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Isolation of Thermotolerant Acetic Acid Bacteria from Fruits for Vinegar Production

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Abstract: Sixty thermotolerant acetic acid bacteria were isolated from 13 kinds of fruit using sterile distilled water supplemented with 4% ethanol (v/v) as an enrichment medium. Successful isolations were obtained from apple, Jamaican cherry, longan, mango, pineapple and rambutan. Morphological and biochemical examinations revealed that 43 isolates were members of the genus *Acetobacter* whereas the remaining 13 isolates were members of the genus *Gluconobacter*. Preliminary screening showed that isolates No. 13, 34, 36 and 37 gave the widest zone of acidity on overoxidation medium. These isolates were identified as *A. aceti* and selected for acetic acid production at 30 and 37°C by shaking culture for 14 days in ethanol-yeast extract medium. It was found that *A. aceti* isolate No. 37 from rambutan gave the highest acetic acid yield of 13.53 and 8.97 g L^{-1} at 30 and 37°C, respectively after 7 days of fermentation.

Key words: Acetic acid bacteria, thermotolerant, vinegar production, enrichment technique, *Acetobacter*, *Gluconobacter*

INTRODUCTION

Acetic acid bacteria are large group of obligate aerobic Gram negative bacteria with the ability to oxidize ethanol to acetic acid (Matsushita *et al*., 1994). They are widely distributed in natural habitats and classified in the family *Acetobacteraceae* (Holt *et al*., 1994). Members of this family are useful in industrial production of vinegar (Adachi *et al*., 2003). The increasing temperature in the recent years poses a serious challenge to fermentation industries since a large cooling system is required for maintaining the optimum temperature. The production of vinegar by thermotolerant acetic acid bacteria attracts many interests due to their economic profits (Saeki *et al*., 1997a). Development in isolation, identification and characterization of this group of bacteria were in progress for oxidative fermentation (Saeki *et al*., 1997b; Adachi *et al*., 2003). Thailand as a tropical country which has long been known for her richness in biodiversity including microbial resources is a great place in search for useful microorganisms especially thermotolerant strains. It is the purpose of this study to isolate thermotolerant acetic acid bacteria from fruits and investigated their potential to produce vinegar.

MATERIALS AND METHODS

Samples

The fruit samples (Table 1) were purchased from local market within and around Chiang Mai University main campus.

Selective Isolation Procedure for Thermotolerant Acetic Acid Bacteria

Acetic acid bacteria were isolated from each fruit sample by enrichment culture technique using sterile distilled water containing 4% ethanol (v/v) as an enrichment broth (Sudsakda *et al*., 2007).

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Approximately 5 g of fruits were added to the medium and incubated at 37°C for 3-5 days to select thermotolerant strains (Saeki *et al*., 1997a). Acetic acid bacteria were isolated by streaking the content of an enrichment broth onto potato agar plate supplemented with 0.003% bromocresol purple and 4% ethanol (v/v). The colonies producing yellow halo were selected and presumptively identified as acetic acid bacteria. All selected colonies were restreaked onto fresh new plate to obtain pure culture and subjected to detailed taxonomic characterization.

Screening of Thermotolerant Acetic Acid Bacteria for Acid Production

All isolates were inoculated onto bromocresol green ethanol agar plate (ethanol 0.2%, yeast extract 0.3%, bromocresol green 0.01%) and incubated at 30°C for 24 h. Acid production was indicated by yellow zone appearance on agar plate. Four isolates that gave the widest yellow zone were selected for acid production.

Acetic Acid Production at 30 and 37°C

Selected isolates of acetic acid bacteria were grown in an ethanol-yeast extract medium (ethanol 2%, yeast extract 0.5%, pH 6.8) at either 30 or 37°C for 2 days. A 10% of these prepared cultures (OD_{660} = 0.475-0.529) was used to inoculate 120 mL of an ethanol-yeast extract medium in 250 mL Erlenmeyer flask. Acetic acid bacteria were grown at 30 and 37°C for 2 weeks on a rotary shaker at 200 rpm. Acetic acid contents were determined by titration with 0.1 N NaOH against phenolphthalein as an indicator.

Taxonomic Characterization

Morphological, physiological and biochemical characteristics of pure isolates were examined according to the ninth edition of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

RESULTS AND DISCUSSION

Acetic acid bacteria were successfully isolated from 6 fruit samples using enrichment culture technique. This culturing technique had proved to be an effective way for isolation of acetic acid bacteria from nature as exemplified in recent literatures (Yamada *et al*., 2000; Lisdiyanti *et al*., 2002; Jojima *et al*., 2004). A total of 60 presumptive acetic acid bacteria were isolated (Table 1). The highest number of isolate was from longan followed by rambutan. Mango seems to be a poor source of acetic acid bacteria as only 4 isolates were obtained. Almost all isolates (95%) were able to produce acid as they formed yellow zone around their colonies on ethanol agar plates.

The ability of acetic acid bacteria to oxidize acetate to CO_2 and H_2O was used to distinguish between members of the genus *Acetobacter* and *Gluconobacter* (Holt *et al*., 1994). *Acetobacter* species are able to oxidize acetate this leads to the change of medium colour from purple to yellow and then to purple again. Most isolates (72%) were identified as members of the genus *Acetobacter* (Table 1). Four isolates namely isolates 13, 34, 36 and 37 which demonstrated the most pronounced yellow zone were selected for acid production at 30 and 37°C. Morphological and biochemical determinations indicated that these 4 isolates belong to *A. aceti* subspecies *aceti* from the criteria in Bergey's Manual of Determinative Bacteriology (Table 2).

Table 1: Acetic acid bacteria isolated from fruit samples

Source	No. of isolates	*Acetobacter* sp.	*Gluconobacter* sp.	Vinegar production
Longan	18	14	4	18
Rambutan	14	10	4	14
Pineapple	11	10	1	10
Apple	7	6	1	7
Jamaican cherry	6	3	3	4
Mango	4	2	2	4

Table 2: Biochemical properties of selected acetic acid bacteria

	Isolate No.				
Biochemical tests	R	13	34	36	37
Growth on ethanol	+	+	+	+	+
Acid production from glucose	+	+	+	+	+
Dihydroxyacetone from glycerol	+	+	+	+	+
Cellulose production	-	-	-	-	-
Production of brown soluble pigment	-	-	-	-	-
Catalase	+	+	+	+	+

R = *Acetobacter aceti* subspecies *aceti*, +: Present; -: Absent

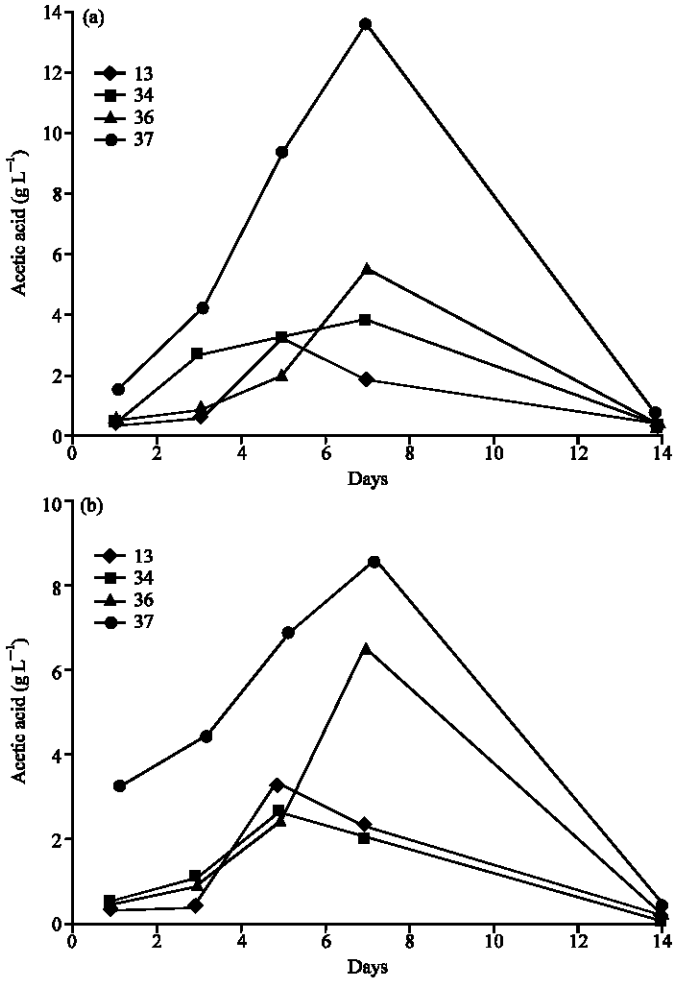

Fig. 1: Time course of acid production by 4 selected isolates of acetic acid bacteria. Acetic acid bacteria were grown on 120 mL of ethanol yeast extract medium in 250 mL Erlenmeyer flask at 30°C (a) and 37°C (b) for 2 weeks on a rotary shaker at 200 rpm. Acetic acid contents were determined by titration with 0.1 N NaOH against phenolphthalein. The data are average based on two trials

It is evident from Fig. 1 that all selected isolates produced maximum acid after 5-7 day of fermentation. The highest yield was obtained from isolate No. 37, a value corresponds to 13.53 and 8.97 g L^{-1} at 30 and 37°C, respectively. There was also no delayed in acetic acid production at 37°C

for these thermotolerant strains. Though, a considerable reduction in yield was observed. *A. aceti* IFO 3283, a well known mesophilic vinegar producer was reported to be hardly grown at 37°C and ethanol oxidation was greatly delayed (Adachi *et al*., 2003). Although it is difficult to compare data for thermotelerant strains directly with mesophilic strains, the usefulness of thermotolerant strains for vinegar production is obvious. This present study also showed that acetic acid bacteria are associated with various kinds of fruit including those not normally use in isolation scheme e.g., longan or rambutan which supported the view that fruits are good sources of acetic acid bacteria.

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