

Diversity of Acetic Acid Bacteria in Indonesia, Thailand, and the Philippines

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A total of 331 strains of acetic acid bacteria were isolated from Indonesian, Thai, and Philippine sources using five enrichment media at pH 3.5. All isolates were subjected to the study of the diversity of acetic acid bacteria in the light of country origins, enrichment media used, and isolation sources. *Acetobacter*, *Gluconacetobacter*, and *Kozakia* strains were enriched in a medium containing glucose-acetic acid-ethanol but *Asaia* and *Frateuria* strains were inhibited by acetic acid. Furthermore, *Acetobacter* strains were mainly isolated from fermented foods, *Gluconobacter* strains from fruits and flowers, *Gluconacetobacter* strains from fermented foods, and most of *Asaia* strains from flowers. *Kozakia baliensis* strains were isolated from ragi (starter for fermented foods) and palm brown sugar in Indonesia, and *Frateuria aurantia* strains were isolated from fruits and flowers in Indonesia. No *Acidomonas* strains were isolated from the sources used in this study. This study showed the diversity of acetic acid bacteria in Indonesia, Thailand, and the Philippines.

Key words: acetic acid bacteria, microbial diversity

INTRODUCTION

Until recently, the genera *Acetobacter* and *Gluconobacter* were the core genera of the acetic acid bacteria (15). A major change in the classification of the acetic acid bacteria was the introduction of the genus *Gluconacetobacter* by Yamada et al. (18, 19) based on partial sequences of the 16S rRNA gene and ubiquinone systems. Since 1994, we have been studying a large number of acetic acid bacteria isolated from traditional fermented foods, fruits, and flowers collected in tropical countries; Indonesia, Thailand, and the Philippines. Investigation of new niches led to the introduction of two new genera *Asaia* (21) and *Kozakia* (11). In addition,

several new species and new combinations have been described since 1998 (2, 3, 4, 5, 7, 9, 10, 13, 14, 16).

Currently, seven genera and 33 species of acetic acid bacteria are recognized. *Acetobacter* (14 species), *Acidomonas* (1 species), *Asaia* (2 species), *Gluconobacter* (3 species), *Gluconacetobacter* (11 species), and *Kozakia* (1 species) are located in the α -*Proteobacteria* lineage; whereas *Frateuria* (1 species) is located in the γ -*Proteobacteria* lineage. Although the genus *Frateuria* is located in a lineage different from acetic acid bacteria, this genus is biochemically similar to acetic acid bacteria with the following characteristics: They are able to grow at pH 3.5, oxidize ethanol to acetic acid, and oxidize D-glucose to D-gluconate, 2-keto-D-gluconate and 2,5-diketo-D-gluconate. Therefore, the genus *Frateuria* was includ-

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Table 1. Enrichment media used in this study

Composition	Enrichment media at pH 3.5				
	I	II	III	IV	V
D-Glucose	1.0 *	–	–	–	0.15
D-Sorbitol	–	2.0	–	–	–
D-Mannitol	–	–	2.0	–	–
Dulcitol	–	–	–	2.0	–
Methanol	–	–	–	–	2.0
Ethanol	0.5	–	–	–	–
Peptone	1.5	0.5	0.5	0.5	0.5
Yeast extract	0.8	0.3	0.3	0.3	0.3
Acetic acid	0.3	–	0.2	–	–
Cycloheximide	0.01	0.01	0.01	0.01	0.01

* The concentration of ingredients is expressed in (w/v) % or (v/v) %.

ed in the category of acetic acid bacteria from the viewpoint of microbial ecology in this study.

This paper deals with the diversity of acetic acid bacteria isolated from fermented foods, fruits, flowers, etc. in Indonesia, Thailand, and the Philippines and discusses the diversity of this group of bacteria in the light of country origins, enrichment media used, and isolation sources.

MATERIALS AND METHODS

Strains used in this study

Three hundred and thirty-one acetic acid bacteria were used in this study. One hundred and thirty-five strains were previously reported (7, 8, 9, 10, 11, 20, 21), while another 196 strains were newly isolated.

Isolation and cultivation of acetic acid bacteria

Isolation of acetic acid bacteria was conducted from 1994 to 1999 in Denpasar, Yogyakarta, and Bogor in Indonesia, Bangkok in Thailand, and Los Banos in the Philippines. Habitats of acetic acid bacteria are known to be sugary and alcohol-containing materials. Therefore, the sources used for the isolation were targeted for fermented foods, fruits, flowers, and other materials such as ragi (starter for fermented foods), palm brown sugar, beverages, etc. Approximately 400 samples were used for the isolation of acetic acid bacteria.

On-site isolation was performed and five enrichment media were employed for the isolation. A sample was incubated on each of the enrichment media at pH 3.5. When microbial growth occurred in the enrichment

media, a simple dilution method was used for isolation. One loopful of a sample was diluted in 1 ml saline and the loop dilution-pour plate technique was used for the isolation of acetic acid bacteria by using the isolation agar medium as described by Yamada et al. (20). The isolation medium contained 2.0 % D-glucose, 0.8 % yeast extract, 0.5 % peptone, 0.5 % ethanol, 0.3 % CaCO₃, and 1.5 % agar. After incubation at 30 °C for 3 days, aerobic colonies, which produced clear zones in the isolation agar medium, were picked up. Isolates were maintained on agar slants of AG medium as described by Katsura et al. (7).

Enrichment media for isolation

The composition of enrichment media used is shown in Table 1. All media contained peptone, yeast extract, and cycloheximide. In addition, Enrichment medium I (EM I) contained glucose, ethanol, and acetic acid, EM II contained D-sorbitol, EM III contained D-mannitol and acetic acid, EM IV contained dulcitol, and EM V contained a small amount of D-glucose and methanol. The pH of the media was adjusted to 3.5 with HCl before autoclaving at 121 °C for 15 min.

Identification of isolates

Polyphasic taxonomy, which assesses the phenotypic characteristics, chemotaxonomic characteristics, and phylogenetic data, was used for the identification of the isolates at the species level. Methods used in this study were mostly those described by Lisdiyanti et al. (9).

Table 2. Diversity of acetic acid bacteria in the light of country origins

Genera	Species	The countries			Total
		Indonesia	Thailand	The Philippines	
<i>Acetobacter</i>	<i>A. pasteurianus</i>	9	11	21	41
	<i>A. orleanensis</i>	11	3	12	26
	<i>A. lovaniensis</i>	27	–	4	31
	<i>A. indonesiensis</i>	20	–	7	27
	<i>A. tropicalis</i>	9	6	–	15
	<i>A. syzygii</i>	4	–	2	6
	<i>A. cibinongensis</i>	2	–	–	2
	<i>A. orientalis</i>	9	–	–	9
		91	20	46	157
<i>Gluconobacter</i>	<i>G. oxydans</i>	17	4	–	21
	<i>G. frateurii</i>	35	8	3	46
		52	12	3	67
<i>Gluconacetobacter</i>	<i>Ga. xylinus</i>	8	–	3	11
	<i>Ga. hansenii</i>	–	–	6	6
	<i>Gluconacetobacter</i> sp.*	–	–	3	3
		8	0	12	20
<i>Asaia</i>	<i>As. bogorensis</i>	25	17	9	51
	<i>As. siamensis</i>	9	2	2	13
	<i>Asaia</i> sp.	3	–	–	3
		37	19	11	67
<i>Kozakia</i>	<i>K. baliensis</i>	4	–	–	4
		4	0	0	4
<i>Frateuria</i>	<i>F. aurantia</i>	16	–	–	16
		16	0	0	16
Total		208	51	72	331

Abbreviations: A., *Acetobacter*; G., *Gluconobacter*; Ga., *Gluconacetobacter*; As., *Asaia*; K., *Kozakia*; F., *Frateuria*.

* *Ga. hansenii* homology group III (12).

RESULTS AND DISCUSSION

Acetic acid bacteria used for this study

As mentioned above, a total of 331 isolates were used for the study of the diversity of acetic acid bacteria in Indonesia, Thailand, and the Philippines. The isolates were all Gram-negative, aerobic, rod-shaped, catalase-positive, oxidase-negative, produced clear zones on basal agar plates containing CaCO₃, and grew on the AG medium at pH 3.5. At the genus level, 157 of the 331 isolates were identified as *Acetobacter* because they oxidized acetate and lactate and had Q-9 as the major ubiquinone. Of the 157 isolates, 91 had already been reported (9, 10). Of the 331 isolates, 67 were identified as *Gluconobacter*

because they did not oxidize acetate and lactate and had Q-10 as the major ubiquinone. Of the 67 *Gluconobacter* strains, 11 isolates had been reported previously (20). Of the 331 isolates, 20 were identified as *Gluconacetobacter* because they oxidized acetate and lactate and had Q-10 as the major ubiquinone. Of the 331 isolates, 67 were identified as *Asaia* because they did not grow in the presence of 0.35 % acetic acid, did not oxidize acetate and lactate, and had Q-10 as the major ubiquinone. Of the 67 *Asaia* strains, 13 had been reported previously (7, 21). Four strains were identified as *Kozakia* because they oxidized acetate and lactate, had Q-10 as the major ubiquinone, and produced mucous substances from

Table 3. Diversity of acetic acid bacteria in the light of enrichment media used

Genera	Species	Enrichment Media					Total
		I	II	III	IV	V	
<i>Acetobacter</i>	<i>A. pasteurianus</i>	23	6	12	–	–	41
	<i>A. orleanensis</i>	21	1	4	–	–	26
	<i>A. lovaniensis</i>	16	7	7	–	1	31
	<i>A. indonesiensis</i>	15	8	4	–	–	27
	<i>A. tropicalis</i>	12	1	2	–	–	15
	<i>A. syzygii</i>	5	–	1	–	–	6
	<i>A. cibinongensis</i>	1	–	1	–	–	2
	<i>A. orientalis</i>	1	6	2	–	–	9
		94	29	33	0	1	157
<i>Gluconobacter</i>	<i>G. oxydans</i>	11	8	2	–	–	21
	<i>G. frateurii</i>	10	24	3	6	3	46
		21	32	5	6	3	67
<i>Gluconacetobacter</i>	<i>Ga. xylinus</i>	8	–	–	–	–	8
	<i>Ga. hansenii</i>	6	–	–	–	–	6
	<i>Gluconacetobacter</i> sp.*	6	–	–	–	–	6
		20	0	0	0	0	20
<i>Asaia</i>	<i>As. bogorensis</i>	–	32	–	17	2	51
	<i>As. siamensis</i>	–	5	–	6	2	13
	<i>Asaia</i> sp.	–	2	–	1	–	3
		0	39	0	24	4	67
<i>Kozakia</i>	<i>K. baliensis</i>	4	–	–	–	–	4
		4	0	0	0	0	4
<i>Frateuria</i>	<i>F. aurantia</i>	–	3	–	4	9	16
		0	3	0	4	9	16
TOTAL		139	103	38	34	17	331

Abbreviations: *A.*, *Acetobacter*; *G.*, *Gluconobacter*; *Ga.*, *Gluconacetobacter*; *As.*, *Asaia*; *K.*, *Kozakia*; *F.*, *Frateuria*.

* *Ga. hansenii* homology group III (12).

sucrose (11). Of the 331 isolates, 16 were identified as *Frateuria* because they did not grow in the presence of 0.35% acetic acid, oxidized lactate but not oxidize acetate, and had Q-8 as the major ubiquinone (8). No isolates were identified as *Acidomonas*.

Diversity of acetic acid bacteria in the light of country origins

Of the 331 isolates, 208 of acetic acid bacteria were isolated from Indonesian sources, 51 from Thai sources, and 72 from Philippine sources. Table 2 presents the distribution of acetic acid bacteria in Indonesia, Thailand, and the Philippines. In general, *Acetobacter* strains were widely distributed over the sources of the three countries.

Diversity of acetic acid bacteria in the light of enrichment media used

Of the 331 isolates of acetic acid bacteria, 139 were isolated by using EM I, 103 by EM II, 38 by EM III, 34 by EM IV, and 17 by EM V. Table 3 presents the diversity of acetic acid bacteria in the light of the enrichment media used.

EM I is recommended for the isolation of *Acetobacter* strains. The addition of acetic acid and ethanol is important for the growth of *Acetobacter* strains. Members of *Gluconacetobacter* and *Kozakia* were also enriched by using EM I. Furthermore, EM II containing D-sorbitol as a sole source of carbon is a suitable enrichment medium for *Gluconobacter* strains.

Table 4. Diversity of acetic acid bacteria in the light of isolation sources

Genera	Species	Sources				Total
		Fermented foods	Fruits	Flowers	Others	
<i>Acetobacter</i>	<i>A. pasteurianus</i>	41	–	–	–	41
	<i>A. orleanensis</i>	9	16	1	–	26
	<i>A. lovaniensis</i>	14	15	–	2	31
	<i>A. indonesiensis</i>	8	14	4	1	27
	<i>A. tropicalis</i>	7	7	–	1	15
	<i>A. syzygii</i>	2	2	2	–	6
	<i>A. cibirongensis</i>	–	1	–	1	2
	<i>A. orientalis</i>	2	5	1	1	9
		83	60	8	6	157
<i>Gluconobacter</i>	<i>G. oxydans</i>	2	8	9	2	21
	<i>G. frateurii</i>	12	16	14	4	46
		14	24	23	6	67
<i>Gluconacetobacter</i>	<i>Ga. xylinus</i>	8	–	–	–	8
	<i>Ga. hansenii</i>	5	1	–	–	6
	<i>Gluconacetobacter</i> sp.*	6	–	–	–	6
		19	1	0	0	20
<i>Asaia</i>	<i>As. bogorensis</i>	2	–	49	–	51
	<i>As. siamensis</i>	–	–	13	–	13
	<i>Asaia</i> sp.	–	–	3	–	3
		2	0	65	0	67
<i>Kozakia</i>	<i>K. baliensis</i>	–	–	–	4	4
		0	0	0	4	4
<i>Frateuria</i>	<i>F. aurantia</i>	–	13	3	–	16
		0	13	3	0	16
TOTAL		118	98	99	16	331

Abbreviations: A., *Acetobacter*; G., *Gluconobacter*; Ga., *Gluconacetobacter*; As., *Asaia*; K., *Kozakia*; F., *Frateuria*.

* *Ga. hansenii* homology group III (12)

EM II and IV containing D-sorbitol or dulcitol without acetic acid were suitable media for the isolation of *Asaia* strains because D-sorbitol and dulcitol were a good source of carbon for members of *Asaia*. In addition, *Asaia* and *Frateuria* strains were not enriched by using EM I and EM III because the growth of the strains of these genera was inhibited by acetic acid. This is characteristic of both genera because members of the genera did not grow in the presence of acetic acid. On the other hand, *Acetobacter* strains were not isolated by using EM IV, which contained dulcitol as a sole source of carbon. This is characteristic of *Acetobacter* because members of the genus did not assimilate dulcitol as a sole source of car-

bon.

No *Acidomonas* strains were isolated even by using EM V in which methanol was supplied. Further modification of the enrichment media for the genus *Acidomonas* is needed. The major habitat of this genus is sludge of wastewater treatment plants, which differed from those of other acetic acid bacteria (22).

Basically, EM I was used for the isolation of acetic acid bacteria (15, 17). In the present study, we modified EM I for enrichment not only of *Acetobacter* and *Gluconacetobacter* strains but also of other acetic acid bacteria. Modification of enrichment media is useful for the isolation of target microorganisms in nature.

Table 5. Habitats of acetic acid bacteria

Species	Fermented foods	Fruits	Flowers	Others
<i>A. pasteurianus</i>	palm vinegar, palm wine, pickle, water of nata, fermented rice tape cassava, rice wine	–	–	–
<i>A. orleanensis</i>	nata de coco, rice wine	guava, sapodilla, starfruit	flower	–
<i>A. lovaniensis</i>	nata de coco, moromi soya, palm wine, tape cassava, pickle	coconut, starfruit, markisa, mangosteen, mango, sapodilla, java grape	–	palm seed, coconut juice
<i>A. indonesiensis</i>	palm wine	banana, papaya, zirzak, mango, durian, coconut starfruit	hibiscus	palm seed
<i>A. tropicalis</i>	palm wine, rice wine	lime, orange, guava, coconut	–	coconut juice
<i>A. syzygii</i>	vinegar	malay rose apple, starfruit	flower	–
<i>A. cibinongensis</i>	–	montana, coconut	–	tofu
<i>A. orientalis</i>	tempe	coconut, starfruit	canna flower	tofu
<i>G. oxydans</i>	nata de coco	sapodilla, papaya, orange, soursop, mangosteen	flower of pea, hibiscus, chili flower, bougenvilla	coconut juice, ragi, palm brown sugar
<i>G. frateurii</i>	nata de coco, moromi soya	sapodilla	ixora, palm flower, african tulip	cincau
<i>Ga. xylinus</i>	nata de coco, vinegar	–	–	–
<i>Ga. hansenii</i>	nata de coco	pineapple	–	–
<i>Gluconacetobacter</i> sp.*	nata de coco	–	–	–
<i>As. bogorensis</i>	tape ketan	–	bauhinia, plumbago ixora, lantana, african tulip, coconut flower, alaman-da	–
<i>As. siamensis</i>	–	–	crown flower, spider lily, ixora, lantana, rose	–
<i>Asaia</i> sp.	–	–	jade vine, new guinea creeper	–
<i>K. baliensis</i>	–	–	–	ragi, palm brown sugar
<i>F. aurantia</i>	–	limus, menteng, cempedak, kemaris	coconut flower	–

Abbreviations: *A.*, *Acetobacter*; *G.*, *Gluconobacter*; *Ga.*, *Gluconacetobacter*; *As.*, *Asaia*; *K.*, *Kozakia*; *F.*, *Frateuria*.

* *Ga. hansenii* homology group III (12).

Diversity of acetic acid bacteria in the light of isolation sources

As the isolation sources of acetic acid bacteria, we focused on traditional fermented foods (nata, vinegar, pickles, etc.), tropical fruits (coconut, mango, guava, sapodilla, etc.), tropical flowers (ixora, lantana, etc.), and other sources such as ragi, tofu, palm sugar, etc. in Southeast Asia because in general, sugary and alcoholic materials are known to be the sources of acetic acid bacteria. The distribution of acetic acid bacteria in the present study revealed that 116 strains were isolated from fermented foods, 101 strains from fruits, 99 from flowers, and 15 from other sources. The diversity and distribution of acetic acid bacteria compiled by isolation source are shown in Tables 4 and 5.

The tables show that most of the *Acetobacter* strains were found in fermented foods such as palm vinegar, palm wine, nata de coco, and pickles as well as in fruits such as guava, mango, zirkak, etc. Interestingly, 41 strains of *Acetobacter pasteurianus* were isolated only from fermented foods such as palm vinegar, palm wine, pickles, water of nata de coco, fermented rice, tape cassava, and rice wine. No *A. pasteurianus* strains were found in tropical fruits and flowers. It is also noted that *Acetobacter aceti*, a typical acetic acid bacterium, was not found in the sources studied.

Most of the *Gluconobacter* strains inhabited fruits and flowers. This agreed with a report of Asai (1) in 1935 that indicated a wide distribution of *Gluconobacter* strains in fruits and flowers. *Gluconobacter cerinus* was not found in the sources studied. Currently, only five *G. cerinus* strains are preserved in culture collections worldwide, and they were isolated from Japanese sources (6).

Gluconacetobacter strains were mostly found in nata de coco. This genus is known to play an important role in the production of fermented foods such as nata de coco and vinegar.

A large number of *Asaia* strains were isolated from tropical flowers such as ixora and lantana collected in Indonesia, Thailand, and the Philippines. *Kozakia baliensis* strains were found in ragi and palm brown sugar so far. *Frateuria aurantia* strains have been found in fruits and flowers in Indonesia.

The above-mentioned data indicate the rich diversity of acetic acid bacteria in Indonesia, Thailand, and the

Philippines.

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インドネシア、タイおよびフィリピンの酢酸菌の多様性

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インドネシア、タイ、フィリピン試料より5種類の増菌培地 (pH3.5) を用いて331株の酢酸菌を分離し、全菌株を対象に分離国、増菌培地の種類、分離源の観点から酢酸菌の多様性を研究した。*Acetobacter*, *Gluconacetobacter* および *Kozakia* はグルコース・酢酸・エタノールを含む増菌培地でよく増菌されたが、*Asaia* および *Frateruria* は酢酸により生育が阻害された。*Acetobacter* は主として発酵食品より分離された。*Gluconobacter* は花や果実より分離され、*Gluconacetobacter* は発酵食品、ほとんどの *Asaia* は花から分離された。*Kozakia baliensis* はインドネシアのラギー (発酵食品のスタータ) および黒糖より分離され、*Frateruria aurantia* はインドネシアの果物および花より分離された。しかし、*Acidomonas* は全試料から分離されなかった。本研究によりインドネシア、タイおよびフィリピンに広く分布する酢酸菌の多様性が明らかになった。