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Antioxidant Properties of Roselle Vinegar Production by Mixed Culture of *Acetobacter aceti* and *Acetobacter cerevisiae*

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ABSTRACT

Roselle (*Hibiscus sabdariffa*) has been used in folk medicine and as a treatment for diseases and cancer. The objective of this study was to enhance the acetic acid production and the antioxidant activities (AOA) of Roselle vinegar using mixed culture fermentation. The Roselle wine was produced in a batch reactor. The kinetic parameters obtained for wine fermentation using *Saccharomyces cerevisiae* were: maximum ethanol production = 63.47 $g.L^{-1}$, ethanol production rate = 2.25 $g.L^{-1}.hr^{-1}$, product (ethanol) yield coefficient = 0.45 and cell mass yield coefficient = 1.53. Using a mixed culture of *Acetobacter aceti* and *Acetobacter cervisiae*, the wine vinegar process optimization ranges for initial concentrations of ethanol and acetic acid as independent variables were 63.47 and 69.21 $g.L^{-1}$, respectively. Roselle was processed into juice fermentation and vinegars. The total phenolics, total anthocyanins, and antiradical activity (1-1 diphenyl-2-picryl hydrazyl radical-scavenging (DPPH) method) were determined. Acetification increased the total anthocyanin content, total polyphenols and antioxidant activities. Correlations indicated that anthocyanins made greater significant contributions than did phenolics to the antioxidant activities of the products. The antiradical activity of wine from Roselle juice was high with an effective concentration which inhibited the DPPH activity by 50% (EC_{50}) of 14.95 $mg.L^{-1}$. The antioxidant activity of the vinegar product was high with an EC_{50} of 0.72 $mg.L^{-1}$. The results indicated that fermentation is a better method for obtaining higher antioxidant activity of Roselle products. Furthermore, acetification significantly increased the anthocyanins and antioxidant activities.

Keywords: Roselle, vinegar, antioxidant activity

INTRODUCTION

Reactive oxygen species or free radicals are generated as byproducts or intermediates of aerobic metabolism and through reactions with drugs and environmental toxins (Devasagayam *et al*., 2004). 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 damage (Simic, 1988) caused by endogenous or exogenous oxidants (Sun, 1990). 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

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are widely distributed in plants, were considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems (Hertog and Feskens, 1993). The study of numerous compounds that could be useful antioxidants has generated increasing interest in the fields of food and medicine. The dried flowers of *Hibiscus sabdariffa* L. (Malvaceae) commonly called "Roselle" have gained importance as a local soft drink and as a medical herb in local regions, as studies have 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 (Tseng *et al*., 1997). Included in foods for centuries, white vinegar, a natural source of distilled acetic acid, provides significant and substantial health benefits when consumed in moderation (Fushimi *et al*., 2006). Anecdotal evidence suggests possible further astonishing benefits, including improving calcium absorption, and moderating type 2 diabetes and even some cancers (Sugiyama *et al*., 2003, Seki *et al*., 2004). The acetic acid process is divided into two fermentation processes according to the characters of the growth and metabolization of *S. cerevisiae* and acetic acid bacteria—namely, primary fermentation for the growth of *S. cerevisiae* and the production of ethanol; and secondary fermentation for the production of acetic acid by acetic acid bacteria (Raspor and Goranovic, 2008). *S. cerevisiae* is a facultative anaerobe, while the acetic acid bacteria are aerobes (Raspor and Goranovic, 2008). Mixed culture systems exhibit great advantages for many processes involving more than one reaction step, such as high productivity (Mendoza *et al*., 2011).

Mixed culture fermentation may become an attractive addition to traditional pure-culture-based biotechnology for traditional fermented foods (Temudo *et al*., 2008). Although the advantages of mixed culture over pure culture have been pointed out (Harrison, 1978), little is known about the application of mixed culture technology in acetic acid production (Tetsuya and Masao, 1996). In the current study, a mixed culture of *Acetobacter aceti* and *Acetobacter cervisiae* was optimized in batch culture to enhance the yield of acetic acid production and enhance the antioxidant capacity of Roselle vinegar.

MATERIALS AND METHODS

Microorganisms

Acetobacter aceti, *Acetobacter cerevisiae* and *Saccharomyces cerevisiae* were obtained from the Thailand Institute of Scientific and Technological Research.

Preparation of the Roselle must

Dried powder (200 g) of Roselle was added with 1 L of distillated water. The physiochemical properties of the extract were measured and are recorded in Table 1. The filtrate was ameliorated using glucose to raise the sugar level to 23.7 ºBrix and ammonium sulfate (111 ppm) added to stabilize the must and to provide a nitrogen source for the yeast. The must was

Table 1 Composition and physiochemical properties of Roselle extract, must and wine.

Parameter	Extract	Must	Roselle wine
Total soluble solids (°Brix)	8.20±0.09^{a}	23.71±0.04^{b}	0.78±0.04^{c}
Acetic acid (g.L^{-1})	3.27±0.06^{a}	2.01±0.07^{b}	6.49±0.01^{c}
pH	4.52±0.02^{a}	5.52±0.06^{b}	5.23±0.02^{c}
Glucose (g.L^{-1})	3.02±0.05^{a}	282.36±0.09^{a}	0.37±0.05^{c}
Ethanol (g.L^{-1})	0.00^{a}	0.00^{b}	63.47±0.20^{b}

Values expressed as mean ± SE (n = 3).

Means with different lowercase superscripts within a row are significantly different ($P < 0.05$).

adjusted to pH 5. The treated must was pasteurized at 63 ºC for 30 min and then allowed to cool to room temperature (25 ºC). The supernatant was supplemented with additional nutrients to give a base medium composition of: 1 $g.L^{-1}$ yeast extract; 2 $g.L^{-1}$ $(NH_4)_2SO_4$ and 1 $g.L^{-1}$ $MgSO_4 \bullet 7H_2O$.

Batch fermentation

Batch fermentation was conducted in a 2 L fermentor with a working volume of 1 L. The fermentation medium was inoculated with 5% volume per volume (v/v) inoculum (20 hr culture, 1×10^7 $cells.mL^{-1}$). 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 fermentation to determine the concentrations of the cell mass, ethanol, acetic acid and residual glucose in the broth. All experiments were carried out in duplicate.

Biomass estimation

The 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 using a glucose assay kit (No. G 3660; Sigma; Saint Louis, MO, USA.).

Acetic acid and ethanol assay

The acetic acid and ethanol assay was determined by gas chromatography (GC). An AT-wax capillary column was used in the GC system. The parameters were set at: carrier gas of nitrogen, flow rate of carrier gas 10 $mL.min^{-1}$, split ratio 10:1, temperature of injection port 220 ºC and temperature of detector 260 °C. The column temperature program, was initially kept at 50 °C for 3 min, then heated up to 80 °C at the rate of 10 $°C.min^{-1}$, immediately heated up to 200 °C at the rate of 30 $°C.min^{-1}$ and then kept at 200 ºC for 1 min. Sample pretreatment was carried out as follows. Samples of 5 mL were centrifuged at 10,000 rpm for 3–5 min. The supernatant was diluted to a suitable concentration by decuple dilution and filtrated through a 0.22 µm microporous membrane. Then, 1.0 mL of each sample was mixed with 10 µL of isobutanol as an internal standard. The standard sample was a mixture of 0.02% isobutanol, 0.02% ethanol and 0.02% acetic acid. When the GC was working in a stable manner, samples were injected with a volume of 1 µL.

Total phenolic content

The total phenolic content (TPC) was determined using a modified Folin-Ciocalteu method (Singleton and Rossi, 1965). A calibration curve was constructed using gallic acid (Fluka; Buchs, Switzerland) as a standard. The TPC was expressed in 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*. (1999). Each test sample (250 µL) and 1.25 mL of distilled water were added and then 75 µL of 5% $NaNO_2$ and 150 µL of 10% $AlCl_3$ were 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 0.125, 0.25, 0.5 and 1.0 $g.L^{-1}$ quercetin as a standard. The total flavonoid content was expressed as milligrams of quercetin per gram of dry extract.

Anthocyanin analysis

The monomeric anthocyanin content was determined using the pH-differential method (Abdel-Aal *et al*., 2006). A pigment content was calculated as cyanidin-3-glucoside and expressed as milligrams per 100 g of fresh weight, using an extinction coefficient of 26,900 $L.cm^{-1}.mol^{-1}$and a molecular weight of 449.2 $g.mol^{-1}$.

1-1 Diphenyl-2-picryl hydrazyl assay

The free radical scavenging activity of different fractions was measured using 1-1 diphenyl-2-picryl hydrazyl (DPPH) according to Shimada *et al*. (1992). The DPPH radical-scavenging activity was calculated according to Equation 1:

Percentage of DPPH scavenging activity = {1- (AbS/AbC)} × 100 (1)

where AbC is the absorbance of the control and AbS is the absorbance in the presence of the test compound. The effective concentration in milligrams of extract per milliliter which inhibits the DPPH activity by 50% (EC_{50}) was used. 1, 1-Diphenyl-2-picrylhydrazyl, butylated hydroxyanisole (BHA) was used for comparison.

RESULTS

Approximately 1.0 L of Roselle extract was poured into a 2 L fermentor and inoculated with 10 mL of the yeast starter culture. The inoculated slurry of Roselle extract was subjected to primary fermentation at ambient temperature for 3 d to produce Roselle wine, which was then filtered after complete primary fermentation. The composition and physiochemical properties of the Roselle extract, must and wine were then determined, and the results of this primary fermentation are presented in Table 1.

As shown in Figure 1, the content of ethanol in culture at 84 hr was 54.95 $g.L^{-1}$ while the maximum ethanol concentration (63.47 $g.L^{-1}$) was at 72 hr. The pH was 5.0, and the biomass of the culture of *S. cerevisiae* increased from 7.36 $g.L^{-1}$ at 0 hr to 92.37 $g.L^{-1}$ at 84 hr. The glucose was utilized by *S. cerevisiae* after inoculation and *S. cerevisiae* reached its maximum growth (97.14 $g.L^{-1}$) at 72 hr.

As shown in Figure 2, the maximum acetic acid concentration (45.12 $g.L^{-1}$) was at 168 hr. The pH was 3.91, while the biomass of *A. cerevisiae* increased from 9.26 $g.L^{-1}$ at 0 hr to 64.36 $g.L^{-1}$ at 168 hr. The ethanol was utilized by the mixed culture after inoculation and the mixed culture reached its maximum growth at 168 hr.

As shown in Figure 3, the maximum acetic acid concentration (49.03 $g.L^{-1}$) was at 168 h. The pH was 3.62 while the biomass of *A. aceti* increased from 9.26 $g.L^{-1}$ at 0 hr to 72.14 $g.L^{-1}$ at 168 hr. The ethanol was utilized by the mixed

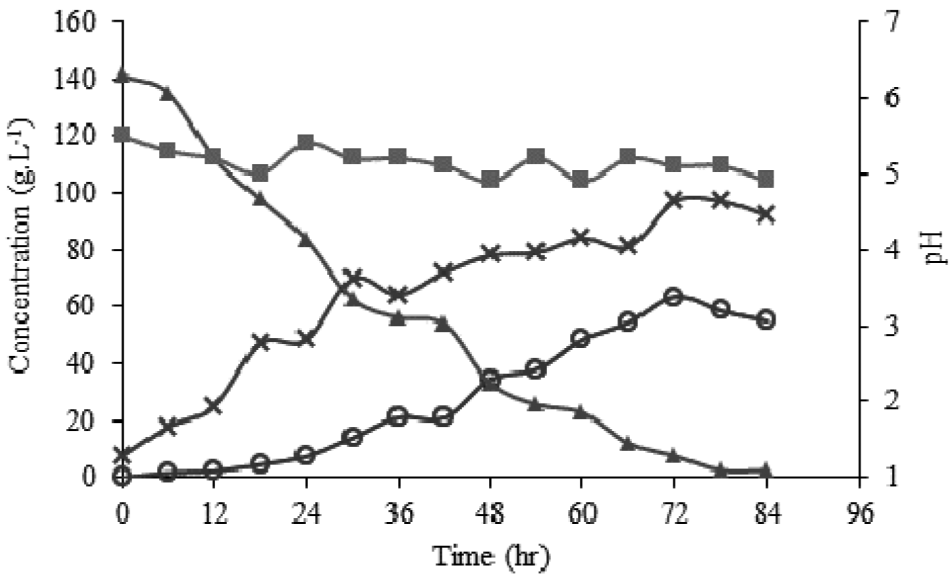

Figure 1 Yeast culture with batch fermentation: ethanol production (○), glucose content (▲), cell growth (×) and pH (■).

culture after inoculation and the mixed culture reached its maximum growth at 168 hr.

As shown in Figure 4, the content of acetic acid in culture at 132 hr was 59.23 $g.L^{-1}$. while the maximum acetic acid concentration (62.21 $g.L^{-1}$) was at 168 hr. The pH was 3.40 while the biomass of the mixed culture of *A. cerevisiae* and *A. aceti* increased from 9.26 $g.L^{-1}$ at 0 hr to 81.47 $g.L^{-1}$ at 168 hr. The ethanol was utilized by the mixed culture after inoculation and the mixed culture reached its maximum growth at 168 hr.

The maximum ethanol production (P_{MAX}), ethanol production rate (Q_E), product (ethanol) yield coefficient ($Y_{P/S}$) and cell mass yield coefficient ($Y_{X/S}$) from *S. cerevisiae* fermentation were 63.47 $g.L^{-1}$, 2.25 $g.L^{-1}.hr^{-1}$ 0.45

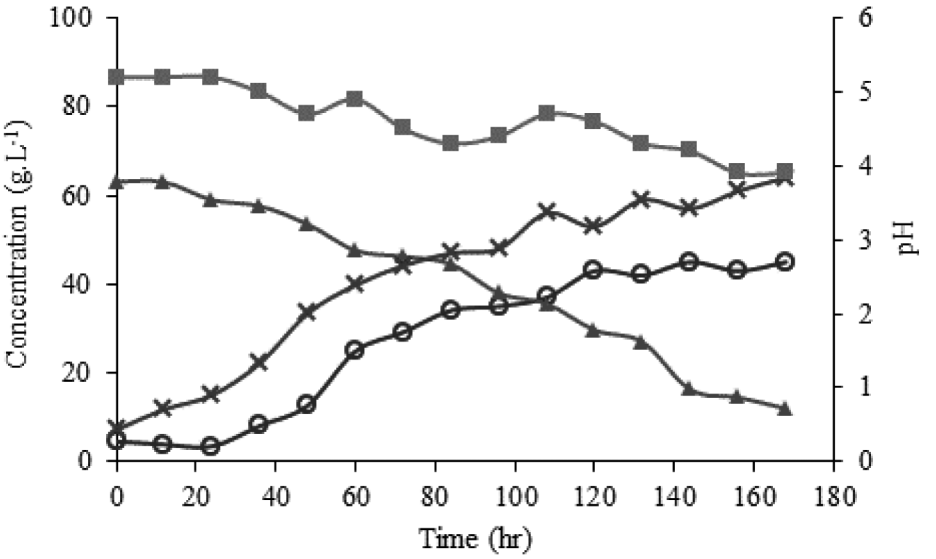

Figure 2 *A. cerevisiae* with batch fermentation: acetic acid production (○), glucose content (▲), the cell growth (×) and pH (■).

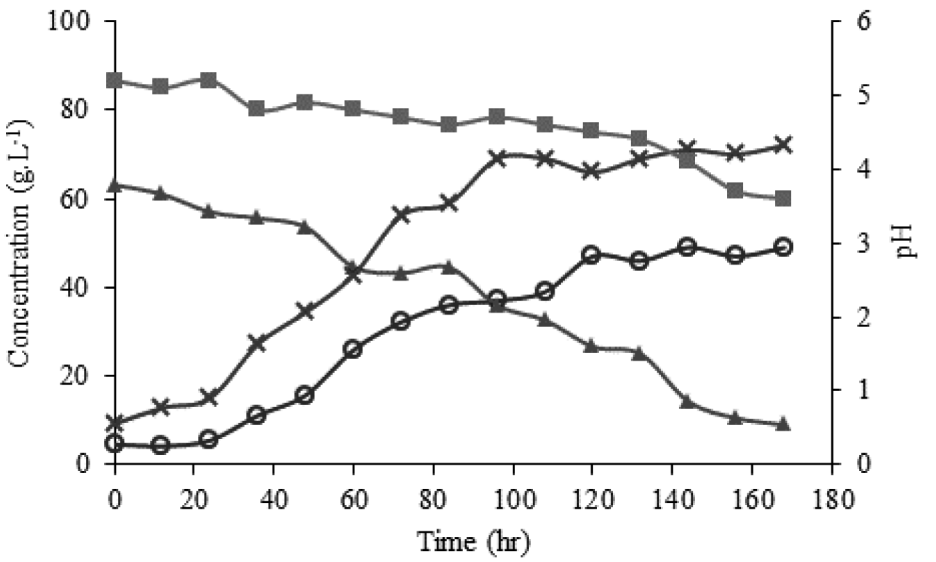

Figure 3 *A. aceti* with batch fermentation: acetic acid production (○), glucose content (▲), the cell growth (×) and pH (■).

g per gram total sugar and 1.53 g per gram cell mass, respectively. For the acetic acid fermentation by the mixed culture of *A. cerevisiae* and *A. aceti*, P_{MAX}, Q_E, $Y_{P/S}$ and $Y_{X/S}$ were 69.21 $g.L^{-1}$, 1.15 $g.L^{-1}.hr^{-1}$, 1.05 g per gram ethanol and 1.17 g per gram cell mass, respectively. The mixed culture of *A. cerevisiae* and *A. aceti* produced a relatively high acetic acid yield (Table 2).

The TAC, flavonoid and anthocyanin contents were significantly ($P < 0.05$) different among the Roselle extract, wine and vinegar (Table 3). Roselle vinegar, with an average TAC of 34.19 ± 0.14 $mg.L^{-1}$, possessed the highest TPC, which was 10.22 and 2.22 times higher than that of Roselle extract (3.42 ± 0.05 $mg.L^{-1}$) and wine (15.74 ± 0.06 $mg.L^{-1}$), respectively (Table 3).

Roselle vinegar, with an average flavonoid content of 3.46 ± 0.04 $mg.L^{-1}$, possessed the highest flavonoid content, which was 6.40 and 3.74 times higher than that of Roselle extract (0.54 ± 0.02 $mg.L^{-1}$) and wine (0.92 ± 0.01 $mg.L^{-1}$), respectively as shown in Table 3. Roselle vinegar, with an average anthocyanin content of 94.75 ± 0.32 $mg.L^{-1}$, possessed the highest anthocyanin content, which was 2.00 and 1.14 times higher than that of Roselle extract (47.22 ± 0.16 $mg.L^{-1}$) and wine (82.63 ± 0.24 $mg.L^{-1}$), respectively (Table 3). The total antioxidant activity of the Roselle vinegar was significantly ($P < 0.05$) higher than that of Roselle extract and wine (Table 3). However, the total antioxidant activities of all the Roselle products were higher than that of the

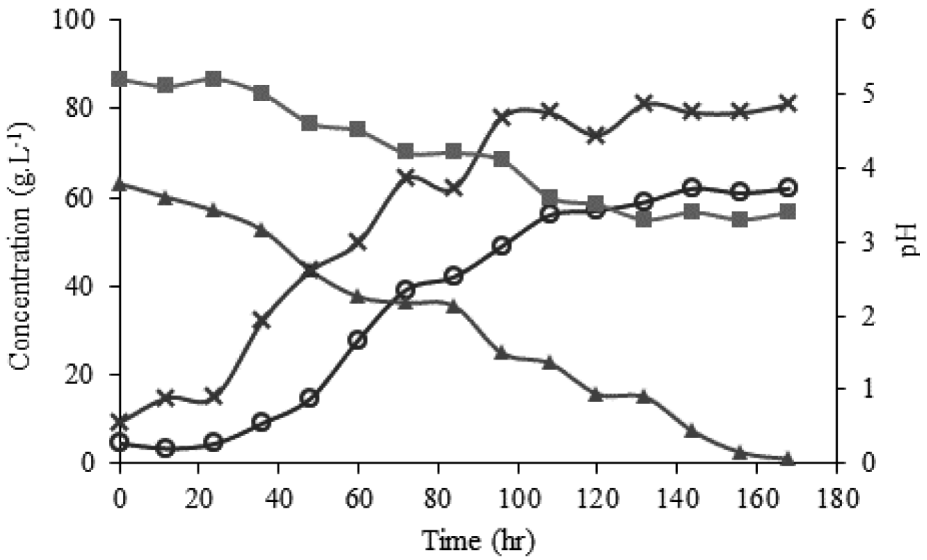

Figure 4 Mixed culture of *A. cerevisiae* and *A. aceti* with batch fermentation: acetic acid production (○), glucose content (▲), the cell growth (×) and pH (■).

Table 2 Ethanol production with *S. cerevisiae,* and acetic acid production with a monoculture and mixed culture of *A. cerevisiae* and *A. aceti*

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

P_{MAX} = Maximum production ($g.L^{-1}$); Q_E = Production rate ($g.L^{-1}.hr^{-1}$), $Y_{P/S}$ = Product yield coefficient (g sugar per gram total substrate); $Y_{X/S}$ = Cell mass yield coefficient (g cell mass per gram total substrate).

positive control BHA (EC_{50} of 27.30 $mg.L^{-1}$).

DISCUSSION

The decrease in the alcohol concentration corresponded to the gradual rise in the acetic acid concentration which accumulated from 4.63 to 65.26 $g.L^{-1}$ over a fermentation period of 168 hr with the mixed culture. Alcohol induces stress in yeast cells causing their death and flocculation (Jimoh *et al*., 2013), but the stress of yeast was more related to acetaldehyde which is the first intermediate product of ethanol which is oxidized to acetic acid by the acetic-acid-producing bacteria and this acetaldehyde disrupts the enzymatic activity of yeast (Claro *et al*., 2007). The beginning of acetic acid formation is related to maximum cellular growth and having sufficient biomass density to start the acetification process (Pan *et al*., 2014).

The pH of the vinegar during the secondary fermentation by the mixed culture decreased from pH 5.2 to 3.43. This initial decrease in pH provided optimal growth conditions for acetification and can be attributed to the accumulation of acetic acid and other volatile short chain organic acids such as propionic, tartaric and butyric acids, which are important in the development of the flavor and aroma of vinegar (Pan *et al*., 2014).

The ethanol content continued to decrease with time from 63.47 to 0.26 $g.L^{-1}$ by 168 hr. This could be interpreted as the ethanol conversion to acetic acid reaching zero when the acetic acid is at its maximum in the medium. The vinegar produced by the mixed cultures using the Roselle extract contained 62.21 $g.L^{-1}$ 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 al* (2010) 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 content of the Roselle, since this is the major antioxidant in vinegar (Bagchi *et al*., 2004). The results indicated that the total antioxidant activity of the Roselle vinegar was higher than that of the Roselle extract and wine due to the higher TPC, flavonoid and anthocyanin contents.

Processing methods are known to have variable effects on the TPC and antioxidant activities (AOA) of plant samples. Effects include little or no change, significant decreases or enhancement in AOA (Nicoli, *et al*., 1999). Food processing can improve the properties of naturally occurring antioxidants or induce the formation of compounds with AOA, so that the overall AOA increases or remains unchanged (Tomaino *et al*., 2005). An increase in AOA following thermal treatment has been reported in tomato (Dewanto *et al*., 2002a), sweet corn (Dewanto *et al*., 2002b) and ginseng (Kang *et al*., 2006). An increase in AOA

Table 3 Variations in total phenolics content (TPC), flavonoid, anthocyanin contents and antioxidant capacities of Roselle extract, wine and vinegar.

Roselle	TPC ($mg.L^{-1}$)	Flavonoid ($mg.L^{-1}$)	Anthocyanin ($mg.L^{-1}$)	Antioxidant capacity[#]
Extract	3.42±0.05[a]	0.54±0.02[a]	47.22±0.16[a]	14.95±0.03[a]
Wine	15.74±0.06[b]	0.92±0.01[b]	82.63±0.24[b]	9.41±0.02[b]
Vinegar	34.19±0.14[c]	3.46±0.04[c]	94.75±0.32[c]	0.72±0.04[c]

Values expressed as mean ± SE (n = 3).

[a-c] = Means with different lowercase superscripts within a column for a specific antioxidant attribute are significantly different ($P < 0.05$).

[#] EC_{50} value = Effective concentration at which the antioxidant activity is 50%; the 1,1-diphenyl-2-picrylhydrazyl radical was scavenged by 50%. EC_{50} value was obtained by interpolation from linear regression analysis.

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 AOA (Tomaino *et al*., 2005).

The most important properties of a production strain in the vinegar industry are tolerance to high concentrations of acetic acid and total concentration, low nutrient requirements, the inability to over oxidize the formed acetic acid and a high production rate. Although any variety of bacteria can produce acetic acid, members of the *Acetobacter* are mostly used commercially and pure cultures are not widely used in the acetic acid fermentation industry (O'Toole and Lee, 2003).

CONCLUSION

The fermentation conditions influenced the level of TPC, the flavoid and anthocyanin contents and the antioxidant activity in Roselle vinegar. After fermentation, the bioactive compounds and antioxidant capacity of Roselle vinegar increased. Roselle vinegar increased in TPC, flavonoid and anthocyanin contents and in total antioxidant activity more than Roselle extract and wine. This finding is important as anthocyanin, TPC and flavonoids are potent and high value antioxidant compounds and the Roselle vinegar product could be a source of these compounds. 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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