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Optimization of Vinegar Production from Nipa (*Nypa fruticans* Wurmb.) Sap Using Surface Culture Fermentation Process

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Abstract

Background and objective: Sap from nipa mangrove palms is rich in nutrition and chemical components. Currently, sap is used for production of fresh juice, syrup, molasses, alcohol and traditional vinegar. The aim of this study was to enhance nutritional values of nipa sap in high-quality vinegar using surface culture fermentation.

Material and methods: Vinegar was produced from nipa sap using a two-step surface culture fermentation process including vinegar starter culture preparation and vinegar production. Vinegar acetic acid, residual alcohol and pH were optimized. Nipa sap vinegar from surface culture fermentation was compared to that from traditional methods for compliance with regulatory standards. Antioxidant activities (total phenolic content, 2, 2-diphenyl-2-picrylhydrazyl radical scavenging and ferric reducing antioxidant power assays) and sensory of the product were assessed.

Results and conclusion: Acidity increased to 6.20% using surface culture fermentation at 2.9-fold, compared to that using traditional methods (2.14%). Alcohol concentration included 11.9% during wine fermentation. The surface culture fermentation converted alcohol to acetic acid using *Acetobacter aceti* TISTR 354 in ten days. A good antioxidant activity was reported for the vinegar. Organoleptic properties scored more than "neither like nor dislike" in each attribute. Therefore, high quality vinegars could be produced fromnipa sap using surface culture fermentation which could be scaled up in the future.

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1. Introduction

Nipa is scientifically known as *Nypa fruticans* Wurmb. It is a monoecious pleonanthic palm, used in native language as "chak" in Thailand, "dua la" in Vietnam and "atap palm" in Singapore [1]. Nipa palm is widely distributed and grows naturally with moderate salt tolerance in coastal areas, river estuaries and mangrove forests as well as managed plantations in Southeast Asia and Northern Australia [2]. In Thailand, xylem collected from cut stalks with fully developed inflorescence includes high yields of 0.5-2.5 liters per day and average sugar contents of 16.4% v v^{-1} with an annual production of 126,000-169,000 l ha^{-1} [3]. These high quantities show potentials as sources of raw materials in south of Thailand. However, nipa sap production is limited to local communities for domestic use only as desserts, syrups and molasses to ferment alcoholic beverages or boiled down to sugar with no industrial uses [4,5]. Tamunaidu et al. [6] showed that nipa sap was rich in chemical components including sucrose, glucose, fructose and organic compounds with high concentrations of minerals, vitamins and antioxidant activities which could be used for various purposes. Therefore, Nipa sap includes interesting physical and chemical properties and is an appropriate source of raw materials for vinegar production.

The traditional nipa vinegar was produced and used as a food preservative agent, food ingredient or beverage [7]. The traditional method involved spontaneous micro-organisms using two steps of alcoholic and acetous fermentations in earthen jars or bamboo tubes. However, vinegar produced by traditional methods includes substandard quality and inappropriate for industrial production with low acetic acids and high residual alcohol

concentrations [8]. Earlier methods used for producing vinegar included Orleans (a traditional method) and submerged culture processes. Quick process and submerged culture process have been developed and used for commercial vinegar production now [9]. Surface culture fermentation (SCF) was described by Saithong et al. [10] using basic inexpensive equipment to improve traditional fermentation process and decrease fermentation time to 7-10 days. High acetic acid concentrations over 6.0% v v^{-1} and low residual alcohol concentrations (0.5% v v^{-1}) were achieved using a starter culture to decrease quality of undesirable microorganisms. The SCF process has demonstrated high potentials to produce vinegar from fruits and agriculture materials using two-step methods as vinegar starter culture preparation (two days) and vinegar production (7-10 days). The SCF process is static and easy to use with low operating costs for vinegar production industries.

To the best of the authors' knowledge, SCF process has been used for vinegar production by the current research group. This process was shown for the production of vinegar with various raw materials. Nipa sap stilled vinegar was not used in industrial or the commercial scales because stilled vinegar used traditional processes only. This is the first report on nipa sap vinegar production using SCF process. Experiments were carried out to assess quality and characteristics of the product including antioxidant and comparative sensory properties of nipa sap vinegar from SCF process, compared to traditional methods based on Food and Drug Administration (FDA) regulations.

2. Materials and methods

2-1 Nipa sap samples

Nipa sap was collected from a local market in Pak Phanang Basin, Nakhon Si Thammarat; the largest area of nipa palms in Thailand. Sap was placed in a clean container and transported in an ice basket before storage in freezers until use. Initial sugar contents included 14.5-16.5 Brix, acidity expressed as lactic acid included 1.5% v v^{-1} and pH 2.1 (at 25°C) with no alcohol contents.

2-2 Alcoholic fermentation

Nipa sap was fermented using stainless steel tank with initial sugar (total soluble solids, TSS) concentration adjusted to 22 Brix with nipa palm sugar. Citric acid was added to preserve pH at 3.5-4.0 to optimize yeast fermentation and growth without nutrient supplements. Nipa sap mush was sterilized at 90°C for 5 min and cooled down to room temperature. Then, 10% v v^{-1} of starter culture (prepared by mixing *S. cerevisiae* var. *burgundy*, *S. cerevisiae* var. *montache*) provided by Department of Applied Microbiology, Institute of Food Research and Product Development (IFRPD), Kasetsart University, Thailand, were added to the sap and mixed well. Fermentation was carried out at 28–30°C for four weeks. A 100-ml sample was collected aseptically during fermentation at appropriate time intervals and filtered using 0.45-µm millipore membranes to remove yeast cells and measure TSS. Total acidity was expressed as lactic acid and ethanol concentration was analyzed. Fermentation was stopped when alcohol concentration reached 10.0% v v^{-1}. Nipa sap wine was stored in a closed container at 4-8°C before further use as raw material for acetous fermentation.

2-3 Acetous fermentation

Acetous fermentation was carried out using SCF process as described by Saithong et al. [10]. The process consists of two steps. In the first step of starter culture preparation, 1000 ml of the starter, containing 600 ml of sterilized nipa sap, were adjusted to initial sugar concentration of 5.0 Brix with nipa palm sugar and then poured into 300 ml of nipa sap wine with an alcohol concentration greater than 10.0% v v^{-1} followed by inoculation with 100 ml of *Acetobacter aceti* TISTR 354 starter culture provided by Thailand Institute of Scientific and Technological Research (TISTR). The bacteria were previously used in a 100 ml volume made up 90 ml of sterilized nipa sap with an initial sugar concentration adjusted to 5.0 Brix using nipa palm sugar, 3 ml of 95.0% ethanol and 7 ml of *A. aceti* TISTR 354. The mixture was incubated at 30°C for 72 h before use. The optimized ratio of vinegar starter culture preparation in stainless steel tray included 600:300:100 ml (sterilized nipa sap:nipa sap wine:starter culture). This was mixed well and covered using plastic sheet with perforations for ventilation and then set for 48 h. After 48 h, acetic acid reached values greater than 3.0%. in the second step of vinegar production, 1000 ml of nipa sap wine with an alcohol concentration greater than 10.0% v v^{-1} were added to the stainless steel tray container (vinegar starter culture preparation) and set for 7-10 days. Samples were collected from the fermentation broth and assessed for acidity. Acetic acid was greater than 4.0% w v^{-1} according to FDA regulations and pH changed with residual alcohol. Fermentation process for each sample was carried out thrice. Final properties of nipa sap vinegar produced using SCF process were compared to those of nipa sap vinegar produced using traditional methods.

2-4 Chemical analysis

2-4-1 Alcohol concentration and total soluble solids (TSS)

Alcohol concentration was assessed using ebulliometer (Model #360, Laboratoires Dujardin-Salleron, Paris, France) by measuring differences in boiling points between water and sample solution. The TSS was assessed at 20°C

using hand refractometer (N-1α, 0-32° Brix, Atago, Japan), which reported quickly on spot °Brix results. All experiments were carried out thrice.

2-4-2 The pH and titratable acidity

Briefly, pH was measured using pH meter (model 430, Corning, NY, USA). Titratable acidity as acetic acid was assessed using titration methods based on a modified procedure of AOAC [11]. Samples of 6 ml were pipetted into a 250-ml titration flask and then three drops of phenolphthalein indicator were added into the flask and titrated with 0.1 N NaOH until a pink color appeared. All experiments were carried out thrice.

2-4-3 Antioxidant activity

The antioxidant activity changed during nipa sap vinegar production. The antioxidant activity was assessed using three different analytical methods to ensure results because antioxidant activity is linked to methods and types of samples. Therefore, three properties were assessed according to the procedures described below.

2-4-3-1 Total phenolic content (TPC)

The TPC of nipa sap vinegar was assessed based on a modified method of Iqbal et al. [12]. Folin-Ciocalteu reagent and gallic acid were used as standards. A 0.2-ml sample was transferred into a 3.5-ml quartz cuvette and then 0.8 ml of Folin-Ciocalteu reagent (10.0% v v^{-1}) was added to the cuvette and mixed. Mixture was incubated at room temperature for 8 min in darkness. Then, 1 ml of sodium carbonate solution (7.5% w v^{-1}) was added to the mixture and incubated at room temperature for 90 min in darkness. Absorbance was measured at 760 nm using UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA). All measurements were carried out thrice.

2-4-3-2 The DPPH radical scavenging assay

Free radical scavenging activity of nipa sap vinegar was assessed based on a modified method of Brand-Williams et al. [13] using 2, 2-diphenyl-2-picrylhydrazyl (DPPH). Samples were prepared at various concentrations by transferring 10, 20, 30, 40 and 50 µl of samples into a 3.5-ml quartz cuvette and adding 40, 30, 20, 10 and 0 µl of distilled water. Solutions were mixed well and 950 µl of 0.0394 g l^{-1} DPPH solution (freshly prepared in methanol solution) were added to the cuvette and incubated at room temperature for 30 min in darkness. Absorbance was measured at 515 nm using UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA). All measurements were carried out in triplicate. Concentration was reported as percentage of inactivation.

2-4-3-3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was modified according to Benzie and Strain [14] and carried out using FRAP reagent and Trolox as standard. Briefly 50 µl of the sample were transferred into a 4-ml quartz cuvette and then 950 µL of the FRAP reagent (300 mM l^{-1} of acetate buffer: 20 mM l^{-1} of ferric chloride: 10 mM l^{-1} of TPTZ (2, 4, 6-tripyridyl-*s*-triazine) were added to the cuvette at a ratio of 10:1:1) and incubated at room temperature for 4 min in darkness. Absorbance was measured at 593 nm using UV-Vis spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA). All measurements were carried out in triplicate.

2-5 Sensory evaluation

Nipa sap vinegar prepared using traditional methods in a local market and vinegar produced using SCF process were compared with each other by dividing samples into two groups of filtered and unfiltered. Filtered group was carried out using 0.45-µm filter membranes for removing bacteria; hence, clarity was greater than that of unfiltered group. Two groups of vinegars were assessed by 35 panelists using 9-point hedonic scale to statistically analyze differences in sensory characteristics between the two vinegar samples. The sensory test assessed appearance, color, flavor, taste and overall acceptance. Coded samples identified by 3-digit random numbers were presented to panelists in random order.

2-6 Statistical analysis

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

3. Results and discussion

3-1 Alcoholic fermentation from nipa sap

Conversion of fermentable sugar of nipa sap to alcohol was assessed using two strains of yeast during four weeks of fermentation (Figure 1a). Alcohol concentration rapidly increased to 10.67% v v^{-1} from the first day of experiment within two weeks and then mildly increased to over 11.00% v v^{-1} within three weeks. Fermentation was stopped when the alcohol concentration stabilized at 11.90% v v^{-1} at Week 4. Thus, TSS showed a sharp steady decrease from 22 (initial sugar concentration) to 7.93 Brix within two weeks and then decreased continuously to 7.60 Brix until fermentation was stopped at Week 4. High fermentation efficiency was reported for the two strains of Saccharomyces species (*S. cerevisiae* var. burgundy and *S. cerevisiae* var. montache), which used sugars for growth and conversion to alcohol. The pH value decreased from 3.80 (initial fermentation) to 3.40, while nipa sap acidity as lactic acid increased from 0.72 to 0.82% v v^{-1} due to malolactic fermentation (Figure 1b). Nipa sap wine from

this process was used as the raw material for acetous vinegar fermentation in the next step.

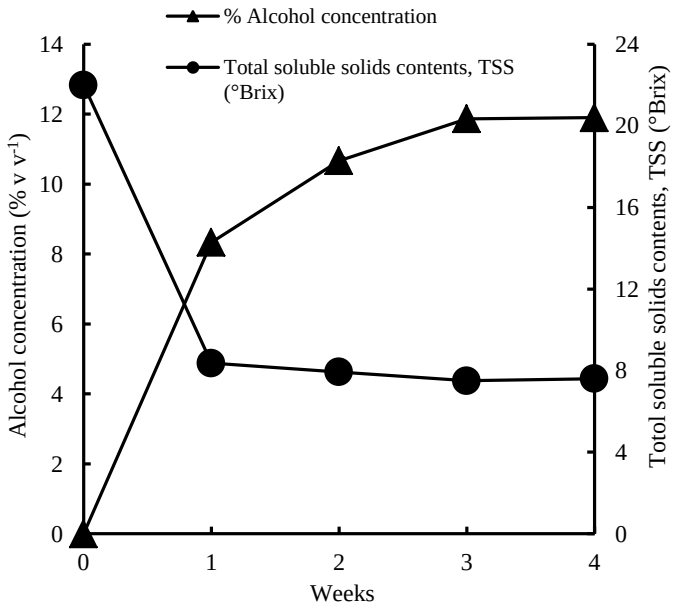

Figure 1a. Change in total soluble solids content, TSS (°Brix) and alcohol concentration (%) during nipa sap wine fermentation by two strains of Saccharomyces species (*Saccharomyces cerevisiae* var. burgundy and *Saccharomyces cerevisiae* var. *montache*) during 4 weeks. All values are presented as mean ± SD (n=3).

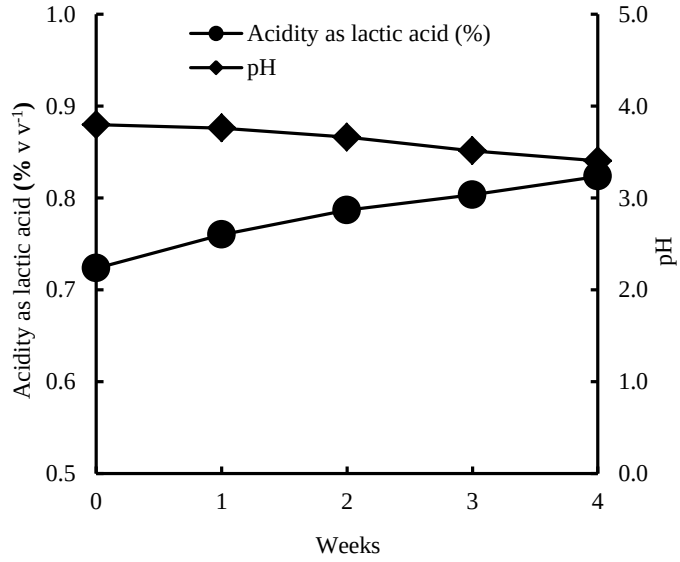

Figure 1b. Change in acidity as lactic acid (%) and pH during nipa sap wine fermentation by two strains of Saccharomyces species (*Saccharomyces cerevisiae* var. *burgundy* and *Saccharomyces cerevisiae* var. *montache*) during 4 weeks. All values are presented as mean ± SD (n=3).

3-2 Acetic acid production from nipa sap wine

Figure 2a shows acetic acid production using SCF process and *A. aceti* TISTR 354 as the starter culture for initial number of cells in 100 ml of liquid starter culture at 9.01 log CFU ml^{-1} at 48 h mixed with 600 ml of sterilized nipa sap (5.00 Brix) and 300 ml of nipa sap wine (11.90% v v^{-1} alcohol concentration), respectively. A total of 1000 ml vinegar starter culture preparation in the first step of SCF process were cultured in stainless steel trays for two days at room temperature. Results showed that acidity as acetic acid increased mildly and reached 3.10% v v^{-1} within two days as the alcohol was oxidized by acetobacter bacteria (*A. aceti* TISTR 354) to produce acetic acid as a reaction product with increasing number of cells that decreased pH from 3.69 to 3.05.

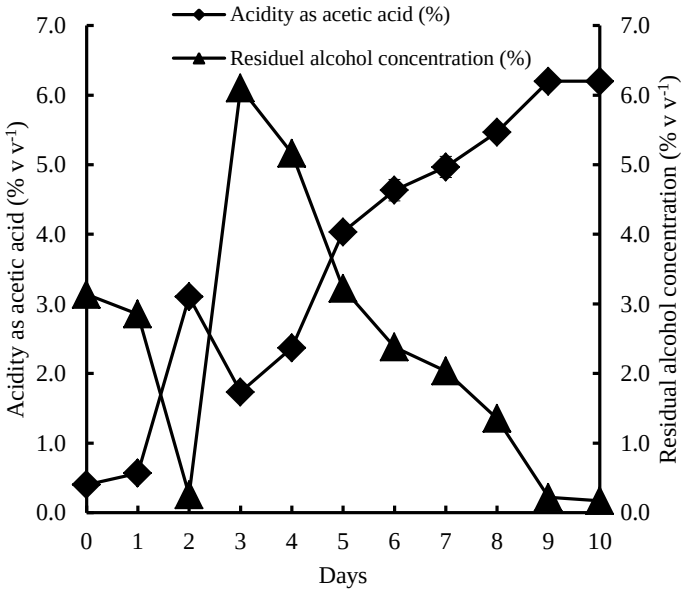

Figure 2a. Change in acidity as acetic acid (%) and residual alcohol concentration (%) during nipa sap vinegar production using the SCF process by *Acetobacter aceti* TISTR 354 starter culture during 10 days. All values are presented as mean ± SD (n=3).

To assess efficiency of high acetic acid production using SCF process, 1000 ml of nipa sap wine were added to a stainless steel tray (vinegar starter culture preparation step) as the second process step (vinegar production). Addition of 1000 ml nipa sap wine resulted in an alcohol concentration of 6.12% and decreased acetic acid from 3.10 to 1.73% v v^{-1} through dilution but increased alcohol concentration for acetobacter bacteria, which oxidized acetic acid as a final product of the process. Results showed that acetic acid gradually increased from 1.73 to 6.20% v v^{-1} after adding nipa sap wine within eight days in the second step, while residual alcohol concentration was lower than 0.5%. Free space volume for oxygen transfer into the liquid media promoted growth and oxidized alcohol to acetic acid rapidly by acetobacter bacteria when using *A. aceti* TISTR 354 as starter culture. Moreover, pH value of all samples decreased from 3.69 to 2.90 within ten days of acetous fermentation during the SCF process (Figure 2b). In vinegar production, 1 g of alcohol yields

1.3 g of acetic acid theoretically but in practice, yield was 15-20% v v^{-1} lower because alcohol, acetaldehyde and acetic acid are all volatile [15]. Furthermore, other organic acids such as lactic acid, citric acid and tartaric acid in nipa sap vinegar production by acetobacter bacteria were reported less than 1.0%, giving vinegar unique flavor and aroma. This finding was similar to finding by Tesfaye et al. [16]. In contrast, nipa sap vinegar from traditional methods included other organic acids but only lactic acid exceeded 3.74% v v^{-1}. Lactic acid bacteria were dominant in traditional methods, while fewer acetobacter bacteria affected production of acetic acid as a final product. Residual alcohol from nipa sap vinegar production using SCF process was 0.17% v v^{-1}. This indicated that these vinegars were acceptable according to FDA regulatory standards. Nipa sap vinegar from traditional methods was purchased from a local market in Nakhon Si Thammarat, Thailand. This vinegar showed acetic acid acidity at only 2.14% v v^{-1} and residual alcohol concentration at 5.89% v v^{-1}; 2.90-fold greater than that nipa sap vinegar produced using SCF process did. Data were reported as sub-standards based on the main criteria of US FDA standard regulations and notification of the Ministry of Public Health, Thailand (No. 204) B.E. 2543 (2000). Compared to previous studies on nipa sap vinegar, Mohamad et al. [17] reported production of 6-8% v v^{-1} of acetic acid from nipa sap wine within four weeks, while 4.26% v v^{-1} of acetic acid were reported by Nagendra et al. using batch-fed fermentation [18]. Recent data show that SCF process using *A. aceti* TISTR 354 as starter culture includes potential to produce high-quality nipa sap vinegar. The raw material (nipa sap) contains high concentrations of minerals and is rich in sugar as previously reported by other researchers [19,20]. This is the main reason that supports yeast and acetobacter bacteria growth for wine and vinegar productions.

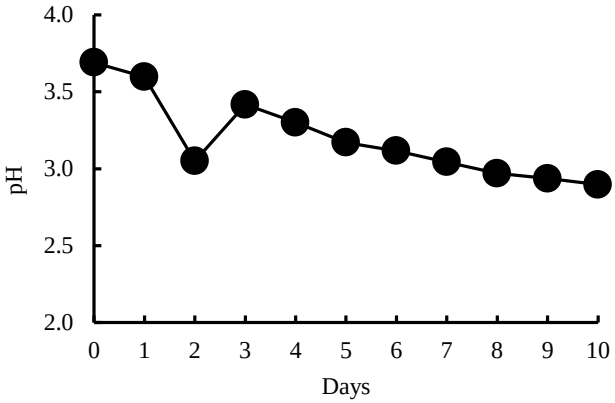

Figure 2b. Change in pH during nipa sap vinegar production using the SCF process by *Acetobacter aceti* TISTR 354 starter culture during 10 days. All values are presented as mean±SD (n=3).

3-3 Antioxidant activity of nipa sap vinegar

Antioxidant activity of the nipa sap vinegar was assessed using three methods of TPC, DPPH scavenging assay and FRAP assay. Results of antioxidant activity of the raw materials (nipa sap), nipa sap vinegar samples from traditional methods purchased from a local market and nipa sap vinegar produced using SCF process were compared to each other (Table 1). Raw materials (448.73 ±0.58 µg ml^{-1}, 80.97±2.94% inactivate and 151.43±0.31 µg ml^{-1}) included a higher activity than that respectively vinegar samples from traditional methods (316.23 ±0.76 µg ml^{-1}, 43.08 ±1.85% inactivate and 60.91 ±0.51 µg ml^{-1}) and nipa sap vinegar produced using SCF process (253.98 ±0.14 µg ml^{-1}, 28.29 ±1.31% inactivate and 40.14 ±0.07 µg ml^{-1}) did. The high antioxidant activity might be resulted from nipa sap vinegar of traditional methods having incomplete fermentation; thus, affecting remaining residual nipa sap. However, antioxidant activity decreased when reaction changed nipa sap into vinegar. Vinegar production showed a lower antioxidant activity in all samples but higher concentrations of acetic acid regulate blood sugar and pressure and treat various diseases such as diabetes and microbial infections in humans [21,22].

Table 1. Antioxidant activities of nipa sap (raw material) and nipa sap vinegar products

Samples	TPC (µg ml^{-1})	DPPH (% inactivated)	FRAP (µg ml^{-1})
Nipa sap (raw material)	448.73±0.58	80.97±2.94	151.43±0.31
Nipa sap vinegar from local market	316.23±0.76	43.08±1.85	60.91±0.51
Nipa sap vinegar by SCF process	253.98±0.14	28.29±1.31	40.14±0.07

TPC= Total phenolic content, DPPH=2, 2-diphenyl-2-picrylhydrazyl, FRAP =Ferric reducing antioxidant power

Table 2. Sensory evaluation of nipa sap vinegar

Sample	Appearance	Color	Flavor	Taste	Overall acceptance
Nipa sap vinegar (SCF process)*	$7.18±0.22^{b}$	$7.18±0.28^{c}$	$6.67±0.30^{b}$	$6.15±0.30^{b}$	$6.64±0.30^{b}$
Nipa sap vinegar (SCF process)**	$4.61±0.29^{a}$	$4.91±0.26^{a}$	$5.48±0.28^{b}$	$5.30±0.28^{b}$	$4.76±0.33^{a}$
Nipa sap vinegar (traditional method)*	$5.52±0.28^{a}$	$6.06±0.26^{b}$	$3.85±0.40^{a}$	$3.58±0.40^{a}$	$3.97±0.42^{a}$
Nipa sap vinegar (traditional method)**	$4.88±0.26^{a}$	$5.09±0.26^{ab}$	$3.55±1.97^{a}$	$3.45±0.35^{a}$	$3.88±0.35^{a}$

*Filtered, **Unfiltered, SCF= Surface culture fermentation
Different letters within columns indicate significant difference (P≤0.05)

3-4 Nipa sap vinegar sensory evaluation

Table 2 shows that nipa sap vinegar from SCF process was accepted more than 'neither like nor dislike' in every attribute (appearance, color, flavor, taste and overall acceptance) and two attributes recorded high acceptance at 'like moderately' of appearance (7.18 ±0.22) and color (7.18 ±0.28) by the 35 panelists. Nipa sap homemade vinegar was most accepted with 'like slightly' only for one attribute of color (6.06 ±0.26) and the lowest level was 'dislike moderately' in three attributes of flavor (3.85 ±0.40), taste (3.58 ±0.40) and overall acceptance (3.97 ±0.42). Filtered and unfiltered vinegars were evaluated for sensory acceptability. Filtered nipa sap vinegar (SCF process) showed acceptable results for every attribute in all nipa sap vinegar samples.

4. Conclusion

The SCF process showed high efficiency for vinegar production from nipa sap with acidity as acetic acid of greater than 6.20 and 0.17% v v^{-1} residual alcohol. This method needed only ten days and converted ethanol to acetic acid at a 2.90-fold higher than that traditional methods did. The antioxidant activity preserved in the product with an acceptable evaluation on nipa sap vinegar of traditional methods. Most importantly, the SCF offers a simple protocol with high performance to produce nipa sap vinegar comparing to traditional methods and is appropriate for local people.

5. Acknowledgements

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6. Conflict of interest

The authors declare no conflict of interest.

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بهینه سازی تولید سرکه از شیره نیپا (*Nypa fruticans* Wurmb.) با استفاده از فرایند تخمیر کشت سطحی

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چکیده

سابقه و هدف: شیره نخل مانگرو نیپا غنی از ترکیبات شیمیایی و مغذی است. اخیراً، این شیره برای تولید آب میوه تازه، شربت، ملاس، الکل و سرکه سنتی مورد استفاده قرار می گیرد. هدف این مطالعه افزایش ارزش تغذیه‌ای شیره نیپا در سرکه با کیفیت با استفاده از تخمیر کشت سطحی می باشد.

مواد و روش ها: سرکه از شیره نیپا با استفاده از فرایند تخمیر کشت سطحی تولید شد که شامل تهیه کشت آغازگر سرکه و تولید سرکه بود. پ هاش، باقیمانده الکل و استیک اسید سرکه بهینه شد. سرکه به دست آمده از شیره نیپا و با استفاده از تخمیر کشت سطحی با روش‌های سنتی به منظور رعایت استانداردهای نظارتی مقایسه شد. فعالیت‌های ضداکسایشی (روش های میزان فنول تام، گیرندگی رادیکال DPPH، و احیای فریک) و ارزیابی حسی محصول مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: اسیدیته با استفاده از تخمیر کشت سطحی تا ۶/۲۰ درصد افزایش یافت، ۲/۹ برابر در مقایسه با استفاده از روش‌های سنتی (۲/۱۴٪). غلظت الکل در حین تخمیر شراب ۱۱/۹٪ بود. تخمیر کشت سطحی با استفاده از *استوباکتر استی* TISTR 354 در مدت ده روز الکل را به اسید استیک تبدیل کرد. فعالیت خوب ضداکسایشی برای سرکه گزارش شده است. امتیاز ویژگی‌های حسی در هر مورد بیشتر از "نه دوست دارم، نه دوست ندارم" بود. بنابراین سرکه های با کیفیت بالا را می توان با استفاده از شیره نیپا و تخمیر کشت سطحی تولید کرد، و ممکن است در آینده بتوان سطح تولید را افزایش داد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- استیک اسید
- شیره نیپا
- تخمیر کشت سطحی
- سرکه

***نویسنده مسئول**

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