การหมักไฮโดรไลเสทกากมะพร้าวเพื่อลดเวลาในการผลิตกรดอะซิติก

Fermentation of Coconut Pulp Hydrolysate to Reduce Time for Production of Acetic Acid

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บทคัดย่อ

กากมะพร้าวเป็นผลพลอยได้จากการผลิตน้ำกะทิ วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาความเป็นไปได้ในการเพิ่มการผลิตกรดอะซิติกโดยเชื้อจุลินทรีย์ผสมระหว่าง *Acetobacter cerevisiae* TN4497 และ *Acetobacter aceti* TISTR102 ในการหมักขั้นแรกเอทานอลถูกผลิตโดยการหมักในระบบการหมักการทำให้เป็นน้ำตาลพร้อมการหมัก (SSF) โดย *Saccharomyces cerevisiae* TISTR5048 โดยใช้ไฮโดรไลติกเอนไซม์ผสมระหว่างอะไมเลส อะไมโลกลูโคซิเดส ไซลาเนส เพกติเนส และเซลลูเลส เพื่อย่อยสลายกากมะพร้าวที่ถูกปรับสภาพด้วยกรดเจือจาง ในการหมักขั้นที่สอง *A. cerevisiae* TN4497 หรือ *A. aceti* TISTR102 และเชื้อจุลินทรีย์ผสมระหว่าง *A. cerevisiae* TN4497 และ *A. aceti* TISTR102 ถูกนำมาหมักเปรียบเทียบผลผลิตกรดอะซิติก ความเข้มข้นกรดอะซิติกที่ได้เท่ากับ 73.62 กรัมต่อลิตร, 92.47 กรัมต่อลิตรand 110.24 กรัมต่อลิตร โดย *A. cerevisiae* TN4497, *A. aceti* TISTR102 และเชื้อจุลินทรีย์ผสมระหว่าง *A. cerevisiae* TN4497 และ *A. aceti* TISTR102 ตามลำดับ จากการศึกษานี้แสดงว่าเชื้อจุลินทรีย์ผสมลดระยะเวลาการหมักและเพิ่มผลผลิตกรดอะซิติก

คำสำคัญ: กรดอะซิติก กากมะพร้าว การหมัก

Abstract

Coconut pulp is byproduct from coconut milk production. The objective of this study was to evaluate the possibility to enhance acetic acid production from coconut pulp by mixed culture fermentation of *Acetobacter cerevisiae* TN4497 and *Acetobacter aceti* TISTR102. For primary fermentation, ethanol was produced by simultaneous saccharification and fermentation (SSF) by *Saccharomyces cerevisiae* TISTR5048 fermentation using mixed hydrolytic enzyme of amylase, amyloglucosidase, xylanases, pectinases and cellulase to hydrolyse the diluted acid pretreated coconut pulp. For secondary fermentation, monoculture of *A. cerevisiae* TN4497 or *A. aceti* TISTR102 and the mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 was compared for acetic acid production. The

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acetic acid concentrations obtained were 73.62 g/L, 92.47 g/L and 110.24 g/L by fermentation with *A. cerevisiae* TN4497, *A. aceti* TISTR102 and mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102, respectively. From this study, the results showed that the mixed culture could shorten the fermentation time, reduce fermentation losses and increase the yield of acetic acid.

Keywords: acetic acid, coconut pulp, fermentation

Introduction

In Thailand, coconut is mostly used for food, and the waste may be used as feed for livestock or organic manure on farm yards. This paper reported an attempt to utilize coconut pulps for the production of vinegar. Vinegar is produced by fermentation of alcohols to acetic acid by bacteria. Generally, the acetic acid bacteria break down the sugars or starch in the food (substrate) converting it to alcohol and then further to acetic acid by membrane bound enzymes (Eram and Ma, 2013). The vinegar can then be used in dressing salads, manufacture of useful medicines, preservation of food stuffs, providing antioxidants or as an antibacterial agent (Soltan and Shehata, 2012). Vinegar is commonly obtained from good wine, cider, fruits and starchy foods (Krusong and Assanee, 2010). These undergo fermentation by acetic acid bacteria during the process of secondary fermentation. Since the coconut pulp has polysaccharide as the major component, it was hypothesized that this can be further processed by fermentation into a valuable product such as vinegar. The production of vinegar from the coconut pulp can be of great value to the country both economically (by increasing the economic value of coconut, providing locally made vinegar on the market and reducing seasonal losses of the fruits) and also provide an avenue to utilize the vast waste produced in form of pulps. Acetic acid is produced from ethanol oxidized through two sequential reactions catalyzed by the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase, both enzymes are membrane-bound and oriented to the periplasmic space of microorganism. Ethanol is produce from pyruvate, byproduct of glycolysis. Pyruvate is catalyzed to ethanol by two sequential reactions of pyruvate dehydrogenase and alcohol dehydrogenase (Eram and Ma, 2013). The most important properties of a production strain in vinegar industry are tolerance to high concentrations of acetic acid and total concentration, low nutrient requirements, inability to over oxidize the formed acetic acid, high production rate. Although a variety of bacteria can produce acetic acid, mostly members of *Acetobacter* are used commercially. Pure cultures are not widely used in the acetic acid fermentation industry. Ethanol is dehydrogenated to acetic acid and the reduced co-substrates are oxidized via the respiratory chain. This fermentation is an incomplete oxidation because the reducing equivalents generated are transferred to oxygen and not carbon dioxide (O'Toole and Lee, 2003). In this study, the possibility to produce acetic acid

from coconut pulp with monoculture and mixed culture fermentation of acetic acid bacteria was evaluated in a stirred tank reactor (STR); the process parameters were optimized and applied to enhance the yield of acetic acid production.

Materials and methods

Microorganisms

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

Medium and culture conditions

Each acetic acid bacterium collected from an agar slant was incubated at 30°C for 24 h in a 200-ml Erlenmeyer flask containing 50 ml of Medium (100 g/L glucose, 1 g/L yeast extract; 2 g/L $(NH4)_2SO_4$; 1 g/L $MgSO_4 \cdot 7H_2O$) on a shaker at 100 rpm. A 10-ml aliquot of the culture broth was inoculated into a 5OO-ml Erlenmeyer flask containing 125 ml of Medium and incubated at 30°C on a shaker at 100 rpm for 24 h to prepare a pre-culture of the acetic acid bacterium in the stationary phase of growth. The pre-cultures of both the ethanol- and acetic acid fermenting organisms were harvested by centrifugation at 10,000xg for 15 min. Collected cells were suspended in 250 ml of growth medium (100 g/L glucose, 1 g/L yeast extract; 2 g/L $(NH4)_2SO_4$; 1 g/L $MgSO_4 \cdot 7H_2O$) to prepare an inoculum. Mixed cultures of acetic acid-fermenting organisms were carried out in a 5-L fermentor with a working volume of 2.5 1**.** Inoculum was added at 5% to medium in fermentor. Cultures were kept at 30°C. The inoculum size of each experiment was controlled by diluting plating count technique and inoculating with the equal number of each microorganism (24 hours culture, 1 x 10^7 cells/mL) into the medium.

Ethanol fermentation of coconut pulp hydrolysate medium

Coconut pulp extract was prepared from dried powder (600 g) of coconut pulp added with 2 L of distillated water. Coconut pulp hydrolysate was prepared from dried powder (600 g) of coconut pulp added with 2 L of 0.1 M hydrochloric acid. Pretreatment and hydrolysate was prepared by autoclaving under 15 lb/inch^2 for 60 min Then, 250 mL filter-sterilize cellulase solution (cellulase activity: 200 Filter paper units (FPU) (g substrate)$^{-1}$, alpha-amylase 100 unit (g substrate)$^{-1}$, amyloglucosidase100 unit (g substrate)$^{-1}$, xylanase activity: 500 unit ((g substrate)$^{-1}$) and pectinase activity: 250 unit ((g substrate)$^{-1}$) in 0.1 M sodium phosphate (pH 5.0) was added to the coconut pulp solution which reacted with the enzyme mixture at 50 °C and 120 rpm for 48 hours for hydrolysis. After the enzymatic reaction, the hydrolysate was centrifuged at 21,000 × g for 10 min. The supernatant was supplemented with additional nutrients to give a base medium composition of: 1 g/L yeast extract; 2 g/L $(NH4)_2SO_4$; 1 g/L $MgSO_4 \cdot 7H_2O$. Batch fermentation was conducted in a 5 L fermentor with a working volume of 2 L. The

fermentation medium was inoculated with 5% v/v *S. cerevisiae* (24 hours culture, 1 x 10^7 cells/mL). The fermentation temperature was kept constant at 30 ± 0.2 °C. The broth was kept under agitation at 50 rpm. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol and residual sugars in the broth. All experiments were carried out in duplicate.

Acetic acid fermentation

The supernatant from the medium of ethanol fermentation was supplemented with additional nutrients to give a base medium composition of: 1 g/L yeast extract; 2 g/L $(NH4)_2SO_4$; 1 g/L $MgSO_4 \cdot 7H_2O$. Batch fermentation was conducted in a 5 L fermentor with a working volume of 2 L. The fermentation medium was inoculated with 5% v/v acetic acid bacterium (24 hours culture, 1 x 10^7 cells/mL). The fermentation temperature was kept constant at 30 ± 0.2 °C. The broth was kept under agitation at 50 rpm. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol and residual sugars in the broth. All experiments were carried out in duplicate.

Analytical methods

Moisture content, crude protein and starch in coconut pulp were determined according to standards (AOAC, 1975). The fibre concentration of coconut pulp was established using the Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Digestible Lignin (ADL) tests. Cellulose, hemicellulose and lignin contents were determined by the detergent extraction method (Robertson and van Soest, 1981). Culture dry weight was measured by centrifugation and drying at 105 °C, until no weight change between consecutive measurements was observed.Total reducing sugar was estimated by using dinitrosalicylic acid (DNS) reagent (Miller, 1959). Glucose was determined by glucose (go) assay kit (Sigma Product No. G 3660).

Acetic acid and ethanol assay

Acetic acid and ethanol was determined by gas chromatography. An AT-Wax capillary column was used in GC system (Chladek *et al*., 2007). The parameters were set as follows: carrier gas nitrogen, flow rate of carrier gas 10 mL/min, split ratio 10:1, temperature of injection port 220ºC, temperature of detector 260ºC. The column temperature program, firstly kept at 50ºC for 3 min, secondly heated up to 80ºC by the rate of 10ºC/min, then immediately heated up to 200˚C by the rate of 30ºC/min and then kept at 200ºC for 1 min. Sample pretreatment was carried out as follows: 5 mL of samples were centrifuged at 10,000 rpm for 3–5 min. The supernatant was diluted to suitable concentration by decuple dilution and filtrated through 0.22 μm microporous membrane. Then 1.0 mL of each sample was mixed with 10 μL of isobutanol as internal standard. Standard sample was the mixture of 0.02% isobutanol, 0.02% ethanol and 0.02% acetic acid. When GC worked stably, samples were injected with the volume of 1 μL.

Results

The average composition of coconut pulp is summarized in Table 1. Primarily, the major constituents of coconut pulp are relatively high carbohydrates (cellulose, hemicellulose and starch). The result indicated that coconut pulp could be a good source for bioconversion.

Table 1 Average composition of coconut pulp

Constituents	% of dry weight
Moisture	3.53±0.02
Hemicellulose	29.17±1.02
Cellulose	43.63±2.65
Lignin	17.34±0.42
Crude protein	0.74±0.02
Starch	2. 03±0.02

Alcoholic fermentation

Approximately 2.0 L of coconut pulp hydrolysate were poured into a 5 L fermentor and inoculated with 20 ml of the yeast starter culture. The inoculated slurry of coconut pulp was subjected to primary fermentation at ambient temperature for 2 days to produce coconut pulp wine, which was then filtered after complete primary fermentation. The compositions and physiochemical properties of the coconut pulp extract, pretreated coconut pulp, hydrolysate and wine were then determined, and the results of this primary fermentation are presented in Table 2.

Table 2 The compositions and physiochemical properties of the coconut pulp extract, pretreated coconut pulp, hydrolysate and wine

Parameter	Coconut pulp extract	Pretreated coconut pulp	Coconut pulp hydrolysate	Coconut pulp wine
Acetic acid (g/L)	0.00	0.00	0.00	4.63±0.01
pH	6.10±0.07	6.40±0.10	5.5±0.10	5.40±0.10
Reducing sugars	6.62±0.30	23.61±0.14	348.28±2.52	2.25±0.02
Glucose	2.71±0.20	9.32±0.10	187.49±4.16	0.37±0.01
Ethanol (g/L)	0.00	0.00	0.00	115.97±0.20
Colour	colorless	Light brown	Light brown	Light brown

Values are means ±SD of triplicate determinations

Acetic acid fermentation

The wine obtained after the alcoholic fermentation contained 115.97 (g/L) alcohol. It was filtered and ethanol wort added for vinegar production. The acetic fermentation was conducted by seeding the wine obtained at alcoholic fermentation with acetic acid bacteria from alcohol. This was a mixture of acetic microorganisms, *A. cerevisiae* TN4497 and *A. aceti* TISTR102. The coconut pulp extract had slightly low amount of sugars. This necessitated amelioration by pretreatment and enzyme hydrolysis in order to raise the levels sugar for yeast fermentation. The acidity of pretreated coconut pulp was adjusted to pH 5.5 for enzymatic hydrolysis. However, pH did not change appreciably upon pretreatment and

enzymatic hydrolysis. However, the pH value did not change after primary fermentation of the wine. The coconut pulp extract color changed from colorless to deep brown after pretreatment and enzymatic hydrolysis. This was possibly due to the brown color of the sugar that was produced. Primary fermentation was carried out at room temperature (30ºC). This resulted in a light brown colored coconut pulp wine with an ethanol concentration of 115.92 g/L. The coconut pulp wine obtained in Table 2 was then subjected to a two step fermentation system using a batch process. This involved enzymatic oxidation where the ethanol substrate was first oxidized to acetaldehyde and subsequently oxidized to the final product, acetic acid. This process was carried out over a period of 7 days. Figure 1-3 shows the trends in pH, accumulation of acetic acid, ethanol concentration and dry matter during fermentation.

As shown in Figure 1, the content of acetic acid in culture at 168 h was 73.62 g/L. Meanwhile, pH value was about 4.10, the biomass of *A. cerevisiae* TN4497 was promoted from 9.27 g/L at 0 h to 43.73 g/L at 168 h. The extracellular ethanol was utilized by the culture after inoculation, and the culture reached the maximum growth at 168h. As shown in Figure 2, the content of acetic acid in culture at 168 h was 92.47 g/L. Meanwhile, pH value was about 3.40, the biomass of *A. aceti* TISTR102 was promoted from 9.27 g/L at 0 h to 57.61 g/L at 168 h. The extracellular ethanol was utilized by the culture after inoculation, and the culture reached the maximum growth at 168 h. As shown in Figure 3, the content of acetic acid in culture at 168 h was 110.24 g/L. Meanwhile, pH value was about 3.20. the biomass of the mixed culture of *A. aceti* TISTR102 and *A. cerevisiae* TN4497 was promoted from 9.27 g/L at 0 h to 69.42 g/L at 168 h. The extracellular ethanol was utilized by the mixed culture after inoculation, and the mixed culture reached the maximum growth (71.64 g/L) at 132 h and maintained at the level until 168 h. From the results, it indicated that the mixed culture increased the yield of acetic acid and shorten the fermentation time when comparing to monoculture fermentation.

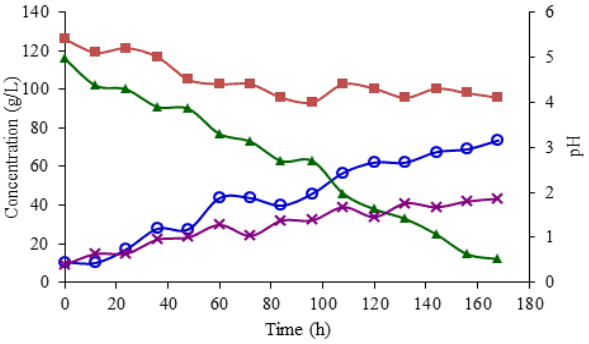

Figure 1 The time courses of acetic acid production (○), glucose content (▲), the cell growth (✕) and pH (■) during fermentation by *A. cerevisiae* TN4497

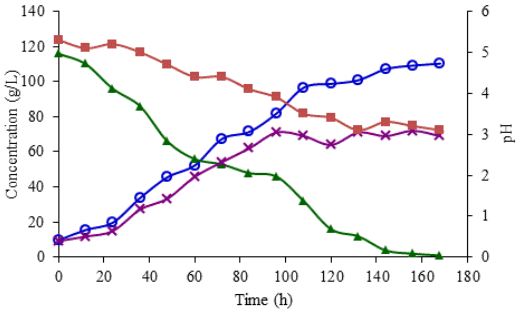

Figure 2 The time courses of acetic acid production (○), glucose content (▲), the cell growth (✕) and pH (■) during fermentation by *A. aceti* TISTR102

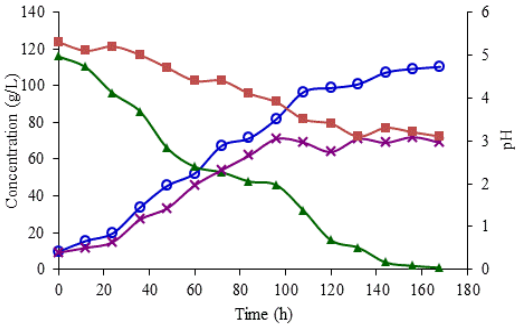

Figure 3 The time courses of acetic acid production (○), glucose content (▲), the cell growth (✕) and pH (■) during fermentation by mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102

Discussion

Acetic acid production in monoculture and mixed cultures was investigated (Fig 1-3). *A. cerevisiae* TN4497 and *A. aceti* TISTR102 was found to be optimum combination with respect to the yield of acetic acid and cultivation time required, 110.24 g/L of acetic acid being produced in 168 h. The $Y_{Ac/Alc}$ value was 0.95g/g (95.06% of the theoretical value), which is much higher than the 0.63 g/g and 0.79 g/g obtained from *A. cerevisiae* TN4497 and *A. aceti* TISTR102, respectively. The results can be explained by the fact that *A. aceti* TISTR102 can ferment ethanol efficiently compared with *A. cerevisiae*, as shown by the finding that $Y_{Ac/Et}$, was higher in Figure 2 than in Figure 1. The acetic acid yields from ethanol (0.64 g/g, 0.80 g/g and 0. 95 g/g, respectively) in monoculture of *A. cerevisiae* TN4497 or *A. aceti* TISTR102 were comparable with those obtained in mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102, indicating that the conversion of ethanol to acetic acid proceeded predominantly in the mixed culture. In *A. cerevisiae* TN4497 and *A. aceti* TISTR102, only 73.62 g/Land 92.47 g/Lof acetic acid, respectively, ($Y_{Ac/ET}$=0.64 g/g, 63.48% of the theoretical value and 0.8 g/g, 79.74 % of the theoretical value, respectively) were produced and 12.1 g/Land 14.4 g/Lof ethanol remained after 168 h. The low yield of acetic acid may be due to weak oxidation of ethanol to acetic acid in *A. cerevisiae* TN4497 and *A. aceti* TISTR102. From these results, the combination of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 was increase oxidation of ethanol to acetic acid. There are two additional advantages in using coconut pulp as carbon source and the combination of *A. cerevisiae* TN4497 and *A. aceti* TISTR102. First, S. *cerevisiae* could grow by using coconut pulp as carbon source but the biomass does not increase to increase amount of ethanol under anaerobic conditions, because of an incomplete TCA cycle (Dickinson, 1999). Second, *S. cerevisiae* accumulates acetaldehyde and acetic acid under anaerobic conditions, although ethanol

productivity increases. Acetaldehyde is also an intermediary metabolite during the oxidation of ethanol to acetic acid by *Acetobacter.* These characteristics of the organisms are beneficial for fermenting acetic acid from glucose from coconut pulp hydrolysate in the mixed culture. When the ethanol concentration fell below 115.97 g/l. Figure 1-3 show that acetic acid began to increase with a decrease in ethanol. On the other hand, as shown in Figure 1-3, ethanol decreased, and acetic acid production started after a lag time of about 24 h. In the mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102, $Y_{Ac/Glc}$ (0.59 g/g, 88.09% of the theoretical value) was higher than that in the monoculture inoculation of *A. cerevisiae* TN4497 (0.39 g/g, 58.82%) or *A. aceti* TISTR102 (0.49 g/g, 73.89%). Thus, a mixed culture of *A. cerevisiae* TN4497 and *A. aceti* TISTR102 was found to be satisfactory for the production of acetic acid. The pH of the vinegar during the secondary fermentation by the mixed culture was observed to decrease from pH 5.30 to pH 3.20. This initial decrease in pH provided optimal growth conditions to acetification. This fall in pH and it can be attributed to accumulation of acetic acid and other volatile short chain organic acids such as propionic, tartaric and butyric acids, which are important in development of the flavor and aroma of vinegar (Pan *et al*., 2013).

The ethanol content continued to decrease with time from 115.92 g/L to about 0 g/L by the 7^{th} day. This interpreted that the ethanol conversion to acetic acid reacheed zero when acetic acid reaches to the maximum in the medium. The vinegar produced from the coconut pulp extract contained 54.71 g/L acetic acid or about 5.4% (v/v) acetic acid and was comparable with 6.33% (v/v) and 6.11% (v/v) vinegar obtained by Torija *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). There was also a significant amount of sugar recorded in the wine vinegar by the end of the fermentation. This implied that there was better utilization of sugar in the production of vinegar than coconut pulp wine (see Table 2). Therefore, the presence of fermentable sugars produced from fruit residues (pulps) can make them ideal substrates for alcoholic fermentation of fruit juice and subsequent secondary fermentation into vinegar.

Conclusions

Coconut pulp hydrolysate was prepared by pretreatment with 0.1 M HCl with autoclaving under 15 $lb/inch^2$ for 60 min and hydrolysed by enzymatic method. The coconut pulp wine vinegar production process by mixed culture took 7 days and had physiochemical characteristics of 54.71 g/L acetic acid and pH of 3.20 which complied with the standard ranges of brewed vinegar after complete fermentation. This study therefore, showed that coconut pulp can be used as an ideal substrate for good vinegar production. This not only increases the economical and food value of coconut pulp but also provides a way of utilizing coconut pulp. In addition, the mixed culture produced average acetic acid

yield that was improved about 50% compared with monoculture fermentation. So, this study showed that the mixed culture could shorten the fermentation time, reduce fermentation losses and increase the yield of acetic acid.

Nomenclature

GLC : initial glucose concentration, g/L

ET : maximum ethanol produced, g/L

AC : maximum acetic acid produced, g/L

$Y_{AC/ET}$: acetic acid yield from ethanol (=AC/ consumed ethanol), g/g

$Y_{AC/GLC}$: acetic acid yield from glucose (=AC/consumed glucose), g/g

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