Isolation and Characterization of Acetic acid Bacteria from Fruits and Fermented fruit juices for Vinegar Production

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Abstract

Acetic acid bacteria are Gram negative aerobic bacteria capable of oxidizing ethanol to acetic acid. They are used in the industrial production of vinegar. The present study aims at isolation of acetic acid bacteria from various kinds of fruits and fermented fruit juices. Thirty varieties of fruits and 4 fermented fruit juices were collected from the northern and eastern areas in Thailand. Ninety-nine isolates of acetic acid bacteria were obtained from 18 varieties of fruits and 4 fermented fruit juices using sterile distilled water supplemented with 4.00% ethanol (v/v) as an enrichment medium. Eighty-nine isolates were identified to be in the genus *Acetobacter* and 10 isolates were in the genus *Gluconobacter*. Fifty-nine isolates were *A.aceti* as determined by biochemical tests. Nineteen isolates ; P1, P4, P6, P8, P12, K4, K5, K6, K7, K8, S1, S2, S3, S4, S5, S6, S7, S8 and S11 gave the widest yellow zone on bromocresol green ethanol agar. They were selected for acetic acid production and compared with *A.aceti* TISTR354 in ethanol-yeast extract medium supplemented with 6.00% (v/v) ethanol. It was found that P1, P4, P6, P12 and *A. aceti* TISTR354 gave the highest yield of acid 4.06%, 3.70%, 3.89%, 4.00% and 4.03%, respectively. All the isolates were tested for their tolerance to ethanol and acetic acid. It was found that they were able to grow at 4% and 6% ethanol. Moreover, isolates P1, P4, P6, P12, K6, K7, K8, S1, S2 and S11 were able to grow at 10.00% ethanol.

Keywords: acetic acid bacteria, acetic acid, *Acetobacter*, fruit, fermented fruit juice

1. Introduction

Acetic acid bacteria (AAB) are Gram negative, rod shape and obligate aerobic bacteria with the ability to oxidize ethanol to acetic acid (Kersters *et al*., 2006; Moryadee and Pathom-aree, 2008; Sharafi *et al*., 2010). In the past, there were two main genera, *Acetobacter* and *Gluconobacter* but at present there are twelve genera which are in the Family Acetobactaceae, Class Alphaproteobacteria i.e. *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacerobacter*, *Asaia, Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia* and *Ameyamaea* (Sengun and Karabiyikli, 2010). The main characteristic between *Acetobacter* and

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Gluconobacter is that *Acetobacter* can oxidize acetate to CO_2 and H_2O, while *Gluconobacter* cannot. Members of the Family Acetobactaceae are useful in the industrial production of vinegar (Hornsey, 1999; Kersters *et al*., 2006; Sharafi *et al*., 2010). AAB are generally found in nature because they can use a variety of substrates (Sharafi *et al*., 2010). These bacteria have been isolated from alcoholic beverage, vinegar, fruits, flowers, honey, sugar cane, fruit juice, soil and water (Maal *et al*., 2010; Sharafi *et al*., 2010). Thailand is a tropical country with a large biodiversity of fruits and microbial resources. Therefore, Thai fruit is a good source for isolation of AAB. This research was focused on isolation and identification of AAB from various kinds of fruits and fermented fruit juices. High acetic acid producing strains as well as tolerant strains against high concentration of ethanol were also looked for.

2. Materials and Methods

2.1 Isolation of acetic acid bacteria

Isolates of AAB were obtained from 4 fermented fruit juices: kaffir lime, Indian gooseberry, pineapple and star fruit and from 30 fruits: apple, black grape, cantaloupe, cherry, chinese pear, dragon fruit, green grape, guava, longan, longkong, lychee, mango, mangosteen, mulberry, muskmelon, papaya, peach, persimmon, pineapple, pisang mas, plum, plum mango, rakum plam, rambutan, red grape, rose apple, santol, strawberry, sugar cane and watermelon. Samples were collected in March-June 2012 in northern area (Chiang Mai province) and eastern area (Chanthaburi province) of Thailand. Approximately 5 g of fruits were cut and incubated in 26 ml of enrichment medium (normal saline containing 1 ml of 95%ethanol) at 30ºC for 7 days. Thirty samples of enriched fruit containing medium and 4 fermented fruit juices were serially diluted and spread on bromocresol purple ethanol agar (0.50% glucose, 1.00% yeast extract, 1.00% peptone, 2.00% glycerol, 0.45% potato extract, 0.03% bromocresol purple, 2.00% agar, 4.00% ethanol 95%) supplemented with 100 ppm of cyclohexamide to inhibit the growth of yeasts and moulds (Moryadee and Pathom-aree, 2008). Plates were incubated aerobically at 30ºC for 2 days. The colonies which produce yellow zone on agar plate were selected and purified. Purified cultures were preserved on potato medium agar (0.50% glucose, 1.00% yeast extract, 1.00% peptone, 2.00% glycerol, 0.45% potato extract, 2.00% agar).

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2.2 Morphological and Biochemical Tests

Bacterial cells were Gram stained. Morphology of cells was examined under light microscope. Catalase and oxidase were examined (Holt *et al*., 1994). The overoxidation of ethanol was examined for the isolates and reference strain (*A. aceti* TISTR 354), on Carr medium (3% yeast extract, 0.0022% bromocresol green, 2% agar, 2% ethanol 95%) (Karin and Kain, 2006). *Acetobacter* changed the medium color from green to yellow and then from yellow to green while *Gluconobacter* changed the medium color into yellow in 14 days at 30ºC.

Ketogenesis from glycerol was determined by inoculating the isolates in test tube containing YG medium (3.00% yeast extract, 3.00% glycerol) incubated at 30ºC for 10 days and adding 8–10 drops of Fehling's solution into the medium (modified from Aydin and Aksoy, 2009). The change of medium color to orange indicated a positive test.

Cellulose production was tested on GYE medium (2% glucose, 0.50% yeast extract, 0.25% ethanol 95%) incubated at 30ºC for 7 days. Cellulose test was carried out using a Lugol's iodine stain followed by 60% sulphuric acid on pellicles from liquid culture, the color of cellulose fiber is blue (Passmore and Carr, 1975; Romero-Cortes *et al*., 2012).

Water-soluble brown pigment was examined by inoculating the isolates on GYC agar (2% glucose, 2% yeast extract, 2% $CaCO_3$, 2% agar) incubated at 30ºC for 2 days (Shimwell and Carr, 1960; Kadere *et al*., 2008).

Acid production from glucose was detected by growing the isolates on modified GYC agar (10% glucose, 3% yeast extract, 3% $CaCO_3$, 2% agar) incubated at 30ºC for 2 days. Acid production was observed from the formation of clear zone around the spotted culture (Shimwell and Carr, 1960).

Growth in ethanol was tested on Hoyer's medium (0.002% $FeCl_3 6H_2O$, 0.01% $K_2HPO_4 3H_2O$, 0.09% KH_2PO_4, 0.10% $(NH_4)_2SO_4$, 0.025% $MgSO_4 7H2O$, 3% ethanol 95%) and incubated at 30ºC for 14 days (Shimwell and Carr, 1960). The presence of turbidity of medium indicated a positive test.

2.3 Alcohol tolerance of isolates

The isolates were tested for their ability to grow in yeast extract agar (0.50% yeast extract, 2% agar) supplemented with 4%, 6%, 8% and 10% (v/v) ethanol.

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2.4 Acetic acid production

Isolates which gave the widest yellow zone on bromocresol green ethanol agar were selected for acetic acid production. AAB were transferred to ethanol-yeast extract medium (0.50% yeast extract, 2% (v/v) ethanol 95%, pH 6.8) and incubated at 30ºC for 2 days on rotary shaker at 150 rpm. A 10% of the prepared cultures (OD660nm = 0.359–0.459) was incubated in 250 ml Erlenmeyer flask containing 120 ml of ethanol-yeast extract medium (Moryadee and Pathom-aree, 2008). Fermentation was carried out at 30ºC for 14 days on rotary shaker at 150 rpm and sample was collected every 48 h. Acetic acid content was determined by titration with 0.1 N NaOH using phenolphthalein as an indicator (Moryadee and Pathom-aree, 2008; Romero-Cortes *et al*., 2012). Isolates that gave the highest acetic acid concentration were selected for acetic acid production in modified ethanol-yeast extract medium supplemented with 6% (v/v) ethanol.

3. Results and Discussion

3.1 Isolation and Identification of Isolates

Acetic acid bacteria were successfully isolated from 4 fermented fruit juices and 18 fruit samples. A total of 99 isolates of AAB, 12 from fermented fruit juices, 87 from fruits. Acetic acid bacteria were found to be high in red grape, cantaloupe, longan, longkong, papaya and strawberry, respectively (Table 1).

Table 1 Acetic acid bacteria isolated from fermented fruit juices and fruits

Source	*Acetobacter* sp.	*Gluconobacter* sp.	Total of isolates
kaffir lime	3	-	3
Indian gooseberry	1	-	1
pineapple	6	-	6
star fruit	2	-	2
apple	1	-	1
cantaloupe	8	-	8
dragon fruit	5	-	5
green grape	2	-	2
longan	4	3	7
longkong	-	7	7
lychee	5	-	5

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Table 1 Acetic acid bacteria isolated from fermented fruit juices and fruits (Cont.)

Source	*Acetobacter* sp.	*Gluconobacter* sp.	Total of isolates
mulberry	3	-	3
papaya	7	-	7
persimmon	1	-	1
peach	6	-	6
pineapple	5	-	5
pisang mas banana	2	-	2
red grape	9	-	9
rose apple	1	-	1
strawberry	7	-	7
sugar cane	5	-	5
watermelon	6	-	6
Total	89	10	99

All the isolates were Gram negative, rod shaped and catalase positive, which belong to the family of the AAB. The ability of acetic acid bacteria to oxidize acetate to CO_2 and H_2O was used to distinguish between members of the genera *Acetobacter* and *Gluconobacter* (Holt *et al*., 1994). The overoxidation was used to separate between *Acetobacter* and *Gluconobacter*. It was found that 89 isolates were identified to belong to the Genus *Acetobacter* while 10 isolates were *Gluconobacter*.

Fifty-nine isolates showed similar biochemical results with the reference strain. They were oxidase negative, ketogenesis from glycerol positive, able to produce acid from glucose and positive growth on ethanol. Moreover, they were unable to form cellulose and water-soluble brown pigment.

3.2 Alcohol tolerance of isolates

Nineteen isolates were tested for their ability to grow in the medium supplemented with ethanol. It was found that they were able to grow at 4% and 6% ethanol. Isolates P1, P4, P6, P8, P12, K4, K5, K6, K7, K8, S1, S2, S3, S6, S8 and S11 were able to grow at 8% ethanol. Moreover, isolates P1, P4, P6, P12, K6, K7, K8, S1, S2 and S11 were able to grow at 10% ethanol (Table 2).

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3.3 Acetic acid production

Nineteen isolates; P1, P4, P6, P8, P12, K4, K5, K6, K7, K8, S1, S2, S3, S4, S5, S6, S7, S8 and S11 were selected for acetic acid production and compared with reference strain in ethanol-yeast extract medium supplemented with 2% (v/v) ethanol. Isolates P1, P4, P6, S3 and *A. aceti* TISTR354 showed the highest acetic acid production of 1.78%, 1.80%, 1.80% 1.81% and 1.81%, respectively (Table 2). Acetic acid production increased within 5–7 days of fermentation. The isolates which produced higher amounts of acetic acid and highest ethanol tolerance were selected for acetic acid production in modified ethanol-yeast extract medium supplemented with 6% (v/v) ethanol.

Table 2 Alcohol tolerance of AAB isolates

Isolate	Alcohol (%)				Acetic acid (%)
	4	6	8	10	
P1	+	+	+	+	1.78
P4	+	+	+	+	1.80
P6	+	+	+	+	1.80
P8	+	+	+	-	1.69
P12	+	+	+	+	1.64
K4	+	+	+	-	1.56
K5	+	+	+	-	1.58
K6	+	+	+	+	1.55
K7	+	+	+	+	1.41
K8	+	+	+	+	1.65
S1	+	+	+	+	1.00
S2	+	+	+	+	1.66
S3	+	+	+	-	1.81
S4	+	+	-	-	1.63
S5	+	+	-	-	1.57
S6	+	+	+	-	1.64
S7	+	+	-	-	1.47
S8	+	+	+	-	1.70
S11	+	+	+	+	1.72
A. aceti TISTR354	+	+	+	+	1.81

Note: + = tolerant; - = non-tolerant

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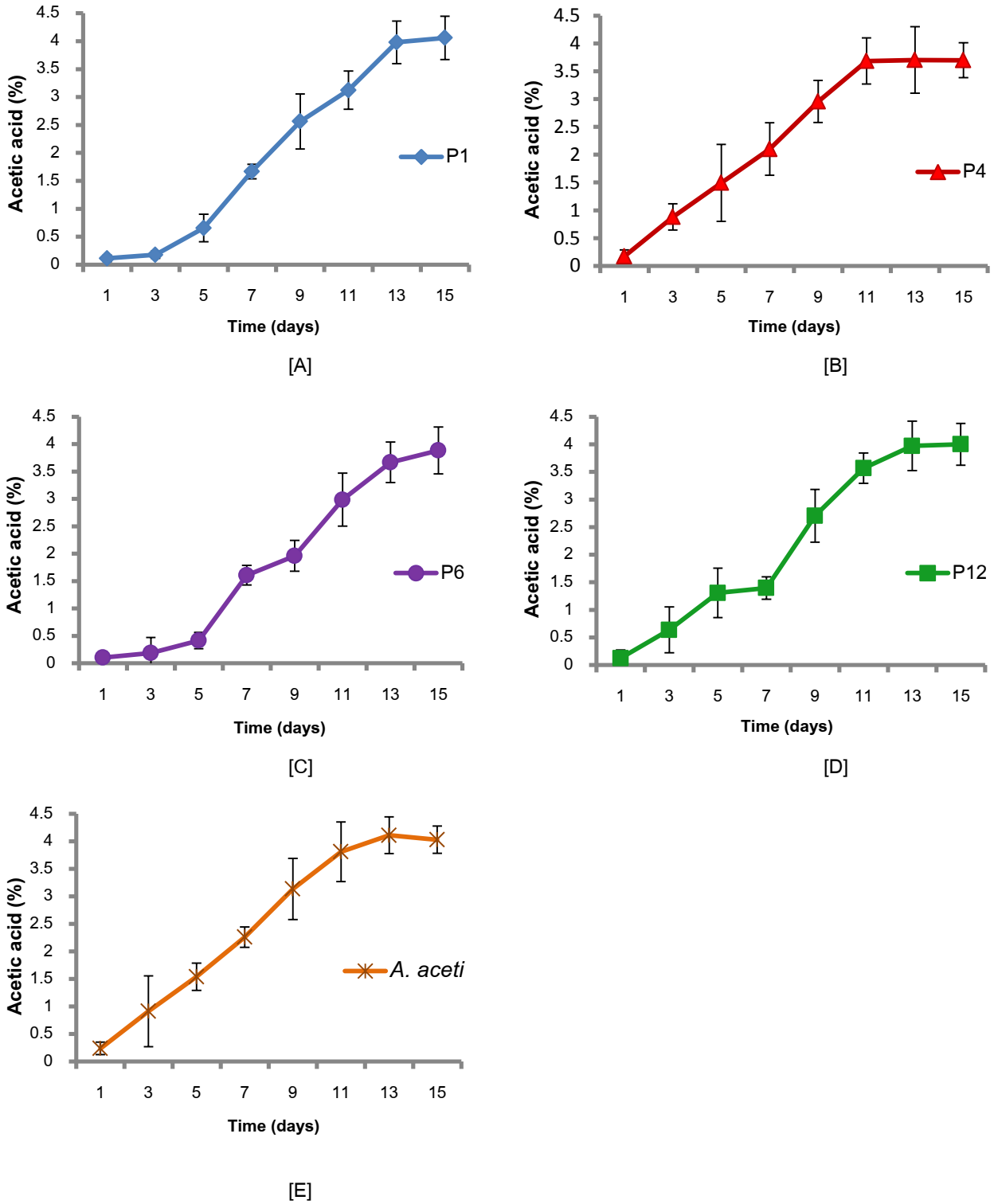

Figure 1 Production of acetic acid by 4 isolates and the reference strain in ethanol-yeast extract medium supplemented with 6% (v/v) ethanol after 15 days. [A]: P1; [B]: P4; [C]: P6; [D]: P12 and [E]: *A. aceti* TISTR354

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Isolates P1, P4, P6, P12 and *A. aceti* TISTR354 were selected for acetic acid production in ethanol-yeast extract medium supplemented with 6% (v/v) ethanol. Figure 1 shows the acetic acid production by these isolates after incubated in 15 days at 30ºC. All of isolates showed high acetic acid yield after 11–15 days. Isolates P1, P4, P6, P12 and *A. aceti* TISTR354 showed the highest acetic acid percentage were 4.06%, 3.70%, 3.89% 4.00% and 4.03%, respectively.

4. Conclusion

In this study, acetic acid bacteria were isolated and identified from fruits and fermented fruit juices. It was showed that the enrichment culture technique is good for promoting the growth of acetic acid bacteria from fruits. It was suggested that these fruits should be ripe fruits which are appropriate for enrichment technique. The isolates could grow at 4–10% ethanol concentrations and produced highest acetic acid content suggesting their suitability for vinegar production.

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References

Aydin. A.Y. and Aksoy. D.N. 2009. Isolation of Cellulose Producing Bacteria from Waste of Vinegar fermentation. Proceeding of the World Congress on Engineering and Computer Science 2009.

Holt. J.G., Krieg. N.R., Sneath. J.T. Stanley and Williams. S.T. 1994. Bergey's Manual of Determinative Bacteriology, 9th Ed., Williams and Wilkins, Baltimore.

Hornsey. I.S. Brewing. 1999. The Royal Society of Chemistry. United Kingdom.

Kadere. T.T., Miyamoto. T., Oniang. R.K., Kutima. P.M. and Njoroge. S.M. 2008. Isolation and identification of the genera Acetobacter and Gluconobacter in coconut toddy (mnazi). African Journal of Biotechology. 7(16): 2963–2971.

Karin. S. and Kain. M. 2006. Acetobacter tropicalis in spontaneously fermented wines with vinegar fermentation in Austria. Mitteilungen Klosterneuburg. 56: 102–107.

Kersters. K., Lisdiyanti. P., Komagata. K. and Swings. J. 2006. The Family Acetobactaceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. Prokaryotes. 5: 163–200.

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Maal. KB., Shafiei. R., and Kabiri. N. 2010. Production of Apricot Vinegar Using an Isolated Acetobacter Strain from Iranian Apricot. World Academy of Science, Engineering and Technology. 71: 177–180.

Moryadee. A., and Pathom-Aree. W. 2008. Isolation of Thermotolerant Acetic Acid Bacteria from Fruits for Vinegar Production. Journal of Microbiology. 3(3): 209–212.

Passmore. S.M. and Carr. G.J. 1975. The ecology of the acetic acid bacteria with particular reference to cider manufacture. Journal of Applied Bacteriology. 38(2): 151–158.

Romero-Cortes. T., Robles-Olvera. V., Rodriguez-Jimenes. G. and Ramirez-Lepe. M. 2012. Isolation and Characterization of acetic acid bacteria in cocoa fermentation. African Journal of Microbiology Research. 6(2): 339–347.

Sengun. IY., and Karabiyikli. S. 2010. Importance of acetic acid bacteria in food industry. Food Control. 22: 647–656.

Sharafi, SM., Rasooli, I., and Beheshti-Maal, K. 2010. Isolation, characterization and optimization of indigenous acetic acid bacteria and evaluation of their preservation methods. Iranian Journal of Microbiology. 2(1): 41–48.

Shimwell. L.J. and Carr. G.J. 1960. The type culture concept in *Acetobacter* and other genera. Reasearch Department, British Vinegars Ltd., Frome, Somerest, England.

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