 1 = dislike extremely; 3 = dislike; 5 = neither like nor dislike; 7 = like; 9 = like extremely.
[3]: Each value is the means ± standard deviation (n = 16). The different superscript letters in the same column are significantly different ($p < 0.05$).

Hunter b values were 1.18-1.45 and 1.46-1.66, respectively. All the values decreased at 15°C (S5: 0.85-1.08; S6: 1.01-1.22) and 25°C (S5: 0.75-0.92; S6: 0.85-1.09) after the aging period. The results from six *M. nitidum* wines fermented by the S5 and S6 yeast groups showed decreasing brightness, blueness, and greenness at two different temperatures, especially 25°C. It is known that phenolic compounds contribute to wine color, taste, structure and make the product suitable for aging. Color is one of the main quality attributes of red wine and a matter of primary importance to winemakers [29]. The initial color of red wine is mainly due to free anthocyanins, self-association of anthocyanins, and co-pigmentation of anthocyanins with other phenols present in the wine. However, during wine aging, grape anthocyanins are gradually displaced by more stable oligomeric and polymeric pigments. The progress of many of these reactions is influenced by factors such as: temperature, oxygen access, pH, acetaldehyde and free SO_2 content [29].

7. Sensory Evaluation

Volatile compounds are important for beverage flavor as they contribute to different desirable sensory characteristics [5]. Table 7 shows the results of the sensory evaluations made by 8 males and 8 females, who were 23 years old on average, of *M. nitidum* wines with 10%-20% sucrose added, and on the color of *M. nitidum* wines fermented by S5 and S6 yeast groups, with no different statistical analysis. As for flavor, the *M. nitidum* wine fermented by the S6 yeast group was more acceptable than that of the S5 yeast group. No matter whether the *M. nitidum* wines were fermented by the S6 yeast groups with 20% sucrose, they had a better taste than those with 10% and 15% sucrose, with differences in statistical analysis. In regard to total preference, *M. nitidum* wine fermented by the S6 yeast groups with 20% sucrose received better evaluation than those with 10% and 15% sucrose, with differences in statistical analysis. Results of sensory evaluations by 11 males and 9 females, who were 22 years of age on average, of *M. nitidum* wines treated at different aging temperatures are displayed in Table 8. The color and taste of *M. nitidum* wines fermented by the S5 and S6 yeast groups at 15 or 25°C after 20 days showed no difference in statistical analysis. The flavor of and total preference for *M. nitidum* wines fermented by the S5 and S6 yeast groups at 15 or 25°C after 20 days showed no difference in statistical analysis. However there was a difference in statistical analysis among the two sets.

The aroma of a wine is one of the most important determinants of its quality. A wine may contain over 800 volatile compounds including alcohols, esters, organic acids, phenols, thiols, monoterpenes and norisoprenoids. The esters, alcohols and acetates are among the volatile compounds derived from

yeast metabolism. The essence of a wine's flavor is formed during alcoholic fermentation. Ethanol and glycerol are the most abundant alcohols, followed by higher alcohols and esters, the combinations of which affect the final aroma of a wine [40]. Arrizon *et al*. [5] indicated that acetaldehyde is directly correlated with yeast metabolism in alcoholic fermentation because this compound is produced by decarboxylation of pyruvate, which is the direct product of glycolysis. Ethyl acetate is produced principally by the action of alcohol acetyl-transferase in *S. cerevisiae*. Conde *et al*. [11] further indicated that compared to other compounds, such as sugars and phenolics, the organoleptic effects of proteins on wine aroma can be considered negligible, but it has been shown that proteins can bind to volatile compounds, increasing the volatility of certain aromatic compounds (such as ethyl octanoate), and reducing it in other cases (such as ethyl hexanoate). Estévez *et al*. [14] studied the must obtained from Palomino grapes inoculated with seven different commercial yeast strains (three *S. cerevisiae cerevisia:* MO5, CEG and IOC BR 8000; and four *S. cerevisiae bayanus*: ALB, KD, REIMS and IOC 2007) and fermented under identical conditions. No differences were found in the gross chemical composition, but the wines had significantly different volatile characteristics and the results demonstrated that large sensory differences were generated by the different yeast strains that had been used in the vinification. The major constituents that contributed to the volatile composition in these wines were the higher alcohols, ethyl esters, acetates, fatty acids and volatile phenols. Vilanova *et al*. [39] also discovered that among Albariño wines that were fermented by 12 different yeast strains isolated from a single winery in Galicia, Spain, the chemical and sensorial properties of the wines differed depending on the yeast strains.

Xu *et al*. [47] studied the effect of *S. cerevisiae* only, and *S. cerevisiae* mixed with *Hanseniaspora* (*H*.) *valbyensis*, on cider fermentation, involving alcoholic fermentation and the formation of flavor compounds. The concentration of major higher alcohols and esters in the cider differed depending on whether *S. cerevisiae* was added to the *H. valbyensis* fermentation on day 3, 9, or 15. Compared to pure fermentation with *S. cerevisiae*, mixed fermentation resulted in an obvious increase in the total concentration of esters of 7.41% to 20.96%, and a decrease in the total concentration of alcohols of 25.06% to 51.38%. The major contributors to variations in total esters were ethyl acetate and phenethyl acetate, which are considered to be the main impact aroma compounds in cider, and those for the alcohols were isoamyl alcohol and isobutyl alcohol. These studies have demonstrated that fermentation conditions and aging affect the final aromatic composition, and then affect the wines sensory value. Moreno and Azpilicueta [30] indicated that during maturation, wine aroma becomes more complex and the color becomes more stable. In wine aging, esters are mainly formed during fermentation by enzymatic conversions. The concentrations attained by esters at the end of fermentation depend on the temperature, the yeast strain that predominates in the fermentation and the nutrients in the medium, especially with concentration of nitrogen compounds and must solids. In maturing wine, esters may be degraded or synthesized through chemical esterification, or remain at constant concentrations. The acetate esters of higher alcohols generally degraded more rapidly than the ethyl esters of fatty acids in both white wine and model solutions. The rate of hydrolysis of fatty acid esters varied in proportion to their molecular weight. Consequently, a rapid degradation of heavy esters was observed. Although the preference for all groups of *M. nitidum* wine after 20 days aging at 15 or 25°C were lower than 7. The acceptances of these wines were still high. The *M. nitidum* wines in this present study possess further value for wine manufacturing.

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