Isolation of acetic acid bacteria for the production of fermented glutinous rice vinegar

การคัดแยกแบคทีเรียกรดอะซิติกเพื่อการผลิตน้ำส้มสายชูหมักจากข้าวเหนียว

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ABSTRACT

Acetic acid bacteria were isolated from ripe fruits by enrichment culture technique. After incubation, each enrichment broth was spread onto potato agar plate supplemented with bromocresol purple and 4% ethanol. Then, the isolates were tested for basic characteristic of acetic acid bacteria. Fifty-nine isolates were obtained from twenty samples. They were Gram-negative, short rod, obligate aerobic bacteria. Fifty-five isolates were identified as member of genus *Acetobacter* due to their ability to oxidize acetate. The five isolates which showed wide clear zone were selected for acetic acid production test. The high yield was obtained from GR-7, GR-14, JF-2, PN-9 and LC-13 as 17.86, 17.20, 17.95, 17.78 and 16.14 g/L respectively. Five isolates were selected for acid and alcohol tolerance test. It was found that isolate LC-13 could able to grown in 16% ethanol and 3% acetic acid (v/v). Isolate LC-13 was chosen for rice vinegar production.

บทคัดย่อ

คัดแยกแบคทีเรียกรดอะซิติกจากผลไม้ด้วยเทคนิคการเพิ่มจำนวนแบคทีเรียในอาหารเลี้ยงเชื้อ จากนั้นทำการทดสอบลักษณะทางชีวเคมีของแบคทีเรียกรดอะซิติกพบว่า เป็นแบคทีเรียแกรมลบ รูปแท่ง ต้องการออกซิเจนในการเจริญ สามารถแยกแบคทีเรียที่ผลิตกรดได้ 59 ไอโซเลตจากผลไม้ทั้งหมด 20 ชนิด ในการทดสอบการเกิดoveroxidation พบแบคทีเรีย 55ไอโซเลตสามารถออกซิไดซ์อะซิเตตเช่นเดียวกับแบคทีเรียในกลุ่ม *Acetobacter* จึงได้คัดเลือกไอโซเลตที่สามารถออกซิไดซ์อะซิเตตได้สูงที่สุดจำนวน 5 ไอโซเลต มาทำการทดสอบการผลิตกรดพบว่า ไอโซเลท GR-7, GR-14, JF-2, PN-9 และ LC-13 ให้ปริมาณกรดน้ำส้ม 17.86, 17.20, 17.95, 17.78 และ 16.14 กรัมต่อลิตรตามลำดับ เมื่อศึกษาความสามารถในการทนต่อเอทานอลและกรดอะซิติก พบว่าไอโซเลต LC-13 สามารถทนต่อเอทานอลที่ความเข้มข้นสูงสุด 16% และทนกรดอะซิติกสูงสุดที่ 3% (v/v) จึงได้เลือกไอโซเลต LC-13 ในการผลิตน้ำส้มสายชูหมักจากข้าวเหนียว

Key Words: Acetic acid bacteria, Vinegar

คำสำคัญ: แบคทีเรียกรดอะซิติก น้ำส้มสายชู

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Introduction

Acetic acid bacteria (AAB) are Gram negative aerobic bacteria, rod shape occurring singly or pairs, and be able to oxidize ethanol to acetic acid rapidly which belongs to class α - Proteobacteria , Family *Acetobacteraceae*. They were classified to 12 genus includes *Acetobacter, Gluconobacter, Acidomonas, Gluconacetobacter, Asaia, Kozakia, Swaminathania, Saccharibacter, Neoasaia, ranulibacter,Tanticharoenia* and *Ameyamaea* (Sengun and Karabiyikli, 2011). They occur in natural habitats such as flowers, fruits, alcohol beverages, vinegar, sugar cane honey bees, soil and water (Saeki et al., 1997; Swings, 1992; Yamada et al., 1999; Seearunruangchai et al., 2004).

AAB are well known for useful in industrial vinegar production because they had ability oxidize acetate and lactate to CO_2 and water by using Kreb's cycle (Klawpiyapamornkun et al., 2014; Kersters et al., 2006; Sievers and Swings, 2006). *Acetobacter* sp. PVB2 was isolated from satoh produced acetic acid in high rate and used in vinegar fermentation gave the highest 4% acetic acid at day sixth (Moonmangmee et al., 2003). Moreover the genus *Gluconobacter* produces dihydroxyacetone, sorbose and 5-keto-gluconic acid (Klawpiyapamornkun et al., 2014).

Thailand is located in tropical area, many previous studies report that many of acetic acid bacteria were found in this country. Seearunruangchai et al (2004) were isolated the acetic acid bacteria from fruits and other sources material in Bangkok and some provinces in Thailand. Kadere et al. (2008) isolated *Acetobacter* strains from grape, date, palm resources and coconut were used in several vinegar productions.

Therefore, this study aims to the isolation and identification of acetic acid bacteria from some fruits and will be used for vinegar production from glutinous rcie. The sources material were ripe fruits such as grape, pineapple, rambutan, salak plum, mango, cantaloupe, watermelon, litchi, apple, custard apple, jackfruit, rose apple, Chinese pear, pitaya, guava, long kong, mangosteen, star apple, monkey apple and banana.

Objective of this study

The aim of this research was to isolate and screen indigenous acetic acid bacteria from fruits for glutinous vinegar production.

Methodology

Isolation of acetic acid bacteria

Fruits samples were collected from local market in Chiang Mai, Thailand. Twenty kinds of fruit samples were washed and chopped into small pieces. Five g of various fruits were added into bottle of distilled water supplement with 4% ethanol (v/v). The samples were incubated at 30°C for 5 days, then serial dilution and spread onto bromocresol purple ethanol agar. All plates were incubated at 30°C for 48 hr. The isolates that showed yellow halo zone around colonies on bromocresol purple ethanol plate were selected and streak on potato medium agar. The pure cultures were restreak on potato agar slant and keep at 4°C until used.

Biochemical characteristic

The colonies of pure isolate were streak on potato agar and incubated at 30°C for 48 h. Gram's stain was tested. Only Gram negative bacteria were collected and used for catalase and oxidase

determination. Hoyer's medium supplement with ethanol was used for growth in ethanol tested. Brown pigment, gluconic acid and cellulose production were also investigated. The nitrate broth was used to nitrate reduction test. *Acetobacter* strain and *Gluconobacter* strain were separated by the ability to oxidise acetate or overoxidation using Carr medium (0.1% bromocresol green, 2.0% ethanol, 3.0% yeast extract and 1.5% agar).

Acid production

All isolate were grown in ethanol–yeast extract medium (2% ethanol, 0.5% yeast extract, distilled water 1L adjusted pH 6.8) and incubated with shaking at 30°C for 48 h. Optical density of ethanol-yeast extract broth was measured by spectrophotometer adjusted 0.4-0.5 at 660 nm. 10% of starter were inoculated in the same fresh medium (120 mL) and grown by shanking at 150 rpm, at 30°C for 14 days. The acid production were determined by titrated with 0.1 N NaOH against phenolphthalein every 48 h. for 7 days.

Acetic acid and ethanol tolerance

Starter was prepared by inoculated AAB into potato medium broth and incubated at 30°C for 48 h by shaking. 10% of starter (OD_{660} = 0.5) was inoculated into potato medium contained with ethanol (4, 6, 8, 10, 12, 14, 16 and 18% (v/v)) and acetic acid (1, 2, 3, 4, 5 and 6% (v/v)). To check the ability of growth, the medium was incubated at 30°C for 5 days. Then, the samples were transfer to potato agar by spread plate technique and incubated at 30°C for 48 h.

Results

Fifty nine isolates were obtained from 20 fruits samples (Figure 1). The isolates were Gram-negative rod shape bacteria. The results of physiological and biochemical test were showed in table 1 suggesting that fifty-five isolates belonged to genus *Acetobacter* and only one isolate was identified as genus *Gluconobacter*.

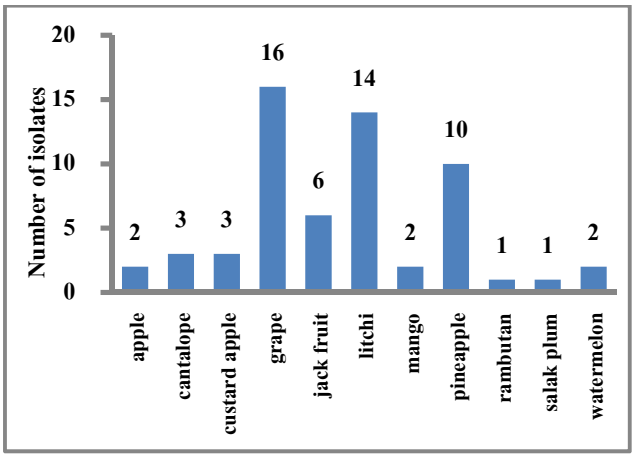

Figure 1 The number of acetic acid bacteria isolated from fruits sample

Table 1 Biochemical characteristic of acetic acid bacteria isolates

Biochemical Test	Bacteria			
	A. aceti TISTR 354	JF-2	GR-14	LC-13
Catalase	+	+	+	+
Oxidase	-	-	-	-
Overoxidation	+	+	+	+
Ketogenesisi from glycerol	+	+	+	+
Growth in ethanol	+	+	+	+
Brown pigment	-	-	-	-
Cellulose production	-	-	-	-
Nitrate reduction	-	-	-	-
Gluconate	+	+	+	+

Sixteen isolates were selected to acid production test from the ability of isolates to oxidized ethanol to acetic acid. The results of high acid production were showed in Figure 2. The acid yields of all isolates were increased from 1 to 7 days continuously except LC-13 and *A. aceti* which decrease at last day.

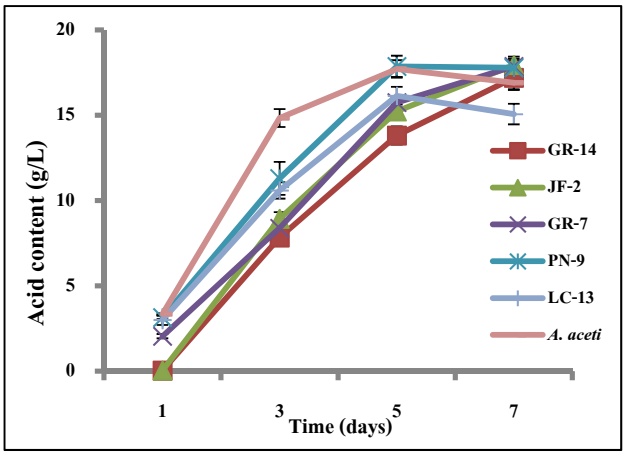

Figure 2 Acid production by acetic acid bacteria isolates

Five isolates include GR-7, GR-14, JF-2, PN-9, LC-13 include *A. aceti* TISTR 354 were produced 16.14 to 17.95 g/L in 7 days. They were then tested for acetic acid and ethanol tolerance.

The isolates were inoculated in potato medium supplement with various concentrations of ethanol and acetic acid. The growth of acetic acid bacteria was decreased when the concentration of acetic acid and ethanol increased.

All of five isolates grown in the seed medium contain with 4 to 12% ethanol (v/v) whereas PN-9 was able to grow at 4 to 8% ethanol (v/v). Therefore, the isolates GR-7, GR-14, JF-2, LC-13 were selected for growth in potato medium supplement with 1 to 6 % acetic acid (v/v) compared with and *A. aceti* TISTR 354. All isolates were grown at 1 to 2 % acetic acid (v/v) however LC-13 and *A. aceti* TISTR 354 were able to grow in 3% acetic acid (v/v).

Isolates GR-7, GR-14, JF-2 and LC-13 were selected for further fermented glutinous rice vinegar production.

Discussion and Conclusions

Acetic acid bacteria are general found in natural but they are not dominance species. Thus, the enrichment culture technique is suitable for isolation of acetic acid bacteria from environment. (Yamada et al., 2000; Lisdiyanti et al., 2002; jojima et al., 2004; Moryadee and Pathom-Aree., 2008). The enrichment broth contain distilled water supplement with 4% ethanol (v/v) was suitable to isolate acetic acid bacteria from fruits due to its simply prepare and low cost (Sudsakda et al., 2007).

The numbers of acetic acid bacteria were found to be high in grape, lychee and pineapple respectively. As previously study report that the acetic acid bacteria have been isolated from fruits, coconut juice, plam juice, and sugarcane juice in Thailand. Therefore, fruits were a good sources of acetic acid bacteria (Seearunruangchai et al., 2004).

The biochemical characteristic of isolated strains were identified according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Classification of genus *Acetobacter* and *Gluconobacter* were based on the ability to oxidize acetate which the genus *Acetobacter* are able to oxidize acetate to CO_2 and H_2O (Kadere et al., 2008).

From the results of acid production, five isolates showed high acid yields. Isolate JF-2, GR-7, PN-9 and GR-14 were produced acetic acid 17.95, 17.86, 17.78 and 17.20 g/L respectively while *A. aceti* TISTR 354 produced 16.90 g/L. The results related to Moryadee and Wasu (2008) report that isolates No. 37 produced 13.53 g/L of acetic acid at 30°C in 7 day of fermentation. While, Nanda et al. (2001) have studied about characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar, it was found that isolate TN-1 strains was produced 4.4% acetate in 3 days.

The various concentration of ethanol and acetic acid test, found that isolates LC-13 and *A. aceti* TISTR 354 could able to grown in 16% ethanol and 3% acetic acid (v/v). The result was very impressed since they showed high ability of alcohol tolerance than reports of Sharafi et al (2010) which could grow only at 11% ethanol. LC-13 is such a mean isolate isolates that will use to produce a high quality of vinegar from glutinous rice syrup.

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