

Saccharification of unripe banana flour using microwave assisted starch degrading enzyme hydrolysis for development of wine and vinegar fermentations

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Abstract

Unripe banana flour (UBF) from *Musa* (ABB) 'Kluai Namwa' was used as the substrate for sugar syrup production by microwave assisted starch degrading enzyme hydrolysis. Results showed that a concentration of 300 g/L of UBF subjected to 800 W microwave power for 2.0 min, with subsequent hydrolysis by a low temperature amylase (iKnowZyme® LTAA) and glucoamylase (iKnowZyme® GA) at 50°C for 9 h yielded highest sugar syrup production at 20 ± 0.89 °Brix of total soluble solids (TSS). The major hydrolysis product from UBF determined by thin-layer chromatography (TLC) was glucose, with reduced amounts of maltose and maltotriose. Fermentation by mixed strains of *Saccharomyces cerevisiae* produced alcohol content at $13.2 \pm 0.07\%$ (w/v) after 10 d at room temperature. Acetic acid fermentation achieved using *Acetobacter aceti* TISTR 354 by surface culture fermentation (SCF) in a stainless-steel tray chamber yielded $5.10 \pm 0.12\%$ (v/v) after cultivation at room temperature for 9 d, corresponding to standard commercial vinegar products at over 4.0%. This is the first report detailing production of sugar syrup, wine, and vinegar from UBF, using microwave assisted starch degrading enzyme hydrolysis at 50°C. Results showed that producing an alternative healthy products from natural material could be feasible with added value through biotechnological processes.

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Keywords

unripe banana flour,
Musa (ABB) 'Kluai
Namwa',
enzyme hydrolysis,
microwave treatment

Introduction

Unripe bananas harvested before ripening have interesting applications in various food products since they contain high dietary fibre (DF), resistant starch (RS), and low soluble sugar concentration (Menezes *et al.*, 2011). Agama-Acevedo *et al.* (2012) reported that using unripe banana flour (UBF) in cookie production increased levels of indigestible carbohydrates and antioxidant compounds.

Bananas are grown in tropical countries for their fruits. They belong to the monocotyledonous order Zingiberales and family Musaceae, and are native to the tropics of Africa, East Asia, Australia, and the South Pacific. *Musa* (ABB) 'Kluai Namwa' is a cheap and abundant crop that is consumed in Thailand and produced for desserts (Naknaen *et al.*, 2016). Banana fruits are a source of DF, vitamins, amino acids, and phenolic compounds. However, excess seasonal production causes oversupply in the

market, and the shelf life of bananas and their application in food products are limited (Naknaen *et al.*, 2016). Thus, it is interesting to find ways to develop new healthy products from bananas.

Microwave assisted saccharification is an alternative method for hydrolysis of starch using irradiation to generate heat and activate the reaction of starch molecules (Matsumoto *et al.*, 2011). Microwave treatment increased the hydrolysis of starch materials to glucose in various substrates such as wheat, rice, potato, and corn in water or dilute acid solutions (Sunarti *et al.*, 2012). Low temperature starch-degrading enzymes directly degrade starch granules at 50 to 60°C as compared to conventional enzymes that usually operate at higher temperatures (85 - 105°C). Lomthong and Saithong (2019) reported the application of starch-degrading enzymes for hydrolysis of Luem Pua glutinous rice powder at 50°C to produce sugar syrup as a substrate for alcoholic vinegar production. This reduced the cost of

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the operation as compared to the conventional process, and as an alternative choice of vinegar product with high antioxidant content.

Vinegar is obtained from various substrates via a two-step fermentation process including alcoholic fermentation by yeast strains and acetic acid fermentation by acetic acid bacteria (AAB) (Lomthong and Saithong, 2019). Vinegar is widely used as seasoning in salads, sauces, and cooking, and in pharmaceutical treatments including anti-infective, antitumor, and hyperglycaemic properties (Johnston and Gaas, 2006; Wongsudarak and Nunium, 2013; Boonsupa *et al.*, 2019). Surface culture fermentation (SCF) is a static low cost and easy to use process for vinegar production using a two-step method which are vinegar starter culture preparation (2 d) and vinegar production (7 - 10 d) (Lomthong and Saithong, 2019).

In the present work, vinegar production with UBF as the substrate was conducted via hydrolysis of starch-degrading enzymes, using the microwave assisted treatment process. Vinegar fermentation was carried out using SCF at room temperature, and chemical compositions of banana wine and vinegar were also investigated.

Materials and methods

Substrates and enzyme preparation

Organic unripe bananas, *Musa* (ABB) 'Kluai Namwa' [hard green, stage 2 of colour index as reported by Aurore *et al.* (2009)] were purchased from a local farmer in Thanyaburi district, Pathum Thani province, Thailand. The bananas were peeled, cut into 0.2 cm slices, and then dried in a hot air oven at 50°C for 2 h. The dried bananas were ground to powder using an electric grinder, passed through 60-mesh sieve (0.25 mm), and stored under dry condition until further use. Chemical compositions (protein, fat, and fibre contents) of UBF were analysed using AOAC methods as described by Helrich (1990). Total starch assays were performed by the Cassava and Starch Technology Research Unit (CSTRU) at Kasetsart University using a starch assay kit (Megazyme International Ireland, Wicklow, Ireland) as described by Lomthong and Saithong (2019).

Starch-degrading enzymes as low temperature amylase (iKnowZyme® LTAA, EC 3.2.1.1) produced by *Bacillus subtilis*, and glucoamylase (iKnowZyme® GA, EC 3.2.1.3) produced by *Aspergillus niger* were purchased from Reach Biotechnology Co., Ltd., Thailand, and stored at -20°C until further use.

Microorganisms and inoculum preparation

Two strains of yeast (*Saccharomyces cerevisiae* var. *montache*, *S. cerevisiae* var. *burgundy*) and vinegar-producing bacteria (*Acetobacter aceti* TISTR 354) were obtained from the Department of Applied Microbiology, Institute of Food Research and Product Development (IFRPD), Kasetsart University, Thailand. Yeast strains were grown separately in YM medium and incubated at 30°C for 16 h; 10% (v/v) was used as inoculum for UBF alcoholic fermentation as described by Lomthong and Saithong (2019). For vinegar fermentation, *A. aceti* TISTR 354 was grown on inoculum liquid medium containing 7.0 g UBF, 7.0 mL of 95% ethanol, and 76.0 mL of distilled water; and incubated at 30°C without shaking for 4 d; 10% (v/v, 10⁸ CFU/mL) was used as the vinegar starter following the method of Saithong *et al.* (2019).

Saccharification of unripe banana flour by microwave treatment and raw starch-degrading enzyme production

Effect of unripe banana flour concentration

UBF was hydrolysed in a 250 mL Erlenmeyer flask containing 50 mL of the reaction (48 mL of distilled water and 1.0 mL of each LTAA and GA) with different concentrations of UBF (100, 150, 200, 250, 300, and 350 g/L). The reactions were incubated at 50°C without shaking for 9 h, and samples were taken at 3, 6, and 9 h for determination of total soluble solids, TSS (°Brix).

Effect of microwave treatment

To study the effect of microwave assisted starch-degrading enzyme hydrolysis on UBF for sugar syrup production, microwave power was investigated at 200 - 800 W using the optimal concentration of UBF in 250 mL Erlenmeyer flasks as described earlier. At each microwave power, the reaction was incubated for 2 min, followed by adding 1.0 mL of each LTAA and GA, and subsequent incubation at 50°C without shaking for 9 h. Samples were taken at 3, 6, and 9 h for determination of TSS (°Brix).

Upscale of unripe banana saccharification

To upscale unripe banana saccharification, UBF was mixed with distilled water at the optimal concentration in a 1.0 L beaker and incubated in a microwave at optimal power for 2 min. The reaction was then moved to a 5.0 L glass jar chamber (18 × 18 × 28 cm) with a 3.0 L working volume of substrate suspension, and added with 60 mL of each LTAA and GA. The reaction was incubated at 50°C without

shaking for 12 h. The reaction without microwave treatment was used as the control (one-step hydrolysis). Samples were taken during the interval times to determine total soluble solids, TSS (°Brix). Scanning electron microscopy (SEM) was used to study morphological changes in native and digested UBF after hydrolysis by iKnowZyme® LTAA and iKnowZyme® GA in a 5.0 L glass jar. All samples were cleaned with distilled water, dried at 50°C for 24 h, and then examined under a scanning electron microscope (Model SU8020; Hitachi, Tokyo, Japan) at 10.0 kV, as reported by Lomthong *et al.* (2015). Hydrolysis products of UBF were qualitatively determined by thin-layer chromatography (TLC), as reported by Sassaki *et al.* (2008) using glucose, maltose, and maltotriose as standards (Lomthong *et al.*, 2016; Lomthong and Saithong, 2019).

Wine fermentation

To prepare unripe banana wine, the obtained sugar syrup from the hydrolysis of UBF in a 5.0 L glass jar chamber was set at initial TSS of 22 °Brix. Diammonium phosphate (DAP) at 1.2 g/L and potassium metabisulfite (KMS) at 350 ppm were added to the reaction, and the pH was adjusted to 4.0 with citric acid. The reaction tanks were maintained at room temperature (30°C) for 24 h before adding the yeast inoculum. For alcoholic fermentation, each 10% (v/v) of yeast strain (*S. cerevisiae* var. *burgundy* and *S. cerevisiae* var. *montache*) was inoculated into the fermentation tank. The fermentation was incubated at 30°C for 10 d, and samples were taken for determination of TSS, pH, titratable acidity, and alcohol content every day (Lomthong and Saithong, 2019). Amino acid profiles and trace elements in unripe banana wine were analysed at the end of fermentation.

Vinegar fermentation

Vinegar production was performed using SCF in a stainless steel deep long tray container at room temperature (30°C), as reported by Saithong *et al.* (2017). Suitable mixture ratios following Saithong *et al.* (2017) included starter culture of *A. aceti* TISTR 354, unripe banana wine, and unripe banana residue from the hydrolysis suspended in distilled water to reach final sugar at 4 °Brix (100:300:600 mL). The fermentation was operated in a stainless-steel tray, and covered with a plastic sheet. After 2 d of cultivation at room temperature (30°C), 1.0 L of unripe banana wine (10%, w/v) was added to the reaction, and left to stand for 7 d. Samples were taken daily for determination of titratable acidity and alcohol contents, as reported by

Lomthong and Saithong (2019).

Analysis

Alcohol and total soluble solids

Alcohol contents were determined following the method of Kocabey *et al.* (2016) and Lomthong and Saithong (2019) using an ebulliometer (Dujardin-Salleron, Paris, France), while TSS (°Brix) were determined using a refractometer (RA-250WE, Kyoto Electronics, Kyoto, Japan).

pH and titratable acidity

The pH values of the samples were measured at 30°C using a pH meter (Model 430; Corning, NY, USA), while the acidity in wine and vinegar was determined by titration with 1 N NaOH using phenolphthalein as an indicator (Helrich, 1990). All measurements were conducted in triplicate.

Amino acid profiles and trace elements

Amino acid profiles (18 amino acids) and trace elements (calcium, iron, magnesium, manganese, potassium, sodium, and phosphorus) were analysed by the Food Quality Assurance Service Centre (FQA), Institute of Food Research and Product Development (IFRPD), Kasetsart University, Thailand based on the methods of Herbert *et al.* (2000) and AOAC (2016), respectively. All values were reported as mg/100 g sample.

Statistical analysis

All results were expressed as mean \pm SD (standard deviation). Mean values, standard deviation, and the data were analysed using one-way analysis of variance (ANOVA) (SPSS software version 21.0, SPSS Inc., USA). Differences among mean values were tested using Duncan's multiple range tests. Values were considered significant at $p < 0.05$.

Results and discussion

Chemical composition of unripe banana

The chemical composition of unripe banana (*Musa* (ABB) 'Kluai Namwa') is shown in Table 1. The major component was starch since the starch in unripe bananas is not converted to sugars as in ripe bananas. Unripe bananas are rich in resistant starch and pectin, which are filling, improve digestive health, and help to reduce blood sugar levels (Birt *et al.*, 2013; Bi *et al.*, 2017). The high starch content indicated feasibility for use as a substrate to produce sugar syrup from the hydrolysis of raw starch-degrading enzyme, as reported by Lomthong

and Saithong (2019). They used Luem Pua glutinous rice powder as the substrate for sugar syrup production by raw starch-degrading enzyme hydrolysis. Starch, protein, and fat contents of unripe Kluai Nam Wah flour were 76.52 ± 0.53 , 2.78 ± 0.02 , and $0.37 \pm 0.01\%$, respectively, while fibre content was $1.22 \pm 0.00\%$ (Table 1). Bangwaek (2014) reported that unripe or green bananas containing starch at approximately 80% were a good source of carbohydrate, and could be used as an alternative for flour production.

Table 1. Chemical composition of unripe banana, *Musa* (ABB) 'Kluai Namwa'.

Component	Analysis (%)
Starch	76.52 ± 0.53
Protein	2.78 ± 0.02
Fat	0.37 ± 0.01
Fibre	1.22 ± 0.00
Ash	1.57 ± 0.02

Values are mean \pm SD of triplicate ($n = 3$).

Saccharification of unripe banana flour

The effect of UBF concentration on sugar syrup production is shown in Figure 1. Maximum TSS in the reaction (8.25 ± 0.35 °Brix) occurred when using substrate concentration at 300 g/L. As substrate concentration increased, TSS content also increased, but beyond 300 g/L, the TSS decreased, as shown in Figure 1a. Lower substrate concentration provided lower starch content in the reaction, while high substrate concentrations increased viscosity causing problems in mixing and hydrolysis by raw starch-degrading enzymes. Bangwaek (2014) reported that UBF increased viscosity in the reaction due to the high starch content. Therefore, UBF concentration at 300 g/L was chosen for further study.

Microwave treatment results showed that 800 W yielded the highest TSS content at 20 ± 0.89 °Brix, as shown in Figure 1b. Many previous research studies have investigated the effect of microwave irradiation on increasing yields of hydrolysis and saccharification from various kinds of starchy materials. The main mechanism of microwave assisted starch-degrading enzyme hydrolysis is that microwave irradiation induces dipole rotation of water molecules, and the delay in molecular motion is converted to heat (Tsubaki *et al.*, 2013). The motion of water molecules during microwave irradiation heating increases

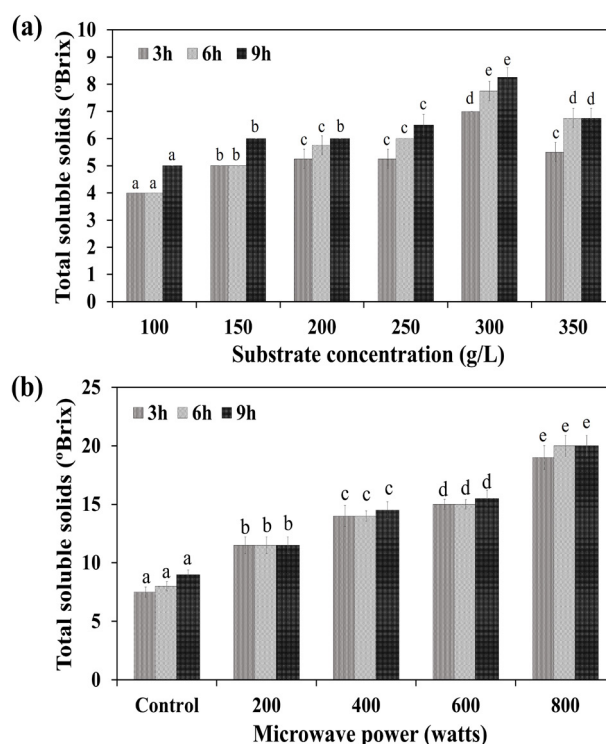


Figure 1. (a) effect of unripe banana, *Musa* (ABB) 'Kluai Namwa' flour concentration on sugar syrup production by the hydrolysis of starch degrading enzyme (LTAA and GA) at 50°C for 9 h, and (b) effect of microwave power on sugar syrup production for 2 min and subsequent hydrolysis with starch degrading enzyme (LTAA and GA) at 50°C for 9 h. Different lowercase letters above columns indicate significant ($p < 0.05$) differences within each parameter.

solubilisation of carbohydrate components in the substrate (Hermiati *et al.*, 2012). Yoshida *et al.* (2010) reported that increasing microwave power raised the temperature of the reaction, and increased the solubilisation of starch from the substrate. In the present work, an increase of microwave power increased sugar syrup production. Therefore, microwave heating solubilised the starch content, with subsequent hydrolysis by starch-degrading enzymes. Findings suggested that microwave treatment improved sugar syrup production by up to 2.42 times as compared to enzyme saccharification without microwave irradiation. Chidi *et al.* (2015) reported that a microwave oven was effective for substrate hydrolysis by improving enzymatic activity and reducing reaction time.

Upscale of unripe banana saccharification

Hydrolysis of UBF was conducted in a 5.0-L glass jar tank as described earlier. Time course hydrolysis of UBF for one-step (without microwave treatment) and two-step (with microwave treatment)

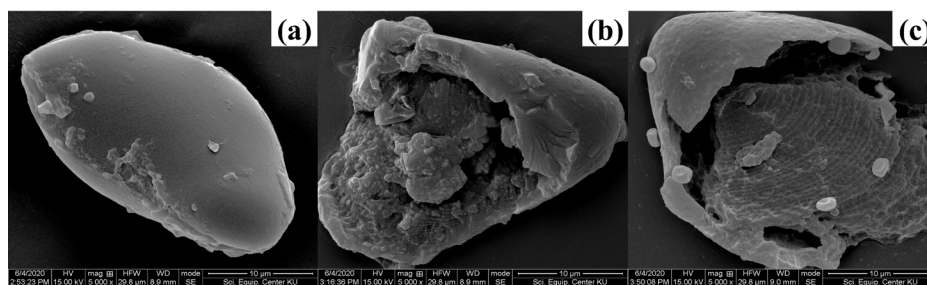


Figure 2. Scanning electron micrographs of unripe banana, *Musa* (ABB) 'Kluai Namwa' flour (a) before hydrolysis, (b) after hydrolysis with iKnowZyme® LTAA and iKnowZyme® GA, and (c) microwave assisted starch degrading enzyme hydrolysis.

are not shown. Maximum yield (19 ± 1.0 °Brix) was found in two-step hydrolysis after incubation at 50°C for 12 h, while maximum yield in one-step hydrolysis was found at 12 ± 0.5 °Brix. The TLC revealed that the major hydrolysis product from UBF was glucose, with minor amounts of maltose and maltotriose.

The SEM showed that native granules of UBF had a flat and oval shape (Figure 2a), similar to results reported by Bi *et al.* (2017). The residue of UBF after one-step hydrolysis of raw starch-degrading enzyme at 50°C is shown in Figure 2b. The granule surface was hydrolysed from outside to inside, corresponding to Cinelli *et al.* (2015) who showed that raw starch was hydrolysed from the outside to the centre of the granule. Granules digested by microwave assisted raw starch-degrading enzyme hydrolysis formed pits with large holes, and loss of structure (Figure 2c), showing more effective degradation than the one-step hydrolysis method by raw starch-degrading enzymes.

Wine fermentation

Alcoholic fermentation was achieved by mixing *S. cerevisiae* species. Results showed that TSS values of unripe banana wine dropped sharply from 22 to 8.0 °Brix within 10 d of fermentation, while alcohol content increased to $13.2 \pm 0.07\%$ (w/v) after 10 d of fermentation at room temperature (30°C), as shown in Figure 3. Amino acid profiles of unripe banana wine are not shown. Proline (95.88 ± 0.08 mg/100 g sample), glutamic acid (30.06 ± 0.01 mg/100 g sample), and aspartic acid (27.17 ± 0.92 mg/100 g sample) were the major amino acids in the product, while major trace elements in unripe banana wine were potassium (148.87 ± 0.08 mg/100 g sample), sodium (120.51 ± 0.02 mg/100 g sample), and phosphorus (31.46 ± 0.02 mg/100 g sample). Deng *et al.* (2016) used banana flour as a substrate for wine production, with heating at 90°C and hydrolysis by pectinase enzyme at 50°C to obtain highest alcohol content at 11.63% (w/v) after 6 d of

fermentation at 30°C . Major amino acids were proline, glutamic acid, and aspartic acid, which are similar to those observed in the present work. This result demonstrated the feasibility of applying enzymatic hydrolysis to UBF for alcoholic fermentation as an alternative healthy product through biotechnological processes.

Vinegar fermentation

The SCF was applied for vinegar fermentation from unripe banana wine. This showed advantages for large-scale industrial application using cheap materials with no need for a fermenter or

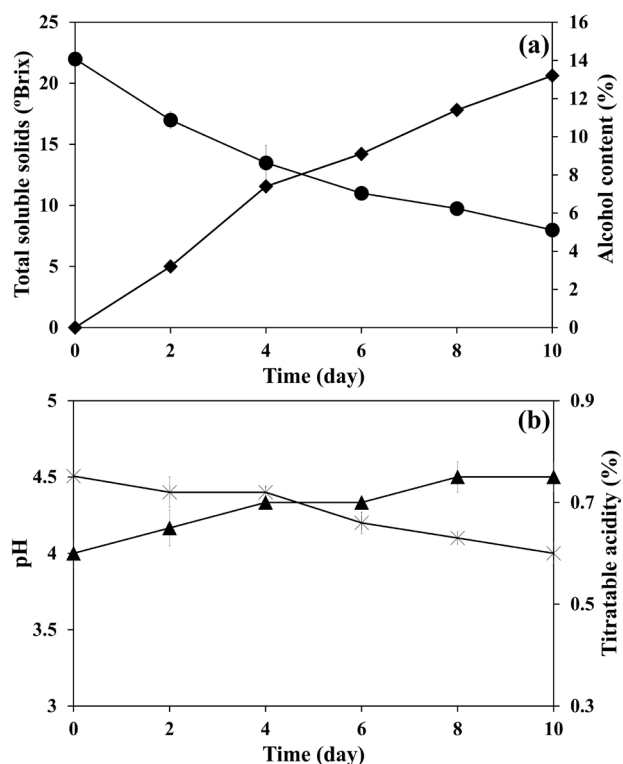


Figure 3. Chemical change during alcoholic fermentation of unripe banana, *Musa* (ABB) 'Kluai Namwa' by mixed strains of *Saccharomyces cerevisiae* at room temperature for 10 d. (a) Change in alcohol and total soluble solids, and (b) pH and titratable acidity.

Table 2. Change in acetic acid, alcohol content, and pH during unripe banana, *Musa* (ABB) 'Kluai Namwa' vinegar fermentation.

Day	Acetic acid (% v/v)	Alcohol content (% v/v)	pH
0	0.35 ± 0.03 ^a	3.2 ± 0.00 ^c	3.7 ± 0.04 ^c
3	2.65 ± 0.10 ^b	6.0 ± 0.14 ^d	3.4 ± 0.07 ^b
6	3.81 ± 0.14 ^c	2.4 ± 0.28 ^b	3.35 ± 0.03 ^b
9	5.10 ± 0.12 ^d	0.45 ± 0.03 ^a	3.0 ± 0.07 ^a

Values are mean ± SD of triplicate ($n = 3$). Means followed by different lowercase superscripts within the same column indicate significant differences at $p < 0.05$.

electric controller, and an easy operation process. Vinegar fermentation was performed in a stainless steel deep long tray at room temperature (30°C) as described earlier. Maximum titratable acidity (%) as acetic acid was found at $5.10 \pm 0.12\%$ acidity from fermentation of *A. aceti* TISTR 354, as shown in Table 2. Vinegar obtained from unripe bananas corresponded to the standard set by the Thai Ministry of Public Health - No. 204 B.E. 2543 (2000) Re: Vinegar (Thai Ministry of Public Health, 2018), with over 4 g of acetic acid per 100 mL. Boonsupa *et al.* (2019) reported that production of vinegar from banana (Phama Heak Kuk cultivar) yielded the highest level of acetic acid at 3.49% from fermentation of *A. pasteurianus* TISTR 521 using a two-step fermentation for 15 d. Findings suggested that unripe banana showed promise for production of banana vinegars with high acetic acid content.

Conclusions

Unripe banana is a cheap and abundant substrate in tropical areas. In Thailand, excess seasonal production causes the value of bananas to decrease. Production of unripe banana wine and vinegar through the application of biotechnological processes could preserve and increase the value of excess bananas. Unripe bananas like *Musa* (ABB) 'Kluai Namwa' were used as a substrate for sugar syrup production by microwave assisted raw starch degrading enzyme hydrolysis (two-step hydrolysis). Highest TSS value was recorded at 20 ± 0.89 °Brix. The obtained syrup could be used as a substrate for wine ($13.2 \pm 0.07\%$) and vinegar ($5.10 \pm 0.12\%$) production by a two-step fermentation process of alcohol and acetic acid. Results indicated that unripe banana could be used as an alternative substrate for sugar syrup production via low-temperature saccharification to produce wine and vinegar as healthy commercial products in the future.

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