

การศึกษาเชื้อผสมของ *Acetobacter aceti* TISTR102 และ *Acetobacter cervisiae* TN4497 เพื่อการผลิตกรดอะซิติก และสารออกฤทธิ์ทางชีวภาพในการหมักน้ำส้มสายชูจากดอกกระเจี๊ยบ

Study on mixed Cultured of *Acetobacter aceti* TISTR102 and *Acetobacter cervisiae* TN4497 on Acetic Acid and Bioactive Compounds Production of Roselle Vinegar Fermentation

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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อเพิ่มผลผลิตสารออกฤทธิ์ทางชีวภาพและกรดอะซิติกของน้ำส้มสายชูดอกกระเจี๊ยบโดยเชื้อจุลินทรีย์ผสม ไวน์ดอกกระเจี๊ยบถูกผลิตในถังปฏิกรณ์แบบกะ ค่าพารามิเตอร์จลนศาสตร์ที่ได้จากการผลิตไวน์โดย *Saccharomyces cerevisiae* TISTR5048 มีค่า P_{MAX} เท่ากับ 63.47 กรัมต่อลิตร, Q_E เท่ากับ 2.25 กรัมต่อลิตรต่อชั่วโมง, $Y_{P/S}$ เท่ากับ 0.45 และ $Y_{X/S}$ เท่ากับ 1.53 การใช้เชื้อจุลินทรีย์ผสมระหว่าง *Acetobacter aceti* TISTR102 และ *Acetobacter cervisiae* TN4497 ในสภาวะที่เหมาะสมจากเอทานอลเริ่มต้น 63.47 กรัมต่อลิตร ตรวจวัดปริมาณสารประกอบฟีนอลิกทั้งหมด แอนโทไซยานินทั้งหมดและคุณสมบัติในการต้านอนุมูลอิสระของน้ำกระเจี๊ยบ ไวน์กระเจี๊ยบ และน้ำส้มสายชูกระเจี๊ยบพบว่ากระบวนการหมักกรดอะซิติกมีผลทำให้ปริมาณสารประกอบฟีนอลิกทั้งหมด แอนโทไซยานินทั้งหมดและคุณสมบัติในการต้านอนุมูลอิสระของกระเจี๊ยบเพิ่มขึ้น ไวน์ และน้ำส้มสายชูกระเจี๊ยบมีความสามารถในการต้านอนุมูลอิสระของ DPPH โดยมีค่า EC_{50} เป็น 16.28 และ 11.89 มิลลิกรัมต่อลิตรตามลำดับกระบวนการหมักกรดอะซิติกมีผลในปริมาณแอนโทไซยานินและคุณสมบัติการต้านอนุมูลอิสระเพิ่มสูงขึ้นผลการศึกษาชี้ให้เห็นว่าว่าการหมักเป็นวิธีช่วยให้กิจกรรมต้านอนุมูลอิสระของผลิตภัณฑ์จากดอกกระเจี๊ยบสูงขึ้น

คำสำคัญ: ดอกกระเจี๊ยบ น้ำส้มสายชู สารออกฤทธิ์ทางชีวภาพ

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Abstract

The objective of this study was to enhance bioactive compounds and the acetic acid production of Roselle Vinegar with mixed culture fermentation. The Roselle wine was produced in a batch reactor. The kinetic parameters obtained for wine fermentation by *Saccharomyces cerevisiae* TISTR5048 were $P_{MAX} = 63.47$ g/L, $Q_E = 2.25$ g/L/h, $Y_{P/S} = 0.45$ and $Y_{X/S} = 1.53$. Using mixed culture of *Acetobacter aceti* TISTR102 and *Acetobacter cervisiae* TN4497, the wine vinegar process optimization ranges found for initial concentrations of ethanol and acetic acid as independent variables were 63.47 g/L and 69.21 g/L, respectively. Total phenolics, total anthocyanins, and antiradical activity (DPPH radical-scavenging method) were determined. Acetification increased total anthocyanin content, total polyphenols and antioxidant activities. The abilities of wine from Roselle juice to antiradical activity were high with EC_{50} of 14.95 mg/L. The antioxidant activity of vinegar product was high with EC_{50} of 0.72 mg/L. Acetification significantly increased anthocyanins and antioxidant activities. The results indicated that fermentation is a better method for obtaining higher antioxidant activity of Roselle products.

Keywords: Roselle, vinegar, bioactive compound

Introduction

Reactive oxygen species (ROS) or free radicals are generated as byproducts or intermediates of aerobic metabolism and through reactions with drugs and environmental toxins. Although almost all organisms possess antioxidant defense and repair systems, which quench or minimize the production of oxygen-derived species, thus protecting them against oxidative damage, these protective systems are insufficient to entirely prevent the damage¹ caused by endogenous or exogenous oxidants.² In view of this, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their actions. The phenolic compounds, which are widely distributed in plants, were considered

to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems.³ The study of numerous compounds that could be useful antioxidants has generated increasing interest in the field of food or medicine. The dried flowers of *Hibiscus sabdariffa* L. (Malvaceae) commonly called "Roselle" have gained importance as local soft drink and medical herb in local regions. Studies revealed that the dried flowers of *Hibiscus sabdariffa* L., a Chinese herbal medicine, have been used effectively in folk medicine against hypertension, pyrexia, and liver disorders.⁴ Included in foods for centuries, white vinegar, a natural source of distilled acetic acid, provides significant and substantial health benefits when consumed in moderation. Anecdotal evidence suggests possible further

astonishing benefits, including improving calcium absorption, and moderating type 2 diabetes and even some cancers. The acetic acid process was divided into two fermentation processes according to the characters of growth and metabolization of *S. cerevisiae* TISTR5048 and acetic acid bacteria: the primary fermentation for growth of *S. cerevisiae* TISTR5048 and the production of ethanol, the secondary fermentation for the production of acetic acid by acetic acid bacteria. *S. cerevisiae* TISTR5048 is facultative anaerobes, while the acetic acid bacteria is aerobic. Mixed culture systems exhibit great advantages for many processes involving more than one reaction step, such as high productivity.⁵

Mixed culture fermentation may become an attractive addition to traditional pure-culture-based biotechnology for the traditional fermented food.⁶ Although the advantages of mixed culture over pure culture have been pointed out by many researchers,⁷ little is known about the application of mixed culture technology in acetic acid production.⁸ In this study, mixed culture of *Acetobacter aceti* TISTR102 and *Acetobacter cervisiae* TN4497 was optimized in batch culture to enhance the yield of acetic acid production and enhance of antioxidant capacity of Roselle vinegar.

Materials and Methods

Microorganisms

Acetobacter aceti TISTR102 and *Saccharomyces cerevisiae* TISTR5048 were from Thailand Institute of Scientific and Technological Research (TISTR). *Acetobacter cerevisiae* TN4497 was from Division of Biochemistry, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Thailand.

Chemicals

Gallic acid, Folin-Ciocalteu's reagent, methanol, linoleic acid, quercetin, 1,1-diphenyl-2-picrylhydrazyl, butylated hydroxyl-toluene, sodium carbonate, sodium hydroxide, aluminium chloride hydrated, sodium nitrite, dibasic sodium phosphate and monobasic sodium phosphate were purchased from Fluka Chemie AG.

Preparation of the Roselle mush

Dried powder (200 g) of Roselle was added with 1 L of distilled water. The physiochemical properties of the extract were measured and recorded in Table 1. The filtrate was ameliorated using glucose to raise the sugar level to 23.7°Brix and ammonium sulphate (111 ppm) added to stabilize the mush and to provide a nitrogen source for yeast. The pH of the mush was adjusted to pH5. The treated mush was pasteurized at 63°C for 30 minutes and then allowed to cool to room temperature of 25°C. The supernatant was supplemented with additional nutrients to give a base medium composition of: 1 g/L yeast extract; 2 g/L (NH₄)₂SO₄; 1 g/L MgSO₄•7H₂O.

Batch fermentation

Alcoholic fermentation

This section describes the different steps and culture media to carry out the fermentation. In first step pre-culture, the composition of the medium was: yeast extract 10 g/L, glucose 20 g/L and peptone 20 g/L. One hundred milliliter of this YPD medium were inoculated and incubated at 30 °C under agitation (50 rpm) during 24 h. The culture medium was composed of: KH_2PO_4 5g/L, $(\text{NH}_4)_2\text{SO}_4$ 2 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g/L Yeast extract 1 g/L. The fermentation medium is a Roselle mush. It was added up to a sugar concentration of 282.36 g/L, KH_2PO_4 5 g/L, $(\text{NH}_4)_2\text{SO}_4$ 2 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4 g/L and yeast extract 1 g/L.

Approximately 1.0 L of Roselle mush was poured into a 2 L fermentor and inoculated with 10 ml of the *S. cerevisiae* TISTR5048 starter culture. The inoculated slurry of Roselle extract was subjected to primary fermentation at 30°C for 3 days to produce Roselle wine, which was then filtered after complete primary fermentation. Batch fermentation was conducted in a 2 L fermentor with a working volume of 1 L. The fermentation medium was inoculated with 10% v/v inoculum (20 hours culture of *S. cerevisiae*, 1×10^7 cells/mL). The fermentation temperature was kept constant at 30 ± 0.2 °C. The broth was kept under agitation at 50 rpm. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol, acetic acid and residual glucose in the broth.

Acetic acid fermentation

This section describes the different steps and culture media to carry out the fermentation. In first step pre-culture, the composition of the medium was: glucose 10 g/L, yeast extract 10 g/L, KH_2PO_4 0.5 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L and ethanol 60 g/L. One hundred milliliter of this medium were inoculated and incubated at 30 °C under agitation (150 rpm) during 24 h. The fermentation broth was composed of: yeast extract 10 g/L, KH_2PO_4 0.5 g/L, MgSO_4 0.5 g/L, Roselle wine (ethanol from fermentation broth 63.47 g/L).

Batch fermentation was conducted in a 2 L fermentor with a working volume of 1 L. The fermentation medium was inoculated with 10% v/v inoculum (20 hours culture of *A. cerevisiae* TN4497 or *A. aceti* TISTR102, 1×10^7 cells/mL for monoculture fermentation; and 20 hours mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 in ratio of 1:1, 1×10^7 cells/mL For coculture fermentation). The fermentation temperature was kept constant at 30 ± 0.2 °C. The broth was kept under agitation at 50 rpm. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol, acetic acid and residual glucose in the broth. The flow chart of alcoholic fermentation acetic acid fermentation was shown in Figure 1.

Biomass estimation

Culture dry weight was measured by centrifugation and drying at 105 °C, until no weight change between consecutive measurements was observed.

Sugar estimation

Glucose was determined by glucose (go) assay kit (Sigma Product No. G 3660)

Acetic acid and ethanol assay

Acetic acid and ethanol was determined by gas chromatography. An AT-Wax capillary column were used in GC system. The parameters were set as follows: carrier gas nitrogen, flow rate of carrier gas 10 mL/min, split ratio 10:1, temperature of injection port 220°C, temperature of detector 260°C. The column temperature program, firstly kept at 50°C for 3 min, secondly heated up to 80°C by the rate of 10°C/min, then immediately heated up to 200°C by the rate of 30°C/min and then kept at 200°C for 1 min. Sample pretreatment was carried out as follows: 5 mL of samples were centrifuged at 15,000 g for 3–5 min. The supernatant was diluted to suitable concentration by decuple dilution and filtrated through 0.22 µm microporous absorbance of each sample was measured against a blank at 750 nm. A calibration curve was constructed using 50, 100, 150, 200 and 250 mg/l gallic acid as a standard. The TPC is expressed as milligrams of gallic acid per gram.

Total flavonoid content

The total flavonoid content was determined using a modified version of the method described by Zhishen et al.¹⁰ Each test sample (250 µl) and 1.25 ml of distilled water were added then 75 µl of 5% NaNO₂ and 150 µl of 10% AlCl₃ was added. After 6 min 0.5 ml of 1 M NaOH was added. The absorbance of the solution was measured against a blank at 510 nm using a spectrophotometer. A calibration curve was constructed using

membrane. Then 1.0 mL of each sample was mixed with 10 µL of isobutanol as internal standard. Standard sample was the mixture of 0.02% ethanol and 0.02% acetic acid. When GC worked stably, samples were injected with the volume of 1 µL.

Total phenolic content (TPC)

The TPC was determined using a modified Folin–Ciocalteu method.⁹ Each test sample (250 µl) was added to a test tube that contained 6.0 ml of distilled water. After vortexing the tubes, 500 µl of Folin–Ciocalteu's phenol reagent was added to each tube. The tubes were vortexed and 2 min later, 2.0 ml of 15% Na₂CO₃ was added to each tube. 1.25 ml of distilled water was added to each tube. The tubes were vortexed again and then allowed to stand for 2 h at room temperature. Thereafter, the 0.125, 0.25, 0.5 and 1.0 g/l quercetin as a standard. The total flavonoid content is expressed as milligrams of quercetin per gram of dry extract.

Free-radical-scavenging activity

Antioxidant activity was measured based on the scavenging of the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). A sample of each fraction (2.0 ml), in methanol, was added to 2.0 of a solution that contained DPPH. After 30 min, the absorbance was measured at a wavelength of 517 nm using UV-visible spectrophotometer.

DPPH assay

The free radical scavenging activity of different fractions was measured by the DPPH (1-1 Diphenyl-2-picryl hydrazyl)

scavenging method proposed by Shimada, et al.¹¹ 2.5×10^{-4} M solution of DPPH in methanol were prepared and 2.0 ml of this solution were added to 2.0 ml of different black rice extracts obtained in different storage conditions. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. The DPPH radical-scavenging activity was calculated according to the following: % of DPPH scavenging activity = $\{1 - (AbS/AbC)\} \times 100$, where AbC was the absorbance of the control and AbS was the absorbance in the presence of the test compound. EC_{50} is the effective concentration in mg extract/ml which inhibits the DPPH activity by 50%. BHA was used for comparison.

Anthocyanin analysis

Anthocyanin content was determined using the pH-differential method.¹² A pigment content was calculated as cyanidin-3-glucoside and expressed as milligrams per 100 g of dry weight, using an extinction coefficient of $26,900 \text{ Lcm}^{-1} \text{ mol}^{-1}$ and molecular weight of 449.2 gmol^{-1} . All experiments were carried out in duplicate.

Results

The compositions and physiochemical properties of the Roselle extract, mush and wine were then determined, and the results of this primary fermentation were presented in Table 1

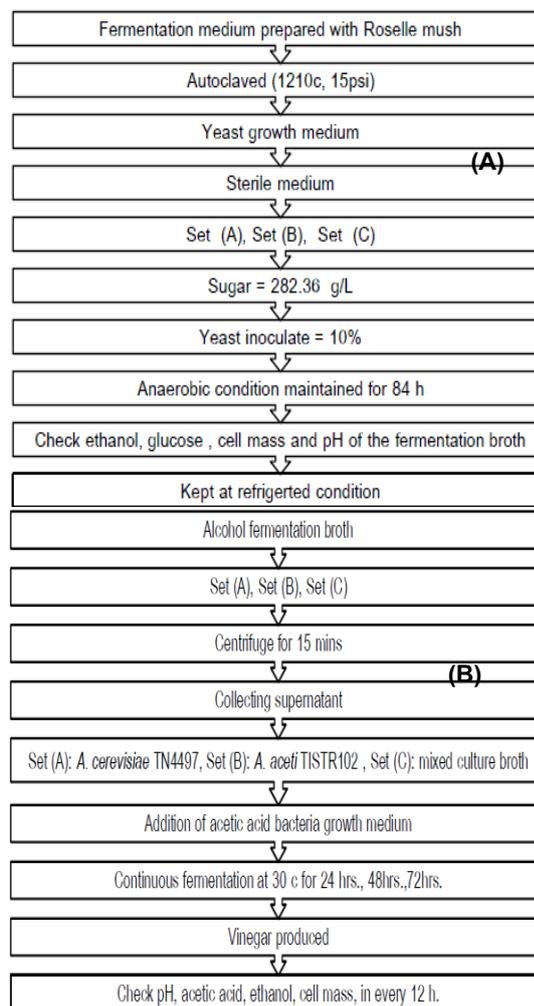


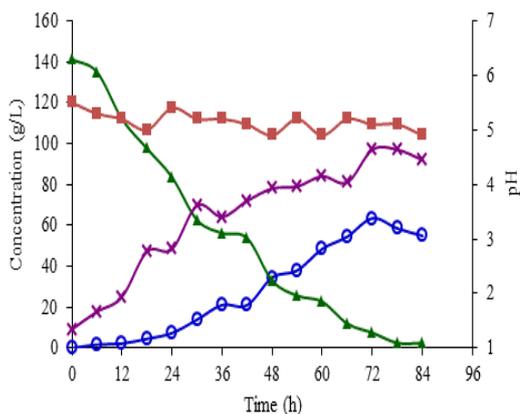
Figure 1 Flow charts of (A): alcoholic fermentation and (B): acetic acid fermentation of Roselle mush

As shown in Figure 2, the content of ethanol in culture at 84 h was 54.95 g/L. while the maximum ethanol concentration (63.47 g/L) was at 72 h. Meanwhile, pH value was about 4.90, the biomass of the culture of *S. cerevisiae* TISTR5048 was promoted from 7.36 g/L at 0 h to 92.37 g/L at 84 h. The glucose was utilized by *S. cerevisiae* TISTR5048 after inoculation, and *S. cerevisiae* TISTR5048 reached the maximum growth (97.14 g/L) at 72 h.

Table 1 The compositions and physiochemical properties of the Roselle extract, mush and wine of *Saccharomyces cerevisiae* TISTR5048

Parameter	Extract	Mush	Roselle wine
Total soluble solids (Brix)	8.2±0.09a	23.7±0.04b	0.78±0.04c
Acetic acid (g/L)	3.27±0.06a	2.01±0.07b	6.49±0.01c
pH	4.52±0.02a	5.5±0.06b	5.2±0.02c
Glucose (g/L)	3.02±0.05a	282.36 ±0.09b	0.37±0.05c
Ethanol (g/L)	0.00a	0.00b	63.47 ±0.20c

Each value is expressed as mean ± SE (n = 3). Means with different small letters within a row are significantly different ($P < 0.05$)

**Figure 2** The time course about culture of *S. cerevisiae* TISTR5048 with batch fermentation of ethanol production (○), glucose content (▲), the cell growth (×) and pH (■)

As shown in Figure 3(A), the maximum acetic acid concentration (45.12 g/L) was at 168 h. Meanwhile, pH value was about 3.91, the biomass of *A. cerevisiae* TN4497 was promoted from 9.26 g/L at 0 h to 64.36 g/L at 168 h. The ethanol was utilized by the mixed culture after inoculation, and the mixed culture reached the maximum growth at 168 h. As shown in Figure 3(B), the maximum acetic acid concentration (49.03 g/L) was at 168 h. Meanwhile, pH value was about 3.62, the biomass of *A. aceti* TISTR102 was

promoted from 9.26 g/L at 0 h to 72.14 g/L at 168 h. The ethanol was utilized by the mixed culture after inoculation, and the mixed culture reached the maximum growth at 168 h.

As shown in Figure 3(C), the content of acetic acid in culture at 132 h was 59.23 g/L. while the maximum acetic acid concentration (62.21 g/L) was at 168 h. Meanwhile, pH value was about 3.40, the biomass of the mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 was promoted from 9.26 g/L at 0 h to 81.47 g/L at 168 h. The ethanol was utilized by the mixed culture after inoculation, and the mixed culture reached the maximum growth at 168 h. The maximum ethanol production (P_{MAX}), ethanol production rate (Q_E), product (ethanol) yield coefficient ($Y_{P/S}$) and cell mass yield coefficient ($Y_{X/S}$) by *S. cerevisiae* TISTR5048 fermentation were found to be 63.47 g/L, 2.25 g/L/h 0.45 g (g-total sugar)⁻¹ and 1.53 g (g-cell mass)⁻¹, respectively. For acetic acid fermentation by the mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102, the maximum acetic acid production (P_{MAX}), acetic acid production rate (Q_E), acetic acid

yield coefficient ($Y_{P/S}$) and cell mass yield coefficient ($Y_{X/S}$): were found to be 69.21 g/L, 1.15 g/L/h, 1.05 g (g-ethanol)⁻¹ and 1.17 g (g-cell mass)⁻¹, respectively. It was found that the mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 could produce relatively high acetic acid yield (Table 2). The TPC, flavonoid and anthocyanin contents were different significantly between Roselle

extract, wine and vinegar (Table 3). In the present study, Roselle vinegar, with an average TAC of 3.42±0.05 mg/L, was found to possess the highest TPC among Roselle extract and wine, which were 10.22 and 2.22 times higher than that of Roselle extract (3.42±0.05 mg/L) and wine (15.74±0.06 mg/L), respectively (Table 3).

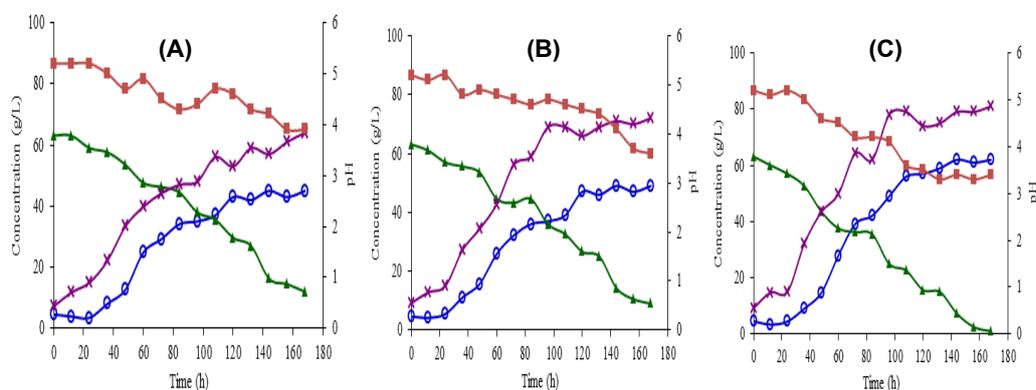


Figure 3 The time course about (A): *A. cerevisiae* TN4497, (B): *A. aceti* TISTR102 and (C): mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 with batch fermentation of acetic acid production (○), ethanol content (▲), the cell growth (×) and pH (■)

Table 2 Ethanol production with *S. cerevisiae* TISTR5048 and acetic acid production with monoculture and mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102

Strain	P_{MAX}	Q_E	$Y_{P/S}$	$Y_{X/S}$
<i>S. cerevisiae</i> TISTR5048	63.47	2.25	0.45	1.53
<i>A. cerevisiae</i> TN4497	45.12	0.07	0.71	1.02
<i>A. aceti</i> TISTR102	49.03	0.08	0.78	1.14
Mixed culture of <i>A. cerevisiae</i> TN4497 and <i>A. aceti</i> TISTR102	62.21	0.57	0.98	1.28

Q_E : Production rate (g/L/h)

P_{MAX} : Maximum production (g/L)

$Y_{P/S}$: Product yield coefficient (g (g-total substrate)⁻¹)

$Y_{X/S}$: Cell mass yield coefficient (g (g-total substrate)⁻¹)

Table 3 Variations in TPC, flavonoid, anthocyanin contents and antioxidant capacities of Roselle extract, wine and vinegar of mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102

Roselle	TPC ^a (mg/L)	Flavonoid ^a (mg/L)	Anthocyanin ^a (mg/L)	Antioxidant capacity ^{a,b} (mg/L)
Extract	3.42±0.05a	0.54±0.02a	47.22±0.16a	43.62±0.06a
Wine	15.74±0.06b	0.92±0.01b	82.63±0.24b	32.28±0.04b
Vinegar	34.19±0.14c	3.46±0.04c	94.75±0.32c	21.79±0.07c

^aEach value is expressed as mean ± SE (n = 3). Means with different small letters within a column at a specific antioxidant attribute were significantly different ($P < 0.05$)

^bEC₅₀ value, the effective concentration at which the antioxidant activity was 50%; the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was scavenged by 50%. EC₅₀ value was obtained by interpolation from linear regression analysis

Roselle vinegar, with an average flavonoid content of 3.46±0.04 mg/L, was found to possess the highest flavonoid content among Roselle extract and wine, which were 6.40 and 3.74 times higher than that of Roselle extract (0.54±0.02 mg/L) and wine (0.924±0.01mg/L), respectively (Table 3). Roselle vinegar, with an average anthocyanin of 94.75±0.32 mg/L, was found to possess the highest anthocyanin content among Roselle extract and wine, which are 2.00 and 1.14 times higher than that of Roselle extract (47.22±0.16 mg/L) and wine (82.63±0.24 mg/L), respectively (Table 3).

The functional properties of fruit juice are attributed to diverse phenolic compounds available in the juice. These functional compounds act as antioxidant, anti-radical, anti-proliferative and lipid peroxidation inhibitor. Some researchers attributed their structural criteria to the potent biological activity of these components to free hydroxyl groups. Phenolic compounds found in fresh pomegranate juice are generally glycosylated with sugars while fermentation of the juice and sugar consumption by micro-organisms

results in deglycosylation and release of free hydroxyl groups and relevant aglycones which can contribute to improve functional properties of pomegranate.¹³ In recent years, fermentation of different fruit was studied by several authors.^{14, 15} Results revealed that since fruit juices may be served as a suitable medium to cultivate bacteria to enhance the health benefits of the food product. The free radical scavenging effect of the juice was increased through fermentation; however, the enhanced effect of fermentation varied with the employed bacteria since acetic acid bacteria exhibited higher improvement in the antioxidant activity of the juice. Improvements in the radical scavenging effect can be related to the increase in the free form of phenolic compounds and the production of other by-products through fermentation. These findings highlight the beneficial effect of juice fermentation by the bacteria able to produce organic acids and improve the antioxidant activity of the fruit juice. Total antioxidant activity of the Roselle vinegar was higher than that of Roselle extract and wine and a significant difference was

determined (Table 3). However, the total antioxidant activities of all the Roselle vinegar were higher than that of the positive control BHT (EC_{50} of 27.30 mg/L).

The DPPH radical is a stable organic free radical with an adsorption band at 517 nm. It loses this adsorption when accepting an electron or a free radical species, which results in a visually noticeable discoloration from purple to yellow. In this study, the high DPPH radical scavenging activity of BHT and the Roselle extract, wine and vinegar were observed in a concentration dependent manner (Table 3). The Roselle extract, wine and vinegar (EC_{50} , 43.62, 32.28 and 21.79 mg/L, respectively) was more active than the positive control BHT (EC_{50} , 27.30 mg/L) Which was similar to the EC_{50} values for DPPH of BHT studied by Hassan et al,¹⁶ and Li and Chen,¹⁷ 25.00 mg/L and 25.92 mg/L, respectively. The high DPPH radical scavenging activity of the Roselle extract, wine and vinegar were attributed to the presence of anthocyanin and acetic acid fermentation.

Discussion

The decrease in alcohol concentration was corresponding to the gradual rise in acetic acid concentration which accumulated from 4.63 g/L to 65.26 g/L over a fermentation progress period of 168 h by the mixed culture. Alcohol induces stress in yeast cells causing their death and flocculation, but the stress of yeast is more related to acetaldehyde which is the first intermediate product of ethanol which is oxidise to acetic

acid by acetic acid producing bacteria. This acetaldehyde disrupts the enzymatic activity of yeast. The beginning of acetic acid formation is related to maximum cellular growth and sufficient biomass density to start the acetification process.¹⁸

The pH of the vinegar during the secondary fermentation by the mixed culture was observed to decrease from pH 5.2 to pH 3.43. This initial decrease in pH provided optimal growth conditions to acetification. This fall in pH and it can be attributed to accumulation of acetic acid and other volatile short chain organic acids such as propionic, tartaric and butyric acids, which are important in development of the flavor and aroma of vinegar.¹⁸

The ethanol content continued to decrease with time from 63.47 g/L to about 0.26 g/L by 168 h. This interpreted that the ethanol conversion to acetic acid reached zero when acetic acid reached to the maximum in the medium. The vinegar produced by the mixed cultures using the Roselle extract contained 62.21 g/L acetic acid or about 6.92 % (v/v) acetic acid and was comparable with 6.33% (v/v) and 6.11% (v/v) vinegar obtained by Vegas et al¹⁹ in their study of two vinegar plants; Laguinelle (B, Banyuls, France) and Viticultors Masd'en gil (P, bellmunt del priorat, Tarragona, Spain). The composition and functional properties of the Roselle vinegar showed potential as a food-grade analog. Therefore, it was of interest to determine the anthocyanin contents of the Roselle, since this was the major antioxidants in vinegar. From the

results, total antioxidant activity of the Roselle vinegar was higher than that of Roselle extract and wine due to higher TPC, flavonoid and anthocyanin contents.

Processing methods are known to have variable effects on TPC and antioxidant activity of plant samples. Effects include little or no change, significant decreases or enhancement in antioxidant activity.²⁰ Food processing can improve the properties of naturally occurring antioxidants or induce the formation of compounds with antioxidant activity, so that the overall antioxidant activity increases or remains unchanged.²¹ Increase in antioxidant activity following thermal treatment has been reported in tomato,²² sweet corn²³ and ginseng.²⁴ Increase in antioxidant activity following thermal treatment has been attributed to the release of bound phenolic compounds brought about by the breakdown of cellular constituents and the formation of compounds with enhanced antioxidant activity.

Fruits and vegetables contain many antioxidant compounds, including phenolic compounds, flavonoids, anthocyanins and tocopherols.²⁵ Most antioxidants are polyphenolic compounds, which act as reducing agents (free radical terminators), metal chelators, singlet oxygen quenchers²⁶ and hydrogen donors.²⁷ Fermentation is a technology used to enhance the shelf-life and nutritional and organoleptic qualities of food.²⁸ Many biochemical changes occur during fermentation, leading to an altered ratio of

nutritive and anti-nutritive components and, consequently, affects the products properties, such as bioactivity and digestibility.²⁹ Recently, this bioprocess has been applied to the production and extraction of bioactive compounds in the food, chemical and pharmaceutical industries.^{30, 31} For example, fermentation has been applied to increase the content of bioactive phenolic compounds in legumes, thus enhancing their antioxidant activity.³² Torino et al³¹ reported that the bioconversion of the conjugated forms of phenolic compounds into their free forms during fermentation improves their health-linked functionality. Increasing the antioxidative activity of plant-based foods by fermentation may be influenced by various factors, including the microorganism species, pH, temperature, solvent, water content, fermentation time, kind of food and aerobic conditions. This study provides an study of the fermentation that influence antioxidative activity that augment antioxidative activities in Roselle vinegar.

The most important properties of a production strain in vinegar industry are tolerance to high concentrations of acetic acid and total concentration, low nutrient requirements, inability to over oxidize the formed acetic acid, high production rate. Although a variety of bacteria can produce acetic acid, mostly members of *Acetobacter* are used commercially. Pure cultures are not widely used in the acetic acid fermentation industry.³³

Conclusions

In conclusion, fermentation condition influenced the level of TPC, flavoid, anthocyanin and antioxidants in Roselle vinegar. After fermentation, the bioactive compounds and antioxidant capacity of Roselle vinegar was increased. Roselle vinegar increased in TPC, flavonoid, anthocyanin and total antioxidant activity higher than that of Roselle extract and wine. This finding is important as anthocyanin, TPC and flavonoids are potent and high value antioxidant compound and Roselle vinegar product could be the source of these compounds. From this study, the results showed that the mixed culture could shorten the fermentation time, reduce fermentation losses and increase the yield of acetic acid.

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