
# Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (2): 194-197, 2007
ISSN 1816-4935


# Identification of Acidotolerant Acetic Acid Bacteria Isolated from Thailand Sources

[1]Watcharee Hanmoungjai, [2]Ekachai Chukeatirote, [1]Wasu Pathom-aree,
[3]Yuzo Yamada and [1]Saisamorn Lumyoung
[1]Department of Biology, Faculty of Science, Chiang Mai University,
Chiang Mai 50200, Thailand
[2]School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand
[3]BIOTEC Culture Collection (BCC), BIOTEC Central Research Unit,
National Center for Genetic Engineering and Biotechnology,
National Science and Technology Development Agency, Pathumthani 12120, Thailand

**Abstract:** In this study, various kinds of fruits and flowers obtained locally in Chiang Mai, Thailand were used as sources for acetic acid bacteria isolation. Using five enrichment media (with pH adjustment to 3.5), a total of one hundred and twenty-four strains were isolated. They were all Gram-negative, rod-shaped and produced clear zones on basal agar plates supplement with calcium carbonate. Of 124 strains, seventy-two (~58%) were derived from fruit samples. These bacterial strains were then characterised based on their cell morphology and biochemical assay; they can be classified in five Genera: *Acetobacter* (~14%), *Acidomonas* (~13%), *Asaia* (~31%), *Gluconacetobacter* (~17%) and *Gluconobacter* (25%).

**Key words:** Acetic acid bacteria, Thailand sources, diversity

## INTRODUCTION

Acetic Acid Bacteria (AAB) are Gram-negative, ellipsoidal to rod-shaped cells. They are strictly aerobic bacteria and commonly found in natural sources. Apart from acetic acid, one of the major products of AAB metabolism, the AAB are able to oxidise different kinds of alcohols and sugars into industrially useful metabolites (i.e., rare sugars). Another distinct feature of the AAB is the capability to produce extracellular cellulose. However, the AAB can also cause food spoilage.

The AAB is placed into the family Acetobacteraceae as a branch of the α-Proteobacteria (De Ley *et al*., 1984; Sievers *et al*., 1994). Recently, the taxonomy of the AAB has been changed and rearranged dramatically. In the past, the study of the AAB taxonomy has been focused on isolates from sources obtained in temperate regions (i.e., Europe and North America). Since the discovery of novel species in the Genera *Acetobacter* and *Gluconobacter* from Indonesian sources (Yamada *et al*., 1999; Lisdiyanti *et al*., 2001), the natural samples of tropical regions have gained an interest as major sources for AAB isolation. The diversity of the AAB group is also now well recognized; they are currently categorized into nine Genera: *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter* and *Neoasaia* (Gonzalez *et al*., 2005; Yukphan *et al*., 2005). Most of these new Genera and species (i.e., *Asaia*, *Kozakia*, *Saccharibacter*, etc.) have only been described from 2000 by studying the AAB isolated from fruits and flowers of the tropical regions. In this study, an attempt was made to isolate the AAB from fruits and flowers collected locally in Chiang Mai, Thailand. It is expected to shed light on the AAB diversity from Thailand sources.

**Corresponding Author:** Watcharee Hanmoungjai, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

## MATERIALS AND METHODS

### Isolation of Acetic Acid Bacteria from Thailand Sources

Several kinds of fruits and flowers were collected locally in Chiang Mai, Thailand. For screening, each sample was cut into small pieces and placed into the test tubes containing enrichment media with pH adjustment to 3.5. In this experiment, five different formula of enrichment media were used: i) glucose-ethanol-acetic acid medium (GAM: 1.5% glucose, 0.5% ethanol, 0.3% peptone, 0.3% yeast extract and 0.3% acetic acid); ii) glucose-ethanol medium (GEM: 1.5% glucose, 0.5% ethanol, 0.3% peptone and 0.3% yeast extract); iii) sorbitol medium (SM: 2% sorbitol, 0.3% peptone and 0.3% yeast extract); iv) sucrose-acetic acid medium (SAM: 2% sucrose, 0.3% peptone, 0.3% yeast extract and 0.3% acetic acid); and v) methanol medium (MM: 0.8% methanol, 0.3% peptone and 0.3% yeast extract). The test tubes were then statically incubated at 30°C for at least 5 days. The samples were subsequently transferred by streaking onto the same enrichment media supplement with 0.7% calcium carbonate. The colonies showing acid production yielding a clear zone were selected and restrict until the pure culture was obtained. For routinely use, the pure cultures were maintained on the GEM agar slants containing 0.7% calcium carbonate and kept at 4°C until use. The bacterial glycerol stock was also prepared and stored at -80°C.

### Acetic Acid Bacteria Reference Strains

The AAB used in this study as reference strains were as follows: *Acetobacter aceti* BCC12455 (IFO14818$^T$), *Gluconobacter oxydans* BCC12337 (IFO14819$^T$), *Gluconobacter frateurii* BCC12341, *Gluconobacter cerinus* BCC12339, *Gluconobacter asaii* BCC12302, *Gluconobacter hensenii* BCC12272, *Gluconacetobacter liquefaciens* BCC12274 (IFO 12388$^T$), *Asaia bogorensis* BCC12264 (IFO16594$^T$) and *Asaia siamensis* BCC12268 (IFO16457$^T$). These microorganisms were obtained from BIOTEC culture collection (BCC), Pathumthani, Thailand.

### Morphological, Physiological and Biochemical Properties

The bacteria were grown on a basal medium (1% glucose, 1% glycerol, 1% ethanol, 1% peptone, 0.5% yeast extract and 1.5% agar) at 30°C for 18-24 h. Microscopic observation was observed after staining by Hucker's modification method (Bartholomew, 1973). Motility was detected by the hanging-drop method. The observation of pigment production was also made. For this, the bacteria were cultured in glucose-calcium carbonate agar slants (3% glucose, 0.2% yeast extract, 0.3% peptone and 0.2% calcium carbonate). The cultures were then incubated for 10 days at 30°C.

To investigate the effect of pH on growth, the bacterial isolates were cultured in GEM broth with pH adjustment to 3, 3.5 and 4, incubated for 7 days at 30°C. For temperature, the isolates were inoculated in GEM broth (pH 3.5) and incubated at 30 and 37°C for 7 days. The ability of these bacteria to grow on glutamate agar was also carried out as described by Iizuka and Komagata (1963). The bacteria were also cultured on mannitol agar (2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 2% agar, pH 6.0), incubated at 30°C for 10 days and observed their growth.

To test the acetate and lactate oxidation, the modified Leifson's method was used (Asai *et al*., 1964). The bacteria were inoculated in the medium containing 0.3% peptone, 0.2% yeast extract, 0.2% sodium acetate (or sodium lactate). Bromthymol blue (0.002%) was used as pH indicator. The cultures were incubated by shaking at 30°C for 24 h. The oxidation/fermentation test was also analysed using Hugh and Leifson's medium containing 1% glucose.

To observe acetic acid formation from ethanol, the dissolution of calcium carbonate around the bacterial colonies in agar plates was used. The agar plates contained 2% absolute ethanol, 1% yeast extract, 2% calcium carbonate and 2% agar. The bacterial capability to produce acid from different carbon sources was also investigated (Asai *et al*., 1964). The medium used contained 0.5% yeast extract

and 1% sugar (including D-xylose, D-galactose, D-sorbitol, D-fructose, D-sorbose, D-mannitol, D-arabinose, D-glucose, D-mannose, sucrose, maltose, melibiose, glycerol and ethanol). Bromocresol purple (0.002%) was used as pH indicator. The cultures were incubated for 7 days at 30°C.

## RESULTS AND DISCUSSION

A total of 124 isolates of acid-tolerant acetic acid bacteria could be isolated from flowers and fruits collected locally in Chiang Mai, Thailand. In this study, five different enrichment media were used; 17 strains were isolated by GAM, 31 by GEM, 39 by SM, 21 by SAMand 16 by MM. It was showed that not all the media were able to support the AAB growth because the numbers of the bacterial isolates were varied indicating the fastidious characteristic of the AAB (Sievers *et al*., 1992). As a result, it is quite reasonable to consider that the isolated strains are only a part of the whole AAB consortia. It should also be noted that, of 124 isolates, 72 (~58%) strains were derived from fruits. All isolates were Gram-negative, rod-shaped and produced clear zone when cultured on GEM supplement with calcium carbonate. They grew aerobically. Most of them were motile and could oxidized acetate and lactate to $CO_2$ and $H_2O$. They also showed good growth in the presence of 0.35% acetic acid at pH 3.5.

The phenotypic identification of the AAB, especially at the species level, is quite difficult. This is due to various reasons including high frequency of spontaneous mutations (Takemura *et al*., 1991; Prust *et al*., 2005). However, taxonomic study of the AAB at the genus level remains practical based on cell morphology and biochemical tests. Although these tests can be laborious and time-consuming if there are a number of bacterial isolates, they still offer a simple and inexpensive means. All AAB isolates were subject to a series of biochemical tests as described in Materials and Methods. The results obtained were then used to compare with the key identification reported by Urakami *et al*. (1989) as well as those obtained from the AAB reference strains to reveal the genus of the AAB isolates. Based on this analysis, 31 isolates belonged to *Gluconobacter* sp., 39 as *Asaia* sp., 21 as *Gluconacetobacter* sp., 17 as *Acetobacter* sp. and 16 as *Acidomonas* sp.

This present study describes the diversity of AAB from Thailand sources. It is expected to shed light on diversity and distribution of the AAB in Thailand environment. It is interesting to note that the Genera *Gluconobacter* and *Asaia* seemed to be widely distributed. Further analysis with molecular methods would be an important tool for detailed study of the AAB taxonomy.

## ACKNOWLEDGMENTS

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

## REFERENCES

Asai, T., H. Iizuka and K. Komagata, 1964. The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. J. Gen. Applied Microbiol., 10: 95-126.

Bartholomew, J.W., 1973. Method for Microbiology. In: Staining Procedures. 3rd Edn., G. Clark (Ed.), Williams and Wilkins, Baltimore, pp: 245-351.

De Ley, J., M. Gillis and J. Swings, 1984. Family VI Acetobacteraceae. In: Bergey's Manual of Systematic Bacteriology. Vol. 1. Krieg, N.R. and J.G. Holt (Eds.), Williams and Wilkins, Baltimore, pp: 267-278.

Gonzalez, A., J.M. Guillamon, A. Mas and M. Poblet, 2005. Application of molecular methods for routine identification of acetic acid bacteria. Intl. J. Food Microbiol., 108: 141-146.

Iizuka, H. and K. Komagata, 1963. An attempt at grouping of the genus *Pseudomonas*. J. Gen. Applied Microbiol., 9: 73-82.

Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura and K. Komagata, 2001. Identification of *Acetobacter* strains isolated from Indonesian sourcesand proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov. and *Acetobacter orientalis* sp. nov. J. Gen. Applied Microbiol., 47: 119-131.

Prust, C., M. Hoffmeister, H. Liesegang, A. Wiezer, W.F. Fricke, A. Ehrenreich, G. Gottschalk and U. Deppenmeier, 2005. Complete genome sequence of the acetic acid bacterium *G. oxydans*. Nat. Biotechnol., 23: 195-200.

Sievers, M., S. Sellmer and M. Teuber, 1992. *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in central Europe. Syst. Applied Microbiol., 15: 386-392.

Sievers, M., W. Ludwig and M. Teuber, 1994. Phylogenetic positioning of *Acetobacter*, *Gluconobacter*, *Rodophilia* and *Acidiphilum* species as a branch of acidophilic bacteria in the α-subclass of Proteobacteria based on 16S ribosomal DNA sequences. Syst. Applied Microbiol., 17: 189-196.

Takemura, H., S. Horinouchi and T. Beppu, 1991. Novel insertion sequence IS*1380* from *Acetobacter pasteurianus* is involved in loss of ethanol-oxidizing ability. J. Bacteriol., 173: 7070-7076.

Urakami, T., J. Tamaoka, K.I. Suzuki and K. Komagata, 1989. *Acidomonas* gen. nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov. Intl. J. Syst. Bacteriol., 39: 50-55.

Yamada, Y., Y. Okada and K. Kondo, 1976. Isolation and characterisation of polarly flagellated intermediate strains in acetic acid bacteria. J. Gen. Applied Microbiol., 22: 237-245.

Yamada, Y., R. Hosono, P. Lisdiyanti, Y. Widyastuti, S. Saono, T. Uchimura and K. Komagata, 1999. Identification of acetic acid bacteria isolated from Indonesian sources, especially of isolates classified in the genus *Gluconobacter*. J. Gen. Applied Microbiol., 45: 23-28.

Yukphan, P., T. Malimas, W. Potacharoen, S. Tanasupawat, M. Tanticharoen and Y. Yamada, 2005. Neoasaia chiangmaiensis gen. nov., sp. nov., a novel osmotolerant acetic acid bacterium in the α-Proteobacteria. J. Gen. Applied Microbiol., 51: 301-311.