



## L-Sorbose Production by Acidotolerant Acetic Acid Bacteria Isolated from Thailand Sources

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Received: 30 June 2006

Accepted: 30 September 2007.

### ABSTRACT

One hundred and twenty four isolates of acidotolerant acetic acid bacteria, isolated from various kinds of fruits and flowers collected in Chiang Mai, Thailand by an enrichment-culture approach were screened for their ability to produce rare ketose sugars. It was found that only 40 isolates (~32%) could produce L-sorbose from sorbitol as detected by cysteine-carbazole reaction. This was then confirmed based on the thin layer chromatography technique. However, when analysed by HPLC, only twenty-two isolates were capable of producing L-sorbose. Of these, the strain CMU 48-1 produced highest accumulation of L-sorbose (0.629%), and was identified as *Gluconacetobacter liquefaciens* based on 16S rRNA gene sequence analysis. This is the first report of biotransformation of L-sorbose from sorbitol by *Gluconacetobacter liquefaciens*.

**Keywords:** *Gluconacetobacter liquefaciens*, acetic acid bacteria, rare sugar, ketose, L-sorbose, acidotolerant.

### 1. INTRODUCTION

Acetic acid bacteria (AAB) are typically classified as Gram-negative, ellipsoidal to rod-shaped and strictly aerobic bacteria. They are widespread in nature and can usually be found in sugary, acid and/or ethanol-containing habitats such as fruits, flowers, fermented foods (i.e., *nata*), and several alcoholic

beverages. The AAB are important in food and chemical industry. However, they are also well-known as food spoilage agents.

The AAB, known as the oxidative bacteria, are able to oxidise a wide range of sugars, sugar alcohols, and sugar acids. This biotransformation is very useful for

production of rare monosaccharides which are important in medicine and food industry [1]. For example, *Acetobacter aceti* IFO3281 can be used to prepare D-xylulose from D-arabitol [2]; L-ribulose, L-ribose and L-arabinose from ribitol [3]. The bioconversion of D-sorbitol to L-sorbose is carried out industrially by *Gluconobacter suboxydans* ATCC621 [4,5]. L- Psicose is produced from allitol using *Gluconobacter frateurii* IFO3254 [6]. Production of tagatose, a ketohexose C4-fructose epimer, from D-galactitol by *Gluconobacter oxydans* DSM2343 has also been reported [7].

L-sorbose is of great importance as the starting material for industrial production of L-ascorbic acid through, the so-called "Reichstein method". Besides, application of L-sorbose as a precursor to synthesise glycosidase inhibitor, 1-deoxygalactonojirimycin, has been reported [8]. Currently, selective oxidation of D-sorbitol to L-sorbose is performed by *Gluconobacter oxidans* on an industrial scale [6]. In this paper, several AAB isolated from flowers and fruits collected in Chiang Mai, Thailand were characterised for their ability to produce rare ketoses, L-sorbose.

## 2. MATERIALS AND METHODS

### 2.1 Acetic Acid Bacteria Reference Strains

The AAB used in this study as reference strains for identification were as follows: *Acetobacter aceti* BCC12455 (IFO14818<sup>T</sup>), *Gluconobacter oxydans* BCC12337 (IFO14819<sup>T</sup>), *Gluconobacter frateurii* BCC12341, *Gluconobacter cerinus* BCC12339, *Gluconobacter asaii* BCC12302, *Gluconobacter henssenii* BCC12272, *Gluconacetobacter liquefaciens* BCC12274 (IFO12388<sup>T</sup>), *Asaia bogorensis* BCC12264 (IFO16594<sup>T</sup>) and *Asaia siamensis* BCC12268 (IFO16457<sup>T</sup>). These microorganisms were obtained from BIOTEC culture collection (BCC), Pathumthani, Thailand.

### 2.2 Isolation of Acetic Acid Bacteria

AAB were preliminary screened and isolated from flowers and fruits collected in Chiang Mai by using four different enrichment culture broths: Glucose-ethanol-acetic acid medium (5% glucose, 0.5% ethanol, 0.3% peptone, 0.3% yeast extract and 0.3% (v/v) acetic acid); Glucose-ethanol medium (1.5% glucose, 0.5% ethanol, 0.3% peptone and 0.3% yeast extract); Sorbitol medium (2% sorbitol, 0.3% peptone and 0.3% yeast extract); Sucrose-acetic acid medium (2% sucrose, 0.3% peptone, 0.3% yeast extract and 0.3% (v/v) acetic acid). Prior to sterilisation, the pH of these media were adjusted to 3.5 with HCl. All ripening fruits and fresh flowers parts: petal, anther or ovaries, were sliced into small pieces (1 x 1 cm<sup>2</sup>) and were then added into those media to enrich the bacterial growth at room temperature for 4 days. Zero point two ml supernatant of enrichment culture was spread on agar media. Morphologically distinct bacterial colonies appeared on the plates were transferred to the new media and subsequently prepared as pure cultures of isolated acetic acid bacterial strains. The isolated bacteria were restreaked on the glucose-ethanol-calcium carbonate agar plates (2% glucose, 0.5% ethanol, 0.3% peptone, 0.3% yeast extract, 0.7% calcium carbonate and 1.2% agar). The acetic acid bacteria were preliminary identified and selected based on the presence of clear zones around colonies on the agar plates. All bacteria strain isolates were cultured at 30°C on the glucose-ethanol-calcium carbonate agar slants and maintained at 4°C until further use.

### 2.3 Characterisation and Identification of Acetic Acid Bacteria

The AAB isolated were then characterised based on their morphology, physiology and biochemistry compared with the reference strains as previously described [9-11]. Growth

in the presence of 0.35% acetic acid (v/v) was also examined at pH 3.5 [12]. Oxidation of acetate and lactate to CO<sub>2</sub> and H<sub>2</sub>O were examined by Leifson's method [9]. Oxidation/Fermentation test was done by the use of Hugh and Leifson's medium that contained 1% glucose [13]. Dihydroxyacetone formation was detected by using Fehling's solution. Acid productions from different carbon sources were tested as described by Asai et al. [9].

#### 2.4 Determination of L-sorbose Production

To determine L-sorbose production, the AAB (OD 0.2) were grown aerobically with continuous agitation (70 rpm) for 24-48 h at room temperature in the basal medium (PYS: 0.5% polypeptone, 0.5% yeast extract, 0.5% NaCl) containing 1% D-sorbitol. After incubation for 48 h at room temperature, the supernatant (OD 0.8) was collected and used to measure the ketose production (i.e., L-sorbose) based on the cysteine-carbazole reaction [14].

The thin layer chromatography (TLC) technique was subsequently used to confirm the sorbose production. The chromatographic developments were carried out using the TLC plates of silica gel 60 F254 (20 × 20 cm<sup>2</sup> glass plates) in a rectangular chamber (10 × 24 × 24 cm<sup>3</sup>). The solvent system employed was phenol-water (4:1). The supernatant samples and standard ketose sugars (D-fructose and L-sorbose) were spotted (5 μl) on the TLC plate; the spotted TLC plate was then placed into the chamber. The development of the chromatogram was then allowed to proceed and the TLC plate was finally sprayed with indicator solution composed of 3% *p*-anisidine in butanol to reveal the ketose sugars.

To accurately determine the concentrations of L-sorbose, further analysis was undertaken from selected strains grown on

the PYS medium with 1% or 3% D-sorbitol (inoculum at OD 0.8, incubation for 48 h at room temperature) using HPLC (Nihonbunko HPLC 880 PU liquid chromatography, Shimadzu RID-6A refractive index detector, Shimadzu LC-6A pump, Shimadzu CTO-6A oven, Shimadzu SCL-6B controller and Shimadzu CR-6A Chromatopac) using a Phenomenex Rezex RCM monosaccharide HPLC column on a Shimadzu model RID-6A liquid chromatograph. The separations were achieved at 80°C using deionised water at a flow rate of 0.5 ml min<sup>-1</sup>.

#### 2.5. Identification of Acetic Acid Bacteria Using 16S rRNA Gene Sequence Analysis

Genomic DNA was isolated from the acetic acid bacteria using the modified method of Marmur [15]. DNA fragment specific for the 16S rDNA was amplified by PCR as described previously [12,16]. Two primers, 20F (5'-GAGTTTGATCCTGGCTCAG-3'; position 9-27) and 1500R (5'-GTTACCTGTTACGACTT-3'; position 1509-1492), were used. The numbers of position in the rDNA gene fragments were based on the *Escherichia coli* numbering system (accession number V00348) [17]. The condition used for thermal cycling was as follows: denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min. The amplified 16S rRNA fragment was then separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (QIAGEN, Germany).

Amplified 16S rDNA were directly sequenced using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit on an ABI PRISM model 310 Genetic Analyser. The following six primers were used: 20F, 1500R, 520F (5'-CAGCAGCCGCGG

TAATAC-3'; positions 519-536), 520R (5'-GTATTACCGCGGCTGCTG-3'; positions 536-519), 920F (5'-AAACTCAAATGAATTGACGG-3'; positions 907-926) and 920R (5'-CCGTCAATTCATTTGAGTTT-3'; positions 926-907).

Multiple alignments of the sequences obtained in comparison with those of microbial reference strains [18] were then carried out with the program CLUSTAL W 1.74 [19]. The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura [20]. The neighbour-joining methods were used for constructing a phylogenetic tree [21]. The robustness for individual branches was estimated by bootstrapping with 1,000 replicates [22].

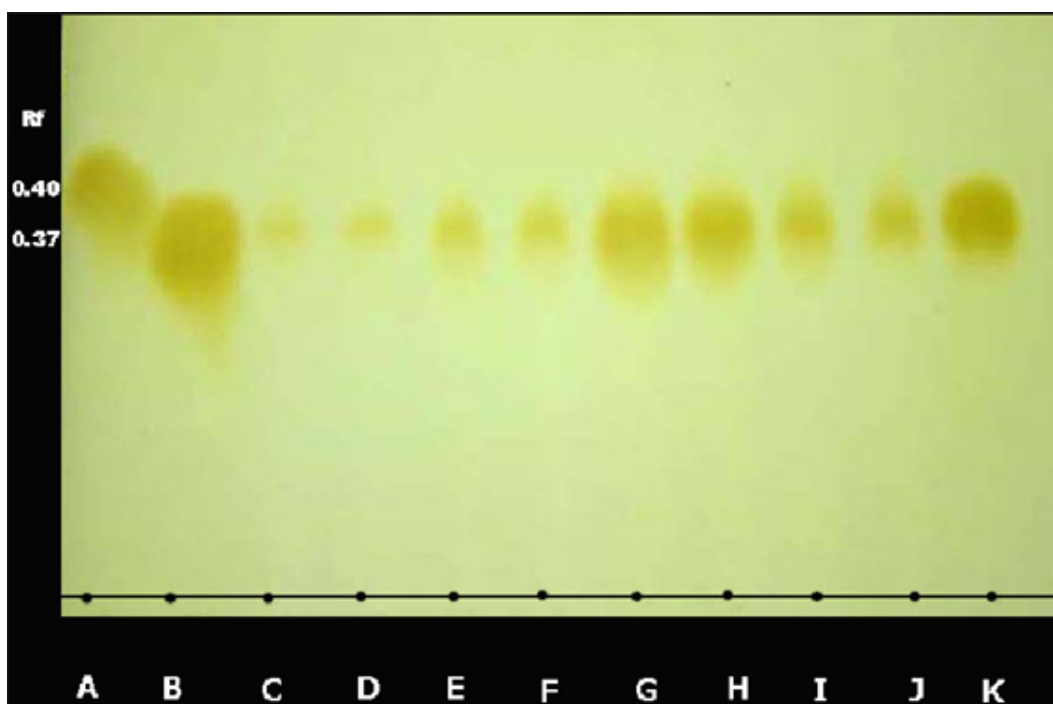
### 3. RESULTS AND DISCUSSION

In the past years, information relevant to the AAB diversity is scarce because of most studies were emphasised on similar sample sources. However, it is now well recognised that the AAB group is so diversified than one ever thought. Currently, nine Genera of the AAB are documented namely *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, and *Neosasaia* [23]; these are expected to increase due to detailed analyses on their taxonomy. Many new AAB species have been isolated from unique sources such as tropical flowers and fruits [23, 24].

In this present work, various kinds of fruits and flowers collected locally in Chiang Mai, Thailand, were used as sources for screening the AAB and a total of 124 of the AAB strains were obtained. The isolation procedure was performed with four different enrichment media in which the results obtained were heterogenous in terms of types and numbers of the AAB (data not shown). This further confirms the fastidious

characteristics of this particular microbial group. On this basis, it therefore suggests that the AAB isolates could only be a part of the whole bacterial population. All AAB isolated were Gram-negative, rod-shaped aerobes and grew well at pH 3.5. They were motile and produced a water-soluble brown pigment when cultured in the glucose-ethanol medium supplement with calcium carbonate. Although most colonies were white, some produced distinct colour pigments such as pink. All isolates were able to produce acid from D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose, D-mannitol, glycerol and ethanol but not from D-fructose, L-sorbose, D-arabinose, D-sorbitol, sucrose and maltose [25].

These AAB strains were then tested for their ability to produce L-sorbose. Initial screening was undertaken based on the cysteine-carbazole method. This assay is rapid and simple in determining the presence of ketoses as observed by the purplish colour development. The sugar quantity can also be estimated based on the standard graph of a series of known concentration of the ketoses tested. It was found that 40 isolates (~32%) were capable of synthesising L-sorbose by this assay. To confirm the results, the TLC technique was introduced. As shown in Figure 1, the existence of L-sorbose could be observed in which the  $R_f$  values of D-fructose and L-sorbose were 0.40 and 0.37, respectively. These supernatants were then subjected to further analysis by HPLC. Our results showed that different AAB strains produced different amounts of L-sorbose. It is interesting to note that, only twenty-two AAB isolates could produce L-sorbose based on HPLC analysis while forty isolates showed positive results when assayed by the cysteine-carbazole method. This emphasises the limited use of the cysteine-carbazole assay, which was incapable of distinguishing fructose from



**Figure 1.** TLC analysis of ketose sugars. A) D-fructose, B) and K) L-sorbose, C-J) the supernatants from the AAB broth culture (5  $\mu$ l) of the following strains: CMU To3-2, CMU To3-1, CMU 38G2, CMU R4-1, CMU 48-1, CMU 48-2, CMU Ma-2, and CMU R1-1, respectively.

sorbose [5]. Of these AAB tested, five strains namely CMU 48-1, CMU R1-1, CMU 48-2, CMU Ma-2 and CMU R4-1 exhibited high yield ( $\geq 0.45$  % (v/v) L-sorbose) of L-sorbose (Table 1) and thus selected for further study.

The five AAB strains (CMU 48-1, CMU R1-1, CMU 48-2, CMU Ma-2 and CMU R4-1) that produced high L-sorbose were selected for transformation reaction of D-sorbitol. After 24 h incubation each strain in PYS medium containing 1% or 3% D-sorbitol, it was found that conversion of 1% and 3% D-sorbitol to L-sorbose were highest for strain CMU 48-1 with 47.49% and 67.66% in concentration, respectively (Table 2). The HPLC analysis also confirmed that the main product was L-sorbose.

To reveal the taxonomic status of these AAB isolates, 16S rDNA sequence analysis

was carried out. Initially, their genomic DNA was extracted and the 16S rRNA genes were then amplified using the universal primers [12, 16]. The 16S rRNA gene sequence data obtained were then aligned with those of the AAB type strains available from the GenBank database. The phylogenetic tree as illustrated in Figure 2 was also constructed from evolutionary distance by using the neighbour-joining method [21]. Based on this analysis, the five AAB strains were located in the lineage of the genus *Gluconacetobacter* and were found to be most identical to *G. liquefaciens*. It should be noted, however that only the 16S rRNA sequences of the AAB strains CMU 48-1 and CMU Ma-2 were submitted to the nucleotide database and can be accessed as follows: CMU 48-1 (DQ486985) and CMU Ma-2 (DQ486986).

**Table 1.** Amounts of L-sorbose produced by 5 selected AAB isolates obtained from various plants. The analysis was performed using HPLC.

AAB isolates	Host plants	% (v/v) L-sorbose
CMU 48-1	Pineapple ( <i>Ananas comosus</i> )	0.629
CMU R1-1	Pineapple ( <i>Ananas comosus</i> )	0.597
CMU 48-2	Mango ( <i>Mangifera indica</i> )	0.537
CMU Ma-2	Rose apple ( <i>Eugenia jambos</i> )	0.502
CMU R4-1	Rose apple ( <i>Eugenia jambos</i> )	0.483

**Table 2.** Amounts of L-sorbose production from 5 selected AAB isolates cultured in basal medium containing 1% and 3% D-sorbitol. The analysis was performed using HPLC.

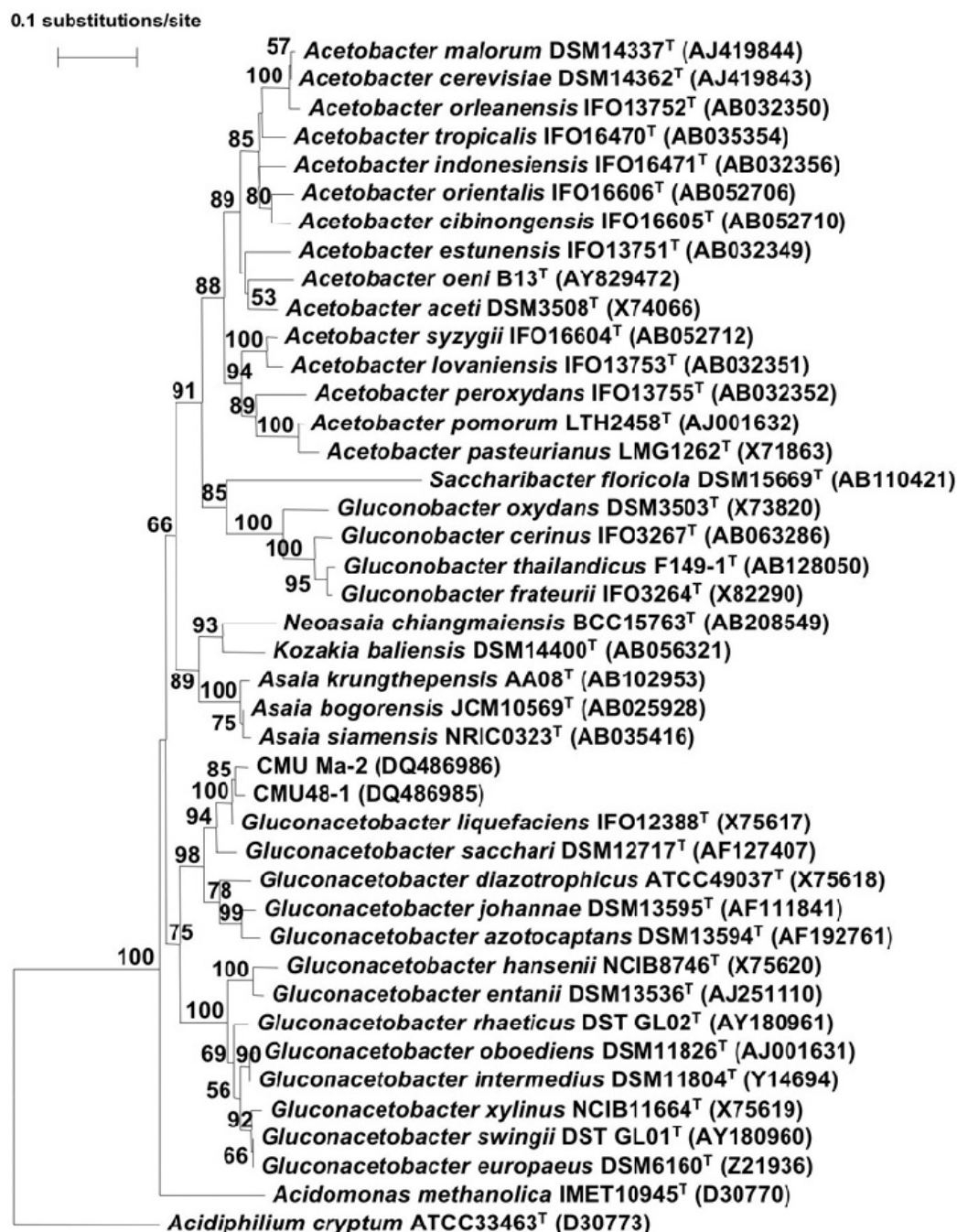
AAB isolates	% L-sorbose production in different substrate	
	1% D-sorbitol	3% D-sorbitol
CMU 48-1	47.49	67.66
CMU R1-1	31.50	65.84
CMU 48-2	42.19	65.48
CMU Ma-2	34.63	56.54
CMU R4-1	38.44	34.92

L-sorbose, a key starting material in the industrial manufacture of vitamin C (ascorbic acid), can be produced by bacterial oxidation of D-sorbitol by *Acetobacter* sp. [26] and *Gluconobacter* sp. [5]. Srivastava and Lasrado [27] cultivated *Acetobacter suboxydans* in fed-batch fermentation by feeding nutrients containing sorbitol exponentially and obtained a productivity of 12.6  $\text{gl}^{-1}\text{h}^{-1}$ . Girdhar and Srivastava [28] also cultivated *Acetobacter suboxydans* NRRL B72 in fed-batch fermentation using an initial sorbitol concentration of 200  $\text{gl}^{-1}$  and demonstrated a productivity of 17.7  $\text{gl}^{-1}\text{h}^{-1}$ . The present work cultivated *Gluconacetobacter liquefaciens* strain CMU 48-1 in batch fermentation containing 10  $\text{gl}^{-1}$  and

30  $\text{gl}^{-1}$  sorbitol yielded productivity of 94  $\text{mg}\text{gl}^{-1}$  and 134  $\text{mg}\text{gl}^{-1}$ , which are lower compared to other bacteria that reported by earlier workers. This present study, however, is the first report of the bioconversion of D-sorbitol to L-sorbose by *Gluconacetobacter* sp. Further study relevant to the metabolic mechanism may offer an alternative to classical L-sorbose synthesis.

#### ACKNOWLEDGEMENTS

The authors wish to thank the BIOTEC Culture Collection, Thailand for supplying reference AAB strains. We also would like to acknowledge the Graduate School of Chiang Mai University for financial support.



**Figure 2.** Phylogenetic relationship based on the 16S rDNA sequence of the AAB strains CMU Ma-2 and CMU 48-1 in comparison with other AAB type strains. Numerals indicate bootstrap values derived from 1,000 replications.

S. Lumyong, P. Lumyong, V. Sahachaisaree and K. Izumori thanks JSPS-NRCT and ISRS Rare sugar project for partial support of this research.

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