

Antioxidant and Anthocyanin-Rich Vinegar Fermented from Thai Colored Rice Varieties

Pramuan Saithong¹, Jirawut Permpool² and Supachai Nitipan^{2,*}

¹Department of Applied Microbiology, Institute of Food Research and Product Development, Kasetsart University, Bangkok 10900, Thailand

²Department of Biology, Faculty of Science, Thaksin University, Phattalung Campus, Phattalung 93210, Thailand

(*Corresponding author's e-mail: nisupachai@tsu.ac.th)

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Abstract

This study aims to produce antioxidant and anthocyanin-rich vinegar using three types of Thai rice through surface culture fermentation (SCF). Three varieties of Thai rice with different colors, including Khao Dawk Mali 105 (white color), Sangyod (brown color) and Hom Nil (black color), were used as a substrate for vinegar fermentation. The different Thai rice varieties were hydrolyzed by boiling and enzymatic hydrolysis. The sweetness of the hydrolyzates was adjusted by adding granulated sugar at 20 °Brix and using it as a substrate. A mixed culture of *Saccharomyces cerevisiae* var. burgundy and *S. cerevisiae* var. kyokai was used as a yeast starter for alcohol fermentation. After 4 weeks of fermentation, *Acetobacter aceti* TISTR 354 required only 10 days to convert alcohol into acetic acid by the surface-culture fermentation method. Vinegar produced from the Thai rice varieties contained acetic acid at a concentration of more than 4.0 % (v/v), with residual alcohol concentrations less than 0.5 % (v/v). Hom Nil vinegar presented an anthocyanin content of 2.39 ± 0.44 mg/L and had the highest antioxidant activity, with 428.47 ± 4.04 g/mL (Total phenolic content; TPC), 71.80 ± 0.00 g/mL (Ferric reducing antioxidant power assay; FRAP), and 49.27 ± 0.27 % inactivation (2,2-Diphenyl-1-picrylhydrazyl; DPPH). A sensory panel rated the vinegar fermented from the three varieties as 'like slightly' on a 9-point hedonic scale.

Keywords: Fermented rice vinegar, Thai rice, *Acetobacter* sp., Surface culture fermentation method, Antioxidant activity, Functional food, Anthocyanin

Introduction

Vinegar is an acidic, fermented beverage produced from various sources, including grains, fruits, sugar or molasses. Acetic acid imparts the characteristic sour taste to vinegar, which is widely used for food preservation and as a flavor enhancer in culinary applications such as salad dressings, mustard, pastries, ketchup and canned goods [1,2]. The production of vinegar involves fermenting sugars or starches with alcohol to generate acetic acid. According to established standards, vinegar must contain a minimum of 4 % (v/v) acetic acid composition, residual alcohol concentration less than 0.59 %, and a pH level between 2.5 and 1.5 [3].

Rice vinegar is distinguished by its primary ingredient: rice. For instance, red rice vinegar, widely used in Chinese cuisine, is produced in the Fujian province. *Monascus* sp. utilizes red koji to ferment rice into red yeast rice vinegar. Kurosu, a Japanese black rice vinegar, is crafted from unprocessed rice, including the bran and germ [4]. Due to its rich nutritional profile, Kurosu finds extensive use in beverages. Additionally, black glutinous rice vinegar is popular in East Asian countries, recognized for its nutritional attributes [5]. Colored rice varieties are known for their abundance of phenolic compounds, with several studies highlighting their potential positive effects, including anti-inflammatory properties [6], inhibition of cancer cell growth [7,8], as well as antioxidative and anti-diabetic properties [7-10].

Many varieties of Thai rice offer valuable nutrients and distinct flavors, characterized by different grain features and colors. Antioxidant activity has been linked to the anthocyanin content in Thai colored rice varieties, such as black glutinous rice and black fragrant rice. Generally, higher anthocyanin concentrations correspond to more potent antioxidant effects. Furthermore, Thai colored rice exhibits a higher anthocyanin concentration compared to non-colored or polished rice [11]. Among the eight types of rice studied, black glutinous rice has shown the highest anthocyanin concentration, featuring cyanidin

3-glucoside and peonidin 3-glucoside as the predominant anthocyanins [6]. Several studies have indicated the positive impact of anthocyanins derived from purple rice on human health. These benefits have been examined through both *in vitro* and *in vivo* research. *In vitro* studies have revealed the advantages of anthocyanins, including their potential to reduce the risk of obesity [12,13], anti-inflammatory properties [14] and antiviral enhancements [15]. In contrast, *in vivo* studies have demonstrated that cyanidin 3-glucoside can mitigate oxidative stress and inflammation in the kidneys [16], alleviate allergic airway inflammation in lung tissue [17] and enhance immune responses in rats [18].

Hence, the objective of this study is to employ the SCF method to produce vinegar using three distinct varieties of Thai rice: polished rice (Khao Dawk Mali 105, also known as Jasmine rice 105), red rice (Sangyod), and black rice (Hom Nil). Our aim is to enhance the health benefits of rice vinegars by determining their antioxidant properties and anthocyanin concentrations. The results obtained may contribute to the development of novel functional foods with potential health benefits.

Materials and methods

Raw material

Three Thai rice varieties including Khao Dawk Mali 105 (KDML105) white rice, Sangyod rice (SYR) red and Hom Nil rice (HNR) black color, which were collected. The rice was kindly supplied by courtesy of Phatthalung Rice Research Center, Phatthalung, Thailand.

Saccharification process by commercial enzyme

Each rice variety was carefully washed and soaked in water for 4 h. Then, a 1:3 ratio of water was added. After heating the rice to 90 °C, 0.1 % (v/v) alpha-amylase from *Bacillus licheniformis*, known as Termamyl®, was added. The temperature was maintained for 30 min. The mixture was then cooled to 50 °C and 0.1 % (v/v) glucoamylase or amyloglucosidase (AMG) from *Aspergillus niger* (Novo Nordisk Co., Bagsvaerd, Denmark) was added. The reaction mixtures were incubated at 50 °C for 20 h. Following this, the total soluble solids (TSS) and anthocyanin contents of boiling and enzymatic rice hydrolysates were measured.

Alcoholic and acetous fermentation

The enzymatic hydrolysate of rice was sugar-adjusted to 22 °Brix (Pearson's square formula). The pH was then adjusted to 3.5 - 4.0 with citric acid, and 0.1 % diammonium phosphate (DAP) was added. The mixture was heated to 90 °C for 5 min. A mixed starter of *S. cerevisiae* var. burgundy and *S. cerevisiae* var. kyokai (supplied by Kasetsart University's Institute of Food Research and Product Development) was added and fermented for 16 h. After inoculating the yeast starter, the rice mixture fermented for 4 weeks or until the alcohol reached a stable state. During the initial week, the mixture was stirred daily. The alcohol content, acidity as lactic acid, and TSS of a sample were monitored weekly.

According to the SCF method reported by [19] acetic fermentation was performed in two stages. Initially, a 100 mL of Thai rice vinegar starter was performed following 90 mL of sterilized hydrolysate was sugar-adjusted to 5 °Brix. Seven milliliters of *A. aceti* TISTR 354 and 3 mL of 95 % ethanol were added. Before usage, the mixture was incubated at 30 °C for 72 h. A sanitized stainless-steel tray containing 600 mL of rice water with a 5 °Brix sugar content, along with 300 mL of rice wine and 100 mL of starter, was covered with a plastic sheet pierced with small holes and left to stand for 48 h at room temperature. In the second stage, 1,000 mL of rice wine with a 10 - 12 % (v/v) alcohol concentration was added into the mixture. The mixture was allowed to stand for 5 - 7 days. Samples were collected and assessed for acidity, pH value and residual alcohol concentration. The obtained rice vinegar was filtered through Whatman No.1 filter paper with pore size 11 µm and stored in a sealed container until required for analysis.

Alcohol concentration and TSS

Alcohol concentration was assessed using an ebulliometer (Model #360, Laboratoires Dujardin-Salleron, Paris, France) by measuring different boiling points between water and the sample solution. TSS was measured at 20 °C using a hand-held refractometer (N-1, 0 - 32 °Brix, Atago, Japan) to report data in °Brix immediately. Each experiment was carried out in triplicate.

The chemical properties and antioxidant activities

The pH was measured using a pH meter (Model 430, Corning, NY, USA). Titratable acidity, expressed as acetic acid, was assessed using titration methods. The total phenolic content (TPC) was determined as follows: 0.2 mL of vinegar samples were mixed with 0.8 mL Folin-Ciocalteu reagent (10.0

% (v/v)) and incubated for 8 min at room temperature in the dark. Then, 7.5 % (w/v) of sodium carbonate solution was added and the mixture was further incubated for 90 min at room temperature in the dark. Absorbance was measured at 760 nm using a UV-vis spectrophotometer (Genesys 10S, Thermo Scientific), with all determinations performed in triplicate.

Antioxidant activity assay

DPPH radical scavenging assay

The DPPH radical scavenging assay was assessed following the modified method of Brand-Williams *et al.* [20] using DPPH. In this assay, 50 μ L of rice vinegar samples were mixed with 950 μ L of a DPPH solution with a concentration of 0.0394 g/L. The reactions were incubated at room temperature for 30 min in the absence of light. Subsequently, the absorbance was measured at 515 nm using a UV-vis spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA), with all determinations performed in triplicate. The concentration was reported as percentage of inactivation.

$$\% \text{ DPPH inactivation} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c = Control absorbent, A_s = Sample absorbent

Ferric reducing antioxidant power (FRAP) assay

The assay was performed using standard chemicals, specifically the FRAP reagent and Trolox. In this procedure, 50 μ L of the vinegar sample was combined with 950 μ L of the FRAP reagent (300 mM/L of acetate buffer: 20 mM/L of ferric chloride: 10 mM/L of 2, 4, 6-tripyridyl-s-triazine). The mixture was then incubated in darkness at room temperature for 4 min. Subsequently, the absorbance was measured at 593 nm using a UV-Vis spectrophotometer, with all determinations performed in triplicate.

Anthocyanin contents

Anthocyanin contents of the hydrolysates and rice vinegar samples were assessed using the pH-differential method. Based on structural changes in chemical forms of anthocyanin and absorbance measurements at pH 1.0 and 4.5, a 1,000 μ L of sample solutions was prepared in two dilutions: one with potassium chloride buffer (pH 1.0) and the other with sodium acetate buffer (pH 4.5). Absorbance at 530 and 700 nm was measured against distilled water blanks. Anthocyanin concentration was expressed as cyanidin-3-glucoside equivalents per 100 g, with all determinations performed in triplicate.

Sensory evaluation

Thai rice vinegar samples were assessed by 40 panelists, including both trained and untrained individuals, using a 9-point hedonic scale to statistically analyze differences in sensory characteristics. The rice vinegar samples were served at 28 - 30 °C in opaque plastic cups coded with 3 random digits. The attributes of color, aroma, viscosity, flavor and overall impression were evaluated through a 9-point hedonic scale (1 = disliked extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither liked nor disliked, 6 = like slightly, 7 = like moderately, 8 = like very much, 9 = like extremely).

Statistical analysis

Statistical analysis of variance (ANOVA) followed by Duncan's multiple range test were used to calculate significant differences between disparate samples ($n = 3$) using SPSS software v.12.0 (IBM Analytics, USA). Means were considered significantly different at $p \leq 0.05$.

Results and discussion

Enzymatic hydrolysis

KDML105, SYR, and HNR could be hydrolyzed by boiling and enzymatic hydrolysis, which included alpha-amylase (Termamyl®) and amyloglucosidase. TSS ranged from 9.13 to 9.83 °Brix in the boiling rice hydrolyzate and from 18.67 to 19.50 °Brix in the enzymatically hydrolyzed rice hydrolyzate. The combination of alpha-amylase and amyloglucosidase could increase the sugar content of rice hydrolyzates due to their synergism. Alpha-amylase and amyloglucosidase can hydrolyze both the 1,4- and the 1,6-alpha links in amylose and amylopectin [21]. Liziane *et al.* [22], who examined the effects of a-amylase, amyloglucosidase, and combinations thereof on porous rice starch, found that treatments enhanced waxy

rice starch pores. As shown in **Table 1**, whereas both KDML105 and SYR lacked any detectable anthocyanins, HNR had a total anthocyanin concentration of 5.24 mg/L after boiling and 11.63 mg/L after enzymatic hydrolysis. Similarly, Pattananandecha *et al.* [23] reported high anthocyanin content in purple rice cultivars cultivated at various altitudes in northern Thailand. Among the three rice varieties tested, HNR exhibited darkest color, and its anthocyanin level was higher. Anthocyanins have been determined to be beneficial to the body, possessing medicinal and antioxidant properties [12,14,24-26].

Table 1 Total soluble solids and total anthocyanin content of boiling and commercial enzymatic Thai rice hydrolysates (Mean \pm Standard Deviation).

| Rice samples | Total soluble solids ($^{\circ}$ Brix) | | Total anthocyanin content (mg/L) | |
|--------------|---|-----------------------|----------------------------------|-----------------------|
| | Boiling hydrolysate | Enzymatic hydrolysate | Boiling hydrolysate | Enzymatic hydrolysate |
| KDML 105 | 9.13 \pm 0.06 | 18.67 \pm 0.06 | ND | ND |
| SYR | 9.60 \pm 0.00 | 19.03 \pm 0.06 | ND | ND |
| HNR | 9.83 \pm 0.06 | 19.50 \pm 0.00 | 11.63 \pm 0.54 | 5.24 \pm 0.39 |

Fermentation conditions of rice wine

Using mixed culture of *S. cerevisiae* var. burgundy and *S. cerevisiae* var. kyokai produced the rice wine. Analysis of alcohol concentration, total soluble solids, pH, and lactic acid content in wine showed that KDML105, SYR and HNR all reached 11.83, 12.40 and 12.07 % (v/v), respectively, after 4 weeks of fermentation. TSS decreased from 22 $^{\circ}$ Brix (initial sugar concentration) to 8.0, 7.8, and 7.5 $^{\circ}$ Brix, respectively, (**Figure 1(a)**). The acidity as lactic acid increased from 0.67, 0.71 and 0.73 % (v/v) (initial fermentation) to 0.81, 0.82 and 0.85 % (v/v), whereas the pH value dropped from 3.83, 3.84 and 3.84 to 3.56, 3.49 and 3.47, respectively (**Figure 1(b)**). Sangngern *et al.* [27] reported that mixed culture of *S. cerevisiae* (*S. cerevisiae* var. montache and *S. cerevisiae* var. kyokai) fermented sugar from rice starch into alcohol with concentration of 13.7 - 14.8 %. Taweekasemsombut *et al.* [28] compared different types of *S. cerevisiae*, including *S. cerevisiae* TISTR 5049 and baker's dried yeast, to produce rice wine with an alcohol content of 3.17 - 4.95 % from a TSS of 11 $^{\circ}$ Brix.

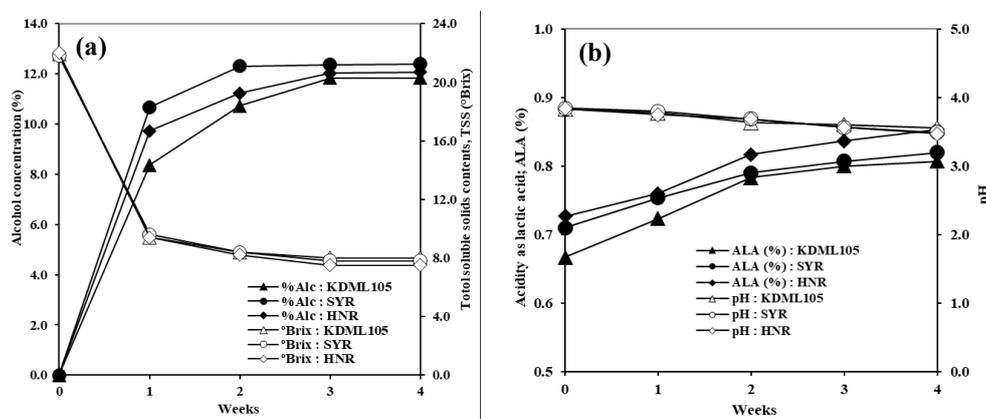


Figure 1 Change in (a) total soluble solids content, TSS ($^{\circ}$ Brix), alcohol concentration (%), (b) acidity as lactic acid (%), and pH during fermentation of 3 varieties of Thai rice wine during 4 weeks. All values are presented as mean \pm SD.

Acetic acid production from Thai rice wine

Saithong *et al.* [19] described the SCF, with the liquid starter *A. aceti* TISTR 354 (range log 9.01 to 9.11 CFU/mL) used for Thai rice vinegar fermentation. Following 72 h of fermentation, the acetic acid content of Thai rice vinegar ranged from 0.23 to 3.47 % (v/v) for KDML105, 0.33 to 3.53 % (v/v) for SYR, and 0.33 to 3.40 % (v/v) for HNR. After adding 1,000 mL of wine from each type of Thai rice (step 2 fermentation), the amount of acidity as acetic acid decreased, ranging 3.47 to 1.57 % (v/v) for KDML105, 3.53 to 1.73 (v/v) for SYR, and 3.40 to 1.73 % (v/v) for HNR. At the end of the fermentation process, the amount of acidity as acetic acid was recorded at 5.14, 5.27 and 6.05 % (v/v) (**Figure 2(a)**). Moreover, pH

values of all samples decreased from 3.76 to 2.69, 3.69 to 2.59 and 3.75 to 2.62, respectively within 10 days of acetous fermentation during the SCF process (**Figure 2(b)**). Maximum residue of alcohol did not exceed 0.5 % (v/v) vinegar in all Thai rice formulae. This work yielded acetic acid concentrations comparable to those reported by Spinoso *et al.* [29] employing submerged fermentation with a commercial starter, the Acetator® (Microdyn, Brazil), for rice vinegar fermentation. The rice vinegar contained 6.85 % (v/v) acidity and 0.17 % (v/v) alcohol. The solid-state fermentation of black rice vinegar using *A. pasteurianus* TISTR 102 [28] yielded 6.68 % (w/v) acetic acid. However, our result was greater than that reported by Sangngern *et al.* [27], who utilized starch-degrading enzymes for Riceberry rice hydrolysis. The hydrolysate was fermented to vinegar by SCF with *A. aceti* TISTR 354, resulting in a 5.4 % (v/v) acetic acid concentration. Data were reported as standard based on the main criteria of United State Food and Drug Administration (U.S. FDA) standard regulations and notifications of the Ministry of Public Health, Thailand (No. 204) B.E. 2543 (2000).

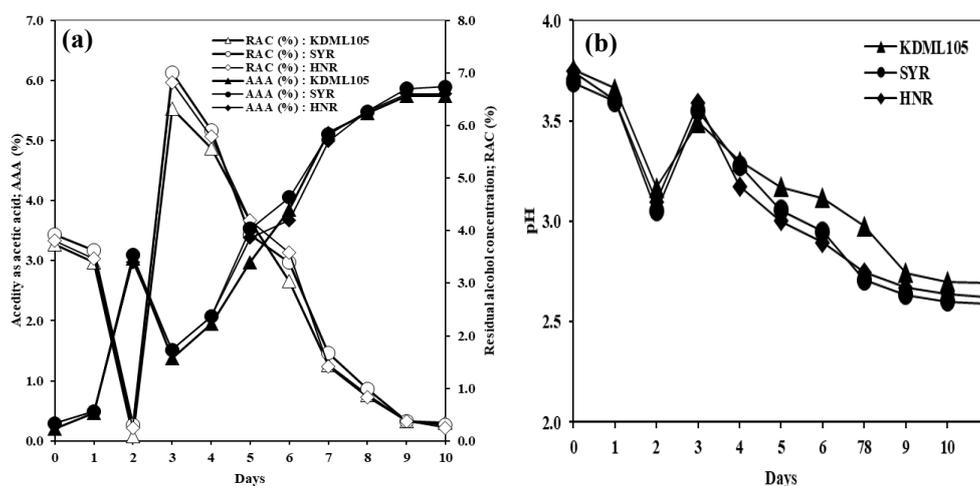


Figure 2 Change in (a) acidity as acetic acid (%), residual alcohol concentration (%), and (b) pH during acetous fermentation of 3 varieties of Thai rice wine following the SCF process. All values are presented as mean \pm SD.

Antioxidant activity of Thai rice vinegar

Analysis of the antioxidant activity in Thai rice vinegar revealed variations among the different rice varieties. HNR vinegar exhibited the highest TPC at 428.47 $\mu\text{g}/\text{mL}$, FRAP at 71.80 $\mu\text{g}/\text{mL}$, and DPPH radical scavenging with 49.27 % DPPH inactivation. Following closely were SYR vinegar with TPC at 363.80 $\mu\text{g}/\text{mL}$, FRAP at 66.88 $\mu\text{g}/\text{mL}$, and 44.71 % DPPH inactivation, and KDML105 vinegar with TPC at 414.47 $\mu\text{g}/\text{mL}$, FRAP at 60.66 $\mu\text{g}/\text{mL}$, and 39.08 % DPPH inactivation. These findings align with a study by Phuapaiboon [30], which observed higher DPPH scavenging activity in germinated Hom Nil vinegar when compared to other colored rice vinegars. However, this study did not identify a direct correlation between anthocyanin content and antioxidant activity. It is worth noting that antioxidant ability may be influenced by various factors, including phenolic compounds [31], flavonoid content, and anthocyanin content [32]. Importantly, HNR vinegar was the only recipe in our study that contained anthocyanin, with a concentration of $2.39 \pm 0.44 \text{ mg}/\text{L}$ (**Table 2**). Reports by Kammapana [33] and Ratsewo *et al.* [34] indicated that Hom Nil rice contained anthocyanin at levels of 10.27 mg/100 g DW and 65.85 mg/100 g, respectively. This result is in line with studies demonstrating higher anthocyanin content in blueberry wine when compared to blueberry extract and blueberry vinegar [35]. It is important to note that variations in anthocyanin content may be attributed to factors such as denaturation during the cooking process and sensitivity to pH and aerobic conditions [30].

Table 2 Antioxidant activities and anthocyanin content of Thai rice vinegar produced through enzymatic hydrolysis (Mean \pm Standard Deviation).

| Rice vinegar | TPC ($\mu\text{g/mL}$) | FRAP ($\mu\text{g/mL}$) | DPPH (% inactivate) | Total anthocyanin content (mg/L) |
|--------------|--------------------------------|-------------------------------|-------------------------------|-------------------------------------|
| KDML 105 | 414.47 \pm 2.52 ^b | 60.66 \pm 0.00 ^a | 39.08 \pm 0.23 ^a | ND |
| SYR | 363.80 \pm 4.58 ^a | 66.88 \pm 0.00 ^b | 44.71 \pm 0.14 ^b | ND |
| HNR | 428.47 \pm 4.04 ^b | 71.80 \pm 0.00 ^c | 49.27 \pm 0.27 ^c | 2.39 \pm 0.44 |

Thai rice vinegar sensory evaluation

The results of the sensory evaluation, conducted by 40 panelists using a 9-point hedonic scale, are presented in **Table 3**. In terms of appearance, KDML105 and HNR vinegar received a satisfaction level of 'like moderately' (7.45 \pm 0.64 and 7.80 \pm 0.41), while vinegar from SYR received a 'like slightly' rating (6.48 \pm 0.51). SYR scored 6.03 \pm 0.73 in the color category, while HNR vinegar was highly rated at 'like very much' (8.03 \pm 0.70). This difference might be attributed to the light orange-yellow color of SYR vinegar, which was less visually appealing compared to the other two formulations resembling the natural color of rice grains. Regarding flavor, taste, and overall acceptance, all three vinegar formulations received similar satisfaction scores: 'like moderately', 'like slightly' and 'like moderately', respectively. It is worth noting that, based on sensory scores, Thai rice vinegar outperformed rice bran vinegar as reported by Pazuch *et al.* [36]. Their study reported sensory means for color (6.34 \pm 1.93), flavor (5.30 \pm 2.36) and overall acceptance (5.74 \pm 2.20).

Table 3 The scores from sensory evaluation of Thai rice vinegar using a 9-point hedonic scale (Mean \pm Standard Deviation).

| Rice vinegar | Appearance | Color | Flavor | Taste | Overall acceptance |
|--------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| KDML 105 | 7.45 \pm 0.64 ^b | 7.75 \pm 0.44 ^b | 7.18 \pm 0.71 ^a | 6.73 \pm 0.64 ^a | 7.05 \pm 0.75 ^a |
| SYR | 6.48 \pm 0.51 ^a | 6.03 \pm 0.73 ^a | 7.30 \pm 0.69 ^a | 6.78 \pm 0.66 ^a | 7.08 \pm 0.66 ^a |
| HNR | 7.80 \pm 0.41 ^c | 8.03 \pm 0.70 ^b | 7.43 \pm 0.64 ^a | 6.98 \pm 0.70 ^a | 7.18 \pm 0.75 ^a |

Different letters within the same column indicate significant difference at $p \leq 0.05$

Conclusions

Production of high-quality vinegar was achieved from three varieties of Thai rice by the SCF process. Vinegar fermentation required 10 days to produce contained acetic acid at higher than 4 %, as 5.14 % (v/v) (KDML105), 5.27 % (v/v) (SYR) and 6.05 % (v/v) (HNR). The vinegar also contained residual alcohol concentration lower than 0.5 % (v/v). Antioxidant activity for vinegar from dark-colored rice (red to purple) was higher than light brown colored rice and white rice. Vinegar fermented from the three varieties of rice was rated on the sensory test scale as not lower than 'like slightly'. The SCF process was determined as a simple method that can produce high quality vinegar.

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