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Physiochemical Characteristics and Antioxidant Properties of Red Rice (Tubtim Chum Phae) Vinegar with a Two-Step Fermentation Process

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Abstract

This study aimed to develop a functional fermented vinegar using Tubtim Chum Phae rice, a red rice hybrid of white Jasmine and Sangyod Phatthalung rice, known for its high antioxidant activity, phenolic content (4,661.05 mg/100 g), and flavonoid content (2,989.21 mg/100 g). Three mixed-culture fermentation formulas, comprising *Amylomyces rouxii* TISTR 3182 and *Saccharomyces cerevisiae* TISTR 5013 at ratios of 1:1, 2:1 and 3:1 (g/mL), were used to ferment the rice over 15 days for alcohol production. *Acetobacter aceti* was added to vinegar production for 7 days. The resulting vinegar showed a pH at 3.03, an alcohol content of 4.1% and an acetic acid concentration of 6.03 g/L. Notably, it exhibited strong antioxidant activity, with DPPH and FRAP values of 46.80 µg TE/mL and 164.50 µg Fe(II)/mL, respectively. The total phenolic content was 187.54 µg GAE/mL, flavonoid content was 87.10 µg RE/mL and total anthocyanin content was 4.10 g/L. Comprehensive profiling revealed the presence of various organic acids and volatile compounds, including acetic acid, ethyl acetate, lactic acid, benzyl alcohol and phenyl ethanol, which contribute to the vinegar's characteristic aroma and flavor. These findings, which highlight the presence of bioactive components, underscore the potential of Tubtim Chum Phae rice vinegar as a natural product with significant nutritional and antioxidant benefits.

Keywords: Rice vinegar, Tubtim Chum Phae rice, Rice fermentation, Functional food

Introduction

Rice (*Oryza sativa*) is a major staple food crop worldwide, serving as a primary source of calories for a large proportion of the global population. While white rice has traditionally dominated the market due to its neutral flavor and long shelf life, there is an increasing interest in pigmented rice varieties such as black, red and purple rice because of their superior nutritional profiles and associated health benefits [1]. These colored varieties, characterized by their distinctive pigmentation, are rich in bioactive compounds such as anthocyanins, flavonoids and phenolic acids. These compounds are largely responsible for the grains' vibrant colors and their potent antioxidant properties, which may contribute to the prevention of oxidative stress-related diseases [2,3].

Tubtim Chum Phae rice is a distinctive pigmented rice variety native to Thailand, primarily cultivated in the Chum Phae district of Khon Kaen Province in the northeastern region of the country. The reddish-purple color of the rice grains is a trait attributed to the high levels of anthocyanins concentrated in the bran layer [4]. This variety belongs to a group of functional or rice types that are gaining increasing attention due to their potential health benefits and their contribution to dietary diversity [5]. Rich in bioactive compounds such as phenolics, flavonoids and particularly anthocyanins, Tubtim Chum Phae rice demonstrates strong antioxidant

properties, which may help reduce oxidative stress and contribute to the prevention of chronic diseases, including cardiovascular disorders, diabetes, hypertension and certain cancers [6-8].

Vinegar is a widely used acidic seasoning and preservative, produced through the fermentation of carbohydrate-rich substrates via a 2-step microbial process. The first stage involves alcoholic fermentation, during which fermentable sugars are converted into ethanol by yeasts, typically strains of *Saccharomyces cerevisiae*. In the second stage, known as acetic acid fermentation, acetic acid bacteria (AAB), primarily from the genera Acetobacter and Gluconobacter, oxidize ethanol into acetic acid in the presence of oxygen [9]. The fermentation process and the resulting quality of vinegar are influenced by several factors, including the raw materials used, microbial strains involved, fermentation conditions (such as temperature, aeration and pH) and the duration of fermentation [10].

A wide variety of agricultural products can serve as substrates for vinegar production, including fruits (e.g., apples, grapes), grains (e.g., rice, barley) and sugary plant extracts (e.g., coconut water, sugarcane juice). These diverse substrates contribute to the broad range of vinegar types found around the world, such as apple cider vinegar, rice vinegar, wine vinegar and balsamic vinegar [11]. Rice vinegars retain various bioactive compounds from the raw material and fermentation, including organic acids (acetic, lactic, succinic, etc.), phenolic compounds, amino acids and vitamins. These components are known to exert antioxidant, antimicrobial, antidiabetic and antihypertensive effects in the body.

Indeed, studies on cereal vinegars have documented their health benefits; for example, fermented rice vinegar has shown antioxidant activity and other bioactivities superior to distilled vinegars lacking such metabolites [9]. This study aims to produce vinegar from Tubtim Chum Phae rice and to evaluate its chemical and nutritional properties. Key parameters analyzed include acetic acid content, pH, residual sugars, phenolic profile and antioxidant capacity. Additionally, the vinegar's volatile compounds and organic acids were identified to better understand its flavor profile and health-related components. This research provides valuable insight into the potential of locally cultivated pigmented rice as a raw material for creating value-added vinegar products with enhanced nutritional and functional benefits.

Materials and methods

This work obtains Tubtim Chum Phae rice (2024 harvest, November) which is sourced from Chum Phae District, Khon Kaen Province, Thailand (16.54°N, 102.12°E). The rice sample was prepared under the condition of low-intermediat moisture (< 14%), colour (L*, a*, b*) and antioxidant screening, plus a plan for a community seed bank.

Microorganisms and chemicals

Amylomyces rouxii TISTR 3182, *Saccharomyces cerevisiae* TISTR 5013 and *Acetobacter aceti* TISTR 102 were obtained from the Thailand Institute of Scientific and Technological Research (TISTR, Pathum Thani, Thailand). Standard Trolox and gallic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Glucose, yeast extract, peptone and malt extract were obtained from Honeywell Fluka (Seelze, Germany). All remaining reagents were analytical grade and were also purchased from Honeywell Fluka (Seelze, Germany).

Preparation of medium and stock cell suspensions

Amylomyces rouxii was maintained on potato dextrose agar (PDA) slants and incubated at 30 °C for 3 days. *Saccharomyces cerevisiae* was maintained on yeast extract peptone dextrose (YPD) agar and incubated at 30 °C for 1 day.

Preparation of Rice Koji

Tubtim Chum Phae rice (4.5 kg) was soaked in water for 20 h and then steamed for 20 min. The steamed rice was rinsed with sterile water to remove excess mucilage and cooled to room temperature. The rice was then distributed into sterilized glass jars at 500 g per jar. Each jar was inoculated with 6 g of rice koji starter. Three formulations of rice koji were prepared by adjusting the mold-to-yeast ratio to 1:1, 2:1 and 3:1, respectively, to optimise amylase release and target approximately 9% ethanol. The inoculated rice was incubated at room temperature for 7 days to allow koji development.

Alcoholic fermentation

After koji development, 500 mL of sterile water was added to each jar to initiate alcoholic fermentation. The fermentation was carried out at room temperature for 15 days. Subsequently, the fermented mixture was filtered to obtain rice wine.

Acetic acid fermentation

A 500 mL aliquot of rice wine from each koji formulation was transferred into a 1,000 mL sterilized glass jar. *Acetobacter aceti* TISTR 102 was inoculated at a concentration of 5% (v/v). The jars were loosely sealed with sterile cotton plugs to permit aerobic fermentation and incubated at 30 °C for 7 days in a shaker incubator at 150 rpm. Upon completion of fermentation, potassium metabisulfite (KMS) was added at a concentration of 200 ppm to terminate microbial activity. Samples were then left at room temperature for 2 days to allow the dissipation of volatile compounds prior to further analysis.

Analysis methods

Physicochemical Analysis

The pH of the paste samples was measured using a digital pH meter (Mettler Toledo FiveEasy Plus FEP20, American Instrument Exchange, USA). Measurements were performed in triplicate at room temperature. Color characteristics were determined using a colorimeter (HUNTER Lab Ultra Scan PRO, USA). Approximately 50 mL of each sample was placed into a cuvette and analyzed for L*, a* and b* values. The L* value denotes lightness on a scale from 0 (black) to 100 (white). The a* value indicates the red-green axis, with positive values representing redness and negative values representing greenness. The b* value reflects the yellow-blue axis, with positive values indicating yellowness and negative values indicating blueness. Total dissolved solids were quantified in degrees °Brix using a handheld refractometer (Master-93H, ATAGO, Japan). All measurements were conducted in triplicate and results were expressed as mean values.

Antioxidant activity and bioactive compounds

2, 2-Diphenyl-1-Picryl Hydrazyl (DPPH) radical scavenging activity

This was determined using a modified version of the previously described methodology of [12]. The antioxidant activity of the sample extract was evaluated using the DPPH radical scavenging assay. Briefly, 20 µL of the extract was mixed with 180 µL of 0.2 mM DPPH solution prepared in 95% methanol. The mixture was incubated in the dark at 30 °C for 30 min. After incubation, the absorbance was measured at 517 nm using a microplate reader. Trolox (0.83 - 84.58 µg/mL) was used as a standard and results were expressed as µg Trolox equivalents (TE) per mL of sample. All experiments were conducted in triplicate.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was conducted based on the modified method of [13]. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ (ferric chloride hexahydrate) in a 10:1:1 (v/v/v) ratio, followed by incubation at 37 °C for 30 min. Subsequently, 20 µL of the sample was added to 180 µL of the FRAP reagent and the mixture was incubated at 37 °C for another 30 min. Absorbance was measured at 593 nm and antioxidant capacity was expressed as µg $FeSO_4$ (ferrous sulfate) equivalents per mL of sample. The experiments were conducted in triplicate with a standard solution of ferrous sulfate (12.5 - 400 µg/mL) used as a standard.

Total Phenolic Content (TPC)

Total phenolic content was assessed using a modified protocol based on the methods of [14,15]. In brief, 20 µL of the sample was combined with 100 µL of 10% (v/v) Folin-Ciocalteu reagent. After a 1-minute incubation, 80 µL of 7.5% (w/v) anhydrous sodium carbonate (Na_2CO_3) solution was added. The mixture was then incubated at room temperature for 30 min. Absorbance was measured at 765 nm and results were expressed as µg gallic acid equivalents (GAE) per mL of sample.

Total Flavonoid Content (TFC)

Total flavonoid content was determined using a modified version of the method described by [16], with adjustments in reagent volumes and assay format to accommodate a 96-well microplate. Briefly, 20 µL of the paste extract (20 mg/mL) was added to 60 µL of distilled water in a well, followed by the addition of 10

µL of 5% sodium nitrite ($NaNO_2$) solution. After 6 min, 10 µL of 10% aluminum chloride ($AlCl_3 \cdot 6H_2O$) solution was added and the mixture was allowed to stand for 5 min. Subsequently, 100 µL of 1 M sodium hydroxide (NaOH) was added and the reaction was incubated for 30 min. Absorbance was measured at 420 nm using quercetin (1,000 µg/mL) as the standard and results were expressed as µg quercetin equivalents (QE) per mL. The experiments were conducted in triplicate.

Acids and compounds

HPLC determination of phenolic acids and flavonoids

The analysis of phenolic acids and flavonoids in vinegar samples was performed using high-performance liquid chromatography (HPLC), following a modified method based on [17]. The HPLC system utilized was a Shimadzu 20 Series [18], equipped with a photodiode array (PDA) detector. Separation was achieved on a C18 column (4.6×150 mm^2, 5 µm particle size). The mobile phase consisted of solvent A: Water containing 1% (v/v) citric acid and solvent B: Acetonitrile. A gradient elution program was employed to achieve optimal analyte separation. The flow rate was maintained at 1.0 mL/min and the column temperature was set at 30 °C. Detection wavelengths were set at 280 and 320 nm to monitor phenolic acids and flavonoids, respectively. Phenolic acids, including gallic acid, syringic acid and p-coumaric acid, as well as flavonoids such as rutin, quercetin, catechin, myricetin and kaempferol, were identified by comparing their retention times and UV spectra with those of authentic standards. Quantification was performed using calibration curves constructed from standard solutions. All analyses were conducted in triplicate to ensure accuracy and reproducibility.

HPLC analysis of organic acids

The organic acid composition of the vinegar samples was analyzed using high-performance liquid chromatography (HPLC), following a modified method based on [19]. The analysis was performed using a Shimadzu HPLC system, model 20 Series [18], equipped with a UV-Vis detector set at 210 nm. Separation was achieved using an Aminex HPX-87H column (300×7.8 mm^2), maintained at 50 °C. The mobile phase consisted of 0.005 N sulfuric acid (H_2SO_4), delivered isocratically at a flow rate of 0.8 mL/min. Vinegar samples were filtered through a 0.45 µm membrane filter and appropriately diluted with deionized water prior to analysis. A 20 µL volume of each sample was injected into the system. Organic acids, including acetic, citric, lactic, malic and succinic acids, were identified by comparing their retention times with those of authentic standards. Quantification was performed using calibration curves obtained from standard solutions. All analyses were conducted in triplicate to ensure accuracy and reproducibility.

GC-MS analysis of volatile compounds

Volatile compounds were analyzed using an Agilent 7890A gas chromatograph equipped with an HP 5MS capillary column (30 m×0.25 mm, 0.25 µm film thickness, fused silica) and coupled to an Agilent 7000B mass spectrometer and Shimadzu GCMS QP2010 SE for headspace GC/MS analysis [18]. Data acquisition and compound identification were performed using Agilent MassHunter Qualitative Analysis Workstation software (version 10) with reference to the NIST MS Search 2.0 library. The autosampler was programmed to begin at 50 °C for 5 min, followed by a temperature ramp from 150 to 250 °C at 4 °C/min. Sample injections were carried out in split mode (5:1) at 250 °C, with helium as the carrier gas at a constant flow rate of 1.0 mL/min. The transfer line was maintained at 250 °C. The oven temperature was set initially to 50 °C (no hold), then increased to 150 °C at 3 °C/min with a 3-min hold and further increased to 250 °C at 10 °C/min with an additional 3-minute hold. The total run time was 60 min. Volatile compounds were semi-quantified based on the peak areas in the total ion chromatogram (TIC). Only compounds with an identification probability of ≥ 80% were included in the analysis to ensure accurate identification and minimize the risk of misclassification due to overlapping spectral features. A heatmap and dendrogram illustrating the distribution of volatile compounds were generated using the online SRPlot tool.

Statistical analysis

All data are measured with the number of samples at least 3 to 5 for more accuracy in scientific research. All results in this work are expressed as average plus/minus standard deviation.

Results and discussion

Physicochemical properties

Tubtim Chum Phae rice wine

The physicochemical properties of Tubtim Chum Phae rice wine (pH, total dissolved solids (TDS) and alcohol content) are shown in **Figure 2**. Tubtim Chum Phae rice was used as the substrate for 2-stage fermentation. The pH values of all 3 formulations (F1, F2 and F3) over the 14-day fermentation period, are shown in **Figure 2(a)**. The initial pH (~7.0) decreased steadily to values below 4.0 by day 14. This trend is characteristic of organic acid accumulation, mainly due to the fermentative metabolic activities of mold and yeasts [20]. Among the 3 formulations, F3 showed a slightly more rapid pH reduction, suggesting a more active microbial fermentation process. [21] reported that fermentation of broken rice with *A. rouxii* significantly enhanced the levels of bioactive compounds, including kojic acid, a known antioxidant and tyrosinase inhibitor, as well as oxalic, ascorbic, gallic, protocatechuic and 4-hydroxybenzoic acids, which provide pH during 3 - 4. In our research, rice wine contained formic acid, acetic acid and propionic acid (**Table 4**).

The °Brix values, representing the concentration of soluble solids (primarily sugars), declined gradually during fermentation in all formulations (**Figure 2(b)**). Starting at ~18 - 19 °Brix, the levels decreased to ~4 °Brix by day 14. This decrease reflects the utilization of fermentable sugars by yeasts and other microorganisms to produce alcohol and metabolic by-products [22]. Formulation F3 exhibited a slightly faster reduction in °Brix, which aligns with its steeper pH drop and may indicate a higher rate of fermentation. Similar patterns have been reported in traditional fermentations, where rapid sugar depletion correlates with robust microbial activity [23].

Alcohol content increased progressively in all formulations, reaching ~8.5% - 9.0% by day 14, is shown in **Figure 2(c)**. The rate of alcohol accumulation corresponds with sugar consumption and microbial growth dynamics. Among the tested formulations, F3 achieved the highest final alcohol concentration, followed closely by F2 and F1. This suggests that F3 supported more efficient conversion of sugars to ethanol, possibly due to more favorable physicochemical conditions or better substrate availability [24]. Higher alcohol yields are often associated with optimized fermentation conditions, which enhance yeast performance and reduce competing microbial growth. Overall, the trends observed in pH, °Brix and alcohol content indicate successful fermentation in all 3 formulations. However, formulation F3 consistently exhibited more favorable fermentation kinetics and final product characteristics, suggesting it may be the most suitable for alcoholic beverage production. These findings underscore the importance of formulation in modulating microbial activity and fermentation outcomes. Further studies, including microbial community profiling and metabolite analysis, could provide deeper insights into the mechanisms underlying these differences.

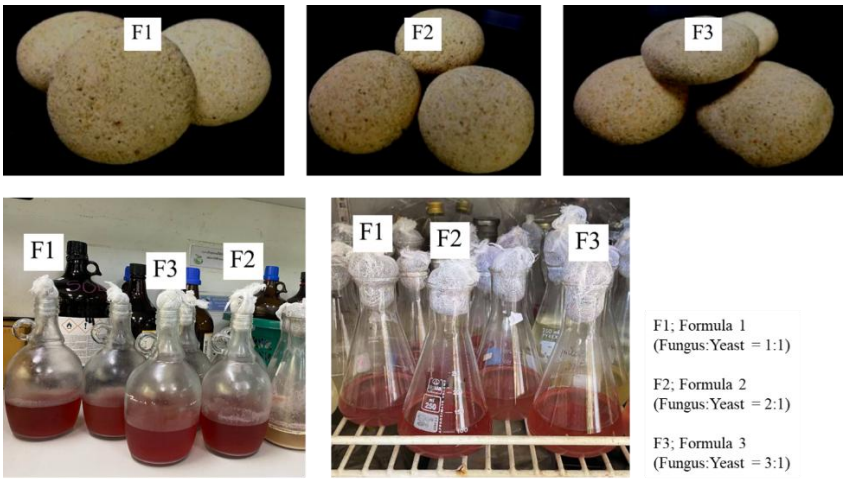

Figure 1 Top panel: Starter cultures for Formulas 1 - 3 from left to right, respectively. Bottom panel: Tubtim Chum Phae rice wine and vinegar are shown on the left and right, respectively.

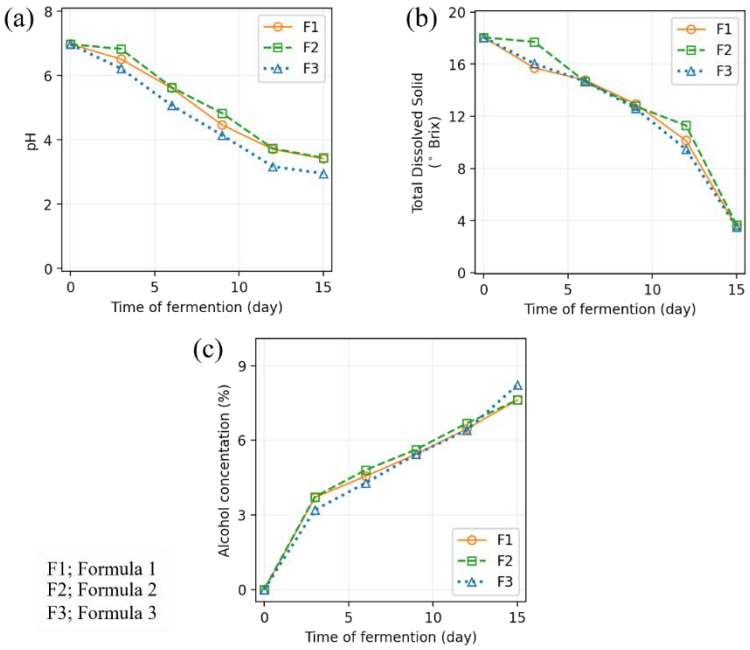

Figure 2 Physicochemical properties of Tubtim Chum Phae rice wine (a) pH, (b) total dissolved solids (TDS), (c) alcohol content). F1 = Fungus:Yeast (1:1), F2 = 2:1, F3 = 3:1.

Tubtim Chum Phae rice vinegar

The apparent color of all vinegar samples was consistently described as purplish-orange. However, significant differences were observed in the color values (L*, a*, b*) among the formulations (**Table 1**). The L* value, representing lightness, was highest in Formulation 2 (35.29 ± 0.04^{a}), indicating a lighter appearance compared to Formulations 1 and 3, which had L* values of 32.62 ± 0.03^{c} and 31.28 ± 0.04^{b}, respectively. Regarding the a* value, which reflects the degree of redness, Formulation 3 exhibited the highest redness (1.58 ± 0.04^{a}), followed by Formulation 2 (1.25 ± 0.03^{a}), while Formulation 1 had the lowest redness (0.57 ± 0.04^{b}). The b* values, which indicate yellowness, did not differ significantly among the formulations, with values ranging from 6.20 ± 0.05^{a} to 6.43 ± 0.01^{a}. These findings suggest that formulation design influences the lightness and redness of vinegar, but has minimal effect on its yellowness.

The pH of all 3 formulations (F1, F2 and F3) decreased progressively over the 7-day fermentation period (**Figure 3(a)**). Formulations F1 and F2 began with similar initial pH values (~3.5), while F3 exhibited a lower starting pH (~3.0). By day 7, F3 had the lowest pH (2.6), followed by F1 (2.9) and F2 (3.0), indicating increased acidity over time due to acetic acid production. As shown in **Figure 1(b)**, total dissolved solids significantly decreased in all formulations, from approximately 12 - 13 °Brix to around 7 °Brix by day 7. This downward trend indicates the consistent utilization of sugars by fermentative microorganisms, with minimal variation among formulations. Total titratable acidity (TTA) increased steadily in all samples, indicating acetic acid accumulation **(Figure 3(c))**. F1 showed the highest final TTA value (~8.5 g/100 mL), followed by F2 (~7.2 g/100 mL) and F3 (~6.5 g/100 mL). This trend reflects the efficiency of ethanol oxidation by acetic acid bacteria.

Figure 3(d) illustrates the decline in alcohol content across all formulations. Initial alcohol concentrations ranged from 11% to 12% (v/v), decreasing to 3 - 4% by day 7. This is consistent with acetic acid fermentation, in which ethanol is oxidized to acetic acid by *Acetobacter* spp. The observed physicochemical changes during the 7-day fermentation underscore the impact of formulation on vinegar production dynamics. The steady decline in pH and increase in titratable acidity confirm the conversion of ethanol into acetic acid, a hallmark of successful vinegar fermentation [25]. F1 demonstrated the most robust fermentation kinetics, with the highest acid production and fastest decline in ethanol, potentially due to a more favorable microbial community or nutrient composition. The decrease in total dissolved solids across all

formulations suggests efficient microbial sugar utilization. The similarity in °Brix patterns implies that sugar availability was not a limiting factor. Interestingly, F3, which began with a lower pH, exhibited slower acid production. This could be attributed to inhibitory conditions affecting microbial performance. Alcohol degradation trends further corroborate the metabolic activity of acetic acid bacteria. The more rapid ethanol depletion in F1 aligns with its greater titratable acidity, indicating a more complete fermentation process.

Collectively, these findings suggest that formulation differences significantly affect fermentation outcomes, particularly in acidity and alcohol content. Formulation F1 may be preferred for industrial vinegar production due to its efficient conversion profile, whereas F3 may be suited for applications requiring milder acidity. Fermentation parameters such as pH, °Brix, TTA and alcohol content changed significantly during the 7 days, with notable differences across formulations. F1 exhibited the most efficient fermentation performance, characterized by high acid production and rapid ethanol depletion. These results provide insights for optimizing vinegar production based on formulation design.

Table 1 Apparent color and color values of vinegar formulation.

Formula	The apparent color	The color values		
		L*	a*	b*
Formula 1	purplish-orange	32.62 ± 0.03^{c}	0.57 ± 0.04^{b}	6.20 ± 0.05^{a}
Formula 2	purplish-orange	35.29 ± 0.04^{a}	1.25 ± 0.03^{a}	6.43 ± 0.01^{a}
Formula 3	purplish-orange	31.28 ± 0.04^{d}	1.58 ± 0.04^{a}	6.37 ± 0.02^{a}

Values are presented as mean ± standard deviation. Different superscript letters within a column indicate significant differences (p-value < 0.05).

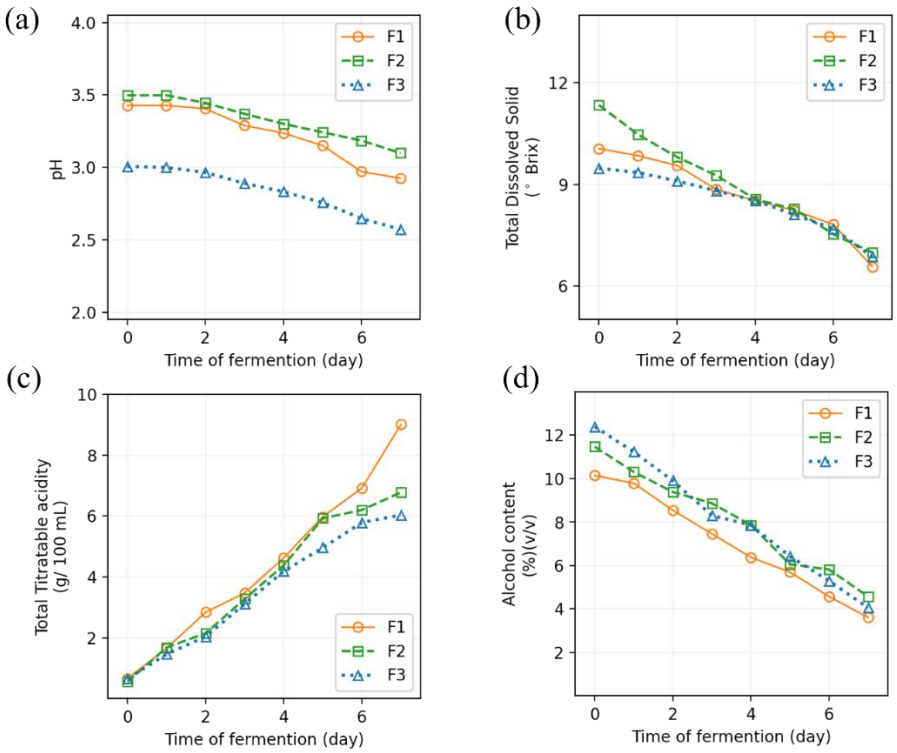

Figure 3 Physicochemical properties of Tubtim Chum Phae rice vinegar (a) pH, (b) total dissolved solids (TDS), (c) total titratable acidity and (d) alcohol content). F1 = Fungus:Yeast (1:1), F2 = 2:1, F3 = 3:1.

Antioxidant activity and bioactive compound

F1 exhibited the highest antioxidant activity (about 10% - 15% higher than other samples), as indicated by both DPPH and FRAP assays, along with the highest levels of TPC and TFC. The respective values were 55.44 ± 0.71 µg TE/mL (DPPH), 174.54 ± 0.42 µg $FeSO_4$/mL (FRAP), 219.47 ± 0.92 µg GAE/mL (TPC) and 99.77 ± 0.51 µg RE/mL (TFC) (**Table 2**).

These findings are consistent with recent studies on naturally fermented vinegars, which report IC_{50} values between approximately 6 - 50 µg/mL and demonstrate enhanced antioxidant capacity linked to substrate phenolics and microbial activity. For instance, a Korean study on *Hovenia dulcis* fruit vinegar showed significant DPPH and ABTS radical scavenging enhancements post-fermentation [26]. Similarly, Thai rice vinegars produced from colored rice varieties exhibited notable DPPH inactivation (~49%) and high total phenolic content (~428 µg/mL) after surface-culture fermentation [27].

In this study, rice vinegar demonstrated quantifiable antioxidant activity and significant concentrations of phenolic and flavonoid compounds, which are recognized for their role in mitigating oxidative stress and enhancing functional food properties. These bioactive components are influenced by the metabolic activity of fermentative microbes, which can modulate the synthesis and retention of antioxidant molecules [20].

The elevated TPC and TFC values suggest a notable presence of bioactive phenolic compounds, either extracted from the rice substrate or synthesized during microbial fermentation. These compounds are known to modulate oxidative pathways and contribute to health-promoting effects [28]. The observed positive correlation between higher TPC and TFC and stronger antioxidant activity aligns with previously established relationships between phenolic content and free radical scavenging capacity [29].

Overall, the results underscore the potential of rice vinegar as a source of natural antioxidants, with phenolic and flavonoid compounds contributing significantly to its bioactivity. The natural fermentation process plays a critical role in determining the final composition of these compounds. Controlled fermentation using selected microbial strains not only enhances acetic acid production but also facilitates the biotransformation of bound phenolics into bioavailable forms [30]. Although prolonged fermentation may lead to oxidative degradation of sensitive compounds, the present study suggests an optimal balance between fermentation time and the preservation of bioactive constituents.

Table 2 Antioxidant activity and bioactive contents of Tubtim Chum Phae rice vinegar.

Formula	DPPH (µg TE/mL)	FRAP (µg $FeSO_4$/mL)	TPC (µg GAE/mL)	TFC (µg RE/mL)
F1	55.44 ± 0.71[a]	174.54 ± 0.42[a]	219.47 ± 0.92[a]	99.77 ± 0.51[a]
F2	48.68 ± 0.97[b]	164.50 ± 0.44[b]	193.27 ± 0.87[b]	90.77 ± 0.33[b]
F3	46.80 ± 0.03[c]	161.29 ± 0.21[c]	187.54 ± 0.38[c]	87.10 ± 0.54[c]

Different superscripts within the same column denote statistically significant differences (p-value < 0.05)
Fungus:Yeast (1:1), F2 = 2:1, F3 = 3:1.

Acids and compounds

Phenolic acid and flavonoid compounds

The composition of individual phenolic acids and flavonoids was also analyzed. Formula 1 contained the highest rutin content (5.54 ± 0.17 mg/mL), while catechin levels showed no statistically significant differences among the 3 formulas. Total phenolic acid concentrations were highest in Formulas 1 and 2, with values of 9.97 ± 0.51 and 9.77 ± 0.33 mg/mL, respectively. Cinnamic acid content did not vary significantly across the formulations (**Table 3**).

These results highlight the influence of fermentation parameters on the retention and transformation of bioactive compounds. The use of selected microbial strains during controlled fermentation not only facilitates acetic acid production but also enhances the conversion of bound phenolics into bioavailable forms [30]. Although prolonged

fermentation can lead to oxidative degradation of sensitive compounds, the findings of this study suggest an optimal balance between fermentation time and the preservation of bioactive components.

Phenolic acids and flavonoid compounds are major contributors to the antioxidant potential and functional properties of fermented foods, including rice vinegar. In this study, vinegar produced using Formula 1 exhibited the highest rutin content, while catechin levels remained statistically similar across all fermentation formulas. Additionally, phenolic acid concentrations were highest in Formulas 1 and 2, with no significant differences observed in cinnamic acid levels among the samples.

The presence and variability of these compounds can be attributed to several factors, including the composition of the raw material, microbial metabolism and fermentation conditions. Rutin, a flavonoid glycoside, has been extensively studied for its strong antioxidant, anti-inflammatory and vascular-protective effects [31]. Its significantly higher concentration in Formula 1 suggests that this fermentation condition may favor the extraction or biosynthesis of rutin, possibly through enzymatic activity from the microbial consortium.

Catechin, commonly found in cereals and plant-based substrates, has been associated with cardiovascular benefits and oxidative stress reduction. Although no significant differences were observed among the formulas, its consistent presence across samples indicates that fermentation preserved this compound effectively. Phenolic acids, including derivatives of hydroxybenzoic and hydroxycinnamic acids, were present in substantial quantities, particularly in Formulas 1 and 2. These compounds are known to act as chain-breaking antioxidants and may play a role in stabilizing free radicals formed during fermentation [28].

Table 3 Phenolic acid and Flavonoid compounds of Tubtim Chum Phae rice vinegar.

Formula	Flavonoid compounds (mg/mL)		Phenolic acid (mg/mL)	
	Rutin	Catechin	Gallic acid	Cinnamic acid
F1	5.54 ± 0.17^{a}	1.74 ± 0.42^{a}	9.97 ± 0.51^{a}	2.19 ± 0.29^{a}
F2	4.86 ± 0.07^{b}	1.64 ± 0.04^{a}	9.77 ± 0.33^{a}	1.93 ± 0.07^{a}
F3	4.68 ± 0.03^{b}	1.12 ± 0.21^{a}	8.71 ± 0.54^{b}	1.87 ± 0.38^{a}

Different superscripts within the same column denote statistically significant differences (p-value < 0.05).
Fungus:Yeast (1:1), F2 = 2:1, F3 = 3:1.

Organic acid compounds

The analysis of organic acids in the 3 vinegar fermentation formulas revealed the presence of several compounds, including citric, tartaric, succinic, lactic, propionic and acetic acids (**Table 4**). These organic acids are key metabolites produced during the fermentation process and significantly influence the sensory profile of vinegar, particularly its sourness, aroma and flavor complexity [32,33].

Acetic acid, the principal organic acid in vinegar, is synthesized via ethanol oxidation by Acetobacter species. It contributes the characteristic sharpness, antimicrobial activity and preservative function of vinegar. Citric and tartaric acids are either naturally present in the rice substrate or formed through microbial metabolism and they contribute to the overall tartness and fruity aroma. Succinic acid, derived from the tricarboxylic acid (TCA) cycle, imparts umami and a mild bitterness, enhancing flavor complexity [34].

Lactic acid, produced mainly by lactic acid bacteria through the fermentation of sugars, contributes to a softer sourness and improved mouthfeel. Its presence often indicates mixed or heterolactic fermentation pathways. Propionic acid, although typically found in lower concentrations, can impart sharp, nutty, or pungent sensory notes and may originate from specific microbial pathways involving propionibacteria or cross-feeding microbial interactions.

The composition and concentration of these organic acids are influenced by the type of microbial strains used, fermentation time, oxygen availability and substrate composition. Modulating fermentation conditions can therefore be a critical strategy for optimizing both microbial activity and the sensory

characteristics of vinegar products [34,35]. Understanding these biochemical pathways offers insights into the development of high-quality rice vinegar with enhanced functional and organoleptic properties.

Table 4 Organic acid compounds of Tubtim Chum Phae rice vinegar.

Formula	Organic acid (g/L)					
	Citricacid	**Tartaric acid**	**Succinic Acid**	**Lactic acid**	**Propanoic acid**	**Acetic acid**
F1	43.90 ± 0.05^{c}	36.50 ± 0.20^{c}	66.10 ± 0.03^{a}	30.50 ± 0.13^{c}	63.60 ± 0.04^{c}	49.87 ± 0.12^{a}
F2	45.10 ± 0.14^{a}	39.40 ± 0.09^{a}	67.50 ± 0.05^{a}	36.47 ± 0.11^{a}	66.73 ± 0.14^{a}	50.30 ± 0.15^{a}
F3	44.50 ± 0.08^{b}	38.10 ± 0.05^{b}	66.50 ± 0.11^{a}	34.20 ± 0.13^{b}	65.40 ± 0.11^{b}	49.32 ± 0.10^{a}

Different superscripts within the same column denote statistically significant differences (p-value < 0.05).
F1 = Fungus: Yeast (1:1), F2 = 2:1, F3 = 3:1.

Volatile compound profile

Volatile compounds play a critical role in defining the aroma and overall sensory quality of rice vinegar. The complex mixture of esters, acids, alcohols and aromatic compounds results from microbial metabolism during fermentation and significantly contributes to the characteristic flavor profile [36]. In the present study, a range of volatile compounds was identified in Tubtim Chum Phae rice vinegar, including esters such as ethyl acetate and ethyl lactate, organic acids like acetic and lactic acids and alcohols such as benzyl alcohol and phenyl ethanol (**Table 5**). This finding is consistent with the study by Zhu *et al.* [37], who analyzed vinegar composition using GC-MS and reported that the dominant aroma compounds in wine vinegar included ethanol, ethyl acetate, 2,3-butanediol, 1-methylnaphthalene, 2-methylnaphthalene and various acids.

These results indicate that such chemical components play an important role in enhancing the aroma and taste of traditional rice wines. Esters, particularly ethyl acetate, are known for their fruity and floral aromas and are typically produced by yeast and acetic acid bacteria through esterification reactions involving ethanol and organic acids [38]. Their presence enhances the aromatic complexity and consumer appeal of rice vinegar. Organic acids such as acetic and lactic acids not only contribute to the sour taste but also act as precursors for volatile esters and other aroma-active compounds [39].

Alcohols, including phenyl ethanol, impart sweet and floral notes and are usually formed during the yeast fermentation stage from amino acid metabolism [38]. The balance and concentration of these volatile compounds are influenced by fermentation conditions, microbial community composition and raw material characteristics.

The volatile profile of rice vinegar is comparable to that of other traditionally fermented vinegars, as reported by [37], where compounds such as ethanol, ethyl acetate and various aromatic hydrocarbons were dominant. These volatiles not only define the sensory quality but may also possess antimicrobial properties that contribute to vinegar's preservative effects [33]. In summary, understanding the volatile compound profile is essential for optimizing fermentation processes to produce rice vinegar with desirable sensory and functional attributes.

Table 5 Volatile compounds (% relative abundance) of Tubtim Chum Phae rice wine and vinegar.

No.	Compound	Area % of total			Retention Time (min)	Formula	Molecular weight
		F1	**F2**	**F3**			
				Rice Wine			
1	Formic acid	5.23	5.29	7.82	1.383	$C_3H_4O_2$	72.06
2	Acetic acid	7.01	7.09	8	1.859	$C_2H_4O_2$	60.05
3	Acetoin	3.41	2.5	0.49	2.650	$C_4H_8O_2$	88.10

No.	Compound	Area % of total			Retention Time (min)	Formula	Molecular weight
		F1	F2	F3			
4	Ethanol	23.45	24.4	25.6	3.658	CH_3CH_2OH	46.07
5	2-Propanol, 1-(2-propenyloxy)	1.08	2.09	1.9	4.392	$C_6H_{12}O_2$	116.16
6	Ethyl N-hydroxyacetimidate	7.75	6	2.86	4.625	$C_4H_9NO_2$	103.12
7	Ethyl acetate	15.7	15.28	17.2	12.687	$C_4H_8O_2$	88.11
8	2,3-Butanediol	13.47	14.7	15.3	13.779	$C_4H_{10}O_2$	90.12
9	Propanoic acid	10.4	9.2	7.2	19.549	$C_3H_8O_2$	74.08
10	Glycerol	5.23	5.29	7.82	20.611	$C_3H_8O_3$	92.09
11	Carbon dioxide	7.01	7.09	8	3.725	CO_2	44.01
				Rice vinegar			
1	Acetic acid	35.26	35.45	37.29	1.859	$C_2H_4O_2$	60.05
2	Ethyl acetate	12.48	11.25	12.1	12.687	$C_4H_8O_2$	88.11
3	Ethylic acid	15.14	15.19	15	12.716	$C_2H_4O_2$	60.05
4	ethyl lactate	19.72	19.4	16.48	13.835	$C_5H_{10}O_3$	118.13
5	Lactic acid	9.38	9.9	9.8	19.833	$C_3H_6O_3$	90.08
6	Propanoic acid	3.27	3.49	3.78	19.549	$C_3H_8O_2$	74.08
7	benzyl alcohol	2.46	2.87	2.96	21.584	C_7H_8O	108.14
8	Phenyl ethanol	2.29	2.45	2.59	22.035	$C_8H_{10}O$	122.16

Conclusions

This study demonstrated that Tubtim Chum Phae rice vinegar produced using different starter cultures exhibited significant variations in antioxidant activity, phenolic and flavonoid content, as well as organic and volatile compound profiles. Formula 1 consistently showed superior retention of bioactive compounds, particularly rutin and phenolic acids, which are closely associated with enhanced antioxidant properties. The identified organic acids and volatile compounds contributed notably to the vinegar's characteristic flavor and aroma, highlighting the crucial role of microbial metabolism and fermentation conditions in shaping its sensory and functional qualities. These findings provide valuable insights into optimizing rice vinegar fermentation to improve its health-promoting attributes and sensory appeal. However, Tubtim Chum Phae costs about THB 60 per kg vs Jasmine rice about THB 35 per kg which is expected retail price of vinegar is 3 - 4 times higher according to functional properties above.

We found that the alcohol content of all 3 formulas is approximately 4.2%, which exceeds the standard limitation for ethanol content in an acetic acid product (less than 0.5%). Future work will focus on optimizing the fermentation conditions to control the use of ethanol by bacteria to produce more acetic acid. Moreover, we then further elucidating the impact on bioactive compound biosynthesis for industrial-scale applications.

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Declaration of Generative AI in Scientific Writing

The authors confirm that generative AI tools (such as QuillBot and ChatGPT by OpenAI) were utilized solely for language refinement and grammatical improvements during the preparation of this manuscript. These tools were not used for generating content or interpreting data. The authors take full responsibility for the content and conclusions of this work.

CRediT Author Statement

Warisara Worawarangkul: Conceptualization, Resources, Methodology, Investigation, Writing original draft, and Visualization. **Vijitra Luang-in:** Conceptualization, Investigation, and Validation. **Siripan Deesilatham:** Conceptualization, Investigation, and Validation. **Sirirat Deeseenthum:** Conceptualization, Supervision, Investigation, Validation, Funding acquisition, and Writingreview & editing.

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