Identification and Characterization of Thermotolerant *Acetobacter* Strains Isolated from Brewing Coconut Vinegar in Sri Lanka

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Abstract

Coconut vinegar made from coconut water or coconut sap is extensively used in many Sri Lankan dishes. As its production is done by natural fermentation, the responsible acetic acid bacteria for Sri Lankan vinegar fermentation is not known. From pellicle formed on the top of the brewing vinegar, three *Acetobacter* strains (SL13E-2, SL13E-3, and SL13E-4) which could grow at temperatures up to 42°C were isolated. All three strains could tolerate up to 8% and 3% ethanol concentration at 41°C and 42°C, respectively. These strains were identified as *Acetobacter pasteurianus* by phenotypic characterization and 16S rDNA sequence analysis. The 16S-23S internal transcribed spacer (ITS) sequence data of two strains (SL13E-2 and SL13E-3) were identical, while the other (SL13E-4) was 6 bp different from them. Acetic acid fermentation of three strains gave their highest production of 4% acetic acid at 37°C with 6% initial ethanol. They gave 2.5% acetic acid production even at 40°C with 4% initial ethanol. Compared with the other strains, SL13E-4 shows slower growth and acetic acid production rate. These strains were successfully used in simultaneous vinegar production with coconut water at higher temperatures.

Key words.. Thermotolerant, Characterization, *Acetobacter*, Coconut Vinegar

Introduction

Vinegar made from fermented coconut water or sap of coconut palm inflorescence is used extensively as a preservative and flavoring agent in pickles and many dishes in Sri Lanka. Like other kinds of vinegar, coconut vinegar is produced by two-stage fermentation process where firstly fermentable sugars are converted into ethanol by yeasts and secondly oxidation of ethanol into acetic acid by acetic acid bacteria (AAB).

AAB are an important group of bacteria with high ability to oxidize carbon sources rapidly and incompletely, and thus widely used in commercial production of vinegar, D-gluconate and L-sorbose. Recently, they have been classified into ten genera belonging to the family Acetobacteraceae. *Acetobacter* and *Gluconacetobacter* are found as main microbes among them in vinegar fermentation due to their strong ability to oxidize ethanol and to tolerate high acetic acid concentration accumulated in the medium. They produce acetic acid from ethanol by two sequential oxidation reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), which are localized on the outer surface of the cytoplasmic membrane (Adachi et al. 2003).

Microbiology and biochemistry of natural fermentation of coconut palm sap was published by Athputharajah (1986). However, the responsible AAB in Sri Lankan vinegar production is not known. So the aim of this study is to isolate, identify and characterize AAB from Sri Lankan vinegar.

Materials and Methods

Samples were collected from the pellicle formed on the top of the brewing vinegar in fermentation tanks of a coconut water vinegar factory in Panadura, Sri Lanka in 2010, where vinegar production is done by natural fermentation. AAB were isolated by streaking a loopful of sample on YPG (0.5 g yeast extract, 0.5 g polypeptone, 1.0 g glycerol, 0.5g $CaCO_3$, and 1.5g agar 3mg bromocresol purple per 100ml) agar plate with 4% ethanol.

Growth of the isolated *Acetobacter* strains was examined on potato agar plates at 30, 37 and 40°C for 5 days. Acetic acid and ethanol tolerance of the identified strains were examined in YPGD agar plates supplemented with either 1-4% acetic acid or 1-10% ethanol, at 30, 37 and 40°C for 5 days. Acetic acid production of those strains were examined in YPGD

medium with 4 and 6% ethanol at 30, 37 and 38°C under static and shaking (200 rpm) culture conditions. Simultaneous coconut vinegar production was examined statically at 37, 39 and 40°C by inoculating powder of commercially obtained dry yeast and isolated strains together or sequentially with using coconut water where Brix value was adjusted to 10% by adding glucose. Growth characters were compared with those of thermotolerant *A. pasteurianus* SKU1108 strain which was isolated from Thailand previously (Saeki et al. 1997).

Phenotypic characterization of the isolated strains were examined according to the *Bergy's Manual of Systematic Bacteriology* (Sievers and Swings, 2005). Discriminating of *Acetobacter* strains were done by abilities of their carbon source utilization (Sokollek et al. 1998). Genotypic characterization was done by 16S rDNA sequencing (Hiraishi, 1992) and 16S-23S rRNA gene intergenic spacer region PCR amplification (Tanasupawat et al. 2011). Acidity of the culture medium was measured by titration with 0.8N NaOH using phenolphthalein as a pH indicator.

Results and Discussion

Using YPG medium with ethanol, three AAB (SL13E-2, SL13E-3 and SL13E-4) strains with different colony sizes, were isolated from pellicle formed in the fermentor tank. They were all Gram-negative, rod-shaped and catalase positive. The three isolates were assigned to the genus *Acetobacter*, since acetate was oxidized to CO_2 and H_2O (Brenner et al. 2005). Physiological and biochemical characters of the three *Acetobacter* strains are summarized in Table 1.

According to the 16S rDNA sequence data, all three strains are having more than 99% identity with those of several *A. pasteurianus* strains. Thus, all three strains were identified as *A. pasteurianus*. Furthermore, nucleotide sequences (784 bp) of 16S-23S ITS sequence data of SL13E-2 and SL13E-3 were identical, while the ITS sequence of SL13E-4 was 6 bp different from others.

Acetic acid production by three isolated strains was examined at 30, 37, 38 and 40°C in YPGD medium with 4

Table 1: Differential characteristics of *Acetobacter* strains isolated from Sri Lankan coconut vinegar

Character	SL13E-2	SL13E-3	SL13E-4	*A. pasteurianus*[1,2]
Production of acetic acid from ethanol/$CaCO_3$ agar	+	+	+	+
Oxidation of acetate to carbonate	+	+	+	+
Growth on potato agar plates at;				
37°C	+	+	+	+
40°C	+	+	+	+
42°C	+	+	+	+
Formation of dihydroxyacetone from glycerol	-	-	-	-
Acid production from				
D-glucose	+	+	+	+
D-fructose	+	+	+	+
D-galactose	+	+	+	+
D-mannose	+	+	+	+
D-xylose	+	+	+	+
Sucrose	w	w	w	w
Melibiose	w	+	+	+
L-rhamnose	-	-	-	-
meso-erythritol	-	-	-	-
Dulcitol	-	-	-	-
L-arabinose	+	+	+	+
Utilization of C source				
D-glucose	(+)	(+)	(+)	(+)
D-mannitol	(+)	(+)	(+)	(++)
Glycerol	(+++)	(+++)	(+++)	(+++)
Ethanol	-	-	-	-
Methanol	-	-	-	-

+, positive; w, weakly positive; -, negative; (+++), excellent growth; (++), moderate growth; (+), poor growth:
[1.] Tanasupawat et al., 2011: [2.] Sokolleck et al., 1998.

or 6% ethanol in either shaking or static culture. With 4% initial ethanol concentration, all three strains showed good growth up to 40°C, although SL13E-2, SL13E-3 and SL13E-4 showed different lag phase, 2, 3 and 6 days respectively, at 40°C. When the initial ethanol concentration was 6%, at 38°C and 40°C, all three isolated strains showed a very poor growth. SL13E-4 showed slower growth and acetic acid production compared to the other strains either in static or shaking culture with 4 or 6% ethanol. At 37°C, all three strains grew and oxidized ethanol more rapidly than at 30°C either with 4 or 6% initial ethanol. When the acetic acid production was compared, all three strains gave their highest acetic acid production at 37°C, with 6% initial ethanol concentration either in shaking or static culture, where the production was more than 4% and is higher than that of SKU1108.

At 38°C, with 4% ethanol, as well as SKU1108 strain, all three isolates produced more than 3% acetic acid, although they showed longer lag phase (about 5 days) than that of SKU1108 (2 days). Even at 40°C, SL13E-2 and SL13E-3 gave 3% acetic acid production while SL13E-4 gave only 2.5% acetic acid production. However, in the medium containing 6% ethanol at 38C and 40C, all three strains didn't show any acetic acid production, while SKU1108 strain gave more than 2% and 3.5 % acetic acid production in shaking and static culture, respectively.

Further, their simultaneous vinegar production from coconut water with baker`s yeast was examined. At 37°C, all three isolated strains gave 4% acetic acid production as well as SKU1108 did. Although they showed acetate oxidation in YPGD medium with 4% ethanol, such decrease of acidity did not occur in simultaneous coconut vinegar production. Both SL13E-2 and SL13E-3 gave about 3% acetic acid production at 39°C and 2.5% at 40°C, a little better than the results of SKU1108. When mixed culture of the three isolates was used, similar acid production was observed to those of SL13E-2 or SL13E-3.

Three thermotolerant AAB strains which could grow and produce acetic acid at temperatures higher than 37°C were isolated from Sri Lankan coconut vinegar. These strains were identified as *A. pasteurianus* by phenotypic and biochemical analyses and 16S rDNA gene sequencing. As well as thermotolerant SKU1108 strain, the isolated strains were successfully used in coconut water vinegar production at higher temperatures. Thus, these strains are useful for vinegar fermentation at higher temperatures, which may reduce the cooling water expenses.

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