

# Isolation of dihydroxyacetone-producing acetic acid bacteria in Vietnam

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## ABSTRACT

Sixty-six acetic acid bacteria (AAB) were isolated from forty-five flowers and fruits collected in Hochiminh City, Vietnam. Of the sixty-six, thirty-one isolates were selected as dihydroxyacetone (DHA)-producing AAB based on the reaction with Fehling's solution and grouped into three groups by routine identification with phenotypic features. Group I composed of fourteen isolates and was assigned to the genus *Acetobacter*, Group II composed of thirteen isolates and was assigned to the genus *Gluconobacter* and Group III was the remaining

four isolates and was assigned to the genus *Gluconacetobacter*. Ten isolates among the thirteen isolates of Group II gave a larger amount of DHA (22.2–26.0 mg/mL) than *Gluconobacter oxydans* NBRC 14819<sup>T</sup> (19.8 mg/mL), promising for the potential use in producing DHA. In phylogenetic analysis based on 16S rRNA gene sequences, six isolates of the ten potential DHA producers were suggested to be candidates for new taxa in the genus *Gluconobacter*.

**Key words:** acetic acid bacteria, dihydroxyacetone-producing, *Gluconobacter*

## INTRODUCTION

The production of dihydroxyacetone (DHA) is of interest in various applications in cosmetic, medicine, pharmaceuticals and food industries and in very cheap cost of glycerol, as the substrate for DHA production, due to the overproduction of this material by the biodiesel industry [10]. In acetic acid bacteria, strains assigned to *Gluconobacter oxydans* are widely used in the production of DHA through a microbiological method [6, 8]. Except for strains of the genus *Gluconobacter*, strains of some other genera of acetic acid bacteria such as the

genera *Acetobacter*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Neoasaia*, *Tanticharoenia*, *Ameyamaea*, *Komagataeibacter* and *Endobacter* were also reported to have the ability to produce DHA [8]. Acetic acid bacteria showed an abundant diversity in tropical countries such as Thailand, Indonesia and the Philippines. Vietnam is also a tropical country, however, there is no research on the microbial DHA producing in Vietnam. Furthermore, it is quite rare report about the diversity of acetic acid bacteria in Vietnam.

This study aims to preliminarily investigate the richness of diversity and the industrial applicability of bacterial resources in Vietnam through the isolation of DHA-producing AAB from fruits and flowers based on physiological and biochemical characterization and on the 16S rRNA gene sequence along with screening for the DHA forming ability.

## MATERIALS AND METHODS

### Isolation of AAB

AAB were isolated from 29 fruit and 16 flower samples collected in Hochiminh City, Vietnam by an enrichment culture approach using pH 3.5 medium [20]. After two days of incubation, a culture showing microbial growth was streaked onto a GEY-agar plate containing 0.3 % CaCO<sub>3</sub> (w/v). The acid-producing bacterial strains that formed a clear zone around the colony on the agar plate were selected for testing the growth at pH 3.5. Isolated strains were examined for their Gram stain, cell shape and catalase/oxidase formation by conventional methods.

### Screening of strains producing DHA from glycerol

The isolates selected as AAB were qualitatively analyzed for the ability to produce DHA. Bacterial cells were incubated in a DHA production medium containing 3.0 % glycerol, 0.5 % yeast extract, 1.0 % peptone (all by w/v) under a shaking condition for seven days at 30 °C and pH 6.0. A DHA-producing ability was detected by the appearance of the orange color in a bacterial supernatant with Fehling's solution [1].

For the quantitative analysis of DHA, potentially selected isolates and the reference strain were cultivated in the DHA production medium for 24 h. One mL of each culture (0.5 optical density at 600 nm) was transferred to a 200 mL beaker containing the same medium and incubated at 30 °C on a rotary shaker (150 rpm)

for 48 h. The supernatant of the cultivated broth was investigated for the amounts of DHA produced by the DNS (3,5-dinitrosalicylic acid) method according to Burner (1964) [3]. Pure dihydroxyacetone was used as standardizer. All the chemical agents was purchased from either Merck (Germany) or Sigma (USA).

The most potent and widely studied bacterium for DHA production is the species *Gluconobacter oxydans* [2, 3, 6, 10, 13]. The type strain *Gluconobacter oxydans* NBRC 14819<sup>T</sup> was used as a DHA-producing reference strain.

### Routine identification of DHA-producing AAB

Physiological and biochemical characterizations including the oxidation of acetate and lactate, the production of acetic acid from ethanol and of water-soluble brown pigments, the growth in the presence of 0.35 % acetic acid (v/v), on 30 % D-glucose (w/v) and on glutamate agar were made, as previously reported [1, 19, 20, 22, 23]. *Gluconobacter oxydans* NBRC 14819<sup>T</sup>, *Acetobacter aceti* NBRC 14818<sup>T</sup>, *Gluconacetobacter liquefaciens* NBRC 12388<sup>T</sup>, *Asaia bogorensis* NBRC 16594<sup>T</sup>, *Kozakia baliensis* NBRC 16664<sup>T</sup> were used as reference strains.

### Phylogenetic analysis of 16S rRNA genes for highly DHA-producing AAB

PCR amplification of 16S rRNA genes was carried out, and amplified 16S rRNA genes were sequenced and analyzed, as described previously [12, 15, 17, 18]. Multiple sequence alignments were done with the program CLUSTAL X (version 1.8) [17]. Alignment gaps and unidentified bases were eliminated. Genetic distances for the aligned sequences were calculated using the two-parameter method of Kimura (1960) [7]. A phylogenetic tree based on 16S rRNA gene sequences of 1,382 bases derived from the neighbor joining method was constructed by the use of the program MEGA 5 (version 5.05) [14, 16]. The robustness for

individual branches was estimated by bootstrapping with 1,000 replications [5].

## RESULTS AND DISCUSSION

### Isolation AAB

Sixty-six isolates were selected as AAB from 45 samples (Table 1). They formed clear zones of CaCO<sub>3</sub> on GEY-agar. Most of isolates gave creamy, brownish or pale yellow when colonies were grown on GECA. There were no isolates with a pink colony. They grew at pH 3.5 and showed positive catalase and negative oxidase. They were Gram-negative and rod shaped. There were 21 isolates from 16 flower samples and 45

isolates from 29 fruit samples. Kommanee et al. (2012) obtained 24 isolates from 22 fruits and 2 flowers samples collected in Thailand. Meanwhile, Moryadee and Pathum-Aree (2008) obtained 60 thermotolerant AAB from 13 kinds of fruits from Thai sources [8, 11]. Yamada et al. (1999) obtained 64 isolates in Indonesia, although they did not mention either the number of isolation sources or the kinds of isolation source [20]. Considering the numbers of 66 isolates obtained from 36 kinds of isolation sources, it can be preliminarily assumed that the presence of AAB in Vietnam is quite general (Table 1).

**Table 1.** Isolates and their isolation sources

No.	Isolation source	Type of isolation source	No. of samples	Isolates
1	Water convolvulus	Flower	1	VTH-AE01
2	Mango	Flower	1	VTH-AE02
3	Gandaria	Flower	1	VTH-AE12
4	Crêpe ginger	Flower	2	VTH-AE18, VTH-AH38, VTH-AH41, VTH-AH42, VTH-AH46
5	Yellow apricot	Flower	1	VTH-AE47
6	Jambu air	Flower	1	VTH-AE57
7	Crape jasmine	Flower	1	VTH-AE65, VTH-AE66
8	Frangipani	Flower	1	VTH-AE70, VTH-AH69
9	Blue pea	Flower	1	VTH-AE77
10	Giant spider lily	Flower	1	VTH-AH52
11	Ponna	Flower	1	VTH-AE83
13	Blue skyflower	Flower	1	VTH-AH71
13	Rose	Flower	1	VTH-AK36
14	Shoeblackplant	Flower	1	VTH-AK16
15	Tonkin jasmine	Flower	1	VTH-AK26
16	Orange.	Fruit	2	VTH-AE28, VTH-AE39, VTH-AK33
17	Strawberry	Fruit	2	VTH-AE44, VTH-AK14
18	Pineapple	Fruit	1	VTH-AE67, VTH-AE73, VTH-AE99
19	Jambu air	Fruit	3	VTH-AE75, VTH-AH49, VTH-AK23, VTH-AK30
20	Mandarin orange	Fruit	1	VTH-AE76, VTH-AH62
21	Avocado	Fruit	1	VTH-AE94
22	Grape	Fruit	3	VTH-AH37, VTH-AH39, VTH-AH47, VTH-AK04, VTH-AK20
23	Star fruit	Fruit	1	VTH-AH55, VTH-AH59
24	Mango	Fruit	2	VTH-AH57, VTH-AK17, VTH-AK18, VTH-AK19
25	Sapodilla	Fruit	1	VTH-AH61, VTH-AH72
26	Paradise apple	Fruit	1	VTH-AH81
27	Coconut	Fruit	2	VTH-AH82, VTH-AH89, VTH-AK15, VTH-

				AK28
28	Gandaria	Fruit	1	VTH-AK05
29	Barbados cherry	Fruit	1	VTH-AK07
30	Buffalo thorn	Fruit	1	VTH-AK21, VTH-AK31
31	White mulberry	Fruit	1	VTH-AK12
32	Oleaster-leafed pear	Fruit	1	VTH-AK22
33	Sugar-apple	Fruit	1	VTH-AK24, VTH-AK25, VTH-AK37
34	Papaya	Fruit	1	VTH-AK29
35	Rambutan	Fruit	1	VTH-AK34
36	Water melon	Fruit	1	VTH-AK32

### Screening of DHA-producing AAB and routine identification of selected DHA-producing AAB

Sixty-six isolates of selected AAB were examined for the qualitative screening of DHA-producing ability by using the Fehling's solution. Of the sixty-six, thirty-one isolates showed orange precipitations in the Fehling's solution and were designated as DHA-producing AAB (Table 1).

The thirty-one DHA-producing AAB were grouped into three groups by the routine identification [21].

Group I showed that the oxidation of acetate and lactate was positive, the acetic acid production from ethanol was positive, the growth was positive in the presence of 0.35 % acetic acid (v/v) but negative on glutamate agar and the production of water-soluble brown pigments was negative. Group I was assigned belonging to the genus *Acetobacter* and included fourteen isolates, comprised of VTH-AE39, VTH-AE76, VTH-AE75, VTH-AH55, VTH-AK62, VTH-AK07, VTH-AK17, VTH-AK18, VTH-AK19, VTH-AK22, VTH-AK26, VTH-AK28, VTH-AK29 and VTH-AK32.

Group II showed the that oxidation of acetate and lactate was negative, the acetic acid production from ethanol was positive, the growth was positive in the presence of 0.35 % acetic acid (v/v) but negative on glutamate agar and the production of water-soluble brown pigments was positive or negative. It was assigned to the genus

*Gluconobacter* and included thirteen isolates, comprised of VTH-AE18, VTH-AE44, VTH-AE57, VTH-AE67, VTH-AE83, VTH-AH39, VTH-AH46, VTH-AH59, VTH-AH69, VTH-AH82, VTH-AK04, VTH-AK12 and VTH-AK36.

Group III showed that the oxidation of acetate and lactate was positive but delayed, the acetic acid production from ethanol was positive, the growth was positive in the presence of 0.35 % acetic acid (v/v) and on glutamate agar and the production of water-soluble brown pigments was positive. It was assigned to the genus *Gluconacetobacter* and included four isolates, comprised of VTH-AH38, VTH-AH41, VTH-AH42 and VTH-AK05.

### Production of DHA by the selected DHA-producing AAB

The selected DHA-producing AAB were examined for the production of DHA. The amounts of DHA produced were from 0.17 to 25.98 mg/mL (Table 2). Instead, *Gluconobacter oxydans* NBRC 14819<sup>T</sup> produced 19.78 mg/mL. Among thirty-one tested isolates, excellent DHA producers were restricted only to ten isolates assigned to the genus *Gluconobacter*, showing 22.20–25.98 mg/mL. When examined on Thai *Gluconobacter* isolate PHD-27 for duration of 96 hours, Kommanee et al. (2012) obtained an amount of approximately 21 g/L (or mg/mL) DHA for 48 hours at 30 °C. These data suggested that yield of DHA production of the ten *Gluconobacter* isolates from Vietnam was similar to that of the Thai isolate.

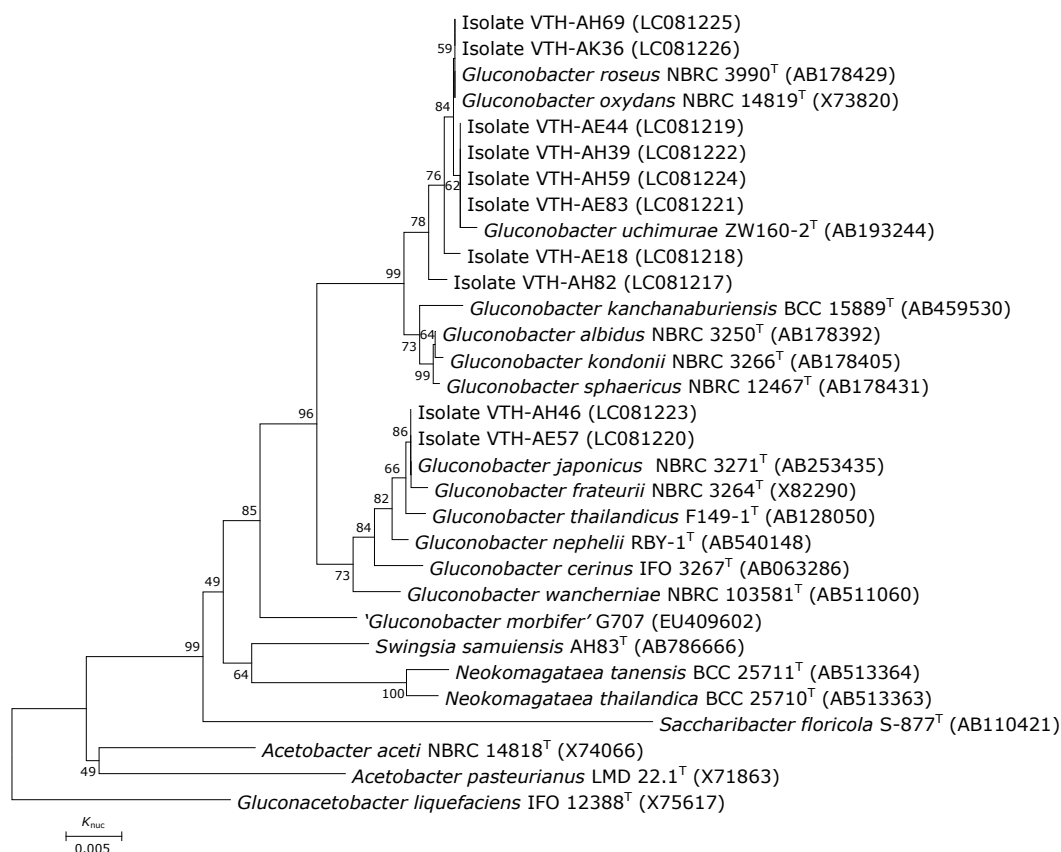
**Table 2.** Amounts of DHA produced by the selected DHA-producing AAB with their groups by routine identification

Group by routine identification	Isolates and their amount of DHA production (mg/mL)	Isolation sources
Group I Identified as <i>Acetobacter</i>	VTH-AE39 (0.26±0.09); VTH-AE75 (1.47±0.15); VTH-AE76 (2.67±0.22); VTH-AH55 (0.69±0.04); VTH-AH62 (2.17±0.39); VTH-AK07 (0.17±0.02); VTH-AK17 (3.10±0.21); VTH-AK18 (3.05±0.28); VTH-AK19 (0.81±0.01); VTH-AK22 (0.29±0.01); VTH-AK26 (3.64±0.19); VTH-AK28 (1.98±0.18); VTH-AK29 (0.43±0.03); VTH-AK32 (1.68±0.10)	Flower of Tonkin jasmine Fruit of Orange, Jambu air, Mandarin orange, Star fruit, Oleaster-leaved pear, Mango, Barbados cherry, Coconut, Papaya and Water melon
Group II Identified as <i>Gluconobacter</i>	<b>VTH-AE18 (22.20±0.47); VTH-AE44 (22.29±0.41);</b> <b>VTH-AE57 (24.73±0.54);</b> VTH-AE67 (0.63±0.04); <b>VTH-AE83 (23.97±0.69); VTH-AH39 (24.77±0.61);</b> <b>VTH-AH46 (22.91±0.32); VTH-AH59 (22.47±0.47);</b> <b>VTH-AH69 (22.64±0.81); VTH-AH82 (25.98±0.54);</b> VTH-AK04 (10.04±0.54); VTH-AK12 (0.93±0.08); <b>VTH-AK36 (23.37±0.41)</b>	Flower of Crêpe ginger, Ponna, Frangipani and Rose Fruit of Strawberry, Mango, Pineapple, Grape, Star fruit, Coconut and White mulberry
Group III Identified as <i>Gluconacetobacter</i>	VTH-AH38 (6.42±0.71); VTH-AH41 (5.91±0.34); VTH-AH42 (5.83±0.36); VTH-AK05 (1.13±0.55)	Flower of Crêpe ginger Fruit of Gandaria
<i>Gluconobacter oxydans</i> NBRC 14819 <sup>T</sup> produced 19.78±0.27 mg/mL DHA, when used as a reference strain.		

#### Phylogenetic relationship of highly DHA-producing selected AAB

The highly DHA-producing ten AAB were examined phylogenetically. As shown in Fig. 1, all the ten isolates were included in the lineage of the genus *Gluconobacter*. Firstly, the two isolates, VTH-AH69 and VTH-AK36 were phylogenetically related to either *G. oxydans* NBRC 14819<sup>T</sup> or *G. roseus* NBRC 3990<sup>T</sup>. Secondly, the four isolates, VTH-AE44, VTH-AE83, VTH-AH39 and VTH-AH59 that were

related to *G. uchimurae* ZW160-2<sup>T</sup> appeared to constitute a separate and independent taxon. Thirdly, the two isolates, VTH-AE18 and VTH-AH82 respectively formed independent clusters and obviously constituted separate taxa. Fourthly, the two isolates, VTH-AE57 and VTH-AH46 were related to *G. japonicus* NBRC 3271<sup>T</sup>. The obtained phylogenetic results suggested that six isolates of the ten are candidates for three new taxa.



**Figure 1.** Phylogenetic relationships of the ten *Gluconobacter* isolates (with the species in the genus *Gluconobacter*). The phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbor-joining method. The type strain of *Gluconacetobacter liquefaciens* was used as an outgroup. The numerals at nodes of the respective branches indicate bootstrap values (%) derived from replications

## CONCLUSION

The production of DHA by acetic acid bacteria has already been known from early days [2, 4, 8]. The thirty-one DHA-producing AAB obtained in Vietnam were distributed only in the three genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*. Especially, the excellent producers of DHA were restricted only to the genus *Gluconobacter* [2, 3, 6, 10, 13]. The ten isolates assigned to the genus *Gluconobacter* produced 22.20–25.98 mg/mL of DHA for the cultivation of two days by shaking at 150 rpm. In a certain Thai isolate, the production of DHA achieved in 21 mg/mL for 48 h cultivation [9].

These data suggested that the ten *Gluconobacter* isolates from Vietnam gave a yield of DHA production similar with that of the Thai isolate.

Phylogenetically, the ten isolates that were potential DHA producers were included in the lineage of the genus *Gluconobacter*. Of the ten, the six isolates, VTH-AE44, VTH-AE83, VTH-AH39, VTH-AH59, VTH-AE18 and VTH-AH82 were suggested to be candidates for three new species. The systematic study of the six isolates will be presented elsewhere.

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# Phân lập vi khuẩn acetic acid sản sinh dihydroxy-acetone ở Việt Nam

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## TÓM TẮT

Sáu mươi sáu chủng vi khuẩn acetic acid đã được phân lập từ 45 mẫu hoa, quả thu thập tại TP. Hồ Chí Minh, Việt Nam. Trong đó, 31 chủng được xác định là vi khuẩn acetic acid có thể sản sinh dihydroxyacetone dựa vào phản ứng với thuốc thử Fehling và được phân chia thành 3 nhóm theo đặc điểm kiểu hình. Nhóm I gồm 14 chủng được xác định thuộc chi *Acetobacter*. Nhóm II gồm 13 chủng được xác định thuộc chi *Gluconobacter*. Nhóm III gồm 4 chủng còn lại thuộc chi *Gluconacetobacter*. Trong 13 chủng

**Từ khóa:** vi khuẩn acetic acid, sinh dihydroxyacetone, *Gluconobacter*

thuộc nhóm II, có mười chủng sản sinh lượng DHA nhiều hơn lượng DHA thu thập được từ chủng *Gluconobacter oxydans* NBRC 14819<sup>T</sup> (22,2–26,0 mg/mL so với 19,8 mg/mL). Mười chủng này được đánh giá có khả năng ứng dụng trong sản xuất DHA. Phân tích mối quan hệ phát sinh loài dựa trên vùng trình tự gene mã hóa 16S rRNA cho thấy có 6 trong 10 chủng vi khuẩn tiềm năng ứng dụng trong sản xuất DHA có khả năng là những đơn vị phân loại mới trong chi *Gluconobacter*.

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