

Isolation of yeast and acetic acid bacteria from palmyra palm fruit pulp (*Borassus flabellifer* Linn.)

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Abstract

The isolation and identification of yeast and acetic acid bacteria from palmyra palm fruit pulp were investigated. Twenty yeast isolates were selected from palmyra palm fruit sample. Ten yeast isolates showed the highest tolerant ability to 10 and 15% (w/v) glucose. To screening for ethanol tolerance with 6 and 8% (v/v) ethanol, the cell viability was obtained at 6% (v/v) ethanol than 8% (v/v) with a range 6.86 - 7.67 log CFU/ml and 2.20 - 4.00 log CFU/ml, respectively. The isolate Y15 produced the highest ethanol content about 7.4% at 15% (w/v) glucose within 4 days. This isolate was *Candida stellimalicola* with identification method of rRNA region and sequence of D1/D2 domain of rDNA. The isolates of acetic acid bacteria from palmyra palm fruit pulp were studied by the biochemistry test. The catalase test showed positive and oxidase test as negative. Microscopic examinations confirmed that the strains were gram negative rod to coccobacilli. All of strains showed negative overoxidation and cellulose formation. For ethanol tolerance, twenty acetic acid bacteria isolates were cultured in 6 and 8% (v/v) ethanol. Ten acetic acid bacteria isolates and tolerant ability to ethanol were subsequently screened with 6 and 8% (v/v) ethanol for acetic acid production. The isolate A10 produced the highest acetic acid content with 5.64% within 55 days at 6% (v/v) ethanol as compared with the other isolates. This isolate was identified as *Acetobacter ghanensis*, a new acetic acid bacteria for using native acetic acid bacteria strain as starter culture for palmyra palm fruit vinegar production.

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Introduction

Palmyra Palm, sugar palm or toddy palm (*Borassus flabellifer* Linn.) is commonly available in the Africa, South Asian (e.g. Sri Lanka and India), Southeast Asia (e.g. Myanmar, Cambodia, Malaysia, Indonesia, Vietnam and Thailand). The mesocarp or pulp ripe is sweet with abundant carotenoid. It had yellow orange as it becomes ripe and can be used for foods such as cakes, jelly, ice cream, jams, cordials, beverages and toffee (Ariyasena *et al.*, 2001; Chakraborty *et al.*, 2011). However, palm fruit has a short shelf-life because of the high moisture content. Several applications the microbiological in aspect for palmyra palm fruit ripe have been studied to increase, its value in use. For the first time, Theivendirarajah and Chrystopher (1986) found that fermented palmyra palm fruit with *Saccharomyces cerevisiae* to make alcohol. Besides, some yeast strains were isolated from palmyra palm fruit ripe and used as starter in cake (Tuntiwongwanich and Leenanon, 2009).

Vinegar has been possibly produced from different fruits such as apple, banana, grape, strawberry and

cherry. There are a variety of fruits appropriate for making a good quality vinegar. Vinegar produced by yeasts and acetic acid bacteria. Several strains of yeasts and acetic acid bacteria were isolated from many fruits such as grape, cherry, strawberry, peach, apricot, mango, rambutan, longan and tamarind. There has been several studies on isolation and identification of microorganisms in spontaneous vinegar products; as a result, the good ones are collected to apply for starter culture (Kadere *et al.*, 2008; Hidalgo *et al.*, 2010; Maal *et al.*, 2010; Maal and Shafiee 2012). The vinegar made from palm fruit pulp is a traditional product with a high content of carotenoids. But it is a few knowledge palmyra palm fruit ripe vinegar. In general, spontaneous fermentation has been conventionally applied in household scale to produce vinegar with less than 4% (w/v) acetic acid. To get the pure cultures acetic acid bacteria are favorable due to the high fermentation efficiency and qualities in final products. Therefore, this research project aimed to isolate microorganisms (e.g. yeast and acetic acid bacteria) in the palmyra palm fruit pulp to make vinegar. The native yeast

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and acetic acid bacteria should be developed the fermentation and produced a vinegar. The properties of the microorganisms, in terms of fermentation performance, were investigated to obtain the best ones.

Materials and Methods

Palmyra palm fruit ripe preparation

The ripe palmyra palm fruits were harvested in Pattani province in southern of Thailand. The fruits were kept at 4°C and transported in boxes with ice to the Food Science and Nutrition Laboratory. The fruits were washed in tap water and peeled before extraction. The pulp was hand-extracted in water at ratio of pulp : water (1:2) until the pulp was dissolved completely as called palmyra palm fruits juice. The extract of palmyra palm was pasteurized at 65°C for 5 min (Leuangnapa, 1990). The aqueous extract of palmyra palm fruits juice after heating and cooling was packed in plastic bottle and kept in refrigerator at -20°C until use. The pH value of palmyra palm fruits juice was determined using digital pH meter (Satorius, Germany). Total soluble solid (TSS) contents were measured with a digital hand-refractometer (Atago, Tokyo, Japan). Acidity was determined according to the method of AOAC (2000) by titration with 0.1 N NaOH. The reducing sugar was analyzed using the Somogyi-Nelson method of Nelson (1944) and Somogyi (1952). The proximate total nitrogen was determined by Kjeldahl method (AOAC, 2000).

Isolation and identification of yeast

Approximately 10 g of palmyra palm fruit pulp in 100 ml of yeast extract peptone dextrose broth (YEPD broth; yeast extract (Merck, Darmstadt, Germany) 10 g, bacteriological peptone (Himedia, Mumbai, India) 20 g, D-glucose 20 g (Ajax, Auckland, New Zealand) in 1000 ml distilled water) were incubated on rotary shaker at 30°C and 110 rpm for 48 h. The culture was streaked on YEPD agar, supplemented with 100 mg/l chloramphenicol (Calbiochem, Darmstadt, Germany) for antibacteria (Li *et al.*, 2011) to obtain the individual colonies. Twenty colonies expressed creamed color and convex shape that all colonies were restreaked on YEPD agar for glucose, ethanol tolerance and ethanol production.

Only the yeast isolated that the highest ethanol production was identified and analyzed by molecular methods. Isolated yeast was inoculated on yeast malt agar (YM agar; Difco, Detroit, MI, USA) and incubated at 17°C for 3-5 days to collect the cells. A full loop of yeast was mixed with saline-EDTA buffer (0.15 M NaCl, 1 M EDTA pH 8.0) in 1.5 ml

microcentrifuge tube to centrifuge at 8,000 rpm at 4°C for 10 min. The pellet was collected, while the supernatant was discarded.

The second method for isolation based on DNA was polymerase chain reaction (PCR) with boiled lysis buffer (Maniatis *et al.*, 1982). A loopful of yeast cell was transferred into 100 µl of lysis buffer (100 mM Tris (pH 8.0), 30 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), which was then boiled on water bath or metal block bath for 15 min. After boiling, 100 µl of 2.5 M potassium acetate pH 7.5 was added and centrifuged at 14,000 rpm, 4°C for 5 min. The supernatant was extracted with 100 µl of chloroform: isopropanol (24:1) two times. DNA was precipitated with cold isopropanol, which was cooled at -20°C for 10 min and centrifuged at 15,000 rpm for 15 min. DNA pellet was rinsed with 70% and 90% ethyl alcohol and dried. The dried DNA was dissolved in 30 µl milli Q water. The cycling parameters were as follows: An initial denaturation step 94°C, 5 min then follows by 30 cycles of 94°C, 1 min for denaturation, 55°C, 1 min 30 seconds for annealing, and 72°C for extension 2 min 30 seconds then follow by the final extension of 72°C for 10 min. PCR products were purified using SUPREC TM-02 (Takara, Otsu, Japan) according to the manufacturer's instruction. Visualization of amplified DNA was performed by electrophoresis using 0.8% agarose in 1X TBE buffer (0.09 M Tris-borate, 0.001 M EDTA, (pH8.0) and staining with ethidium bromide and visual the amplified DNA under the UV illuminator.

Sequencing was performed by using ABI Prism™ Big Dye™ Terminator cycle sequence ready reaction kit (Perkin Elmer, Japan) according to the manufacturer's instruction. Analysis of DNA sequencing reaction was performed on ABI PRISM 310 Genetic analyzer (Perkin-Elmer, Japan). The primers used for DNA sequencing consisted of primer: F63: (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and LR3: (5'-GGT CCG TGT TTC AAG ACG G-3'). The strain of yeast identified by Thailand Institute of Scientific and Technology Research (TISTR).

Glucose tolerance, ethanol tolerance and ethanol production

The tolerance of isolated strains to glucose concentration was investigated using YEPD broth with supplementation of 10% and 15% (w/v) glucose and incubated on rotary shaker at 30°C and 110 rpm for 72 h. The growth of yeast was evaluated after 72 h by checking the optical density at 600 nm absorbance by spectrophotometer (Thermo Madison, USA) and counting the colony on YEPD agar by spread plate

technique. Moreover, the ethanol content produced by these isolated strain was measured every 2 days by ebullimeter (Dujardin Salleron, France).

For ethanol tolerance, the isolated strains were inoculated in YEPD broth with 6% and 8% (v/v) ethanol supplementation. The cultures were incubated on a rotary shaker at 30°C and 110 rpm for 72 h to measured optical density at 600 nm absorbance and colony counting on YEPD agar.

Ethanol production from palmyra palm fruits juice by yeast isolates

Starter culture preparation, it was prepared by inoculating the pure yeasts into 200 ml palmyra palm fruits juice, incubated on rotary shaker at 30°C and 110 rpm for 18 h. For ethanol production, the starter culture at 10% (v/v) was inoculated in to 600 ml palmyra palm fruits juice with the TSS 10 and 15 °Brix and ammonium sulphate 500 mg/l supplementation. The fermentation was carried out for 7 days at 30°C and 110 rpm. The ethanol content produced by these isolated strain was daily measured ethanol by ebullimeter.

Isolation of acetic acid bacteria

Approximately 10 g of palmyra palm fruit pulp in glucose ethanol yeast extract (GEY; 10 g yeast extract, 20 g D-glucose and 50 ml ethanol in 1000 ml distilled water) were incubated at 30°C for 3-5 days. The culture was streaked on GEY agar plate. (GEY agar; 10 g yeast extract, 20 g D-glucose, 3 g calcium carbonate and 15 g agar in 1000 ml distilled) to collect the individual colonies. Twenty colonies appeared with clear zone on GYC agar were collected to restreak on GEY agar to obtain the pure cultures (Kommanee *et al.*, 2008). The morphological properties were performed by microscope (Zeiss, Germany). Several biochemistry tests (e.i. gram stain, catalase, oxidase, over-oxidation and cellulose) were subsequently investigated (Zahoor *et al.*, 2006 ; Maal *et al.*, 2010).

Ethanol tolerance and acetic acid production

The isolated strains were inoculated in glucose yeast extract medium (GYE broth; 100 g D-glucose and 10 g yeast extract in 1000 ml distilled water) with 6% and 8% ethanol, incubated on rotary shaker at 30°C and 110 rpm for 2 months. After 72 h, the microbial biomass was evaluated by measuring optical density at 600 nm absorbance, together with colony counting on glucose yeast extract calcium carbonate agar (GYC agar; 100 g D-glucose, 10 g yeast extract, 20 g calcium carbonate and 20 g agar in 1000 ml distilled water). Otherwise, acetic acid production was determined every 5 days during

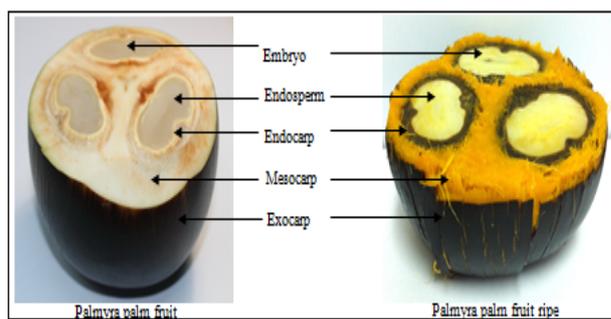


Figure 1. The palmyra palm fruit young (left) and palmyra palm fruit ripe (right)

2 months. Acetic acid content was measured by titration with 0.1N NaOH (AOAC, 2000).

The experimental results were expressed as mean \pm standard deviation (SD) of triplicate.

Results and Discussion

Physical and chemical property of palm fruit pulp

This research used the mature palm fruit pulp showed in Figure 1. The ripe palmyra palm fruit pulp was extracted in water showed the yellow-orange color. The palmyra palm juice proximate composition of wet basic show in table 1 moisture content $91.79 \pm 0.02\%$, protein $0.15 \pm 0.10\%$, ash $0.29 \pm 0.05\%$, crude fiber $6.5 \pm 0.05\%$, TSS 5.1 ± 0.15 °Brix, total acidity 0.53 ± 0.02 , pH 4.47-5.1 and reducing sugar 1.38 ± 0.02 g/l.

The palmyra palm fruit juice showed the TSS 5.1 ± 0.15 °Brix and reducing sugar 1.38 g/l. It was closely to previous research such as TSS 7.5 °Brix (Leuangnapa, 1990). The TSS depend on the ripeness of the fruits. In general, glucose is the major carbon source of yeast for ethanol production. Therefore, the glucose was addition to 10-15 °Brix and to obtain 6-8% alcohol in fermentation.

Isolation and identification of Yeast

The result of the total yeast counts performed by plating about 6.28-9.66 log CFU/ml. A total of yeast 81 isolates and selection 20 isolates circle cream by microscope showed colony with monopolar bipolar and multipolar budding. The next examination in measuring the glucose tolerance of 20 isolate strains in YEPD broth with 10% and 15% (w/v) on rotary shaker at 30°C and 110 rpm for 72 h. It found that showed the growth cells viability about 1.49-7.75 logCFU/ml and 0.7-8.88 logCFU/ml, respectively. The 9 yeast isolates showed growth cells viability at 15% (w/v) glucose more than 10% (w/v) glucose. All of strain about 10 isolates have cells viability more than 6 logCFU/ml in both 10% and 15% (w/v) glucose. Then, the ten isolates were inoculated

Table 1. Chemistry property from palmyra palm fruit pulp

| Chemistry property (% wet basic) | Result | Leuangnapa (1990) | Boonthrapong <i>et al.</i> , (2001) |
|-------------------------------------|------------|----------------------|--|
| Moisture content | 91.79±0.02 | 89 | 93 |
| Protein | 0.15±0.10 | - | 0.14 |
| Ash | 0.29±0.05 | - | 0.38 |
| Crude fiber | 6.5±0.05 | - | 2.73 |
| Total soluble solid (°Brix) | 5.1±0.15 | 7.5 | - |
| Total acidity | 0.53±0.02 | - | 0.59 |
| pH | 4.47-5.1 | 5-6 | 3.56 |
| Reducing sugar (g/l) | 1.38±0.02 | - | - |

in YEPD broth with 6% and 8% (v/v) ethanol and incubated on rotary shaker at 30°C and 110 rpm for 72 h. The yeasts showed the growth cells viability about 6.86-7.67 logCFU/ml at 6% (v/v) ethanol concentration. But 8% (v/v) ethanol concentration the cells viability were decrease to ethanol 8% (v/v) 2.20-4.00 log CFU/ml.

Several studies have revealed that isolation yeast from fruits such as, grape, banana, papaya, orange, guava and oil palm fruit. The yeast strain was not able to tolerate ethanol at concentration greater than 5% (v/v) (Siriwattanawimolchai *et al.*, 2013). The yeast isolates showed the growth well at 6% (v/v) ethanol more than 8% (v/v) ethanol (Maragatham and Panneerselvam, 2011). But, isolation yeasts from wine fermentation showed the growth cells at 10-16% (v/v) ethanol concentration (Li *et al.*, 2011). Isolation yeast from different kinds of fruits were collected randomly from different areas. It will effect on ability to glucose and ethanol tolerance.

The majority of isolated strains were able to grow at difference concentration of glucose and ethanol. The isolate Y15 produced the highest ethanol content about 5.0% and 7.4% at 10% and 15% (w/v) glucose within 2 and 4 days, respectively (Figure 2). It had the highest ethanol as compared with the other isolates.

It found that ethanol production in palmyra palm fruit juice showed in Figure 3. The isolate Y15 produced the highest ethanol content about 5.4% at 10 °Brix glucose within 7 days, but 15 °Brix glucose concentration the highest ethanol content 4.7% only. This isolate Y15 was *Candida stellimalicola* with identification method of rRNA region and sequence of D1/D2 domain of rDNA.

In this study isolation and identification of yeast species, *Candida stellimalicola* is a anamorphic yeast. This strain was first isolated from Ma-Fueng

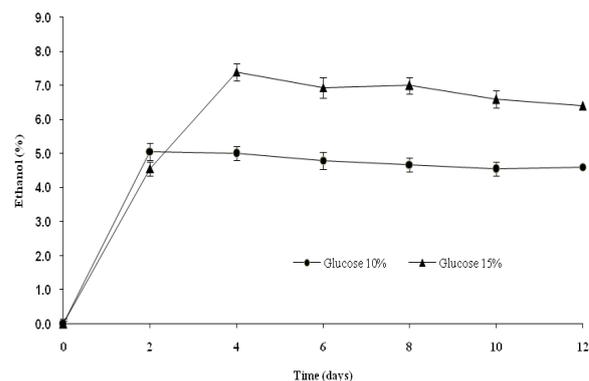


Figure 2. The ethanol production of isolate yeast Y15 in YEPD broth with 10% and 15% (w/v) glucose for 12 days cultivation

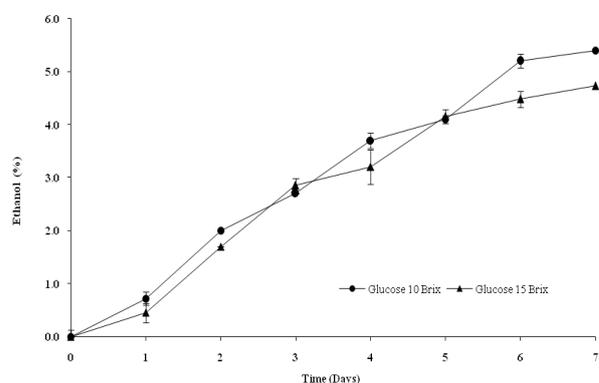


Figure 3. The ethanol production of isolate yeast Y15 in palmyra palm fruit juice with 10 and 15 °Brix glucose for 7 days cultivation

(star apple) in Thailand (Suzuki *et al.*, 1994). *Candida stellimalicola* showed the ubiquinone-7 (Q-7) and similar group of the base on 18S ribosomal DNA sequence divergence (Suzuki and Nakase 2002).

Candida stellimalicola is common non-*Saccharomyces* yeasts. It has been found in many fruits and in other fermentation, *Ghanaian cocoa* fermentation (Nielsen *et al.*, 2005); spontaneous alcoholic fermentation (Ocon *et al.*, 2010); Dadih fermentation (Jatmiko *et al.*, 2012). Similar, isolation of yeasts from toddy palm in Thailand the genera *Candida* and *Kloeckera* yeasts were the main species (Tuntiwongwanich and Leenanon 2009). In the present study it was found that *Candida stellimalicola* produced the low ethanol. Ariyasena *et al.* (2000) found that palmyra palm fruit ripe contained the flabelliferin (F_B). The results showed F_B was the bioactive, anti-yeasts and anti-bacteria.

Acetic acid bacteria isolation and identification

The bacteria showed the clear zone on GEY medium after incubation for 24-48 h at 30°C. These strains belonged to gram negative or gram-variable,

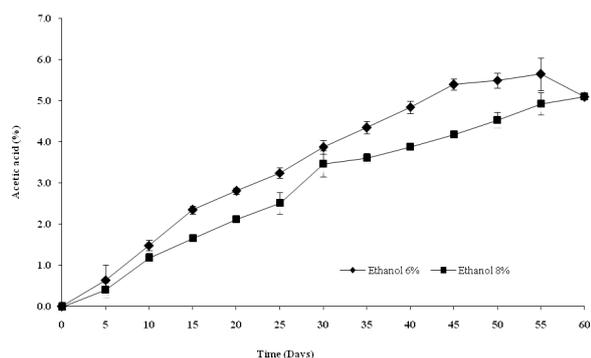


Figure 4. The comparison of acetic acid production of isolate A10 in GYE broth with 6% and 8% (v/v) ethanol at 60 days

morphologically long, rod and coccobacillus shape. Biochemical catalase tests were positive and negative. The result was recorded for oxidase test (Sokollek *et al.*, 1998; Seearunruangchai *et al.*, 2004; Gullo and Giudici 2008; Mall and Shafiee 2009). The bacteria were cultured in bromocresol green ethanol agar plate to observe the changing in color. The initially green color of media changed into yellow color after 24 h and stably remained for 14 days. The color change was the main indicator for acid production and no overoxidation. The isolates bacteria were cultured in GYE broth at 30°C for 24 h to test the cellulose formation. The result showed all strains did not produce cellulose after 4-5 days.

The problem in the vinegar spontaneous fermentation is overoxidation because the acetic acid bacteria were able to oxidize acetic acid into CO_2 and H_2O . The twenty acetic acid bacteria showed negative overoxidation. In addition, overoxidation of acetic acid has been used for the differentiation between *Acetobacter* and *Gluconobacter* genera (Sokollek *et al.*, 1998). Result to the cultivation of acetic acid bacteria 20 isolates in GYE broth with 6% and 8% ethanol (v/v) concentrations on rotary shaker at 30°C and 110 rpm for 72 h showed the cells viability 4.61-6.15 and 2.11-5.87 logCFU/ml, respectively. The 10 isolates were high cells viability about 5.70-6.40 logCFU/ml in GYE broth with 6% and 8% ethanol (v/v) concentrations about 4.20-5.99 logCFU/ml. Several studies have performed acetic acid bacteria isolated from fruits such as, cherry, mango etc and characterized their properties. For examples, the *Acetobacter* strain grew well in modified Caar media with 4% and 5% ethanol, but the growth decreased at higher 6% (v/v) ethanol concentration (Maal and Shafiee 2010). Kommanee *et al.* (2012) found that the growth of *Acetobacter pasteurianus* increased maximally at 4% (v/v) ethanol and the cells decreased at 6% and 8% ethanol concentration. However,

ethanol tolerance strain is conditioned depend trait on the temperature, pH and oxygen (Drysdale and Fleet, 1988; Du Toit and Pretorius 2002; Gullo and Giudici 2008).

Among the 10 isolates of acetic acid bacteria were important for acetic acid production. They were cultivated in GYE broth with 6% and 8% ethanol (v/v) concentrations at 60 days. The isolate A10 produced the highest acetic acid content with 5.64% and 5.10% at 6% and 8% (v/v) ethanol within 55 and 60 days, respectively (Figure 4). This isolate was *Acetobacter ghanensis* with identification method sequence of 16S rDNA.

In this study, The *Acetobacter ghanensis* is an acetic acid bacteria belongs to genus *Acetobacter*. *A. ghanensis* was firstly isolated from *Ghanaian cocoa* beans in Ghana (Cleenwerck *et al.*, 2007). It also has been found in many fruits jujube, kaffir lime, pineapple, rose apple in Thailand (Kommanee *et al.*, 2012). In this study, it was the first time that the *A. ghanensis* was isolated from palmyra palm fruit ripe. Obviously, this bacterium could produce acetic acid faster than spontaneous fermentation performed, which was possible to use it as the starter culture for vinegar fermentation. It produced the fast acetic acid than spontaneous fermentation, also to the isolation acetic acid bacteria from other fruits for example, strawberry (Hidalgo *et al.*, 2013).

Conclusion

The strain *C. stellimalicola* could produce a high ethanol with 5.4% at 10 °Brix palmyra palm fruit within 7 days. Moreover, a new *Acetobacter ghanensis* was isolated and identified strain from palmyra palm fruit ripe from Thailand. This strain could produce high acetic acid 5.64% in ethanol 6% after 55 days and showed negative overoxidation and cellulose. Therefore, *C. stellimalicola* and *Acetobacter ghanensis* will useful and efficiency to fermentation the palmyra palm fruit juice vinegar. This product will produce in ours research for the healthy food.

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