Journal of Food Science and Agricultural Technology

International peer-reviewed scientific online journal

Published online: http://rs.mfu.ac.th/ojs/index.php/jfat

Original Research Article

Potential of Natural Isolated Yeasts from Thai Vineyard and their Ability to Growth in Limited Nitrogen Source of Wine Production

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ARTICLE INFO

Article history:

Received 31 July 2018

Received in revised form 31 December 2018

Accepted 08 January 2019

Keywords:

Grape varieties

Hydrogen sulfide

Isolated yeast

Low nitrogen

Wine production

ABSTRACT

In wine fermentation process, yeast species are essential for providing good taste of wine. Effective yeast also can be grown under limited essential nutrients especially nitrogen. However, limitation of nitrogen source leads to stress condition which induce yeast to produce hydrogen sulfide (H_2S) resulting in unacceptable quality of wine. Moreover, it may lead to incomplete fermentation process. This research focused on isolation of natural yeasts in five grape varieties (Syrah Granmonte, Syrah J&J, Durif, Viognier and Chenin blanc) growing in Thai vineyard and evaluation their ability to grow in low nitrogen condition during fermentation without H2S production and providing a good flavor of wine. The relationship between isolated yeasts on yeast assimilable nitrogen (YAN), viable cell count, soluble solid and sensory test were determined during four week of fermentation. The results showed that isolated yeast from Viognier grape variety was the best performance for longest fermentation without H2S production. The highest viable cell in certain isolate was found at 1.02 x 10^8 CFU/ml with highest YAN as 262.5 mgN/L. In addition, sensory evaluation by cultivation of grapes and wine director, viticulture and winemaking from Asoke valley winery, found that isolated yeast from Viognier grape provided the best flavor with rich alcohol taste from the second week of fermentation while the sugar taste was relatively low. Furthermore, nucleotide sequencing data at 26S RNA gene showed that potential isolated yeasts all belong to *Saccharomyces cerevisiae* NRRL Y-12632 (100% similarity) which is GRAS (General Regard as Safe) species. Therefore, this suggests that three isolated yeasts (Granmonte, Viognier and J&J Syrah varieties) have potential for further development in wine fermentation process with minimal nitrogen requirement.

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Published by School of Agro-Industry, Mae Fah Luang University

ISSN: 2408-1736

INTRODUCTION

Fermentation problems are often vineyard-specific especially in nitrogen deficiency in grapes juice. Many juice or musts lack sufficient assimilable nitrogen and other components needed by yeast for fermentative growth (Morris et al., 2016) Therefore, addition of nitrogen source like diammonium phosphate (DAP) or specific media before wine production result in increased operating's time and cost. Yeast strain may be significant in terms of nitrogen requirements for uptake and release of specific amino acids and relative to sugar fermentation (Henschke and Jiranek, 2013). For commercially *Saccharomyces cerevisiae* wine strains, concentrations of yeast assimilable nitrogen (YAN) should be 250–500 mg N/L available yeast to produce cellular proteins needed to meet the worst environmental conditions (Gump et al., 2001; Vilanova et al., 2017). Moreover, insignificant nitrogen source to be amino acid precursors result in off-flavors of hydrogen sulfide (H2S) compounds derived from yeast metabolism during alcoholic fermentation (Boudreau et al., 2017; Kinzurik et al., 2016; Ugliano et al., 2011). In wine fermentations, H2S is formed by reduction of exogenous sulfate during biosynthesis of the sulfur-containing amino acids, cysteine and methionine (Franco-Luesma and Ferreira, 2016; Moreira et al., 2002). Hydrogen sulfide only 1.6 µg/l in wine can be detected (Ugliano et al., 2009). In addition, previous studies suggested that the addition of YAN to wine fermentations may not reduce H2S formation, but it appears to relate with individual yeast strain (Giudici et al., 2013; Hine and Mitchell, 2015). Isolated yeast by natural method was considered to obtain a specific wine characteristics. Furthermore, isolated yeasts from wine growing area have been shown to be better adapted to specific environmental conditions and substrates (Esteve-Zarzoso et al., 2000; Clemente-Jimenez et al., 2004). Natural yeasts have been reported to contribute either positively sensory characteristics of wine and useful or beneficial for wine production (Carrau, 2008). The aim of this study was to investigate ability of isolated natural yeast from Thai vineyard to growth in limited nitrogen source during fermentation of wines from different grape varieties with acceptable flavor in finished wine.

MATERIALS AND METHODS

Isolation of natural yeasts

Six varieties of healthy and undamaged grape berries (Vitis vinifera) include Viognier, Chenin blanc, Verdelho, Durif, Granmonte Syrah and J&J Syrah were collected at Granmonte vineyard, Nakhon Ratchasima, Thailand (Figure 1) by aseptic technique. They were squeezed and the juice collected in separate sterile flasks (500 ml/flask). The sample was spontaneously fermented (26OC) and the samples were collected 3 times per week (2-4 weeks) during the whole fermentation process. Undiluted samples were plated on yeast extract peptone-dextrose agar medium (YEPD) supplemented with 250 mg/ml streptomycin sulphate (Scacco et al., 2012). The single colonies were obtained by Koch's dilution method (2007). The yeasts were purified by subsequent streaking on YEPD medium. Pure culture of each natural strains was kept on glycerol stock and stored at -20oC until used.

Figure 1. Six varieties of grape berries included Viognier (A), Chenin blanc (B), Verdelho (C), Durif (D), Granmonte Syrah (E) and J&J Syrah (F) were used in this experiment.

Screening of Yeast for ethanol tolerance

Each identified indigenous strains were used for the screening of the yeast for ethanol tolerance. Ethanol tolerance was tested in 5 mL of YPD medium supplemented by 5, 9, 13, and 17% (v/v) ethanol and the tubes were inoculated by cell concentration at 6 log CFU/mL (Osho, 2005). Inoculated tubes were cultivated at 26 ◦ C for 10 days. The culture was streak on YPD medium and incubated at 26°C for 48 h. The growth of colonies was screened. Only the yeast strains that showed growth in 9% ethanol (v/v) were further examined.

Screening of Yeast for hydrogen sulfide (H2S) production

H2S production by selected ethanol tolerance strains was tested on indicator Biggy agar (Himedia, India). After incubation at 26°C for 3–5 days, the zone surrounding the colony was evaluated by the color of the colonies as the follows: + white colonies; ++ light brown; +++ brown; ++++ dark (Suranská et al., 2016). Only the yeast strains that showed white until light brown colonies as were further examined.

Wine fermentation

The fermentation potential of two yeast strains from each grape varieties was evaluated in Laboratory-scale comparing with commercial yeast strain (code C) (IOC R 9008, *Saccharomyces cerevisiae*, Lallemand Australia Pty Ltd, Australia). White wine must was prepared from Viognier (yeast's code: VC, V3 and V4) and Chenin blanc (yeast's code: CC, C10 and C13) while Red wine must was prepared from Durif (yeast's code: DC, D7 and D8), Granmonte Syrah (yeast's code: GC, G1 and G2) and J&J Syrah (yeast's code: JC, J5 and J6). For red wine, after crushing the must was macerated at 0°C for 24 h. All musts were treated with potassium metabisulfite (30 mg/l) before used (Longo et al., 2018). Each wine was produced by mono-culture of isolated yeast strain in duplicate at 18OC for 4 weeks with 2000 ml each grape varieties must (°Brix below 24, adjust pH to 3.5) without added diammonium phosphate (DAP) as nitrogen source. The starter inoculate of each fermentation was 6 log CFU/ml. The fermentations were carried out under static conditions.

Chemical and microbiological analyses of wines

Yeast assimilable nitrogen (YAN) consumption profiles for each potential strains were determined during fermentation using the formaldehyde method (Gump et al., 2001). Soluble solids content (°Brix) was determined by digital refractometer (ATAGO, Tokyo, Japan). Viable cell count at different stages of fermentation was determined by microscope count using a hemocytometer. The fermentation progress was monitored during four week of fermentation. Each analysis was performed in duplicate.

Sensory analysis

Fifteen judges were recruited from Asoke valley winery co.th, ranging ages from 24 to 45. Candidates were already trained their sensory performance on basic tastes and the aromas of wines (UNE 87-013:1996). Five of candidates are wine director, viticulture and winemaking experts. The panels selected descriptive attributes

regarding color appearance, odor and taste of wine with five-point intensity scale (ISO, 2003) every 7, 14, 21, 28 days. The wines were tested in triplicate. Forty milliliters of each wine was served at 22 °C±1 °C and covered to prevent volatile loss.

Identification of potential yeasts isolation by molecular analyses

Three potential yeasts from different wines include Viognier (V3), Granmonte Syrah (G2) and J&J Syrah (J5) were DNA sequencing analyzed. Genomic DNA was isolated, and amplification of D1/D2 domain of 26S ribosomal RNA sequence genes. The divergent D1/D2 domain of 26S rDNA was amplified with primers NL-1 (59- GCA TAT CAA TAA GCG GAG GAA AAG-39) and NL4 (59-GGT CCG TGT TTC AAG ACG G-39) (Kurtzman and Robnett, 1998). Sequencing was carried out on an ABI PrismTM BigDyeTM Terminator Cycle Sequencer (Applied Biosystems, Stafford, USA) using Thailand Bioresource Research Center service. Subsequently, the sequences of D1/D2 domain of 26S rDNA were compared by BLASTn Homology Search (http://www.ncbi.nlm.nih.gov/blast).

Statistical analysis

ANOVA and Duncan's multiple range tests of differences for strains inoculated in fermentation performance included cell growth, YAN consumption, soluble solid and sensory analysis. Statistical analyses were performed using the SPSS version 16.0 (SPSS Inc., Chicago, Illinois). Significant differences with a value of $P < 0.05$.

RESULTS AND DISCUSSION

Isolation of natural yeasts

The 232 different morphological isolated natural yeast colonies were obtained from the fermenting each grape juices during 2-4 week. For white wine fermentation, Viognier musts showed highest isolated yeast colony (52 colonies) within 2 weeks fermentation follow by Chenin blanc (31 colonies) and Verdelho (18 colonies) respectively. For red wine fermentation, highest isolated yeast colony belonged to Granmonte Syrah (64 colonies) follow by J&J Syrah (42 colonies) and Durif (25 colonies). Only 25 of the 232 isolates were able to grow above 9% (v/v) ethanol concentration and lower hydrogen sulfite (H2S) production (Figure 2). Hydrogen sulfite (H2S) production relative to levels of sulphite reductase activity in yeast strains can be estimated by colony color (Ugliano et al., 2011). Two colonies from each grape musts and one commercial strain (IOC R 9008, *Saccharomyces cerevisiae*) were selected to further study for wine production. However, none of yeast colonies from Verdelho musts growth above 9% (v/v) ethanol concentration.

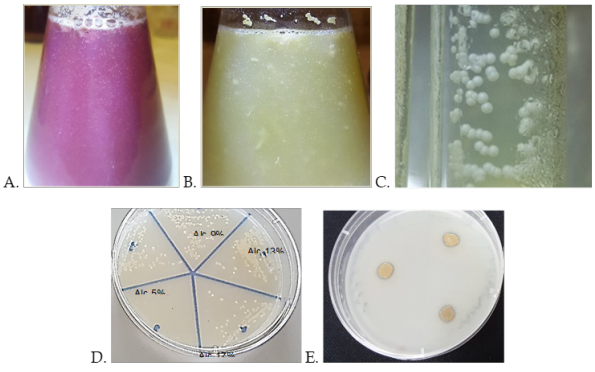

Figure 2. Two week fermentation of Granmonte Syrah musts (A); Viognier grape musts (B); colony characteristic (C), different of ethanol-tolerance (D) and H2S production (E) by G1 isolated yeasts.

Viable cell count during wine fermentations

The microbiological monitoring performed first period of wine fermentation (48 h) every three hours and second period for twenty-eight day until the end of fermentation (Figure 3). The result in first period of fermentation showed that J&J Syrah wine with commercial strain (8.01 log CFU/ml) and J5 strain (8.09 log CFU/ml) reached maximum cellular concentration within first day of fermentation without significant difference. However, in Durif wine (C10 and C13), yeast strains cannot grew than 4.78 and 4.85 log CFU/ml within 24 h and grew less than 1 log CFU/ml in 48 h. During fermentation, found that most of the sample could maintain cellular concentration between 5.20 (C10) - 8.26 (J6) log CFU/ml at the third day of fermentation until final day (28 days) with 7.29 (D8) – 8.14 (G1) log CFU/ml. Highest cell growth during fermentation found at 8.38 log CFU/ml (1.02 x108 CFU/ml) with Granmonte Syrah wine in G1 strain on day 25. The rapid growth of isolated yeasts found in Granmonte Syrah wine inoculated with strain G1 while the lowest growth found in Chenin blanc white wine inoculated with C10 and C13 strain. The result indicated that those isolate strain had the longest lag phase and needed time almost two week for adaptation. Therefore, some of isolated yeast strain have potential to growth as same as commercial strain especially in Granmonte Syrah, J&J Syrah and Viognier varieties consistently with Scacco et al (2012) reported that isolated indigenous yeast can be extended of fermentation to 22 days as same as commercial strain.

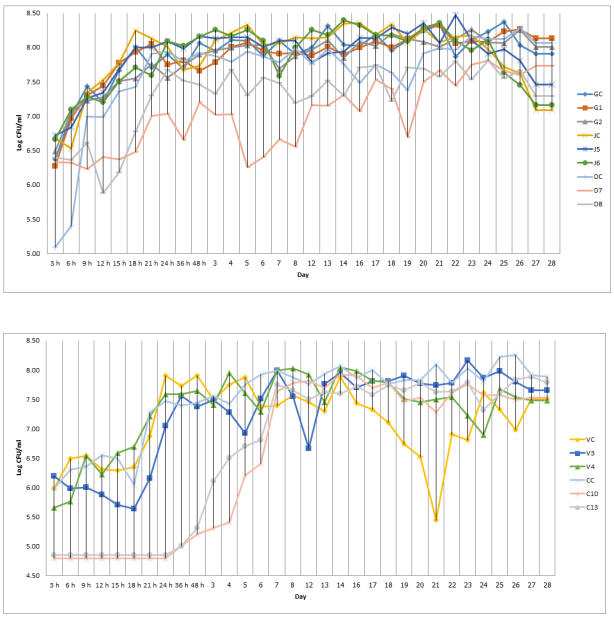

Figure 3. Viable cell count of six isolated yeasts in red wine (A), four isolated yeasts in white wine (B) compare with commercial yeast strain (GC, JC, DC, VC and CC) during fermentation.

Soluble solid content during wine production

The soluble solid content (^{0}Brix) can be refer to trend of sugar content in wine (Comitini et al., 2011). Grape juice usually contains approximately equivalent concentrations of glucose and fructose sugars. However, glucose is fermented preferentially to fructose (Gump et al., 2011). The soluble solid content during wine fermentation time is shown in Figure 4. At the first period of fermentation, viognier wine (V3) showed the highest soluble solids at 29^{0}Brix followed by Durif wine (D5) at 27.2 ^{0}Brix. When related with viable cell count found that the optimum of soluble solid content for maximum growth of isolated strain should be range from

19-20^{0}Brix. Suarez-Lepe and Morata (2012) reported that when sugar levels range from 25 to 30°Brix, yeast starters should be prepared at greater than 106 yeast cells/ml due to high osmotic pressure associated with high sugar concentrations which could inhibit yeast growth. The reduction of soluble solid content found along with wine fermentation. Viognier wine (V3) showed the highest reduction of soluble solid content from 29 to 9 °Brix. In the end of fermentation (28 day), Viognier wine (VC) showed lowest soluble solid at 2.8 ^{o}Brix. Chenin blanc (C13) found highest soluble solid at 11.3 ^{0}Brix relate to slowly growth of yeast cell (4.75 –7.79 log CFU/ml) result in lower sugar consumption. This indicated that soluble solid content affect the growth factor of isolated strain especially in white wine that had lower soluble solid than red wine.

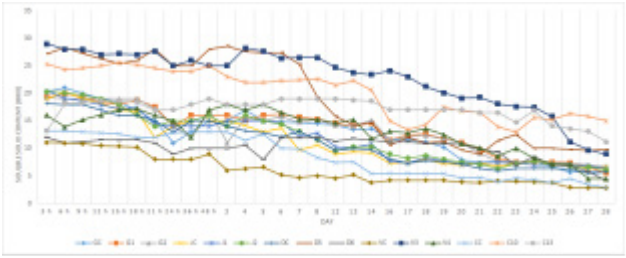

Figure 4. The soluble solid content during white and red wine fermentation inoculated with isolated yeast and commercial yeast.

Yeast assimilable nitrogen (YAN) during wine fermentation

YAN was calculated as the sum of ammonia-derived nitrogen (Ugliano et al., 2009). Moreover, nitrogen supplementation strong related with amount of H2S produced by yeast. Mendes-Ferreira et al. (2014) reported that DAP supplementation can be disadvantage since it strongly affect to up-regulation of MET genes and maximum H2S formation. In the non-supplemented wine fermentations found content of YAN ranging from 105 - 262.5 mg/l YAN at inoculation day (Figure 5). YAN content in red wine fermentation (21.14 - 262.5 mgN/L) higher than white wine (8.75 – 196.88 mgN/L) from increased viable cell count during fermentation. Among of graph verities, Granmonte Syrah wine and J&J Syrah wine showed the highest YAN content followed by Viognier, Durif and Chenin blanc, respectively. However, YAN content in Granmonte Syrah and J&J Syrah wine were rapidly decreased in the first week of fermentation (7 day). The faster cell growth rate in those wines result in higher YAN consumption. In the meantime, Durif wine (D5) and Viognier (V3) wine showed the increase of YAN content increase in second week of fermentation and maintained YAN content until third and fourth week. This resulted indicated that there are some nitrogen resource left on raw material or yeast strain slowly used of nitrogen. For commercial strain found low assimilable nitrogen and trend to produce H2S at the end of fermentation.

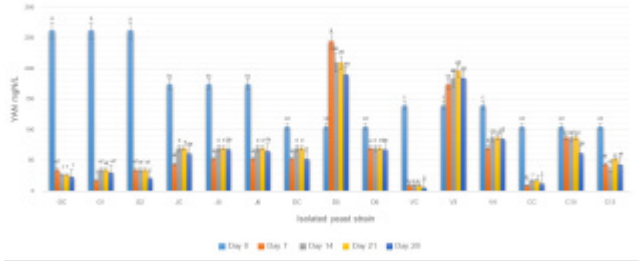

Figure 5. YAN content during white and red wine fermentation with isolated and commercial yeast.

Sensory analysis of wines

Scores of the attributes of the sensory profiles for each sample are reported in Figure 6-7. Red and white wines were described by referring to appearance (color intensity), seven to aroma and tastes.

For Wine appearance, color intensity of red and white wines was determined by appearance score (Figure 6). For red wine, the score was divided into pale red (1), bright red (2), red-purple (3), purple (4) and dark purple (5). For white wine, the score was divided into pale yellow (1), yellow-gray (2), yellow (3) and dark yellow (4). In the first week of red wine fermentation, Granmonte Syrah and Durif wines appeared pale and bright red color, while J&J Syrah provided darker color (Figure 6A). During 2-4 week fermentation, color appearance of wines were significance developed from pale to bright red color in Durif wines whereas Granmonte Syrah and J&J Syrah wines developed from red to dark purple. In contrast, white wines showed no significant differences in color intensity during 4 week fermentation (Figure 6B).

Aroma of red wines were determined by seven factors including hydrogen sulfide, fruity, greenish, floral, yeast, pepper and leather, while white wine factors included hydrogen sulfide, fruity, greenish, floral, yeast, grape and oxidized aroma.

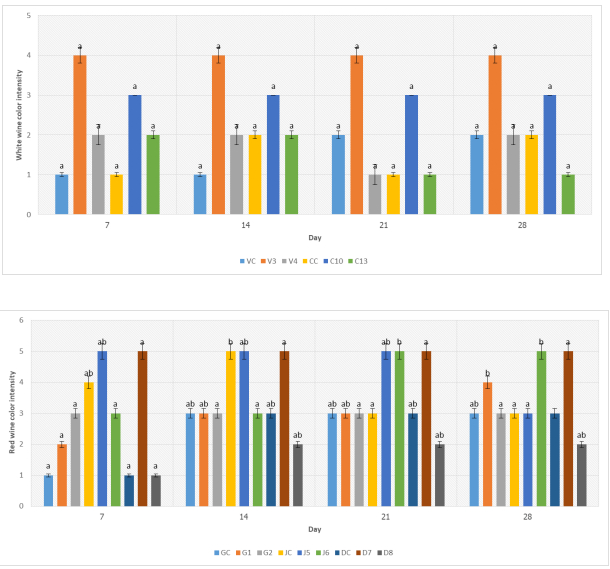

Figure 6 Color Intensity of red wine (A) and white wine (B) during four week of fermentation.

The aroma profiles during fermentation of the different wines are shown in Figure 7-8. Red wines fermented by commercial yeast strains (GC, JC and DC) were distinguished by hydrogen sulfide, greenish odor and pepper aroma due to low YAN content in grape musts. Whereas, red wines fermented with isolated yeast strains provided remarkable sense of fruity especially berry aroma. However, some of them like D8 strains still produced hydrogen sulfide sense. Red wine from Granmonte Syrah (G2) and J & J Syrah (J5) wine showed moderate intensity of floral aroma and low sense of hydrogen sulfide. As same as in white wine, commercial yeast strains (VC and CC) were detected by hydrogen sulfide and greenish odor, while isolated strain could produced fruity aroma especially in Viognier (V3 and V4) wine. Intense floral aroma could be found in Chenin blanc (C10) wine. However, both C10 and C13 wine still maintained grape aroma, yeast and greenish odor since its low

fermentation rate until final week of fermentation. The results indicated that different grape varieties and yeast strain could lead to diverse sensory profiles in wine, depending on the function of yeast strains and nutrient in raw material during wine fermentation process. Moreover, longer fermentation had an effect to increase all aroma sense in both red and white wine. Swiegers et al. (2009) studied isolation of natural strains fermented in Sauvignon Blanc wine found that those wines developed more overall sensory complexity and reinforced the global aromatic intensity when comparison with the wines inoculated with commercial strain alone.

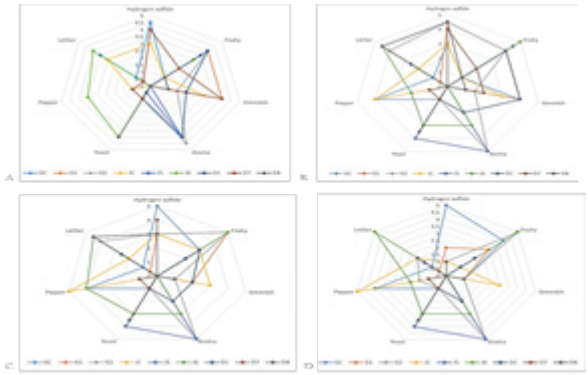

Figure 7 Aroma profiles of red wine during 7(A), 14(B), 21(C) and 28(D) of fermentation day.

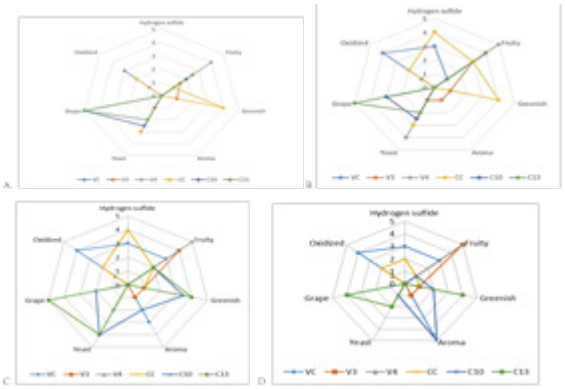

Figure 8 Aroma profile of white wine during 7(A), 14(B), 21(C) and 28(D) of fermentation day.

Sensory profiles of wine taste were distributed to seven tastes including bitterness, greenish, hydrogen sulfide, fermented, fruity, acidity and ethanol taste among four week fermentation (Figure 9-10). For red wine, the strongest bitterness taste from tannin in grape musts found in both Syrah wines until end of fermentation. Only commercial strain (DC) in Durif wine showed bitterness taste. Greenish tastes were detected only with Granmonte Syrah wine in the first period of fermentation (7 day). However, taste of H2S showed less detected than aroma factor in Granmonte Syrah (G1) in second and fourth week of fermentation. This resulted from low YAN content since second week of fermentation (17.5 -35 mg N/L). Whereas Durif wine can maintained YAN content (52.1-210.5 mg N/L) along fermentation period, without H2S detection. All of isolated strains still maintained their ability to produce fruity taste in wine better than commercial strain which developed fruity taste in final period of fermentation. Acidity taste could be found only with red wine fermented with commercial strain. Meanwhile, alcohol taste found no significant differences among each wine samples.

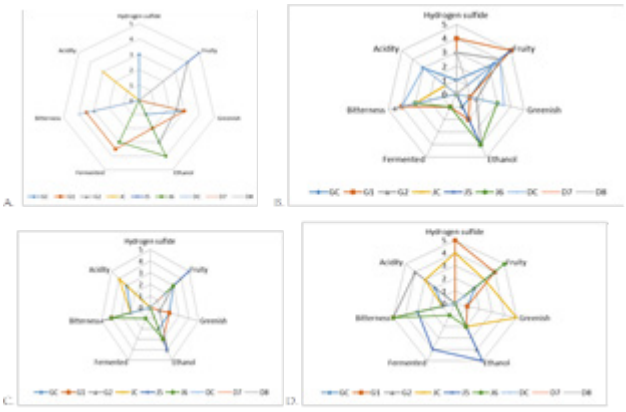

Figure 9 Taste profiles of red wine during 7(A), 14(B), 21(C) and 28(D) of fermentation day.

In white wine, strong bitterness taste found in wine fermented by isolated yeast (V3, V4 and C13) wine except last week of fermentation found in Viognier wine fermented with commercial strain (VC). Hydrogen sulfide taste was detected only with Chenin blanc variety with commercial strain (CC) as low YAN content (8.75-17.55 mg N/l) during second until four week of fermentation. Viognier (V3) wine had significant fruity flavor (best flavor) with rich alcohol and low sugar taste from the second until four week of fermentation. In contrast, Chenin blanc wine showed rich acidity flavor and still had sugar taste due to low fermentation rate. In addition, Viognier wine with commercial strain (VC) provided tasteless wine.

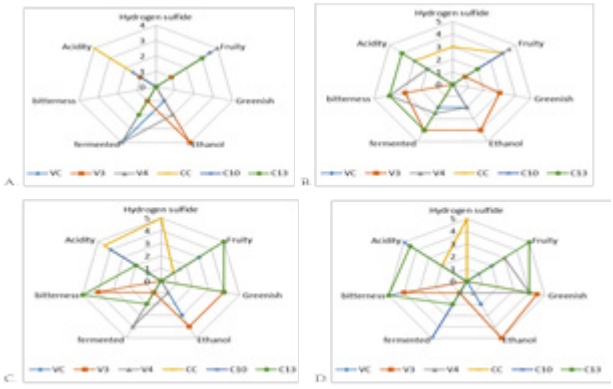

Figure 10 Taste profile of white wine during 7(A), 14(B), 21(C) and 28(D) days of fermentation day.

In both red and white wines, the complexity and intensity of taste and aroma results varied during fermentation time. For red wine, Granmonte Syrah wine with G2 strain and J&J Syrah wine with J5 strain provided good flavor with intensive aroma and signature fruity taste. Viognier wine fermented with V3 strain also showed ability to produced good taste and aroma of white wine.

Identification of potential yeast strains

Potential of isolated yeast strains in different wines (Granmonte Syrah (G2) wine and J&J Syrah (J5) wine and Viognier (V3)) before further industrial used was determined. These potential yeast isolates were identified according to D1/D2 domain of 26S ribosomal RNA sequence genes polymorphisms. Each isolated yeast strain showed PCR product around 600 bp. The PCR products were

sequenced and compared to the available DNA sequence databases. Three isolated yeast showed 100% similarity sequence homology to *Saccharomyces cerevisiae* NRRL Y-12632T (NG042623) with 578 nucleotide sequence. This yeast strain was descripted by previous studies as ethanologenic yeast (Liu and Moon, 2009), invertase production strain for confectionaries and food industries (Rashad and Nooman, 2009), fermented yeasts in traditional sorghum beer (N'guessan et al., 2011). Moreover, this strain identified as GRAS (General Regard as Safe) species (Guzzon et al., 2014). Therefore, this suggests that three isolated yeast has potential for employing in wine fermentation process with minimal nitrogen requirement.

CONCLUSIONS

Studies on fermentation efficiency and assimilable nitrogen content requirement of isolated yeast from Thai-vineyard on five graph varieties (Syrah, Durif, Viognier, Verdelho and Chenin blanc). Yeast isolated from Verdelho grape musts found lack of ethanol-torlence. The high efficiency of fermentation found in grape varieties of Granmonte and J&J Syrah with the highest viable cell during fermentation. Meanwhile, Chenin blanc grape variety prolonged lag phase to five days before the start of fermentation. Whereas, other isolated yeast can be adapt and began to ferment within 6-15 h after inoculation. YAN content of different wines depended on viable yeast cell and different grape musts. The sensory evaluation indicated that YAN content in each fermentation period related to hydrogen sulfide odor and taste. Commercial yeast frequently produced hydrogen sulfide odor due to lack of nitrogen source while isolated yeast strains did not. During wine fermentation, specialists sensory found that Durif wine developed unacceptable taste even none of hydrogen sulfide production. Isolated yeasts from Granmonte (G2) and J&J Syrah varieties (J5) began to produce hydrogen sulfide in the two week of fermentation, but those strains developed well-established flavor and relatively high alcohol content. Moreover, isolated yeast (V3) from Viognier variety was faster fermentation, which is considered to development of color (yellow gold) and flavor in positive way with relatively high alcohol content. Molecular identification of those strains belonged to safe alcohol production strain (*Saccharomyces cerevisiae*). Therefore, three isolated yeast can be recognized strain for highly fermentation efficacy with limited nitrogen source and provided good taste product. Those strains can be application to further development for Thai wine industries.

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