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Full Paper

Isolation of acetic acid bacteria from honey

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Abstract: Four thermotolerant acetic acid bacteria designated as CMU1, CMU2, CMU3 and CMU4 were isolated from six honey samples produced by three native bee species in northern Thailand, namely the dwarf honey bee (*Apis florea*), Asian honey bee (*A. cerena*) and giant honey bee (*A. dorsata*). All isolates were tested for their tolerance to acetic acid and ethanol at 30°C and 37°C. It was found that they grew only in a medium containing 1% (v/v) acetic acid at 30°C. However, isolate CMU4 showed the highest toleration to ethanol, viz. 10% (v/v) and 9% (v/v) at 30°C and 37°C respectively. Morphological and biochemical examination indicated that all isolates were members of the genus *Gluconobacter*.

Keywords: acetic acid bacteria, honey, enrichment culture technique, *Gluconobacter*

Introduction

Honey is a nectar and sweet deposit from plants which is gathered, modified and stored in the honeycomb by honey bees [1]. There are five major bee species used in honey production industry in the northern part of Thailand. Four native species are the dwarf honey bee (*Apis florea*), small dwarf honey bee (*Apis adreniformis*), Asian honey bee (*A. cerena*) and giant honey bee (*A. dorsata*). The remaining European honey bee (*A. mellifera*) is the only introduced species [2].

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Acetic acid bacteria are a large group of obligate aerobic Gram-negative bacteria which are commonly found in association with various kinds of sugary material. They are divided into four groups, namely *Acetobacter*, *Acidomonas*, *Frateuria* and *Gluconobacter*, these being slightly different in some physiological characteristics [3]. The two most studied acetic acid bacteria are *Acetobacter* and *Gluconobacter* due to their economical importance. Acetic acid bacteria are found in association with honey and honey bees [4-6]. However, to date, apparently no studies attempting to isolate these bacteria from Thai honey have been reported. In this study, the selective enrichment procedure for the isolation of acetic acid bacteria of particular thermotolerant strains from honey was carried out.

Materials and Methods

Selective isolation of acetic acid bacteria by enrichment culture technique

A total of six honey samples from dwarf honey bees (*Apis florea*), Asian honey bees (*A. cerena*) and giant honey bees (*A. dorsata*) were collected from local honey farms in San Kamphang district, Chiang Mai, Thailand. Approximately 1 ml of each sample was added to the enrichment broth (20 ml) containing 0.5% glucose, 2% glycerol, 1% yeast extract, 1% peptone, 1.5% potato extract, 4% ethanol and 1% Acti-dione (an antifungal). The mixture was incubated at 37 ^{o}C for up to one week. After 1, 3 and 5 days of incubation, one loop-full of enrichment broth was streaked onto a potato agar plate (containing 0.5% glucose, 2% glycerol, 1% yeast extract, 1% peptone, 1.5% potato extract, 4% ethanol (v/v) and 0.003% bromcresol purple). All colonies showing a yellow halo zone were collected for further study. The change of pH in the enrichment broth was also observed after 1, 3, 5 and 7 days of incubation.

Phenotypic characterisation of isolates

Morphological, physiological and biochemical characteristics of all pure isolates were examined according to Bergey's Manual of Determinative Bacteriology [3]. The ability of the isolate to oxidise acetate to CO_2 and H_2O was used to distinguish between members of the genera *Acetobacter* and *Gluconobacter*.

Acid and alcohol tolerance of isolates

All isolates were tested for their ability to grow in seed culture agar supplemented with 1-4 % (v/v) acetic acid and that containing 5-15% (v/v) ethanol at 30 and 37 ^{o}C. Inoculum was prepared by growing each isolate on seed culture medium for 48 h and then transferred to the test plates. All plates were incubated for up to 5 days. The growth of each isolate was compared between the two temperatures.

Results and Discussion

Four isolates of acetic acid bacteria (Table 1) were successfully recovered from honey samples using enrichment technique. Two isolates, CMU1 and CMU2 were isolated from honey of *A. florae* whereas isolate CMU3 and CMU4 were from honey of *A. cerena* and *A. dorsata* respectively. The

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successful isolation was due to the efficacy of enrichment culture to promote the growth of acetic acid bacteria present in the samples. It is evident from Figure 1 that the numbers of acetic acid bacteria in the culture broth increased with time during the enrichment period. As a result, the pH values were decreased accordingly (Figure 1). Many reports have also addressed the usefulness of the enrichment culture technique in selective isolation of targeted microorganisms [7-10].

Table 1. Morphological characterisation of acetic acid bacterial isolates

Isolate	Source of honey	Days of enrichment	Gram stain and morphology
CMU1	*Apis florae*	3-7	Gram-negative, short rod
CMU2	*Apis florae*	3-7	Gram-variable, short rod
CMU3	*Apis cerena*	7	Gram-variable, short rod
CMU4	*Apis dorsata*	7	Gram-variable, short rod

As shown in Table 1, Gram staining revealed that the isolates were either Gram-negative or Gram-variable, short rod bacteria, which is a typical character of acetic acid bacteria. All of them were identified as *Gluconobacter* sp. due to their inability to oxidise acetate (Table 2). All isolates recorded the same phenotypic profile among themselves, thus suggesting that they belonged to the same taxa. Most acetic acid bacteria are known to be mesophilic with an optimum temperature for growth around 30°C. A slight increase in temperature results in a dramatic decrease in growth of these organisms [11,12]. However, all isolates obtained in this study grew well at 37°C, the character which suggested that these isolates may be thermotolerant strains. No members of the genus *Acetobacter* were obtained from any of the samples tested.

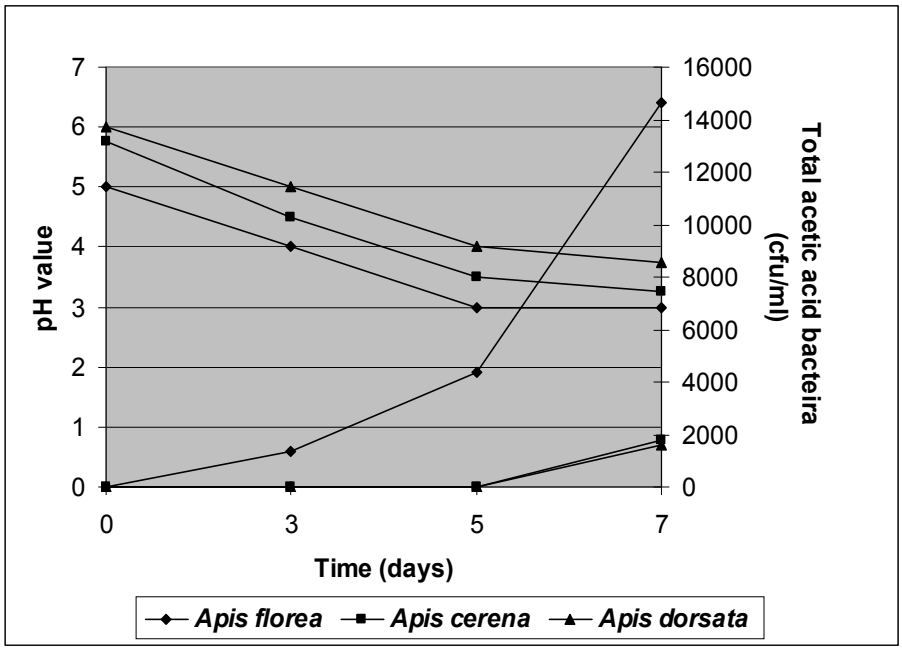

Figure 1. Change in numbers of acetic acid bacteria in culture broth and pH value during enrichment period

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Table 2. Biochemical characterisation of acetic acid bacterial isolates

Biochemical test	CMU1	CMU2	CMU3	CMU4
Overoxidation	-	-	-	-
Catalase	+	+	+	+
Growth in ethanol	+	+	+	+
Production of gluconate	+	+	+	+
Ketogenesis from glycerol	-	-	-	-
Cellulose production	-	-	-	-
Soluble pigment	-	-	-	-

Note: + = positive; - = negative

The four isolates were tested for their ability to grow in broths supplemented with acetic acid and alcohol at 30 and 37°C. It was found that all isolates could grow in 1% (v/v) acetic acid only at 30°C. However, these isolates showed remarkable tolerance to ethanol, particularly isolate CMU4, which was able to grow well in a medium containing as high as 10% ethanol (v/v) at 30°C (Table 3). In general, the alcohol tolerance decreased with higher temperature (Tables 3, 4). It seems that there may be some relationship between thermotolerance, acetic acid resistance and ethanol resistance characteristic in this group of bacteria. This suggestion is supported by the observation that thermotolerant growth in the presence of acetic acid and high ethanol concentration is an outstanding characteristic of acetic acid bacteria from Thailand [11]. However, little has so far been reported about the mechanism of either acetic acid or ethanol resistance. Further investigation has to be done to test this hypothesis.

Table 3. Alcohol tolerance of acetic acid bacterial isolates at 30°C

Isolate	Percentage of alcohol (v/v)				
	6	7	8	9	10
CMU1	++++	++++	++++	+++	-
CMU2	++++	++++	+++	-	-
CMU3	++++	++++	++++	+++	-
CMU4	++++	++++	++++	++++	+++

Note : ++++ = very good growth; +++ = good growth; - = no growth

Surprisingly, in this study, the use of enrichment medium with ethanol which usually favours the development of *Acetobacter* yielded only *Gluconobacter*. This is probably due to the nature of honey with very high concentration of sugar, which allows only the growth of osmotolerant or osmophilic microorganisms including member of the genus *Gluconobacter*. This result is in agreement

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Table 4. Alcohol tolerance of acetic acid bacterial isolates at 37°C

Isolate	Percentage of alcohol (v/v)				
	6	7	8	9	10
CMU1	++++	++++	+++	-	-
CMU2	++++	+++	-	-	-
CMU3	++++	++++	+++	-	-
CMU4	++++	++++	++++	+++	-

Note : ++++ = very good growth; +++ = good growth; - = no growth

with earlier reports by Ruiz-Argueso and Rodriguez-Navarro [4,5] who examined the microorganisms of ripening honey from an apiary in Madrid, Spain. They found that *Lactobacillus viridescens* and *Gluconobacter* were two main groups of bacteria present in honey. These authors concluded that the microbial flora of honey appears to be the result of chance as honey bees come into contact with several microorganisms during their visit to various niches. Though *Gluconobacter* was found in association with the honey bees, it is not known whether the presence of the acetic acid bacteria has any significance for the bees or any hive products including honey [6].

Conclusions

The first isolation of acetic acid bacteria from honey in Thailand was reported in the present study, the results of which have also provided evidence that the enrichment culture technique is useful in promoting the growth of acetic acid bacteria. These bacteria present in honey also show high tolerance to ethanol, which suggests their usefulness in fermentation industry.

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