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Feasibility of Leum Pua glutinous rice substrate for sugar syrup and vinegar production by raw starch degrading enzyme hydrolysis

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Abstract

Raw starch degrading enzyme (RSDE) from *Laceyella sacchari* LP175 was applied for hydrolysis of Leum Pua glutinous rice powder (200 g/L) and compared with other brown and black rice varieties in 250 mL Erlenmeyer flasks at 50°C for 12 h. Results showed that Leum Pua glutinous rice gave the highest liberated sugar syrup and anthocyanin contents at 11°Brix and 82.27 mg/L, respectively. Leum Pua glutinous rice powder (250 g/L) was up-scaled for hydrolysis in 5.0 L glass jar by simultaneous liquefaction and saccharification at 50°C for 12 h and obtained liberated sugar syrup at 13°Brix. Sugars were fermented by two mixed strains of *Saccharomyces cerevisiae* for 10 d without shaking at room temperature. During fermentation, total soluble solids decreased from 22 to 10°Brix while alcohol content increased to 15% (w/v). Leum Pua glutinous rice wine was subjected to acetic acid surface culture fermentation (SCF) with *Acetobacter aceti* TISTR 354 at room temperature. After 6 d of fermentation, acidity of Leum Pua glutinous rice vinegar increased to 5.7%. Antioxidant activity and vinegar concentration were determined during acetic acid fermentation for 6 d. Results suggested the feasibility of producing Thai rice vinegar using cold hydrolysis with raw starch degrading enzyme without the heating process.

Introduction

Leum Pua glutinous rice (*Oryza sativa* L., variety Leum Pua) is a local Thai glutinous rice with purple seeds and high nutrition value as compared to white and other coloured rice varieties (Pornputtapitak *et al*., 2018). Boonsit *et al*. (2010) reported that Leum Pua glutinous rice contains higher antioxidants and bioactive compounds such as anthocyanins, γ-oryzanol and phenolic compounds than white rice. Raw rice seeds contain high starch content which can be used as a carbon source for microbial growth and fermentation of various end products (Joo *et al*., 2009).

Vinegar is obtained from microbial fermentation through biotechnological processes. Production of vinegar consists of two steps which are alcoholic and acetic fermentations of liquid juice substrates (Kulkarni, 2015). Currently, various substrates are used for vinegar fermentation such as mushroom (Li *et al*., 2014), apple (Qi *et al*., 2017), plum (Williams *et al*., 2016), tomato (Koyama *et al*., 2017), onion (Lee *et al*., 2017) and purple sweet potato (Wu *et al*., 2017). Vinegar has several advantages, including application as a food additive for preservation against spoilage (Budak *et al*., 2014). Ali *et al*. (2016) reported that vinegar could be used as a medicine with a positive effect on biomarkers for diabetes, cancer and heart diseases. A recent report described vinegar production by surface culture fermentation (SCF) using a stainless steel deep long tray as a container at room temperature which gave high contents of acetic acid in a short time period (Saithong *et al*., 2017).

Cold hydrolysis by raw starch degrading enzyme at low temperature (40 - 50°C) has been compared to the conventional hydrolysis process at above gelatinization temperature (90 - 105°C) (Cinelli *et al*., 2015; Lomthong *et al*., 2018). Hydrolysis of starch-based materials at low temperature without the heating process can reduce energy consumption

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and cost. Recently, cold hydrolysis by raw starch degrading enzyme for the production of various kinds of fermentation products was reported for bioethanol (Lomthong *et al*., 2016) and lactic acid (Okano *et al*., 2018). However, scant information exists regarding vinegar or acetic acid fermentation by raw starch degrading enzyme. One advantage of hydrolysis at low temperature by rice starch degrading enzyme (RSDE) is the preservation of enzyme activity in the rice grains. Takahashi *et al*. (1971) reported that the presence of α-glucosidase in rice grains increased glucose hydrolysis. Moreover, hydrolysis at low temperature also maintains bioactive compounds which are denatured at high temperature. Therefore, the production of vinegar from Thai rice was investigated in the present work via low temperature saccharification by RSDE from *Laceyella sacchari* LP175, and reported as the first application of raw starch degrading enzyme to produce a healthy product through a biotechnological process.

Materials and methods

Substrates

Four varieties of brown and black rice (Riceberry, Leum Pua glutinous, Black Jasmine and Sangyod) were purchased from the Or Tor Kor fresh market, Bangkok, Thailand. Each substrate was ground to powder using an electric grinder and stored in dry condition until further use. The total starch assay (Megazyme International Ireland, Wicklow, Ireland) was performed by the Cassava and Starch Technology Research Unit (CSTRU) at Kasetsart University.

Microorganism and inoculum preparation

Laceyella sacchari LP175, a thermophilic filamentous bacterium, was obtained from the Department of Microbiology, Kasetsart University, Bangkok, Thailand. Raw starch degrading enzyme production by the LP175 strain was carried out in a 3.0 L airlift fermenter using a 2.0 L working volume of optimized medium: 4.93 g/L cassava starch, 2.8 g/L yeast extract, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, and 1.0 g/L $CaCl_2$ (pH 6.5) (Lomthong *et al*., 2015). Fermentation was operated at 45°C with an aeration rate of 0.5 vvm for 36 h (Lomthong *et al*., 2016).

Two strains of yeast (*Saccharomyces cerevisiae* var. *montache* and *S. cerevisiae* var. *kyokai*) and *Acetobacter aceti* TISTR 354 were obtained from the Department of Applied Microbiology, Institute of Food Research and Product Development (IFRPD), Kasetsart University, Thailand and used for wine and vinegar fermentation, respectively. The yeast strains were grown separately in a 500 mL Erlenmeyer flask containing 100 mL of YM medium (3 g/L yeast extract, 3 g/L malt extract, and 5 g/L peptone; supplemented with 10 g/L glucose). Cultures were incubated at 30°C overnight, and 10% (v/v) was used as inoculum for wine fermentation. *A. aceti* TISTR 354 at 10% (v/v, 10^8 CFU/mL) was grown on a Leum Pua glutinous rice inoculum liquid medium containing 7.0 g Leum Pua glutinous rice powder, 7.0 mL of 95% ethanol and 76.0 mL of distilled water, and incubated at 30°C without shaking for 96 h for use as the vinegar starter in acetic fermentation.

Hydrolysis of Leum Pua glutinous rice powder by raw starch degrading enzyme

The hydrolysis of Leum Pua glutinous rice (250 g/L) was performed in a 50 mL reaction mixture of 300 U/mL RSDE produced by *L. sacchari* LP175 in 0.1 M phosphate buffer (pH 6.5) in a 250 mL Erlenmeyer flask. Other varieties of brown and black rice including Riceberry, Black Jasmine, and Sangyod were used to compare the yield of sugar syrup production and anthocyanin contents. Reaction mixtures were incubated at 50°C without shaking for 12 h, and the total soluble solid and anthocyanin contents of the samples were subsequently determined.

Production of sugar syrup in a 5.0 L glass jar tank

Hydrolysis of Leum Pua glutinous rice was up-scaled in a 5.0 L glass tank containing 3.0 L reaction mixture of enzyme and Leum Pua glutinous rice powder (250 g/L). The reaction was incubated at 50°C for 12 h. Samples were taken at time intervals to determine total soluble solid and anthocyanin contents. Scanning electron microscopy was used to study the native and digested rice powder with crude enzyme at optimum conditions. All samples were washed with pure water, dried at 50°C overnight and then examined under a scanning electron microscope (model SU8020, Hitachi, Tokyo, Japan) at 10.0 kV. Hydrolysis products were qualitatively detected by thin-layer chromatography (TLC) (Sassaki *et al*., 2008). A silica gel TLC plate (Merck, 20 × 20 cm) was coated with a solvent mixture of ethyl acetate:2-propanol:glacial acetic acid:water (4:2:2:1, v/v), developed by spraying with a solution of orcinol-sulfuric acid and then heated at 100°C for 10 min. Glucose and maltose were used as standards.

Wine fermentation

Sugar syrup from the Leum Pua glutinous rice residue was applied as substrate for wine fermentation in a medium containing Leum Pua glutinous rice, with initial sugar concentration

adjusted to 22°Brix by addition of sucrose powder. For alcoholic fermentation, 5% (v/v) of both strains was inoculated into the same tank which contained hydrolysed Leum Pua glutinous rice sugar syrup in 1:1 ratio of *S. cerevisiae* var. *kyokai* and *S. cerevisiae* var. *montache*. The fermentation was incubated at 30°C for 10 d. Fermentation was stopped when alcohol content exceeded 10% (w/v) as the optimum concentration for application to the Surface Culture Fermentation (SCF) process for acetic acid fermentation as described by Saithong *et al*. (2017).

Scanning electron microscopy was used to study the growth of Saccharomyces strains on Leum Pua glutinous rice powder after cultivation on the medium for 10 d. Fermented residue was separated from the bioethanol fermentation by centrifugation at 4,032 g for 15 min at 4°C. The solid fermented residue was packed into Whatman filter paper No. 1 and soaked through a series of 15 - 100% (15, 30, 60, 80 and 100% (w/v)) ethanol for 15 min at each stage (Thirunavukarasu *et al*., 2016). The substrate was finally placed in a critical point drying (CPD) machine (Polaron Range CPD 7501, Quorum Technologies Ltd., England) with liquid CO_2, prior to examination by scanning electron microscope at 10.0 kV.

Preparation of vinegar

Vinegar production was performed by surface culture fermentation (SCF) as reported by Saithong *et al*. (2017). The SCF process usually requires two steps which are vinegar starter culture preparation (within 2 d) and vinegar production (3 - 4 d) at room temperature as a static fermentation process. Vinegar starter culture preparation was performed with sterile Leum Pua glutinous rice at an initial sugar concentration of 5°Brix by addition of sucrose and Leum Pua glutinous rice wine with 10.0% (w/v) and liquid starter culture of *A. aceti* TISTR 354. Suitable mixture ratio followed Saithong *et al*. (2017) who modified the process of vinegar starter culture preparation of 100:300:600 mL (vinegar starter of *A. aceti* TISTR 354:Leum Pua glutinous rice wine:rice residue suspension with adjusted sugar to 4°Brix) which was then poured into the stainless-steel tray and covered with a plastic sheet. A hole was punched in the plastic sheet and the presence of water vapour on the sheet was observed for 2 d (starter culture preparation). Vinegar production was then performed, and 1 L of the clear Leum Pua glutinous rice wine (10.0% w/v) was added into the same tank (starter culture) as described above which was covered with a plastic sheet. A hole was punched in the plastic sheet and the culture was left to stand for 3 - 4 d.

Analysis

Alcohol and soluble solid contents

An ebulliometer (Dujardin-Salleron, Paris, France) was used to assess the total alcohol content by measuring the difference in boiling points between pure water and the wine (Kocabey *et al*., 2016). Based on the comparison, percentage alcohol (v/v) was determined as a percentage. Soluble solid contents were investigated at 20°C using a refractometer (RA-250WE, Kyoto Electronics, Kyoto, Japan). All readings were conducted in triplicate and reported as the percentage alcohol content and °Brix for soluble solid contents.

pH and titratable acidity

Samples were centrifuged (5.0 min at 3,500 g), and pH measured at 30°C using a pH meter (model 430, Corning, NY, USA). Titratable acidity is a measure of acid content in wine and vinegar. This was determined as acetic acid for vinegar by titration with 1 N NaOH (AOAC, 1990). A sample (6.0 mL) was pipetted into a 250 mL Erlenmeyer flask, and 50 mL of distilled water and 2 - 3 drops of phenolphthalein were added. Sample solution was titrated with 1 N NaOH until a pink colour appeared and percentage of acetic acid in the samples was calculated. All measurements were conducted in triplicate.

Anthocyanin content

Total anthocyanin content was determined by the pH-differential method, following Wrolstad *et al*. (2005) with slight modifications. A total of 1,000 µL of sample solution was transferred into a test tube to prepare two sample dilutions. One dilution contained 1,000 µL of potassium chloride buffer, pH 1.0 and the other contained 1,000 µL of sodium acetate buffer, pH 4.5. Absorbance was measured for each dilution at 530 and 700 nm against a blank cell filled with distilled water. Absorbance readings were made against water blanks. All samples should be prepared within 15 min and measured within 1 h of sample preparation. Anthocyanin pigment concentration was calculated and expressed as cyanidin-3-glucoside equivalents per 100 g. All determinations were performed in triplicate.

DPPH radical scavenging assay

Antioxidant assay was reported as half-maximum effective concentration (EC_{50}) which is defined as mL sample/g DPPH, following the modified procedure of Brand-Williams *et al*. (1995) using 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay. The 50 µL appropriated diluted samples of Leum Pua glutinous

rice vinegar were mixed with 950 µL of 0.0394 g/L DPPH solution, and reactions were incubated at room temperature for 30 min in darkness. Absorbance was measured with a UV-vis spectrophotometer at 515 nm.

Enzyme activity

RSDE activity was determined following the method of Mitsuiki *et al*. (2005) by analysis of reducing sugars released during hydrolysis of raw cassava starch, as previously described by Lomthong *et al*. (2015). One unit of RSDE activity was defined as the amount of enzyme releasing 1 µg of glucose equivalent per min under standard assay conditions. The DNS method (Miller, 1959) was used to determine the amount of reducing sugars released from the hydrolysis reaction.

Statistical analysis

All results were calculated as mean ± SD (standard deviation). Mean values, standard deviation and analysis of variance (ANOVA) were computed using a commercial statistical package SPSS 21.0 (USA). Differences among mean values were tested using the least significant difference multiple range test. Values were considered significant when $p < 0.05$.

Results and discussion

Hydrolysis of brown and black rice powder by raw starch degrading enzyme

Results of brown and black rice powder hydrolysis by raw starch degrading enzyme from *L. sacchari* LP175 are shown in Table 1. Leum Pua glutinous rice showed the highest total soluble solids (°Brix) content of liberated sugars by hydrolysis at 50°C for 12 h, with significant differences ($p < 0.05$) as compared to other substrates, therefore Leum Pua glutinous rice was chosen for further analysis. Results in Table 1 show that Leum Pua glutinous rice (raw material) contained the highest starch content (86.5%). This was the major reason why it could liberate high concentrations of sugar syrup. Liberated sugar syrup of each brown and black rice in Table 1 confirmed that raw starch degrading enzyme from *L. sacchari* LP175 could hydrolyse and produce sugar syrup at a lower temperature and did not require high temperature (gelatinization) before hydrolysis (Sun *et al*., 2010; Cinelli *et al*., 2015). Raw starch degrading enzyme from *L. sacchari* LP175 directly digested starch granules at low temperature by adsorption on the surface and granule degradation from outside to inside (Lomthong *et al*., 2016). The use of raw starch degrading enzyme decreased heating process cost for gelatinization as compared to the conventional process (Lomthong *et al*., 2015; 2018).

Table 1. Starch content, total soluble solids and anthocyanin content of Thai rice powder after hydrolysis with RSDE from *L. sacchari* LP175 at 50°C for 12 h.

Thai rice	Starch content (%)	Total soluble solids (TSS) (°Brix)	Anthocyanin content (mg/L)
Leum Pua glutinous rice	86.5 ± 0.16^{d}	11 ± 0.77^{c}	82.27 ± 7.7^{c}
Riceberry rice	69.2 ± 0.24^{a}	10 ± 0^{b}	35.96 ± 3.4^{b}
Black jasmine rice	70.3 ± 0.04^{b}	10 ± 0^{b}	11.08 ± 1.2^{a}
Sangyod rice	74 ± 0.49^{c}	9 ± 0.77^{a}	ND

ND: not detected. Data are means of three determinations ($n = 3$) ± standard deviation. Different letters within the same column indicate significant difference at $p < 0.05$.

Leum Pua glutinous rice powder gave the highest anthocyanin content (82.27 mg/L) which showed significant differences ($p < 0.05$) as compared to other substrates (Table 1). The name 'black rice' is related to the accumulation of anthocyanins but the hydrolysis efficiency also affects the liberated anthocyanins from the granule of rice powder. From other reports, Leum Pua glutinous rice contained a purple seed and high nutrition value as compared to white rice and other coloured rice (Pornputtapitak *et al*., 2018). Boonsit *et al*. (2010) reported that Leum Pua glutinous rice has higher antioxidants and bioactive compounds such as anthocyanin content.

To up-scale sugar syrup production, hydrolysis of Leum Pua glutinous rice was performed in a 5.0 L glass jar tank. The maximum yield (13°Brix) was obtained when incubated at 50°C for 12 h (Figure 1). SEM images of native Leum Pua glutinous rice powder and rice digested with RSDE produced by *L. sacchari* LP175 are shown in Figure 2. Rice powder granules showed regular and globular shapes (Figure 2a). Damage to the granules was observed after digestion with RSDE with pits formed on the surfaces and some small particles showed loss of structure (Figure 2b). The major hydrolysis product was glucose with small amounts of maltose and maltotriose. Hydrolysis of rice powder at low temperature also maintained starch degrading enzymes such as glucoamylase (GA) in native rice seed as confirmed by the TLC chromatogram (Figure 2c). Takahashi *et al*. (1971) reported that natural rice grains contained starch degrading enzymes such as α-glucosidase which could react and produce glucose. Therefore, the application of RSDE without high temperature heating supports the activity of natural starch degrading glucoamylase in rice grains while also reducing industrial cost for glucoamylase enzyme preparation.

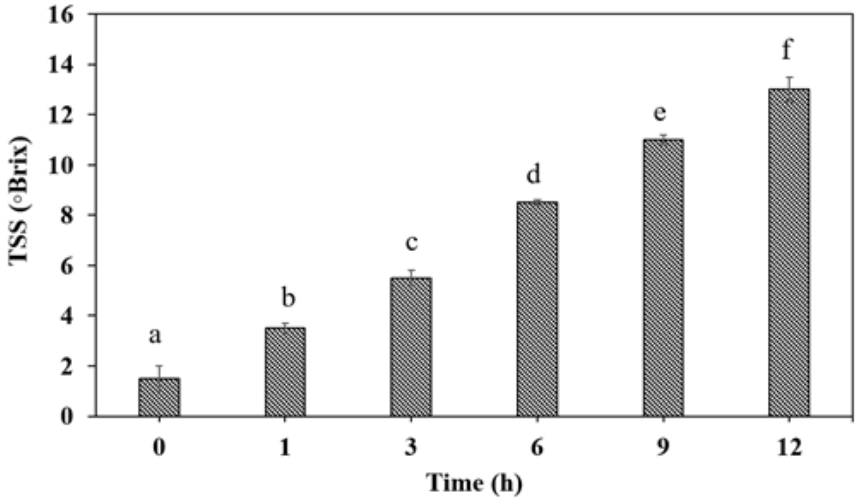

Figure 1. Time course hydrolysis of Leum Pua glutinous rice by RSDE from *L. sacchari* LP175 at 50°C for 12 h in a 5.0 L glass jar. Data are means of three determinations ($n = 3$) ± standard deviation. Different letters indicate significant difference at $p < 0.05$.

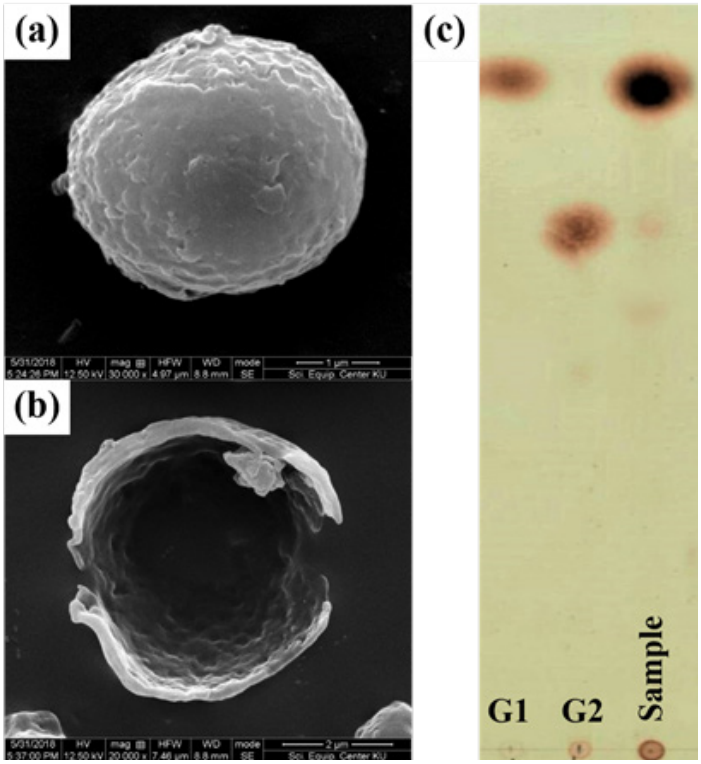

Figure 2. (a) Scanning electron micrograph of Leum Pua glutinous rice before hydrolysis; (b) after hydrolysis with RSDE from *L. sacchari* LP175; (c) TLC chromatogram of Leum Pua glutinous rice hydrolysis products at different times; G1, glucose; G2, maltose.

Wine fermentation

During fermentation, alcohol content increased from 0% to 15.1% while soluble solid contents decreased from 22 to 10°Brix (Figure 3a). Yeast utilized liberated reducing sugars in the reaction for growth and fermentation to alcohol. Alcohol content rapidly increased after 2 d of fermentation (10.8% (w/v) and then slightly increased. Fermentation stopped when alcohol content exceeded 15%. Alcoholic fermentation of Leum Pua glutinous rice syrup gave high ethanol concentration due to simultaneous fermentation of the two strains of Saccharomyces species (*S. cerevisiae* var. *montache* and *S. cerevisiae* var. *kyokai*). Previous research mentioned that these strains were suitable for rice wine fermentation (Koguchi *et al*., 2010) of liberated

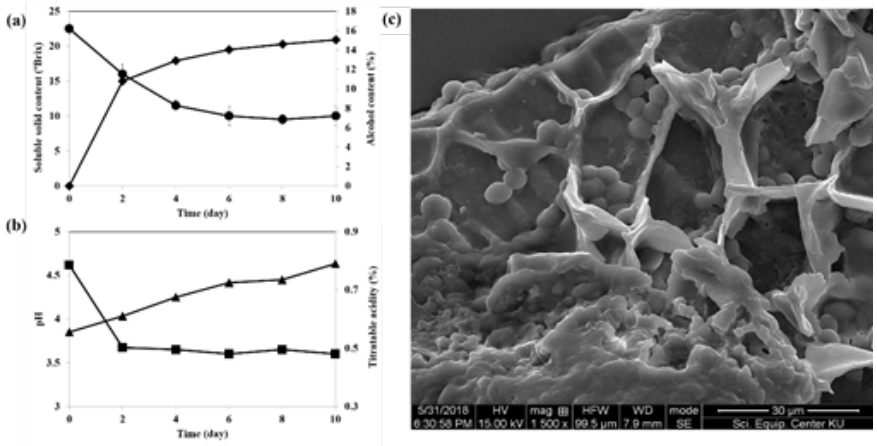

Figure 3. (a) Change in alcohol and total soluble solid contents; (b) pH and titratable acidity during acetic fermentation of Leum Pua glutinous rice wine (♦: alcohol content;●: total soluble solid; ▲: %acidity; ■: pH); (c) Scanning electron micrograph of solid residues of Leum Pua glutinous rice

sugar syrup (glucose) from hydrolysis of raw starch degrading enzyme by LP175 strain. The pH of samples was determined to check for contamination by lactic acid bacteria during wine fermentation, as described by Cappello *et al*. (2017). The pH decreased from 4.5 to 3.7, while acidity (as lactic acid) of Leum Pua glutinous rice wine increased from 0 to 0.4%. Figure 3 (c,d) shows electron micrographs of Leum Pua glutinous rice wine residue after fermentation. Yeast cells adsorbed onto the solid fibre residue to protect against stress conditions at high osmotic pressure, leading to an increase in ethanol production. Solid fibre hydrolysis residue aids the viability of yeast for fermentation of ethanol at high concentration (Trakarnpaiboon *et al*., 2017). Hydrolysis residues of cassava pulp increased alcohol fermentation efficiency by two-fold as compared to fermentation without hydrolysis residues (Wattanagonniyom *et al*., 2017). The obtained Leum Pua glutinous rice wine was used in the next step for vinegar fermentation. Anthocyanin content of the rice wine at the end of fermentation was 30.4 mg/L.

Vinegar fermentation

Vinegar was obtained from the fermentation of *A. aceti* TISTR 354 using Leum Pua glutinous rice wine as substrate. Leum Pua glutinous rice is well known as a good source of bioactive compounds and antioxidant activities with applications as active ingredients of cosmetics and health food products (Thitipramote *et al*., 2016). Alcohol in the reaction was oxidized to acetic acid by acetic acid producing bacteria within 6 d of fermentation. Changes in alcohol content of Leum Pua glutinous rice vinegar during acetic fermentation are shown in Figure 4. Alcohol content gradually decreased during acetic fermentation and reached 0.8% after 6 d. Acidity of the reaction rapidly increased after 4 d to 4.0% and achieved 5.7% after 6 d of fermentation. Different acidity yields in vinegar products can be attributed to variations in raw materials, amount of acetic acid bacteria added, fermentation time and dilution (Li *et al*., 2014). However, SCF techniques showed efficiency with the advantage of vinegar production at shorter fermentation times over traditional

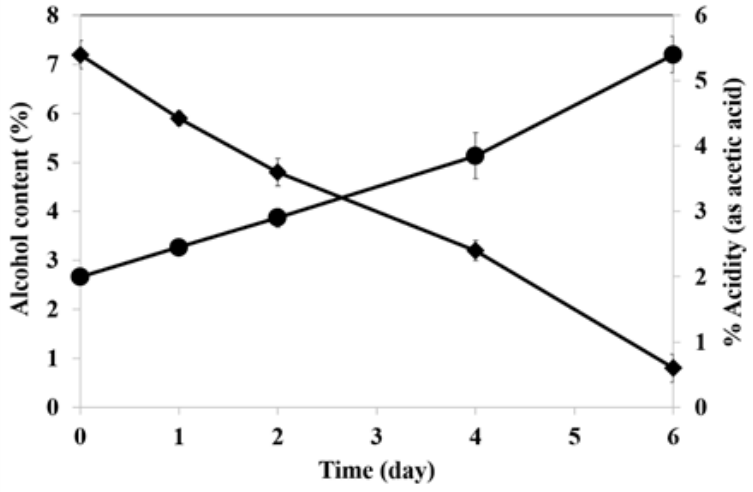

Figure 4. Time course of Leum Pua glutinous rice vinegar fermentation (♦: alcohol content; ●: %acidity as acetic acid).

methods, high acetic acid content, and low risk of contamination (Saithong *et al*., 2017). Vinegar obtained from Leum Pua glutinous rice corresponded to the standard set by the Notification of the 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. Maximum residue of alcohol did not exceed 0.5% and other parameters such as heavy metals or sulphur dioxide were within the prescribed parameters. To compare with other studies, Phuapaiboon (2017) obtained 3.18% of vinegar from fermented Riceberry rice at room temperature for 13 d. Anthocyanin content of Leum Pua glutinous rice vinegar was determined at the end of fermentation at 25.4 mg/L as compared to reported anthocyanin contents in brewed Riceberry rice vinegar at 1.62 mg/L (Phuapaiboon, 2017). Antioxidant assay was reported as half-maximum effective concentration (EC_{50}) as described above. The EC_{50} of Leum Pua glutinous rice vinegar was 1,218.76 ± 11.19 mL sample/g DPPH, which decreased about 30% as compared to the EC_{50} of Leum Pua glutinous rice wine at the beginning of fermentation (1,717.78 ± 10.16 mL sample/g DPPH). This result showed the possibility of producing healthy vinegar using Leum Pua glutinous rice as substrate without high temperature heating as an alternative health product from black rice in Thailand through biotechnological processes.

Conclusion

Leum Pua glutinous rice is a cheap alternative substrate in Thailand for sugar syrup and vinegar production. Raw starch degrading enzyme from the thermophilic filamentous bacterium, *L. sacchari* LP175 showed high efficiency for sugar syrup production at low temperature (50°C) without the heating process (gelatinization) for liquefaction and saccharification. Wine fermentation produced high alcohol and anthocyanin content with 5.7% acidity obtained from acidic fermentation of Leum Pua glutinous rice wine using SCF with *A. aceti* TISTR 354 starter culture. High levels of anthocyanin content in the finished vinegar product were produced in a short time. Results indicated that Leum Pua glutinous rice vinegar can be used as a new product via low temperature saccharification by raw starch degrading enzyme from *L. sacchari* LP175 without the heating process, with comparable antioxidant activity and anthocyanin content to other vinegar brands on the market.

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