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และผลิตผลที่ได้จากการออกซิเดชัน

ของสายพันธุ์ในสกุล *Acetobacter* และ *Gluconobacter*

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TAXONOMY OF ACETIC ACID BACTERIA AND OXIDATIVE PRODUCTS OF *ACETOBACTER* AND *GLUCONOBACTER* STRAINS

Miss Jintana Kommanee

A Dissertation Submitted in Partial Fulfillment of the Requirements
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จินตนา คำมณี: อนุกรมวิธานของแบคทีเรียที่สร้างกรดแอซีติกและผลิตผลที่ได้จากการออกซิเดชัน ของสายพันธุ์ในสกุล *Acetobacter* และ *Gluconobacter* (TAXONOMY OF ACETIC ACID BACTERIA AND OXIDATIVE PRODUCTS OF *ACETOBACTER* AND *GLUCONOBACTER* STRAINS)
อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.สมบูรณ์ ธนาศุภวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.ภัทรพร ยุคแผน , 153 หน้า.

การคัดแยกแบคทีเรียผลิตกรดแอซีติกจำนวน 147 ไอโซเลต จากผลไม้ ดอกไม้ และวัสดุอื่น จากผลการศึกษาลักษณะทางฟีโนไทป์ อนุกรมวิธานเคมี และการวิเคราะห์ลำดับเบสในช่วงยีน 16S rDNA และ 16S-23S rDNA (ITS) ร่วมกับการใช้เทคนิค RFLP และ DNA-DNA Hybridization สามารถแบ่งได้เป็น 4 สกุล คือ *Acetobacter* (จำนวน 86 ไอโซเลต) *Gluconobacter* (จำนวน 42 ไอโซเลต) *Asaia* (จำนวน 15 ไอโซเลต) และ *Gluconacetobacter* (จำนวน 4 ไอโซเลต) พบว่าสายพันธุ์ของ *Acetobacter* มีระบบยูบิควิโนนเป็นชนิด Q-9 ส่วนสายพันธุ์ในสกุลอื่นเป็น Q-10 ปริมาณ DNA G+C ของสายพันธุ์อยู่ในช่วง 52.2-64.3 โมล% การพิสูจน์เอกลักษณ์ของ *Acetobacter* พบว่าเป็น *A. pasteurianus* (กลุ่มที่ 1 จำนวน 26 ไอโซเลต) *A. orientalis* (กลุ่มที่ 2 จำนวน 13 ไอโซเลต) *A. lovaniensis* (กลุ่มที่ 3 จำนวน 10 ไอโซเลต) *A. indonesiensis* (กลุ่มที่ 4 จำนวน 13 ไอโซเลต) *A. tropicalis* (กลุ่มที่ 5 จำนวน 4 ไอโซเลต) *A. ghanensis* (กลุ่มที่ 6 จำนวน 8 ไอโซเลต) *A. orleanensis* (กลุ่มที่ 7 จำนวน 4 ไอโซเลต) *A. syzygii* (กลุ่มที่ 8 จำนวน 4 ไอโซเลต) และ *Acetobacter* sp. (กลุ่มที่ 9 จำนวน 3 ไอโซเลต) ไอโซเลตของ *Gluconobacter* เป็น *G. frateurii* (กลุ่มที่ 10 จำนวน 8 ไอโซเลต) *G. japonicus* (กลุ่มที่ 11 จำนวน 8 ไอโซเลต) *G. thailandicus* (กลุ่มที่ 12 จำนวน 6 ไอโซเลต), *G. oxydans* (กลุ่มที่ 13 จำนวน 13 ไอโซเลต) *Gluconobacter* sp. (กลุ่มที่ 14 จำนวน 3 ไอโซเลต) และ *Gluconobacter* sp. (กลุ่มที่ 15 จำนวน 4 ไอโซเลต) ไอโซเลตของ *Asaia* เป็น *As. bogorensis* (กลุ่มที่ 16 จำนวน 8 ไอโซเลต) *As. siamensis* (กลุ่มที่ 17 จำนวน 5 ไอโซเลต) และ *As. sphathodeae* (กลุ่มที่ 18 จำนวน 2 ไอโซเลต) และ *Gluconacetobacter* เป็น *Ga. liquefaciens* (กลุ่มที่ 19 จำนวน 4 ไอโซเลต) การวิเคราะห์ลำดับเบสในช่วงยีน 16S ของ G360-1 G361-1 และ G362-1 (กลุ่มที่ 9) พบว่าใกล้เคียงกับ *A. orleanensis* NBRC 13752^T 99.7% และให้รูปแบบแตกต่างจากสปีชีส์อื่นของ *Acetobacter* เมื่อตัดด้วยเอนไซม์ *Hpa*II และ *Ava*II และมีความคล้ายคลึงของ DNA ต่ำ (15-38%) ดังนั้นจึงควรจัดเป็นสปีชีส์ใหม่ในสกุล *Acetobacter* การวิเคราะห์ลำดับเบสในช่วงยีน ITS ของ RBY-1^T PHD-1 และ PHD-2 (กลุ่มที่ 15) พบว่าใกล้เคียงกับ *G. japonicas* NBRC 3271^T 98.1% และให้รูปแบบแตกต่างจากสปีชีส์อื่นของ *Gluconobacter* เมื่อตัดด้วย *Taq*I, *Alu*I, *Hpa*II และ *Ava*II และมีความคล้ายคลึงของ DNA ต่ำ (11-38%) ดังนั้นจึงถูกเสนอเป็นสปีชีส์ใหม่ว่า *Gluconobacter nephelii* การวิเคราะห์ลำดับเบสในช่วงยีน ITS ของ ZW16-2 LC155-1, LG156-2 และ JJ157-2 (กลุ่มที่ 15) พบว่าใกล้เคียงกับ *G. oxydans* NBRC 14818^T 97.3% และให้รูปแบบแตกต่างจากสปีชีส์อื่นของ *Gluconobacter* เมื่อตัดด้วย *Taq*I, *Alu*I, *Hpa*II และ *Ava*II และมีความคล้ายคลึงของ DNA ต่ำ (11-26%) ดังนั้นจึงควรจัดเป็นสปีชีส์ใหม่ในสกุล *Gluconobacter* การวิเคราะห์ลำดับเบสในช่วงยีน 16S ของ GB23-2^T และ GB23-3 (กลุ่มที่ 18) พบว่าใกล้เคียงกับ *As. siamensis* NBRC 16457^T 99.9% และให้รูปแบบแตกต่างจากสปีชีส์อื่นของ *Asaia* เมื่อตัดด้วย *Sty*I, *Bsa*JI, *Sna*BI, *Hpa*II และ *Hpy*AV และมีความคล้ายคลึงของ DNA ต่ำ (21-48%) จึงถูกเสนอเป็นสปีชีส์ใหม่ว่า *As. spathodeae*

การวิเคราะห์กิจกรรมเอนไซม์แอลกอฮอล์ดีไฮโดรจีเนสของ *Acetobacter* พบว่ามีกิจกรรมของเอนไซม์ในช่วง 2.05-7.52 unit/mg ที่อุณหภูมิ 30°C ไอโซเลต PHD-23 สามารถผลิตกรดแอซีติกได้สูงสุดเมื่อเติมเอทานอล 4% แต่ไม่เติมกรดแอซีติกในอาหาร การคัดกรองการผลิตไดไฮดรอกซีอะซิโตนและแอล-ซอร์โบส ของ *Gluconobacter* จำนวน 42 ไอโซเลต พบว่า ไอโซเลตสามารถผลิตไดไฮดรอกซีอะซิโตนในช่วง 20.05-42.52 กรัมต่อลิตร ที่อุณหภูมิ 3 0°C ไอโซเลต PHD-27 มีอัตราการผลิตไดไฮดรอกซีอะซิโตน 0.52 กรัมต่อลิตรต่อชั่วโมงที่อุณหภูมิ 30°C พบว่าไอโซเลตผลิตแอล-ซอร์โบสในช่วง 19.99 to 48.39 กรัมต่อลิตร ที่อุณหภูมิ 30°C และไอโซเลต AP59-1 ผลิตแอล-ซอร์โบสได้สูงสุด

สาขาวิชา.....เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ	ลายมือชื่อนิสิต..
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JINTANA KOMMANEE: (TAXONOMY OF ACETIC ACID BACTERIA AND OXIDATIVE PRODUCTS OF *ACETOBACTER* AND *GLUCONOBACTER* STRAINS) THESIS ADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D., THESIS CO-ADVISOR: PATTARAPORN YUKPHAN, Ph.D.153 pp.

One hundred and forty-seven isolates of acetic acid bacteria were isolated from fruits, flowers, and other materials in Thailand. They were divided into 4 genera, *Acetobacter* (86 isolates), *Gluconobacter* (42 isolates), *Asaia* (15 isolates), and *Gluconacetobacter* (4 isolates) based on their phenotypic and chemotaxonomic characteristics including RFLP-ITS analysis and the 16S rDNA phylogenetic analysis. *Acetobacter* isolates contained Q-9 as major quinone while the other genera contained Q-10. The DNA G+C contents ranged from isolates 52.2-64.3 mol%. *Acetobacter* isolates were identified as *A. pasteurianus* (Group 1, 26 isolates), *A. orientalis* (Group 2, 13 isolates), *A. lovaniensis* (Group 3, 10 isolates), *A. indonesiensis* (Group 4, 13 isolates), *A. tropicalis* (Group 5, 4 isolates), *A. ghanensis* (Group 6, 8 isolates), *A. orleanensis* (Group 7, 4 isolates), *A. syzygii* (Group 8, 4 isolates) and *Acetobacter* sp. (Group 9, 3 isolates). *Gluconobacter* isolates were identified as *G. frateurii* (Group 10, 8 isolates), *G. japonicus* (Group 11, 8 isolates), *G. thailandicus* (Group 12, 6 isolates), *G. oxydans* (Group 13, 13 isolates), *Gluconobacter* sp. (Group 14, 3 isolates) and *Gluconobacter* sp. (Group 15, 4 isolates). *Asaia* isolates were identified as *As. bogorensis* (Group 16, 8 isolates), *As. siamensis* (Group 17, 5 isolates) and *As. sphathodeae* (Group 18, 2 isolates), and *Gluconacetobacter* isolates were identified as *Ga. liquefaciens* (Group 19, 4 isolates). The isolates G360-1, G361-1 and G362-1 (Group 9) showed 99.7% 16S rDNA sequence similarity to *A. orleanensis* NBRC 13752^T and discriminated from known *Acetobacter* species when digestion with *Hpa*II and *Ava*II and low DNA-DNA relatedness (15-38%). Therefore, they should be proposed as a new species in the genus *Acetobacter*. The isolates RBY-1^T, PHD-1 and PHD-2 (Group 14) showed 98.1% ITS genes sequence similarity to *G. japonicus* NBRC 3271^T and discriminated from other *Gluconobacter* species when digestion with *Taq*I, *Alu*I, *Hpa*II and *Ava*II and low DNA-DNA relatedness (11-38%) Therefore, the name *Gluconobacter nephelii* sp. nov. was proposed for them. The isolates ZW160-2, LC155-1, LG156-2 and JJ157-2 (Group 15) showed 97.3% ITS genes sequence similarity to *G. oxydans* NBRC 14818^T and discriminated from other *Gluconobacter* when digestion with *Taq*I, *Alu*I, *Hpa*II and *Ava*II and low DNA-DNA relatedness (11-26%). Therefore, they should be proposed as a new species in the genus *Gluconobacter*. The isolates GB23-2^T and GB23-3 (Group 18) showed 99.9% 16S rDNA sequence similarity to *As. siamensis* NBRC 16457^T and discriminated from other *Asaia* species when digestion with *Sty*I, *Bsa*JI, *Sna*BI, *Hpa*II and *Hpy*AV and low DNA-DNA relatedness (21-48%). Therefore, the name *Asaia spathodeae* sp. nov. was proposed for them.

ADH activity was determined in *Acetobacter* isolates and the activity ranged from 2.05 to 7.52 unit/mg at 30°C. The isolate PHD-23 could produce highest acetic acid when the medium composed of ethanol 4% and with out addition of acetic acid. Forty-two *Gluconobacter* isolates were screened for DHA and L-sorbose production. The isolates could produce DHA ranged from 20.05 to 42.52 g/l at 30°C. Isolate PHD-27 could produce the DHA with a maximum of 44.08 g/l at 30°C by conversion time of 84 h and generated DHA at a rate of 0.52 g/l/h at 30°C. The tested isolates produced L-sorbose ranged from 19.99 to 48.39 g/l at 30°C and *G. frateurii* AP59-1 produced the largest amount of L-sorbose.

Field of Study: Pharmaceutical Chemistry and Natural Products………. Student's Signature..
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LIST OF ABBREVATIONS

AAB	=	Acetic acid bacteria
ADH	=	Alcohol dehydrogenase
ALDH	=	Aldehyde dehydrogenase
Bp.	=	Base pair
BCC	=	BIOTEC Culture Collection
°C	=	Degree Celsius
DDBJ	=	DNA Data Bank of Japan
DHA	=	Dihydroxyacetone
EDTA	=	Disodiummethylenediamine tetraacetate
EtoAc	=	Ethyl acetate
g	=	Gram
GC	=	Gas chromatography
GenBank	=	National Institute of Health genetic sequence database
g/l	=	Gram/litter
g/l/h	=	Gram/litter/hour
h	=	Hour
HCl	=	Hydrochloric acid
HPLC	=	High performance liquid chromatography
ITS	=	Internal transcribes spacer region
kDa	=	Kilo Dalton
l	=	Liter
lb/min	=	Pound/minute
M	=	Molar
MEGA	=	Molecular Evolutionary Genetics Analysis
MeOH	=	Methanol
mg	=	Milligram
ml	=	Mililiter
mM	=	Milimole
NBRC	=	NITE Biological Resource Center
nm	=	Nanometer
PCR	=	Polymerase chain reaction
Q	=	Quinone
rDNA	=	Ribosomal deoxynucleic acid

LIST OF ABBREVATIONS

RFLP	=	Restriction fragment length polymorphism
rpm	=	Round per minute
rRNA	=	Ribosomal ribonucleic acid
rpm	=	Round per minute
sp.	=	Species
sec	=	Second
TEM	=	Transmission electron microscope
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
µl	=	Microliter
µg	=	Microgram
U	=	Unit
v/v	=	Volume/volume
w/v	=	Weight/ volume
%	=	Percent
α	=	Alpha

CHAPTER I

INTRODUCTION

Acetic acid bacteria (AAB) are α-*Proteobacteria* in the family *Acetobacteraceae* and they are classified into twelve genera: *Acetobacter* (Beijerinck, 1898) *Gluconobacter* (Asai, 1964), *Acidomonas* (Urakami *et al*., 1989), *Gluconacetobacter* (Yamada *et al*. 1997), *Asaia* (Yamada *et al*., 2000), *Kozakia* (Lisdiyanti *et al*., 2002), *Swaminathania* (Logonathan and Nair. 2004), *Saccharibacter* (Jojima *et al*., 2004), *Neoasaia* (Yukphan *et al*., 2005), *Granulibacter* (Greenberg *et al*., 2006), *Tanticharoenia* (Yukphan *et al*., 2008) and *Ameyamaea* (Yukphan *et al*., 2009). They are gram negative, ellipsoidal to rod-shaped. They are motile due to the presence of flagella, which can be either peritrichous or polar. They do not form endospore as a defensive resistance. AAB occur in sugar and alcoholics, slightly acid niches such as flowers, fruits, beer, wine, cider, vinegar, and souring fruit juices.

AAB species have conventionally been identified by testing physiological and chemotaxonomic properties (Delay *et al*., 1984). The identification of the AAB, using phenotypic characteristics, especially on the species level, is difficult and is not only inaccurate, but also very time-consuming. Therefore, the application of the molecular methods based on the restriction fragment length polymorphism (RFLP) analysis and sequence analysis, could be a proper solution for a quick and accurate identification of these microorganisms. Recently, the 16S-23S rDNA internal transcribed spacer (ITS) region was genetic variation on 16S rDNA in the AAB and other microorganisms (Trček and Teuber, 2002). In addition, the RFLP of ITS was not useful for identification of AAB at the species level although it might be a variation spacer on 16S rDNA similar with the other regions but as well as these details were useful for generic level identification (Trček and Teuber, 2002). At the specific level identification of AAB, the phenotypic characteristics were traditionally utilized. Therefore, we selected the restriction pattern or RFLP analysis and sequence analysis for identification of AAB at the species level.

The strains in genus *Acetobacter* are classified into 19 species; *A. aceti*, *A. indonesiensis*, *A. cerevisiae*. *A. cibinongensis*, *A. pasteurianus*, *A. lovaniensis*, *A. orleanensis, A. estunensis*, *A. malorum*, *A. orientalis*, *A. peroxydans*, *A. pomorum*, *A. syzygii*, *A. tropicalis*, *A. oeni, A. ghanensis, A. nitrogenifigen, A. senegalensis* and *A. fabarum* (Cleenwerck *et al*., 2008). They are frequently used for the industrial vinegar (Saeki *et al*., 1997). They are well known as vinegar producers from ethanol by two sequential catalytic reactions of membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The oxidation

reactions are called "oxidative fermentation" (Matsushita *et al*., 1994). Vinegar fermentation by AAB is usually carried out at 30°C and strict, temperature control is necessary because if a temperature increase of 2-3°C causes a serious in both the fermentation rate and fermentation efficiency (Saeki *et al*., 1997). Therefore, if favorable strains of AAB that can work optimally at 37-40°C were available, the cooling expensive would be reduced greatly.

The genus *Gluconobacter* is classified into 11 species; *G. oxydans, G. cerinus, G. frateurii, G. thailandicus, G. albidus, G. sphericus, G. kondonii, G. japonicus, G. roseus, G. kanchanaburiensis* and *G. wancherniae* (Yukphan *et al*., 2010). *Gluconobacter* strains generally produce acid during growth on several carbohydrates and show a pronounced and efficient ketogenesis from polyhydroxyalcohols via a single-step oxidation. The latter property finds numerous applications in industry. They produce gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, L-sorbose, tagatose, dihydroxyacetone (DHA), fructose, etc. (Manzoni *et al*., 2001; Adachi *et al*., 2003)

AAB occur in sugar and alcoholics, slightly acid niches such as flowers, fruits, beer, wine, cider, vinegar and souring fruit juices. On these substrates they oxidize the sugars and alcohols, resulting an accumulation of organic acids as final products. When the substrate is ethanol, acetic acid is produced, and this is where the name of the bacterial group comes from. However, these bacteria also oxidize glycerol to DHA, D-galactitol to tagatose, L-sorbitol to L-sorbose, etc. Some of these transformations carried out by AAB are of considerable interest for the biotechnological industry.

Recently, many new AAB have been reported in Thailand, including the genera *Neoasaia*, *Tanticharoenia, Ameyamaea* and the species *Asaia siamensis*, *Asaia krungthepensis, Asaia lannensis, Gluconobacter thailandicus*, *Gluconobacter kanchanaburiensis*, *Neoasaia chiangmaiensis, Tanticharoenia sakaeratensis* and *Ameyamaea chiangmaiensis* (Katsura *et al*., 2001; Malimas *et al*., 2008a, 2009b; Tanasupawat *et al*., 2004; Yukphan *et al*., 2004, 2005, 2008, 2009).

Thailand is located in the tropical area that is hot and humid, additionally, the relatively diverse fruit, flower types and natural high biodiversity of this region which is highly conducive for microbial growth. This work deals with the isolating, identifying and classifying AAB in Thailand based on phenotypic, chemotaxonomic characteristics, ITS-RFLP analysis, DNA sequencing analysis and DNA-DNA hybridization including study the oxidative products of *Acetobacter* and *Gluconobacter* strains.

The main objectives of this presence study are as follows:

1. To isolate, screen and identify acetic acid bacteria based on the phenotype and genotypic characteristics.
2. To study oxidative products of *Acetobacter* and *Gluconobacter* strains.

CHAPTER II

LITERATURE REVIEWS

2.1 General characteristics of acetic acid bacteria

AAB are gram negative, ellipsoidal to rod-shaped. They are motile due to the presence of flagella, which can be either peritrichous or polar. They do not form endospores as a defensive resistance. They have an obligate aerobic metabolism, with oxygen as the terminal electron acceptor. The optimum pH for the growth of AAB is 5-6.5 (Holt *et al.*, 1994). However these bacteria can grow at lower pH values of between 3-4. They have wide ranged from 0.4-1 µm and 0.8-4.5 µm of length. They are catalase positive and oxidase negative. The ubiquinone system was Q-9 or Q-10. The optimum temperature was 30°C. The colonies were arounded with clear zone when they grown on calcium carbonate agar medium. The base composition ranges from 54.0-65.0 mol% of G+C content. AAB some strain can present pigmentation in solid cultures and can produce different kinds of polysaccharides (De Ley *et al.*, 1984). AAB occur in sugar and alcoholics, slightly acid niches such as flowers, fruits, beer, wine, cider, vinegar and souring fruit juices. On these substrates they oxidize the sugars and alcohols, resulting an accumulation of organic acids as final products. Acetic acid was produced when the substrate is ethanol, and this is where the name of the bacterial group comes from. However, these bacteria also oxidize glycerol to DHA, D-galactitol to tagatose, L-sorbitol to L-sorbose etc. Some of these transformations carried out by AAB are of considerable interest for the biotechnological industry.

2.2 Taxonomy of acetic acid bacteria

The French scientist name Pasteur should be considered as the first taxonomist of this bacterial group. Studying the Orléans method of vinegar production, he demonstrated that the acetic acid came from ethanol oxidation and that long-term oxidation of acetic acid converted it into CO_2 and water. His results led him to formulate the involvement of the microorgansism in the process of transforming alcohol into vinegar, and confirmed the existence of *Mycoderma aceti* which Persoon had already described in 1822. Subsequently, in the year 1879, Hansen observed that the microbial flora which converted alcohol into acetic acid was not pure and consisted of various bacterial species. The genera *Acetobacter* was proposed later by Beijerinck (1898).

The first classifications were proposed by Hansen in 1894, based on the occurrence of a film in the liquid media, and its reaction with iodine. The first to propose a classification

based on biochemical and a physiological criterion was Vissert Hooft (1925). Asai (1934-1935) formulated the proposal of classifying AAB into two genera: *Acetobacter* and *Gluconobacter*. The main differences between these two genera were both cytological and physiological. The main physiological difference was that *Acetobacter* oxidized ethanol into acetic acid and, subsequently, completed the oxidation of acetic acid into water and CO_2. *Gluconobacter* species, on the other hand, were unable to complete this oxidation of acetic acid.

It was Frateur (1950) who formulated a classification based mainly on five physiological criteria: the presence of catalase, gluconic acid production from glucose, the oxidation of acetic acid into CO_2 and water, the oxidation of lactate into CO_2 and water, and the oxidation of glycerol into hydroxyacetone. On the basis of these criteria he divided *Acetobacter* genera into four groups: *peroxydans*, *oxydans*, *mesoxydans* and *suboxydans*.

Leifson (1954) grouped those AAB that had peritrichous flagella and were able to oxidize ethanol into the geneus *Acetobacter* and those that had polar flagella and unable to perform the complete oxidation into the genera *Gluconobacter*. The taxonomical keys for bacteria taxonomy have been historically collected in Bergey's Manual of Systematic Bacteriology. In its last edition (De Ley *et al*., 1984), some molecular techniques were included as fatty acid composition, soluble protein electrophoresis, mol% of G+C content, and DNA-DNA hybridization.

Nowadays the AAB were classified into twelve genera: *Acetobacter* (Beijerinck, 1898) *Gluconobacter* (Asai, 1964), *Acidomonas* (Urakami *et al*., 1989), *Gluconacetobacter* (Yamada *et al*. 1997), *Asaia* (Yamada *et al*., 2000), *Kozakia* (Lisdiyanti *et al*., 2002), *Swaminathania* (Logonathan and Nair. 2004), *Saccharibacter* (Jojima *et al*., 2004), *Neoasaia* (Yukphan *et al*., 2005), *Granulibacter* (Greenberg *et al*., 2006), *Tanticharoenia* (Yukphan *et al*., 2008) and *Ameyamaea* (Yukphan *et al*., 2009). Their characteristics are shown in Table 2.1

Table 2.1 Characteristics of the genera belonging to family *Acetobacteraceae* (Yukphan *et al*., 2009)

Characteristic	*Am*	*T*	*A*	*G*	*Ac*	*Ga*	*As*	*K*	*S*	*Sa*	*N*	*Gr*
	1	2	3	4	5	6	7	8	9	10	11	12
Flagellation	po	n	pe	po	po[e]	pe	pe	n	pe	n	n	n
Oxidation of												
Acetate	+	-	+	-	+	+	w	w	w	-	-	w
Lactate	w	-	+	-	-	+	w	w	w	w	-	+
Growth on												
30% D-Glucose (w/v)	-	+	-	-	+[e]	-	+	w	+	+	+	nd
Glutamate agar	w	w	-	-	-	+	+	-	+	+	+	+
Mannitol agar	+	+	vw	+	w	+	+	+	+	+	+	w
Methanol	w	-	-	-	+	-	-	-	-	-	-	+
Growth in the presence of												
0.35% acetic acid (w/v)	+	+	+	+	+	+	-	+	+	-	+	nd
1% KNO_3	-	-	-	-	+	-	-	-	+	nd	-	nd
Production of acetic acid from ethanol	+	+	+	+	+	+	-	+	+	w/-	+	vw
Water-soluble brown pigment production	-	+	-	-	-	+	-	-	+	-	-	nd
Production of dihydroxy-acetone from glycerol	w	+	+	+	-	+	w	w	+	-	w	-
Production of levan-like polysaccharide	-	-	-	-	-	-	-	+	nd	-	-	nd
Assimilation of ammoniac nitrogen on												
D-Glucose	vw	-	-	w	w	-	+	-	nd	-	-	+
D-Mannitol	vw	-	-	w	w	-	+	-	nd	-	w	nd
Ethanol	vw	-	+	-	w	-	-	-	nd	-	-	nd
Production of												
2-Keto-D-gluconate	+	+	+	+	nd	+	+	+	nd	+	+	nd
5-Keto-D-gluconate	+	+	+	+	nd	+	+	+	nd	+	+	nd
2,5-Diketo-D-gluconate	-	+	-	-	nd	+	-	-	nd	nd	-	nd
Acid production from												
D-Mannitol	-	-	-	+	w	-	+	-	-	+	w	-
D-Sorbitol	-	-	-	+	-	-	+(d)	-	+	-	+(d)	-
Dulcitol	-	-	-	w	-	-	+(d)	-	v	-	w	-
Glycerol	w	+	+	+	+	+	+	+	+	-	+	w/-
Ethanol	+	+	+	+	+	+	-	+	+	-	+	+
16S rRNA gene restriction pattern with[j]												
*Taq*I	*Gal*	*Ts*	*Aa*	*Go*	*Acm*	*Gal*	*Asb*	*Kb*	*Asb*	*Saf*	*Nc*	*Grb*
*Hin*6I	*Amc*	*Ts*	*Aa*	*Go*	*Acm*	*Gal*	*Asb*	*Kb*	*Ss*	*Saf*	*Nc*	*Grb*
Major isoprenoid quinone	Q-10	Q-10	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
DNA G+C mol%	65.6	65.6	58.6	60.6	62	64.5	60.2	57.2	57.6-59.9	52.3	63.1	59.1

po, polar; pe, peritrichous; n, none; +, positive; -, negative; w, weakly positive; vw, very weakly positive; d, delayed; v, variable; nd, not determined; *Acm*, *Acidomonas methanolica*; *Gal*, *Gluconacetobacter liquefaciens*; *Asb*, *Asaia bogorensis*; *Kb*, *Kozakia baliensis*; *Ss*, *Swaminathania salitolerans*; *Saf*, *Saccharibacter floricola*; *Nc*, *Neoasaia chiangmaiensis*; *Grb*, *Granulibacter bethesdensis*

Abbreviations: *Am*, *Ameyamaea*; *T*, *Tanticharoenia*; *A*, *Acetobacter*; *G*, *Gluconobacter*; *Ac*, *Acidomonas*; *Ga*, *Gluconacetobacter*; *As*, *Asaia*; *K*, *Kozakia*; *S*, *Swaminathania*; *Sa*, *Saccharibacter*; *N*, *Neoasaia*; *Gr*, *Granulibacter*; 1, *Ameyamaea chiangmaiensis*; 2, *Tanticharoenia sakaeratensis* isolate AC37^T; 3, *Acetobacter aceti* NBRC 14818^T; 4, *Gluconobacter oxydans* NBRC 14819^T; 5, *Acidomonas methanolica* NRIC 0498^T; 6, *Gluconacetobacter liquefaciens* NBRC 12388^T; 7, *Asaia bogorensis* NBRC 16594^T; 8, *Kozakia baliensis* NBRC 16664^T; 9, *Swaminathania salitolerans* strain PA51^T; 10, *Saccharibacter floricola* strain S-877^T; 11, *Neoasaia chiangmaiensis* isolate AC28^T; 12, *Granulibacter bethesdensis* CGDNIH1^T

In the family *Acetobacteraceae*, they are eight monotypic genera, i.e., *Acidomonas, Kozakia, Swaminathania, Saccharibacter, Neoasaia, Granulibacter, Tanticharoenia* and *Ameyamaea*, in which only one species is described. The occurrence of strains assigned to some of the eight genera is rather rare in common isolation sources such as vinegar, wine, fruits and flowers. For example, any additional *Kozakia* isolated from palm brown sugar and ragi collected in Indonesia (Lisdiyanti *et al*., 2002), *Neoasaia* isolated from flower of red ginger in Thailand (Yukphan *et al*., 2005) and *Swaminathania* isolated from the rhizosphere, roots and stems of salt-tolerant, mangrove-associated wild rice (Logonathan and Nair. 2004) strains have not yet been isolated from the common isolation sources. In addition, *Acidomonas* strains were isolated mostly from sludge (Yamashita *et al*., 2004).

In the remaining four genera *Acetobacter, Gluconobacter, Gluconacetobacter* and *Asaia*, the genus level identification is not impossible phenotypically by combination of only two conventional tests comprised of acetate and lactate oxidation and acetic acid production from ethanol (Asai *et al*., 1964; Yamada *et al*., 1976).

In strains to be assigned to the genus *Acetobacter*, a deep blue color appears fast and clearly in the acetate and lactate oxidation test, and acetic acid is produced in the acetic acid production test. In acetate and lactate oxidation, strains to be assigned to the genus *Gluconobacter* generally show a clear yellow color, and the color change to blue is not so vigorous in strains to be assigned to the genus *Gluconacetobacter*, in contrast to *Acetobacter* strains. Strains to be assigned to the genus *Asaia* show no or little production of acetic acid from ethanol, differing in this respect from strains of the above-mentioned three genera, and the color change is very slow in acetate and lactate oxidation. The combination of the two phenotypic features mentioned above is very useful, especially when a large number of isolates are routinely classified at the generic level (Yamada and Yukphan., 2008).

2.2.1 *Acetobacter*

Acetobacter strains exhibit the capability for producing acetic acid from ethanol. They oxidize acetate and lactate to carbon dioxide and water and contain the major ubiquinone with nine isoprene units (Q-9) (Yamada *et al*., 1997; Cleenwerck *et al*., 2002). Colonies are smooth, raised, beige to brown color, and regular too smaller. The DNA base composition range from 56.0-60.7 mol % of G+C content (Cleenwerck *et al*., 2008). They can produce vinegar from ethanol and carried out by sequential membrane bound alcohol and aldehyde dehydrogenase functions.

The genus *Acetobacter* comprises 19 species; *A. aceti*, *A. indonesiensis*, *A. cerevisiae*. *A. cibinongensis*, *A. pasteurianus*, *A. lovaniensis*, *A. orleanensis, A. estunensis*, *A. malorum*, *A. orientalis*, *A. peroxydans*, *A. pomorum*, *A. syzygii*, *A. tropicalis*, *A. oeni, A. ghanensis, A. nitrogenifigen, A. senegalensis* and *A. fabarum* (Cleenwerck *et al*., 2008). Their differential characteristics are shown in Table 2.2

Acetobacter sp. were reported in Thailand; *A. pasteurianus*, *A. lovaniensis*, *A. orientalis*, *A. syzygii*, and *A. tropicalis* (Kommanee *et al*., 2008; Tanasupawat *et al*., 2009).

Table 2.2 Differential characteristics of *Acetobacter* species (Cleenwerck *et al*., 2008)

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
5-Keto-D-gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	+	+	-
2-Keto-D-gluconic acid	-	+	-	-	v	-	-	+	+	+	+	+	+	+	+	ND	-	+	+
Growth in ammonium with ethanol	v(+)	+	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	+	+
Growth on																			
Glycerol	+	+	w	+	v	+	-	+	+	+	+	+	+	+	+	+	+	+	v
Maltose	-	-	-	+	v	-	+	+	w	-	+	-	v	-	-	+	-	v	-
Methanol	+	+	-	-	-	-	-	-	w	-	-	-	-	+	-	-	-	-	-
Groeth in 10% ethanol	v(-)	-	v	-	+	-	-	-	-	-	-	+	-	+	ND	+	+	-	-
Growth on YE+30% D-glucose	-	-	+	-	v	-	-	-	-	+	-	+	-	+	-	+	-	-	-
Catalase	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
DNA G+C (mol%)	56.8 - 58.0	57.1 - 58.9	56.9 - 57.3	54.3 - 55.4	53.2 - 54.3	52.1	59.7 - 60.7	54.0 - 54.2	52.0 - 52.8	53.8 - 54.5	55.6 - 56.2	55.6 - 56.0	55.7 - 58.1	57.2	56.0 - 57.6	64.1	58.1	56.9 - 58.3	59.2

1, *A. fabarum*; 2, *A. lovaniensis*; 3, *A. ghanensis*; 4, *A. syzygii*; 5, *A. pasteurianus*; 6, *A. pomorum*; 7, *A. peroxydans*; 8, *A. indonesiensis*; 9, *A. orientalis*; 10, *A. cibinongensis*; 11, *A. tropicalis*; 12, *A. senegalensis*; 13, *A. orleanensis*; 14, *A. malorum*; 15, *A. cerevisiae*; 16, *A. nitrogenifigens*; 17, *A. oeni*; 18, *A. aceti*; 19, *A. estunensis*. +, Positive; -, negative; W, weakly positive; V, variable

2.2.2 *Gluconobacter*

Gluconobacter strains are incapable for completely acetate and lactate oxidation to carbon dioxide and water. They contain ubiquinone-10 (Q-10) as major ubiquinone in oxidation respiratory system (Yamada *et al*., 1997). Some species no exhibited motility but some motile with polar flagella. They are able to oxidize ethanol to acetic acid and utilize various sugars, sugar acids and alcohol sugars such as D-glucose, D-sorbitol, D-arabitol, *meso*-erytritol, glycerol and so on (Matsushita *et al*., 2003). *Gluconobacter* species are very important for L-ascorbic acid or vitamin C, cost valuable sugars production and so on. *Gluconobacter* strains are mostly found in fruits and flowers. The DNA base composition range from 54-60 mol% of G+C content (Katsura *et al*., 2002).

The genus *Gluconobacter* comprises 11 species; *G. oxydans, G. cerinus, G. frateurii, G. thailandicus, G. albidus, G. sphericus, G. kondonii, G. japonicus, G. roseus, G. kanchanaburiensis* and *G. wancherniae* (Yukphan *et al*., 2010). Their differential characteristics are shown in Table 2.3.

Table 2.3 Differential characteristics of *Gluconobacter* species (Yukphan *et al*., 2010)

Characteristics	*Gj*	*Gf*	*Gt*	*Gc*	*Go*	*Ga*	*Gko*	*Gr*	*Gs*	*Gkn*	*Gw*
Growth without nicotinic acid	+	+	+	+	-	-	-	-	-	-	+
Water-soluble brown pigment	-	-	-	-	-	-	-	-	+	+	+
2,5-diketo-D-gluconate formation	-	-	-	-	-	-	-	-	+	+	+
Dihydroxyacetone from glycerol	w	+	+	+	+	+	+	+	+	+	+
Acid production from											
meso-Erythritol	+	w	+	+	+	w	+	vw	vw	w	-
Maltose	-	w	-	-	+	-	-	vw	-	vw	-
Raffinose	+	+	w	w	w	+	+	+	+	w	vw
Growth on											
L-Sorbose	+	-	-	-	-	-	+	-	-	+	+
meso-Erythritol	+	-	w	+	w	-	+	+	vw	+	w
Raffinose	w	+	w	-	-	w	-	-	-	w	-
D-Arabitol	+	+	+	+	-	-	w	w	w	+	+
L-Arabitol	w	+	w	-	-	-	-	-	vw	-	-
meso-Ribitol	+	+	w	w	-	-	-	-	-	-	-
ITS gene restriction pattern with											
*Mbo*II	*Gf*	*Gf*	*Gf*	*Gc*	*Go*	*Ga*	*Gk*	*Go*	*Go*	*Go*	*Gw*
*Bsp*1286I	*Gf*	*Gf*	*Gf*	*Go*	*Go*	*Go*	*Gk*	*Gr*	*Go*	*Gk*	*Go*
DNA G+C (mol%)	56.4	55.1	55.8	55.9	60.3	60.0	59.8	60.5	59.5	59.5	56.6

Gj, *G. japonicus* NBRC 3271^T; *Gf*, *G. frateurii* NBRC 3264^T; *Gt*, *G. thailandicus* BCC 14116^T; *Gc*, *G. cerinus* NBRC 3267^T; *Go*, *G. oxydans* NBRC 14819^T; *Ga*, *G. albidus* NBRC 3250^T; *Gko*, *G. kondonii* NBRC 3266^T; *Gr*, *G. roseus* NBRC 3990^T; *Gs*, *G. sphaericus* NBRC 12467^T; *G.kn*, *G. kanchanaburiensis* BCC 15889^T; *Gw*, *G. wancherniae* BCC 15775^T.

+, positive; w, weakly positive; -, negative

Since 1984, Yamada and Akita studies forty-three strains of *Gluconobacter* species by used electrophoretic comparison of six-enzyme produced in this genus. The agarose gel electrophoretic pattern of enzymes presented to separate this genus into two groups, Group I and II. By the similarity value of relation between of two groups was 0 %. Group I described to a high G+C content of DNA ranging from 58.1 to 62.8 mol%. Group II included of organism with a distinctly lower G+C content of DNA ranging from 54.2-57.6 mol%. This study proposed a new species, *Gluconobacter cerinus*, sp. nov., nom. rev, classified into Group II. Subsequently, Yamada and Akita (1984) were examinated of DNA homology for twenty strains of *Gluconobacter* species with other genera relation. From this study, used for the characteristics for identification and classification in the current time.

Gluconobacter species have been isolated in Thailand; *G. thailandicus* were isolated from flower of Glossy ixora, flower of Barbados pride, flower of Indian cork tree and flower of China box tree (Tanasupawat *et al*., 2004); *G. kanchanaburiensis* were obtained from spoiled fruits of *Artocarpus heterophllus* (jackfruit) collected at Tong Pha Phum, Kanchanaburi, Thailand. (Malimas *et al*., 2009b); *G. wancherniae* were isolated from an unknown seed and an unknown yellow fruit collected in Sakaerat, Thailand (Yukphan *et al*., 2010).

2.2.3 *Acidomonas*

Acidomonas strains are facultative methylotrophic bacteria. They utilizes methanol, ethanol, acetic acid, D-Glucose, glycerol and pectin as carbon and energy source. Colonies characteristics are shiny, smooth surface, raised elevation, entire edge and white to pale-yellow colors. The major ubiquinone system is along between Q-10 with Q-9 and minor ubiquinone was Q-11 components (Yamada *et al*., 1968). They can not produce water-soluble pigment and unable to reduce nitrate to nitrite but produce ammonia. They can not produce dihydroxyacetone from glycerol. The DNA base composition range from 63.0-65.0 mol % of G+C content (Urakami *et al*., 1989). This genus comprise only one species, *Acidomonas methanolica* (Urakami *et al*., 1989).

2.2.4 *Gluconacetobacter*

Gluconacetobacter strains are ellipsoidal to rod and straight or slightly curved, 0.8-1.2 µm by 1.3-1.6 µm, non motile, occurring singly, in chains and mainly in pairs and non spore-forming. The major ubiquinone is Q-10. (Yamada and Kondo, 1984). They grow only on the acetic acid presented, ethanol and glucose medium by the concentration exceed about 6.0 %. They can oxidize acetic acid from ethanol but did not over-oxidization. They high

grow on glucose, fructose and sucrose presented in AE agar medium. Unassimilated gluconate, glycerol and lactate. They can not produce ketogluconic acid from glucose and can not detect formation cellulose on solid media or in broth. The DNA base composition rang from 55-63 mol % of G+C content.

The genus *Gluconacetobacter* comprises 16 species; *Ga. diazotrophicus*, *Ga. europaeus*, *Ga. hansenii*, *Ga. xylinus*, *Ga. liquefaciens*, *Ga. sacchari*, *Ga. intermedius*, *Ga. oboediens*, *Ga. entanii*, *Ga. azotocaptans*, *Ga. johannae, Ga. rhaeticus, Ga. swingsii, Ga. nataicola, Ga. saccharivorans,* and *Ga. kombuchae* (Dutta and Gachhui 2007).

Gluconacetobacter sp. was reported in Thailand; *Ga. liquefaciens*, the isolates were isolated from sugar cane juice, plam juice, and coconut juice (Seearunruangchai *et al*., 2004).

2.2.5 *Asaia*

Asaia strains are various characteristics such as white, yellowish, pale-pink to pink colors, shiny, smooth, and raised. They can oxidize acetate and lactate completely to carbon dioxide and water. The major ubiquinone is Q-10. They can not produce water-soluble brown pigment. Most member not produced acetic acid from ethanol. They can not grow in the present of 0.35% of acetic acid and on methanol. They can produces 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose but can not produce 2,5-diketo-D-gluconate. The DNA base composition range from 59.3-61.0 mol% of G+C content. Nowadays, genus *Asaia* comprises 4 species; *As. bogorensis*; *As. siamensis*; *As. krungthepensis* and *As. lannensis* (Malimas *et al*., 2008b).

Asaia species have been isolated in Thailand; *As. siamensis* was isolated from a flower of crown flower (Katsura *et al*., 2001), *As. krungthepensis* was isolated from heliconia flowers collected in Bangkok, Thailand (Yukphan *et al*., 2004), *As. lannensis* was isolated from flowers of the spider lily collected in Chiang Mai, Thailand (Malimas *et al*., 2008a).

Table 2.4 Differential characteristics of *Asaia* species (Malimas *et al*., 2008a)

Characteristic	*As. bogorensis* BCC 12264^T	*As. siamensis* BCC 12268^T	*As. krungthepensis* BCC 12978^T	*As.lannensis* BCC 15733^T
Oxidation of acetate and lactate	w	w	w	w
Production of acetic aicd from ethanol on ethanol/$CaCO_3$	-	-	-	+
Dihydroxyacetone production from glycerol	w	w	+	w
Acid production from				
Dulcitol	w	-	+	w
L-Arabitol	+	+	+	w
Ethanol	+	w	-	w
Maltose	-	-	w	-
16S rRNA gene restriction group	A	B	C	F
DNA G+C content (mol%)	60.2	59.3	60.3	60.8

+, positive; w, weakly positive; -, negative

2.2.6 *Kozakia*

The *Kozakia* species differentiated from other members of *Acetobacteraceae* as the capable production levan-like mucous substance from sucrose or D-fructose. They are non motile. The major ubiquinone is Q-10. They can not produce gelatinase, hydrogen sulfide, indole or ammonia from L-arginine and no grow on 30 % of D-glucose. They are weakly activity when oxidize acetate and lactate to carbon dioxide and water and production acetic acid from ethanol. They can grow on mannitol agar but not on glutamate and on 30% of D-glucose. The DNA base composition range from 56.8-57.2 mol% of G+C content (Lisdiyanti *et al*., 2002). This genus has only one species; *K. baliensis* (Lisdiyanti *et al*., 2002).

K. baliensis was reported in Thailand by Kommanee *et al* (2008), the two isolates CT8-1 and CT8-2 showed the same phenotypic feature as the type strain of *K. baliensis*. However, the isolation sources of the two isolates were quite different from those of the four strains isolated in Indonesia (Lisdiyanti *et al*., 2002). The former was from fruit of sapodilla collected in Thailand however, the latter was either palm brown sugar or ragi (starter) collected in Bali or Java, Indonesia.

2.2.7 *Swaminathania*

Swaminathania has straight rods with round ends, approximately 0.7–0.9 x 1.9–3.1 mm, possesses peritrichous flagella, oxidase-negative and catalase-positive. They can oxidize ethanol to acetic acid in neutral and acid conditions. They can oxidize acetate and lactate.

They can able to produce water-soluble brown pigments on GYC agar medium. They do not hydrolyse gelatin and starch. They grows well in the presence of 0.35% acetic acid, 3% NaCl and 1% KNO_3. They can able to fix nitrogen and solubilize phosphate. DNA G+C content is 57.6–59.9 mol% and the major quinone is Q-10. This genus has only one species; *Swaminathania salitolerans* (Logonathan and Nair. 2004).

Sw. salitolerans was reported in Thailand by Kommanee *et al* (2008), the isolation sources of the two isolates SI15-1 and SI15-2 were quite different from those of the two strains isolated at Pichavaram, Tamil Nadu, India (Logonathan and Nair. 2004). The former was from ixora collected in Thailand, however, the latter was mangrove-associated wild rice collected at Pichavaram, Tamil Nadu, India. Accordingly, it is of interest that the two strains identified as *Sw. salitolerans* were isolated from seeds of ixora, a kind of flowers other than materials such as mangrove-associated wild rice.

2.2.8 *Saccharibacter*

Cells are Gram-negative, non-motile, straight rods measuring 0.8–1.0 x 2.5–4.0 µm. Non-pigmented. They produces neither cellulosic pellicles nor water-soluble mucous substances. The optimum pH for growth is around 5.0–7.0; no growth above pH 8.0 or below pH 4.0. They can not assimilate ammonia on Hoyer–Frateur medium with glucose, mannitol or ethanol. They can not utilize methanol and can not produce dihydroxyacetone from glycerol. The DNA G+C content is about 52–53 mol%. The major quinone type is Q-10. This genus has only one specie; *Saccharibacter floricola*. (Jojima *et al*., 2004)

2.2.9 *Neoasaia*

During the course of studies on microbial diversity of AAB in Thailand, Yukphan *et al* (2005b), proposed *Neoasaia chiangmaiensis* gen. nov., sp. nov. in the family *Acetobacteraceae*, which was isolated from a flower of red ginger (khing daeng in Thai; *Alpinia purpurata*) collected in Chiang Mai, Thailand. The isolated an osmotolerant AAB, colonies were pink, shiny, smooth, raised, and with an entire margin. They oxidized acetate and lactate. They produced acetic acid from ethanol, dihydroxyacetone from glycerol and 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose. They grew on glutamate agar, mannitol agar, 30% D-glucose and in the presence of 0.35% acetic acid but did not grow in the presence of 1.0% KNO_3. They did not produce either a water-soluble brown pigment on a glucose/peptone/yeast extract/$CaCO_3$ medium or a levan-like polysaccharide on a sucrose medium. DNA base composition was 63.1 mol% G+C. This genus has only one specie*; Ne. chiangmaiensis* (Yukphan *et al*., 2005).

2.2.10 *Granulibacter*

Cells are non-motile and coccobacillus to rod-shaped. They produce a yellow pigment. They have optimum temperature for growth is 35–37 °C, optimum pH for growth is 5.0–6.5. They can oxidize lactate and acetate to carbon dioxide and water, but the activity of the latter is weak. They can produce little acetic acid from ethanol but can not produce dihydroxyacetone from glycerol. They can use methanol as a sole carbon source. They grow on glutamate agar and on mannitol agar. DNA base composition is 59.1 mol% G+C. This genus has only one specie; *Granulibacter bethesdensis* (Greenberg *et al*., 2006).

2.2.11 *Tanticharoenia*

During the course of studies on microbial diversity in the natural environment of Thailand, Yukphan *et al*. (2008), proposed three strains, AC37^T, AC38, and AC39, as *Tanticharoenia sakaeratensis* gen. nov., sp. nov. in the family *Acetobacteraceae.* They were isolated from soil collected at Sakaerat, Nakhon Ratchasima, Thailand. Strains were rods, non-motile, measuring 0.6-0.8 x 1.0-1.6 µm. Colonies were creamy and smooth with entire margin when grown on glucose/ethanol/peptone/yeast extract/calcium carbonate agar. They did not oxidize acetate or lactate. They produced acetic acid from ethanol, dihydroxyacetone from glycerol and produced a water-soluble brown pigment intensely on glucose/peptone/yeast extract/calcium carbonate agar. They grew on glutamate agar (weakly positive), mannitol agar, 30% D-glucose but did not grow on methanol. They grew in the presence of 0.35% acetic acid but not of 1% KNO_3. The major quinone was Q-10. DNA base composition is 64.5–65.6 mol%. This genus has only one specie; *Tanticharoenia sakaeratensis* (Yukphan *et al*., 2008).

2.2.12 *Ameyamaea*

Cells are Gram-negative, motile with polar flagella, straight rods measuring 0.6–0.8 x 1.0–1.8 µm. Colonies are creamy and smooth with entire margin when grown on glucose/ethanol/peptone/yeast extract/calcium carbonate agar. They show intensely oxidize acetate to carbon dioxide and water but weakly oxidize lactate. They produced acetic acid from ethanol, dihydroxyacetone from glycerol and produced a water-soluble brown pigment intensely on glucose/peptone/yeast extract/calcium carbonate agar. They grow on glutamate agar (weakly positive), mannitol agar but can not grow on 30% D-glucose and in methanol. They grow in the presence of 0.35% acetic acid. The major quinone is Q-10. DNA base composition is 66.0–66.1 mol%. This genus has only one specie; *Ameyamaea chiangmaiensis* (Yukphan *et al*., 2009).

2.3 Molecular analysis for taxonomy of acetic acid bacteria

2.3.1 DNA sequencing analysis

Ribosomal RNA represents only a small part (about 0.3-0.4%) of the genome and the cistrons coding for it are highly conserved. They have evolved less rapidly than the rest of the chromosome. The three rDNAs in bacteria are classified by their sedimentation rates as 23S, 16S, and 5S, which have chain lengths of about 3,300, 1,600, and 120 nucleotides, respectively (Stent, 1981). Bacterial 16S rDNA sequences are attractive targets for developing identification methods because they represent conserved regions in all bacteria and species having 70% or greater DNA similarity usually have more than 97% sequence identity (Stackebrandt and Goebel, 1994, Stackebrandt *et al*., 2002). But this recommendation has been questioned (Fox *et al*., 1992). Recently, it has been suggested that a difference rate of >0.5% could be considered indicative of a new species within a known genus (Palys *et al*., 1997). In the present study, in the absence of an accepted cut-off value, which 99% similarity as a suitable cutoff for identification at the species level and 97% similarity as a suitable cutoff for identification at the genus level (Drancourt *et al*., 2000).

Automated DNA Sequencing; one of the major advances in sequencing technology in recent years is the development of automated DNA sequencers. There are based on the chain termination method and use of fluorescent labels. The fluorescent dyes can be attached to the sequencing primer, to the dNTPs or the terminators and are incorporated into the DNA chain during strand synthesis reaction mediated by DNA polymerase. During the electrophoresis of the newly generated DNA fragments on a polyacrylamide gel, a laser beam excites the fluorescent dyes. Detectors collect the emitted fluorescence and the information analyzed by the computer. The data are automatically converted to nucleotide sequence (Fig. 2.1). Several automated DNA sequencers are now commercially available and are becoming increasingly popular.

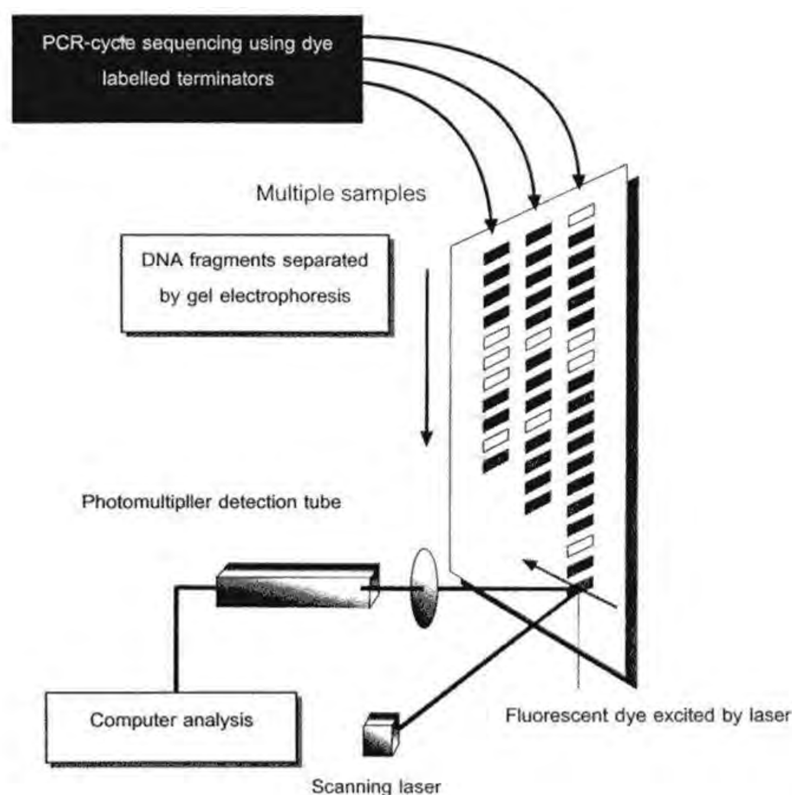


Fig. 2.1 Automated DNA sequencer

2.3.1.1 Analysis of nucleotide sequence and phylogenetic tree

Analysis of nucleotide sequence

a. Homology search: The most frequent analysis of an unknown sequence is to perform homology search, which is a search for sequence similarity with known sequence in the database. There are three international DNA databases available that provide sequence information as no cost over the internet (Table 2.5). All databases exchange their data daily to update the sequence information.

Table 2.5 Lists of international primary sequence databases.

Database	Sponsor	Location (URL)
GenBank	National Center for Biotechnology in formation	http://www.ncbi.nlm.nih.gov
EMBL Data Library	European Molecular Biology Laboratory	http://www.ebi.ac.uk/embl.html
DNA Data Bank of Japan (DDBJ)	National Institute of Genetics	http://www.ddbj.nig.ac.jp

b. Phylogenetic analysis: phylogenetic analysis is the technique of methodically demonstrating a family relationship between species. The sequences are pairwisely compared by BLAST Homology Search (http://www.ncbi.nlm.nih.gov/). Multiple alignments of the sequences were carried out with the program CLUSTAL X (Version 1.81) (Thompson *et al*., 1997). Distance matrices for the aligned sequences were calculated by using the two-parameter method of Kimura (1980) The neighbour-jointing (NJ) method was used to constructed a phylogenetic tree (Saitou & Nei, 1987). Sites where gaps existed in any sequences are excluded. Bootstrap analysis is performed from 1,000 random re-samplings (Felsenstien, 1985).

2.3.2 Restriction fragment length polymorphism (RFLP) analysis

RFLP analysis is method for identification and typing of microorganism by used restriction enzymes are specific locus sites to cutting nucleotide fragment for differently DNA profiles on electrophoresis. PCR-RFLP method are initialed by amplification of target DNAs with *Taq* polymerase and oligonucleotide primers at the specific locus sites thus as 16S, 23S and 16S-23S rDNA spacer regions (ITS). After amplified DNAs following to cleaving PCR products by restriction enzyme and examine DNA fragment by agarose gel electrophoresis. This method is application for identify microorganism and useful for DNA genomic fingerprinting in plant identifications (Olive and Bean, 1999; Griffiths *et al*., 2000).

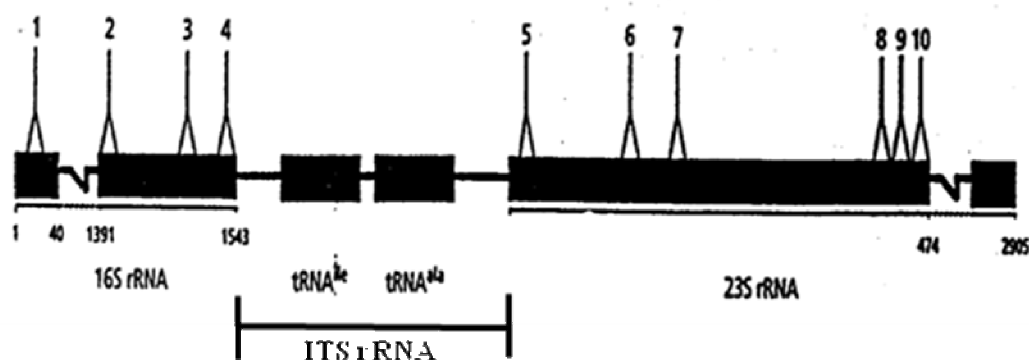


Fig. 2.2 The 16S-23S rDNA internal transcribed spacer regions (ITS)
(Gürtle and Stanisich, 1996)

The ribosomal operon is a classic marker used to trace genetic relationships and to identify strains rapidly. Of all the different regions of the ribosomal operon, the ITS region between 16S and 23S ribosomal DNA are frequently used as molecular markers to identify microbial species and analyze the phylogenetic relationship between strains. The ITS regions are generally found in multiple copies in most bacterial genomes. Since ITS regions are hypervariable with respect to adjacent genes, due to a higher mutation rate, they can differentiate between multiple operons in the cell. The ITS contains genes for encode 0, 1, or 2 tRNA such as $tRNA^{Ile}$ and $tRNA^{Ale}$ addition to the antitermination box B-box A motifs which prevent premature termination of transcription and also have a role in holding in secondary structure of the nascent rRNA for processing to mature rRNAs (Trček and Teuber, 2002).

Trček and Teuber (2002) reported the genetic and restriction analysis of the ITS gene of the AAB. Fifty-seven strains were amplified the ITS regions and digested with *Hae*III and *Hpa*II. The results of these enzymes affected belong to 12 distinct groups of restriction types. All the restriction profiles obtained after analysis of microbial populations from vinegar matched one of the 12 groups.

Yukphan *et al*. (2004a), examined new approach for identification of *Gluconobaceter asai* 1935 based on ITS regions restriction and sequence analysis. This study included thirteen reference strains such as the type strains of the *G. oxydans* NBRC 14819^T, *G. cerinus* NBRC 3267^T, *G. asaii* NBRC 3276^T, and *G. frateurii* (IFO 3264^T). Several restriction endonucleases discriminating the three species from one another were selected by computer analyses such as *Bsp*1286I, *Mbo*II, *Sap*I, *Bpu*10I, *Ear*I, *Bsi*HKAI and *Fat*I. On digestion of the PCR products with restriction endonucleases *Bsp*1286I and *Mbo*II, all the restriction patterns coincided with those of the type strains of the three species except for strain NBRC 3251. This strain gave a different pattern from *G. frateurii*, when digested with *Mbo*II. However, they proposed strain 3251 was included phylogenetically in the *G. frateurii* cluster. In addition, the results of this study showed ability to identify the *Gluconobacter* species at the species level.

Yukphan *et al*. (2005), proposed *Neoasaia chiangmaiensis* gen. nov., sp. nov. in the family *Acetobacteraceae* based on phenotypic, biochemical characteristic and genetic analysis. In the RFLP analysis, when ITS region PCR products of isolate $AC28^T$ for 769 bp were digested with the four restriction endonucleases (*Tsp*509I, *Mbo*II, *Bcc*I and *Hin*6I,), the resulting restriction fragments by digestion with *Tsp*509I and *Mbo*II, the type strains of the three *Asaia* species gave identical restriction patterns, designated as the *As. bogorensis* type of patterns. Other restriction patterns were designated as the *Ne. chiangmaiensis* type of patterns and the *K. baliensis* type of patterns. By digestion with *Bcc*I and *Hin*6I, respective restriction

patterns were given, designated as the *As. bogorensis* type of patterns, the *As. siamensis* type of patterns, the *As. krungthepensis* type of patterns, the *K. baliensis* type of patterns, and the *Ne. chiangmaiensis* type of patterns. The restriction data obtained above differentiated isolate $AC28^T$ molecular biologically from the type strains of *As. bogorensis*, *As. siamensis*, *As. krungthepensis*, and *K. baliensis*.

Malimas *et al*. (2008a), proposedd new specie; *As. lannensis* based on phenotypic, chemotypic including sequencing and RFLP analysis. The 16S rDNA PCR products of the two isolates, $AB92^T$ and AB93, were analyzed by digestion with four restriction endonucleases, *Sty*I, *Bsa*JI, *Sna*BI, and *Hpa*II. The two isolates were practically discriminated from the type strains of *As. siamensis* and *As. krungthepensis* by the absence of a 214-bp fragment in *Sty*I digestion; the type strain of *As. siamensis* by the absence of a 327-bp fragment in *Bsa*JI digestion; the type strain of *As. krungthepensis* by the absence of both 850-bp and 561-bp fragments in *Sna*BI digestion; and the type strains of *As. bogorensis* and *As. siamensis* by the presence of a 311-bp fragment in *Hpa*II digestion.

Malimas *et al*. (2009b) proposed new species; *G. kanchanaburiensis*. The two isolates, $AD92^T$ and AD93 in *G. kanchanaburiensis* isolated from spoiled fruits of *Artocarpus hetterophyllus* (jackfruit). They were identified based on phenotypic, chemotaxonomic and sequencing including RFLP-ITS analyses. Several restriction endonucleases *Bsp*1286I, *Mbo*II, *Bst*NI, *Bsa*JI and *Bso*BI discriminating $AD92^T$ and AD93 isolates from one another.

2.3.3 DNA-DNA hybridization

The DNA-DNA hybridization technique is the hybridization of the total genomic DNA of one organism to that of another. The relatedness of strains can be expressed as a percent homology. Strains related at the species level should show homology in the 60 % to 90 % range, whereas strains with increasing taxonomic divergence show progressively less homology. These finding are now a major factor in decisions on the taxonomic classification of all microorganism, allowing species, genus, and higher taxonomic groupings to be assessed by means that are not subject to the phenotypic variation inherent with classical methods (Ryan, 1994).

Bacterial DNA-DNA homology was done by the hybridization of the total genomic DNA of one strain to that of another. The relatedness of strains can be expressed as a percent homology. Strains related at the species level should show homology over 70% (Wayne *et al*., 1987), whereas strains with increasing taxonomic divergence show progressively less homology. These finding were now a major factor in decisions on the taxonomic classification of all microorganism, allowing species, genus, and higher taxonomic groupings

to be assessed by means that were not subject to the phenotypic variation inherent with classical methods

2.4 Oxidative fermentation of acetic acid bacteria

AAB well known as vinegar producers and also known to be able to oxidize various sugars and sugar alcohols such as D-glucose, glycerol, D-sorbitol, and so on, in addition to ethanol (Fig. 2.3). Such oxidation reactions are called oxidative fermentation, since they involve incomplete oxidations of such alcohols or sugars accompanied by an accumulation of the corresponding oxidation products in large amounts in the culture medium.

Of the two genera of AAB, *Gluconobacter* species extensively catalyze the oxidation of sugars and sugar alcohols except for ethanol, while *Acetobacter* species have a high ability to oxidize ethanol to acetic acid. These oxidation reactions of sugars or sugar alcohols seem to be carried out by membrane-bound dehydrogenases linked to the respiratory chain located in the cytoplasmic membrane of the organism (Matsushita *et al*., 1994).

The membrane-bound dehydrogenases can be divided into quinoproteins and flavoproteins that have pyrroloquinoline (PQQ) and covalently-bound flavin adenine dinucleotide (FAD) as prosthetic groups, respectively. These quinoproteins and flavoproteins dehydrogenases have been shown to function by linking to the respiratory chain which transfers electrons to the final electron acceptor, oxygen, and generating energy for growth. In addition, the enzymes are involved in production of the oxidation products, carboxylic acids and sugar acids, in the culture medium. Thus, AAB are also important for the fermentation industries to produce biomaterials such as vinegar, L-sorbose, dihydroxyacetone, ketogluconic acid, L-erythrulose (Saeki *et al*., 1997; Moonmangmee *et al*., 2000; Hekmat *et al*., 2003; Matsushita *et al*., 2003; Moonmangmee *et al*., 2002).

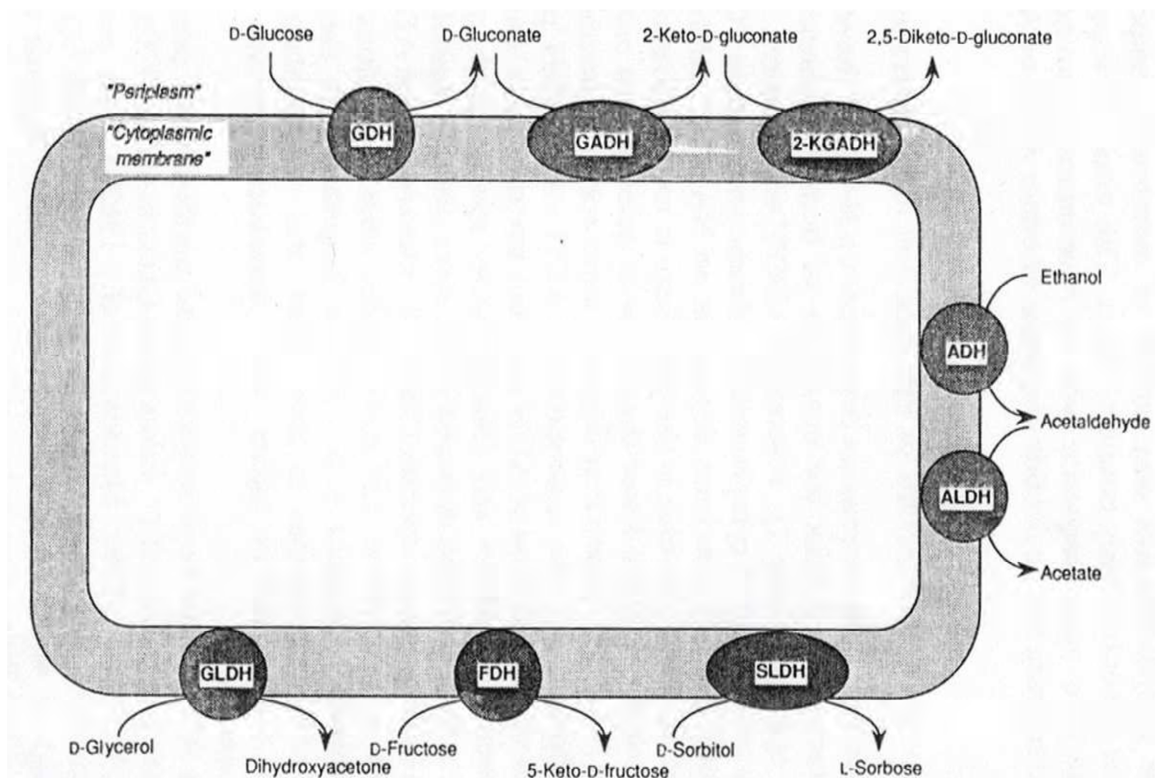


Fig. 2.3 Primary dehydrogenases in alcohol- and sugar-oxidizing system of AAB (Matsushita *et al*., 1994)

2.4.1 Acetic acid production

Acetic acid produced from a wide variety of substances, the main requirement is sufficient alcohol or sugar from which alcohol can be derived with a satisfactory flavoring substance. Louis Pasteur (1862) was the first who discovered the tiny creature, the vinegar bacteria that convert alcohol into acetic acid. *Acetobacter* strains were frequently used for the industrial vinegar (Saeki *et al*., 1997).

Vinegar is an acidic liquid processed from the fermentation of ethanol in a process that yields its key ingredient, acetic acid (ethanoic acid). Commercial vinegar is produced from 2 fermentation processes, 1) slow methods are traditional vinegars and fermentation proceeds (Orleans process), this method show slowly over the course of weeks or months in vinegar production, 2) fast methods will add mother of vinegar (i.e. bacterial culture) to the source liquid and incubated in fermenter, called "Frings generator". Mother of vinegar is *Acetobacter*, their catalyses the biological oxidation of ethanol in acetic acid by the association of two enzymes (primary dehydrogenases) membrane bound: a quinoprotein ADH and ALDH (Fig. 2.3) (Matsushita *et al*., 1994).

Ethanol oxidation by AAB takes place in two steps. In the first one, ethanol is oxidized to acetaldehyde and in the second step acetaldehyde is oxidized into acetate. In both reactions, electrons are transferred and these are later accepted by oxygen (Adachi *et al.*, 1978). The ADH consists of two or three subunits, which include the dehydrogenase (72-78 kDa) and cytochrome *c* (48 kDa) that are essential for the activity of the enzyme. The third subunit is a protein of 20 kDa, found in *A. aceti* and *A. pasteurianus*. The two larger subunits play a role in the intramolecular transport of electrons from the ADH to ubiquinone and further to the terminal cytochrome oxidase during the oxidation of ethanol. The smallest one helps the two functional subunits with their association to the membrane (Saeki *et al.*, 1997).

This membrane-bound alcohol dehydrogenase has pyrroloquinoline as a cofactor and is independent of $NADP^+$. A cytoplasmatic $NADP^+$- dependent alcohol dehydrogenase has also been identified, although it has a much lower specific activity than the membrane-bound alcohol dehydrogenase and a higher optimal pH (6-8), which limits its contribution to the oxidation process of ethanol (Adachi *et al.*, 1978; Matsushita *et al.*, 1994). The $NADP^+$ independent enzyme has an optimal pH of 4, but it is still active at lower pH levels. The ADH activity of *Acetobacter* is more stable under acetic conditions than that of *Gluconobacter*, which explains why *Acetobacter* produces more acetic acid (Matsushita *et al.*, 1994).

The other enzyme involved in the oxidation of ethanol is aldehyde dehydrogenase. It is also a $NADP^+$ independent enzyme and located in the cytoplasmatic membrane. Its optimum pH is between 4 and 5, although it can catalyse the oxidation of acetaldehyde to acetate at lower pH values (Adachi *et al.*, 1980). It is an enzyme that is sensitive to oxygen concentrations, and when these are low its activity decreases, accumulating acetaldehyde. It is also more sensitive to the presence of ethanol than ADH.

These bacteria can produce high concentrations of acetic acid, up to 150 g/l (Sievers *et al.*, 1997; Lu *et al.*, 1999), which makes them very important to the vinegar industry. The enzyme citrate synthase plays a key role in this resistance, because it detoxicates acetic acid by incorporation into the tricarboxylic or glyoxylate cycles, but only when ethanol is not present in the media. Menzel and Gottschalk (1985) reported that *Acetobacter* strains decrease their internal pH in response to a lower external pH. However, an adaptation to high acetate concentrations seems to be a prerequisite for high tolerance (Lasko *et al.*, 2000).

Vinegar fermentation by AAB is usually carried out at 30°C and strict, temperature control is necessary irrespective of whether the culture is static or submerged. A temperature increase of 2-3°C causes a serious problem in both the fermentation rate and fermentation efficiency. Therefore, if favorable strains of AAB that can work optimally at 37-40°C were available, the cooling expensive would be reduced greatly.

Saeki *et al*. (1997) reported a thermotolerant AAB that were collected from Thailand, *A. rancens* subsp. *pasteurianus*, *A. lonaniensis* subsp. *lovaniensis*, *A. aceti* subsp. *liquefaciens*, and *A. xylinum* subsp. *xylinum*. These isolates were show rapid ethanol oxidation, rapid acetate oxidation, growth at 40°C, growth in 3% acetic acid, growth in 8% ethanol, formation of thermotolerant alcohol and aldehyde dehydrogenase. They produced acetic acid at high temperature, 38 to 40°C and they still oxidized ethanol to acetic acid when initially acetic acid added to 4%. They oxidized higher concentrations of ethanol up to 9% without any lag phase. Vinegar production with thermotolerant strains at 38 to 40°C was almost the same as mesophilic strains at 30°C.

Lu *et al*. (1999) reported a thermotolerant bacterium with high production of acetic acid was isolated from spoiled from banana in Taiwan. The isolate, I14-2 was identified as *Acetobacter* sp. Cultural conditions to produce acetic acid were studied under cultivation in a medium containing 2 mg/l acetic acid and 5% ethanol at 37°C, I14-2 can produced 41 g/l of acetic acid after 3 days incubation. This bacterium is thermotolerant and retained 68% of acetic acid-producing activity compare with that when incubated at 30°C

Ndoye *et al*. (2006) isolated AAB from mango and cereals. Two isolates, CWBI-B418 and CWBIB419 were selected for their ability of growth and acetate production at higher temperatures. They have confirmed as *A. tropicalis* and *A. pasteurianus*, respectively. In another hand, their ability of growth and acetification with those of the wild-type strain *A. pasteurianus* LMG 1625^T showed that CWBI-B418 and CWBI-B419 strains developed an optimum of growth without any appreciable lag phase and a high level of acetic acid production at 35 and 38°C, respectively. The two strains exhibited thermoresistance and acidoresistance property. The measure of ADH and ALDH activity realized on a range of temperatures from 25 to 40°C, showed an increasing denaturation more marked in the wild type strain. The inactivation constant of the enzymes ADH and ALDH of these strains was indeed much lower than that of the wild strain. By taking into account of all these physiological potentialities, the use of the CWBI-B418 and CWBI-B419 strains in industrial vinegar production in Sub-Saharan Africa would make it possible to reduce in a considerable way the cooling water expenses.

2.4.2 Dihydroxyacetone (DHA) production

DHA is the oxidation product of glycerol (Salusjärvi *et al*., 2004) and is industrially produced by *G. oxydans*. This product is commonly used as a tanning agent in the cosmetics industry. Moreover, DHA constitutes a building block for several chemical compounds, e.g., methotrexate, which is used in the chemotherapeutical treatment of cancer patients (Claret *et al*., 1994). *G. oxydans* has been most extensively used in industrial processes to produce DHA (Deppenmeier *et al*., 2002). Yields of around 90% DHA (in g/g glycerol) have been reported; the highest initial substrate concentration tested was 200 g/l glycerol (Claret *et al*., 1994).

Cornelia *et al* (2007), studied biotransformation of glycerol to dihydroxyacetone by recombinant *G. oxydans* DSM 2343. They demonstrated the impact of *sld*AB over-expression on glycerol oxidation: Beside a beneficial effect on the transcript level of the *sld*B gene, the growth on glycerol as a carbon source was significantly improved in the over-expression strains compared to the control strains. Furthermore, the DHA formation rate, as well as the final DHA concentration, was affected so that up to 350 mM of DHA was accumulated by the over-expression strains when 550 mM glycerol was supplied (control strain: 200 to 280 mM DHA). Finally, they investigated the effect on *sld*AB over-expression on the *G. oxydans* transcriptome and identified two genes involved in glycerol metabolism, as well as a regulator of the LysR family.

DHA production was improved by genetic engineering (Gatgens *et al*., 2007). The final DHA concentration by *G. oxydans* in shake-flask experiments reached 30 g/l while the wild type produced 18–25 g/l of DHA from 50 g/l glycerol.

2.4.3 L-Sorbose production

L-Sorbose has been used as an intermediate for synthesis of vitamin C following the method of Reichstein Grussner (Hancock and Viola., 2002). L-Sorbose is produced from D-sorbitol by *Gluconobacter suboxydan* (Kim *et al*., 1999). Since D-sorbitol is oxidized to L-sorbose by D-sorbitol dehydrogenase, which is bound to the cell membrane, the oxygen demand is relatively large (Shinnagawa., 1982). D-Sorbose is the oxidation product of D-sorbitol, produced by the action of membrane bound SDH (Shingawa *et al*., 1982). L-Sorbose is further converted to sorbosone by SNDH (Hoshino *et al*., 1990, 1991).

Shinagawa *et al*. (1982) described the solubilization, purification and characterization of D-sorbitol dehydrogenase from the membrane fraction of *G. suboxydans* var. IFO3254. The production of L-sorbose by cells of *G. suboxydans* ATCC621 immobilized in polyacrylamide gel has been carried out in a continuous process. The entrapped cells almost completely converted D-sorbitol into L-sorbose at a rate of about 7 $kg/m^3/h$ over a long period of time (Stefanova et al., 1987).

Rosenberg *et al*. (1993) studied the parameters influencing the conversion of D-sorbitol to L-sorbose by stationary phase cultures of an industrial strain of *G. oxydans* from Farmakon, Czechoslovakia. The D-sorbitol concentration influenced the O_2 consumption rate according to Michaelis Menton Kinetics. D-sorbitol at 20-200 g/l had no inhibitory effect. However, high concentration of L-sorbose inhibited the O_2 consumption rate. Optimum D-sorbitol conversion occurred at pH 5.0 and 35-40°C. L-sorbose formation occurred in a batch culture lasting 34 h (sorbitol concentration 200 g/l), and the yield was 96%. In the fed-batch cultures (30°C, 1 vvm aeration, 0.1 mM pressure, 5% vol. inoculum) having a D-sorbitol concentration of 410g/l, the yield was found to be 92%.

Kim *et al*. (1999), report conversion of D-sorbitol to L-sorbose by immobilized *Gluconobacter suboxydans* cells, they found that L-sorbose yield was increased when bead size was reduced and holes were made in the beads as a means of reducing the thickness of the oxygen diffusion layer.

Girdhar and Srivastava (2000) cultivated *G. oxydans* NRRL B72 in fed-batch fermentation using an initial sorbitol concentration of 200 g/l and demonstrated a productivity of 17.7 g/l/h.

Moonmangmee *et al*. (2000) isolated and screened thermotolerant *Gluconobacter* for D-fructose and L-sorbose production. In the part of L-sorbose production, fermentation efficiency and fermentation rate of the strain CHM54 was quite high and rapidly oxidized D-sorbitol to L-sorbose at almose 100% within 24 h at 30°C. The efficiency of L-sorbose fermentation by the strain CHM54 at 37°C was superior to that observed at 30°C.

CHAPTER III

EXPERIMENTAL

Instruments, materials, chemical reagents and media

Name list of all Instruments, materials, chemical reagents and media were shown in Appendix A, C and D.

Methods

3.1 Sample collection and isolation of acetic acid bacteria

AAB were isolated from 30 fruits, 10 flowers, 1 fermented starch, 1 khao-mak and 1 seed-ixora collected in Thailand. The sample was cut into small pieces and placed in 10 ml of 4 enrichment culture approach using 1) Glucose-ethanol medium, 2) Sorbitol medium, 3) Sucrose-acetic acid medium and 4) Methanol-peptone-yeast extract medium (Appendix A) in 25x250 mm test tube and were incubated on a rotary shaker at 200 rpm, 30°C for 5 days and then the cultures were streaked onto GEY-$CaCO_3$ agar plates (Appendix A) and incubated at 30°C for 5 days. (Yamada *et al*., 1976, 1999).

The bacteria that formed clear zones around colonies on the agar plates were selected as acid-producing bacterial strains. They were streaked for purification on GEY-$CaCO_3$ agar plate and were preserved mainly at -4°C. Twenty % of Glycerol in Glucose- Peptone-Yeast extract was used as a cryoprotectant.

3.2 Identification methods

3.2.1 Cell morphology and cultural characteristics

The colonies grown on GEY-$CaCO_3$ agar medium at 30°C for 2 days were examined for their cell shape and colonial appearance, motility, and pigmentation as described by Barrow and Feltham (1993).

3.2.1.1 Gram staining: Thin smear of bacterial colony on a clean slide was fixed by passing through flame and stained with Gram's crystal violet for 30 sec, rinsed with water, followed by covering with Gram's iodine solution for 30 sec then rinsed with water, decolorized with 95% (v/v) ethanol and washed with water. Then, the smear was countered stained with safranin for 30 sec, blot dried and examined under microscope at 1000X magnification.

3.2.1.2 Flagella staining: Standard microscopic slides, precleaned by the manufacturer, were used. The slide briefly flamed and drawn a thick line with a wax pencil

across its width to confine a stain to be two-thirds of the slide surface. Three drops of sterile distilled water was added to this area and gently mixed with cells. There was no visible opalescence. The suspension was smeared over the staining area and then tapped off onto a disinfectant-soaked gauze sponge, and air dried on a level surface. Staining method by Forbes (1981), staining (Appendix A) was timed for 1 min with 1 ml of stain at ambient temperature. The slide was washed in tap water, counterstained with the Hucker and Conn (1923) modification of Gram crystal violet for 1 min, washed, blotted, and examined under oil immersion starting near the wax line.

3.2.2 Physiological and biochemical characteristics

3.2.2.1 Oxidation/Fermentation catabolism test: The isolates were inoculated in Hugh and Leifson's medium (Appendix A) by stabbing with a straight needle. After inoculation, overley the medium in only one tube with approximately 2 ml of sterile liquid paraffin. The incubation was done at 30°C. Interpret the results as follow, if only the aerobic tube is acidified, the organism catabolizes the carbohydrate by oxidation. If both the aerobic and anaerobic tubes are acidified, the organism is capable of fermentation. If neither tube becomes acidified, the organism is unable to catabolized the carbohydrate.

3.2.2.2 Catalase test: A small amount of pure growth was transferred from agar onto the slide. Hydrogen peroxide (H_2O_2) 3% (v/v) was immediately placed onto a portion of a colony on the slide. The evolution of gas bubbles indicating a positive test was observed.

3.2.2.3 Growth at different pH: The isolates were suspended in 0.85% NaCl and inoculated into the different pH medium (Appendix A). The culture was incubated with out shaking for 7 days at 30°C. The pH was tested in this study are followed: pH 3.0, 3.5, 4.0, 4.5 and 5.0.

3.2.2.4 Oxidation of acetate and lactate: The isolates were inoculated into oxidation of acetate and lactate test medium (Appendix A). The cultures were incubated without shaking for 7 days at 30°C. A positive was indicated by blue color whereas a negative was by yellow color.

3.2.2.5 Growth in the media containing 30% D-glucose: The isolates were inoculated into the medium containing 30% D-glucose (Appendix A). The cultures were incubated without shaking for 7 days at 30°C.

3.2.2.6 Formation of water-soluble brown pigment: The isolates were inoculated on the GEY-$CaCO_3$ agar medium. After incubating at 30°C for 7-15 days, water soluble brown to dark brown pigments were observed.

3.2.2.7 Dihydroxyacetone from glycerol: Cell were inoculated on the Glucose-Glycerol-Yeast extract (GGY) medium (Appendix A) and incubated at 37°C for 5 days, then flooded with Fehling's solution (Appendix A). Yellow or yellow orange colonial appearance indicated a positive test.

3.2.2.8 Growth and acid production from different kind of carbohydrates: The isolates were suspended in 0.85% NaCl and inoculated into the growth and acid production test medium (Appendix A). The culture were incubated without shaking for 7 days at 30°C, and observed the results everyday. The carbon sources used in this study were as followed: D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, D-xylose, D-arabinose, L-arabinose, L-rhamnose, D-mannitol, D-sorbitol, dulcitol, *meso*-erythritol, glycerol, maltose, lactose, melibiose, sucrose, raffinose and ethanol.

3.2.2.9 Production of ketogluconate from D-glucose: The isolates were inoculated into 5 ml medium (appendix A) and shaken at 30°C for 14 days. The cell suspensions were centrifuged and 10 µl supernatant was spotted on microcrystalline cellulose plates (TLC aluminium sheets 20 x 20 cm, CelluloseF). Between each elution, the plates were dried completely in a cold air flow. The plates were sprayed with detection reagent (appendix A) and dried at 105°C. The results of different colour were read under UV light: glucose produces a brown-red colour, 2-ketogluconic acid produces blue-fluorescent colour, 5-ketogluconic acid produces green-colour and 2,5-diketogluconic acid produces yellow-fluorescent colour.

3.2.2.10 Transmission electron microscope: Cells were coated with copper grids and were stained by submerging the grids in 2% (w/v) uranyl acetate for 1 min. The grids were rinsed with distilled water (Dijkstra and De Jager, 1998) and examined with a TEM (JSM-1230, JEOL LTD., Japan).

3.2.3 Chemotaxonomic characteristics

3.2.3.1 Ubiquinone analysis: The representative strians of each group were extracted and quanlitative determined. The isolates were cultured in GEY medium (Appendix A) with shaking condition at 30°C for 48 h. Cells were collected by centrifugation and approximately 1 g wet cell were suspended in 100 ml of absolute ethanol: diethylether (1:3) and shaked for 20 min. After filtration, the extract was completely evaporated and dissolved in a small amount of acetone. The acetone solution was applied to silica gel TLC (0.5 mm silica gel, $60F_{254}$ layers on 20x20 cm glass plate, Merck) and developed with hexane:diethyl ether

(85:15) about 30 min. A yellow band, corresponding to a yellow spot of the reference standard that also visualize as a dark band under short wave UV light, is scrapped off. The silica gel powder is transferred to a tube and extracted with 1 ml of acetone. The solution is filtered with a 0.2 μm membrane filter and concentrated by N_2 gas. This sample could be stored at -20°C until use. Ubiquinone homologues are separated and identified by HPLC, using Cosmosil column (Waters, 5C18, 4.6 mm x 250 mm), methanol:isopropyl alcohol (2:1) as mobile phase at the flow rate of 1 ml/min. Ubiquinones is detected at 275 nm and with known ubiquinones as standards.

3.2.3.2 DNA base composition

DNA was extracted by the method described by Saito and Miura (1963). Briefly, log phase cells grown in the complex agar medium at 37°C for 1 day were harvested by scraping and suspended in 10 ml of saline-EDTA buffer pH 8.0 (Appendix C). Bacterial cell lysis was induced by 20 mg/ml lysozyme in 0.1 M Tris buffer pH 9.0 and 10% (w/v) sodium dodecyl sulfate (SDS) (Appendix C) at 55°C for 10 min. After cell lysis, the suspension became turbid to opalescent and viscous. Protein was denatured by extracting with 4 ml of phenol-chloroform (1:1 v/v) for 30 sec, and then centrifuged at room temperature, 12,000 rpm (9,200 g) for 10 min. DNA was precipitated by adding cold 95% (v/v) ethanol into supernatant. DNA was spooled with a grass-rod, rinsed with 70% then 95% (v/v) ethanol, air dried, then dissolved in 5 ml of 0.1X SSC (Appendix C). RNase A (Appendix C) solution was added into the DNA solution, incubated at 37°C for 20 min, then extracted by 2 ml of phenol-chloroform (Appendix C). After centrifugation at room temperature at 9200 g 10 min, the upper layer was transferred to the new tube. The DNA was precipitated by adding cold 95% (v/v) ethanol and spooled with a grass-rod then rinsed with 70% then 95% (v/v) ethanol. DNA was air dried and dissolved in 5 ml of 0.1X SSC. The purity and quality of DNA were determined from the ratio of an absorbance at 260 and 280 nm (A_{260}/A_{280}) as described by Marmur (1961).

DNA base composition was analyzed by the method described by Tamaoka and Komagata (1984). DNA was hydrolysed into nucleosides by nuclease P1 (EC 3.1.3.30) and alkaline phosphatase (EC 3.1.3.1). DNA sample (0.5-1.0 g of DNA/l distilled water; OD_{260} = 10-20) was boiled in boiling water bath for 5 min and immediately cooled in ice water. Ten microliter of the DNA was mixed with 10 μl of nuclease P1 solution (Appendix C), incubated at 50°C for 1 h, and then 10 μl of alkaline phosphatase solution (Appendix C) was added and kept at 37°C for 1 h. DNA base composition of DNA hydrolysate was analyzed by HPLC using conditions as shown in Table C-1 (Appendix C).

3.2.4 Molecular characteristics

3.2.4.1 The ITS restriction fragments length polymorphism analyses (ITS-RFLP)

DNA of isolates were extracted by the method described previously. The extracted DNA of AAB were amplified with two primers, 1522F-16S (5'-TGC GG(CT) TGG ATC ACC TCC T-3', position 1522 –1540) and 38R-23S(5'-GTG CC(AT) AGG CAT CCA CCG-3', position 38-22 based on the *Escherichia coli* numbering system, Brosius *et al*., 1981). Each of polymerase chain reaction (PCR) contained 10 µl of 2mM dNTP, 6 µl of 25 mM $MgCl_2$, 10 µl of 10X *Taq* buffer, 2 µl of 20 pmol 1522F and 38R, 0.5 µl of *Taq* DNA polymerase, 4 µl of DNA template, mixed well and adjusted volumn as 100 µl by dH_2O. The PCR reaction were amplified with PCR cycling program started with an initial denaturation of DNA at 94ºC for 5 min, followed with 30 cycles of 92ºC for 30 sec, 56ºC for 45 sec, 72ºC for 1 min and following by final extension at 72ºC for 7 min, end temperature of PCR products collected at 4ºC by Peltier Thermal cycle model MJ Research DYAD ALD 1244.

The PCR products were checked by 0.8% agarose gel electrophoresis with submerged in 1X TAE buffer. The 1-kb DNA ladder was used as standard marker. The electrophoresis was analyzed at 100 V for 40 min. The agarose gel were stained with ethidium bromide solution, washed by distilled water and observed under UV light by BIORAD Gel Doc UV transilumination.

The PCR products were purified by QIAquick column as follows: 900 µl of PB solution was added into 1.5 ml microfuge tube and mixed with 200 µl of PCR product. Then placed the solution into the QIAquick column and centrifuged at 6,000 rpm for 30 sec. Discarded the filtrate and added 750 µl of PE buffer into the column, centrifuged at 8,000 rpm for 30 sec. The filtrate was discarded and centrifuged at 8,000 rpm again for 1.0 min. After that moved the column to the new 1.5 ml microfuge tube, then 40 µl of EB buffer was pipette into the center of the column filter, incubated at room temperature for 10 min and performed at centrifuge at 14,500 rpm for 1.0 min. The purified PCR products were stored at 4°C. The purified DNA was checked to electrophoresis same on described above.

The purified PCR products were digested by each appropriate restriction endonucleases separately. The restriction reaction contained 1 µl of restriction enzyme, 13 µl of DNA, 2 µl of appropriate buffer, mixed well and adjusted volumn as 20 µl by dH_2O. Then, incubated the restriction reaction at optimal temperature of each enzyme for overnight. The restriction patterns were checked by 2.5% agarose gel electrophoresis with 10 µl of restriction products. 50 bp DNA ladder was used as DNA size marker.

3.2.4.2 Gene sequencing and phylogenetic tree analysis

DNA of isolates were extracted by the method described previously. The extracted DNA of isolates were amplified for the 16S rDNA regions with two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3'; positions 9–27 by the *Escherichia coli* numbering system, accession number V00348; Brosius et al., 1981) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'; positions 1509-1492). Each of polymerase chain reaction (PCR) contained 10 µl of 2mM dNTP, 8 µl of 25 mM $MgCl_2$, 10 µl of 10X *Taq* buffer, 4 µl of 40 pmol 20F and 1500R, 0.5 µl of *Taq* DNA polymerase, 4 µl of DNA template, mixed well and adjusted volumn as 100 µl by dH_2O. The PCR reaction were amplified with PCR cycling program started with an initial denaturation of DNA at 94ºC for 3 min, followed with 30 cycles of 94ºC for 1 min, 50ºC for 1 min, 72ºC for 2 min and following by final extension at 72ºC for 3 min, end temperature of PCR products collected at 4ºC by Peltier Thermal cycle model MJ Research DYAD ALD 1244. The ITS regions were amplified with two primers, 1522F-16S and 38R-23S. PCR condition and reaction for ITS regions described as previously.

Amplified 16S rDNA and ITS were directly sequenced with an ABI PRISM BigDye Terminator V3.1 Cycle sequencing kit on an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, Foster, California, USA). For the direct sequencing for 16S rDNA, the following six primers were used: 20F, 1500R, 520F (5'-CAG CAG CCG CGG TAA TAC-3'; positions 519-536), 520R (5'-GTA TTA CCG CGG CTG CTG-3'; positions 536-519), 920F (5'AAA CTC AAA TGA ATT GAC GG-3'; positions 907-926), and 920R (5'-CCG TCA ATT CAT TTG AGT TT-3'; positions 926-907). For ITS gene sequencing, the following four primers were used; 1522F, 38R, Talaf (5' AGA GCA CCT GCT TTG CAA 3') and Talar (5' ACC CCC TGC TTG CAA A 3').

Multiple sequence alignments were performed with a program CLUSTAL X (version 1.83) (Thompson *et al*., 1997). Gaps and ambiguous bases were eliminated from calculation. Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenetic tree based on 16S and ITS gene sequences was constructed by the neighbor-joining method of Saitou and Nei (1987) with the program MEGA 4 (Tamura *et al*., 2007). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1,000 replications.

3.2.4.3 DNA-DNA hybridization: The DNAs of the isolates were extracted and purified from their whole cells by the phenol method of Saito and Miura (1963). DNA-DNA hybridization was carried out by the photobiotin-labeling method (Ezaki *et al*., 1989). Briefly;

under optimal condition, 100 μl of heat-denatured, purified DNA solution of unknown and type strains were incubated at 37°C for 2 h in microdilution plates (Nunc Corp., Denmark). Photobiotinylation of DNA was performed by mixing 10 μg/ml of photobiotin with an equal volume of DNA solution (10 μg of DNA/ml), and then irradiated by sunlamp (500 W) for 25 min. After irradiation, free photobiotin was removed by n-butanol extraction. The biotanylated DNA was used for hybridization immediately.

For quantitative detection of biotinylated DNA in microdilution wells, 200 μl of a prehybridization solution (Appendix C) was added to microdilution plates and incubated at 37°C for 1 h. Then, the prehybridization solution was discarded and replaced by 100 μl of hybridization solution (20X SSC, 5% (v/v) Denhardt solution, 3% (w/v) dextran sulfate, 50% (v/v) formamide, 10 μg of denatured salmon sperm DNA/ml) containing 10 μg of biotinylated DNA.

The microplates were then sealed, and incubated for 15 h at 40-52°C. After hybridization, the microdilution wells were washed three times by 200 μl of 0.2X SSC buffer, and 100 μl of streptavidin peroxidase conjugate solution (Boehringer, Germany) was added, and incubated at 37°C for 30 min. After incubation, the wells were washed three times by phosphate buffered. Then the enzyme solution was discarded and 100 μl substrate, 3.3',5.5' – tetramethyl benzidine – H_2O_2 solution (Wako, Japan), was added to each well. The plates were incubated at 37°C for 10 min. The reaction was stopped by addition of 2 M H_2SO_4 and color intensity was measured by Microplate Reader Bio-Rad, Model 3350 (CA, USA) at wavelength of 450 nm. The homology values for the DNA-DNA hybridization were calculated. In practice, a DNA-homology above 70% indicates a relationship in the species level as reported by Wayne *et al*. (1987).

3.3 Oxidative products of *Acetobacter* and *Gluconobacter* strains

3.3.1 Acetic acid production

3.3.1.1 Alcohol dehydrogenase (ADH) activity assay: The activities of ADH was measured colorimetrically with potassium ferricyanide as an electron acceptor described by Adachi *et al*. (1978).

Acetobacter strains were inoculated into 200 ml of potato medium (Appendix D) at 30°C for 48 h on a rotary shaker 200 rpm. Cells were harvested by centrifugation at 7500 rpm for 10 min and washed twice with cooled 5 mM potassium phosphate buffer (KPB), pH 6.0 (Appendix D). The washed cells were resuspended in the same buffer and passed through a sonicator (16,000 lb/min) for 10 min. Cells were removed by using centrifugation at 7500

rpm for 10 min and then the supernatant was used as ADH assay. The reaction mixture contained enzyme solution, McIlvaine buffer (McB), pH 5.0 (Appendix D), substrate (1M ethanol) 100 µl, 0.1M ferricyanide solution (Appendix D). After 5 min, 0.5 ml of dupanol reagent (Appendix D) was added to the medium and was incubated for 20 min. After 20 min, 3.5 ml of dH_2O was added and well mixed. The absorbance was measured at 660 nm with an UV spectrophotometer. One unit of enzyme activity was defined as the quantity of enzymes catalyzing the oxidation of 1 µmol of the ethanol per minute under the operating conditions.

The specific activity was expressed as units per milligram of proteins and the protein content was determined by Lowry method (Lowry *et al*., 1951) with BSA (bovine serum albumin) as standard (Appendix D).

3.3.1.2 Effect of ethanol and initial acetic acid concentration on acetic acid production

Inoculumn preparation: The selected strain (show highest ADH) was cultivated in 100 ml Potato medium at 30°C on a rotary shaker 200 rpm for 24 h.

Effects of initial ethanol concentration: Ten ml of inoculmn was transferred into 90 ml YE medium (Appendix D) in 500 ml embossed flask and cultivated at 30°C on a rotary shaker 200 rpm for 3 days. The YE medium was varied ethanol concentrations as 0, 2%, 4%, 6%, 8%, 10% and 12% (v/v). Samples were taken for biomass evolution by using the turbidimetrical method (the optical density, OD) at 600 nm. Acetic acid determination was analyzed by a gas chromatography (GC).

Effects of initial acetic acid concentration: Ten ml of inoculmn was transferred into 90 ml YEA medium (Appendix D) in 500 ml embossed flask and cultivated at 30°C on a rotary shaker 200 rpm for 3 days. The YEA medium was varied acetic acid concentrations as 0%, 1%, 1.5%, 2%, 2.5%, 3% (v/v). Samples were taken for biomass evolution and acetic acid accumulation. The biomass evolution and the acetic acid determination were analyzed same on described above.

3.3.2 Dihydroxyacetone production (DHA)

3.3.2.1 Preliminary screening for DHA production

Forty-two isolates of *Gluconobacter* were cultivated in Potato medium at 30°C on a rotary shaker 200 rpm for 24 h and were transfered to DHA production medium (Appendix D) and cultured at 30°C on a rotary shaker 200 rpm for 4 days. The quantitative DHA were analyzed by diphenylamine reaction (Navratil *et al*., 2001). The strain as show hightest DHA production was selected for futher study.

The diphenylamine reaction (Navratil *et al*., 2001) as following; sample was centrifuged and 250 µl of supernatant was diluted by dH_2O, 2.5 µl of diphenylamine was added and well mixed. The reaction was incubated at 80◦C in water bath for 20 min and the reaction was stopped with ice box. The absorbance was measured at 615 nm with an UV spectrophotometer.

3.3.2.2 DHA production

The tested strain was cultivated in 10 ml Potato medium at 30°C on a rotary shaker 200 rpm for 24 h and adjusted cell density to 0.5 (OD_{600}). One ml of inoculum was transferred to 200 ml DHA production medium and incubated at 30°C on a rotary shaker 200 rpm for 96 h. Sample was taken every 12 h for biomass evolution by using the turbidimetrical method (the optical density, OD) at 600 nm and quantitative DHA was analyzed by diphenylamine reaction as described above.

3.3.3 L-sorbose production

3.3.3.1 Preliminary screening for L-sorbose production

Forty-two isolates of *Gluconobacter* were cultivated in Potato medium at 30°C on a rotary shaker 200 rpm for 24 h and were transfered to L-sorbose production medium (Moonmangmee et al., 2000; Appendix D) and were incubated at 30°C on a rotary shaker 200 rpm for 48 h. The quantitative L-sorbose was analyzed by resorcinol reaction.

The resorcinol reaction as following; sample was centrifuged and 250 µl of supernatant was diluted by dH_2O, 2.5 ml of resorcinol was added and well mixed. The reaction mixer was incubated at 80°C in water bath for 10 min and the reaction was stopped with ice box. The absorbance was measured at 480 nm with an UV spectrophotometer.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and sample collection of acetic acid bacteria (AAB)

The AAB were isolated from 30 fruits, 10 flowers, 1 fermented starch, 1 khao-mak and 1 seed-ixora collected in Thailand, using 4 enrichment culture 1) Glucose-Ethanol medium, 2) Sorbitol medium, 3) Sucrose-Acetic acid medium and 4) Methanol-Peptone-Yeast extract medium. One hundred and forty-seven isolates produced acid and showed clearance zone around their colonies on GEY-$CaCO_3$ medium. The source of isolation and isolate number were listed in Table 4.1 and Appendix B-5.

Table 4.1 Source, location, and isolate number of AAB

Isolate no.	**Source**	**Location**
AP59-1*, AP59-2*	Apple[2]	Bangkok
BB90-1*	Banana[2]	Bangkok
F143-1	*Canna indica*[1]	Bangkok
GU1-1*, GU1-3*, G375-3*, G375-4*, G377-1*, G377-2*, G378-1*, G378-2*, G378-4*, G379-2*, G379-3*, G379-4*, G380-1*, G380-2*, G362-1*, G374-3*, G380-4*, G360-1*, G361-1*, G363-1*, G379-6*, G379-5*	Fermented starch[3]	Uttaradit
PHD-3, PHD-4, PHD-5	Guava[2]	Kanchanaburi
PHD-6, PHD-7	Guava[2]	Ubon
GB222-1*	Guava[2]	Bangkok
PHD-8	Hog Plum[2]	Nongkhai
PHD-9	Ixoria / Ixora[1]	Rayong
PHD-10, PHD-11	Jackfruit[2]	Nongkhai
PHD-12, PHD-13, PHD-14	Jujube[2]	Trad
JJ157-2*	Jujube[2]	Bangkok
PHD-15, PHD-16	Kaffir lime[2]	Changmai
PHD-17, PHD-18	Kaffir lime[2]	Saraburi
KL13-2*, KL13-3*	Kaffir lime[2]	Bangkok
PHD-19, PHD-20	Kaffir lime[2]	Khon Kaen
G352-1* G355-1*	Khao-mak (sweeted rice)[3]	Nakonpathom
PHD-21, PHD-22	Langsat[2]	Chantaburi
LC155-1*	Lichi[2]	Bangkok
PHD-1, PHD-2	Litchi[2]	Roie
PHD-23, PHD-24	Little Yellow Star[1]	Rayong
PHD-25, PHD-26	Longan[2]	Rayong
LG5-1*, LG6-1*	Longan[2]	Bangkok
PHD-27, PHD-28, PHD-29	Longan[2]	Changmai
PHD-30, PHD-31	Longan[2]	Trad
LG156-2*	Longan[2]	Bangkok

Table 4.1 Source, Locations, and isolate number of AAB

Isolate no.	Source	Location
LP120-2*	Loog-pang khaomak[3]	Pathumthani
PHD-32, PHD-33	Mango[2]	Khon Kaen
PHD-34, PHD-35, PHD-36	Mango[2]	Bangkok
PHD-37, PHD-38	Mango[2]	Nontaburi
MG70-1*	Mango[2]	Bangkok
PHD-39, PHD-40, PHD-41	Mangosteen[2]	Trad
GB132-1*	Manila tamarind[2]	Bangkok
PHD-42, PHD-43	Musk-melon[2]	Bangkok
PHD-44, PHD-45	Musk-melon[2]	Saraburi
MM129-2*	Musk-melon[2]	Bangkok
PHD-46, PHD-47	Night Jasmine[1]	Rayong
PHD-48	Night Jasmine[1]	Saraburi
PHD-49, PHD-50, PHD-51	Orange[2]	Khon Kaen
PHD-52	Pagoda flower[1]	Rayong
PHD-53, PHD-54	Palm juice[2]	Nongkhai
PHD-55	Papaya[3]	Phuket
PHD-56, PHD-57	Peach[2]	Bangkok
PH108-1	Peach[2]	Pathumthani
PHD-58	Periwinkle[1]	Rayong
PHD-59, PHD-60	Petunia[1]	Chonburi
PHD-61, PHD-62, PHD-63	Pineapple[2]	Chantaburi
GB125*, GB126*, GB127*	Plum mango[2]	Nakonpathom
PHD-66, PHD-67	Pum melo[2]	Ubon
PHD-64, PHD-65	Plumeria flower[1]	Chonburi
PHD-68, PHD-69	Quassia[1]	Rayong
ZW160-2*	Rakam (*Zalacca wallichiana*)[2]	Bangkok
RBY-1, RBY-2	Rambutan[2]	Bangkok
PHD-70, PHD-71	Red Grape[2]	Rayong
PHD-72	Rose apple[2]	Ubon
PHD-73, PHD-74, PHD-75	Rumbutan[2]	Khon Kaen
PHD-76, PHD-77, PHD-78	Salas[2]	Rayong
PHD-79, PHD-80	Sapodilla[2]	Chantaburi
PHD-81, PHD-82	Sapodilla[2]	Trad
SL21-2*, SL22-1*	Sapodilla[2]	Bangkok
PHD-83	Seed Ixora[3]	Rayong
GB23-1*, GB23-3*	*Spathodea campanulata*[1]	Bangkok
PHD-84, PHD-85	Star fruit[2]	Chantaburi
PHD-86, PHD-87, PHD-88	Strawberry[2]	Trad
PHD-89, PHD-90	Sugar apple[2]	Chantaburi
GB223-3*	Sugar apple[2]	Bangkok
GB223-2*	Sugar apple[2]	Bangkok
PHD-91, PHD-92	Tamarind[2]	Chantaburi
TM7-1*, TM7-3*	Tomato[3]	Bangkok

[1]Flower, [2]Fruit, [3]Other materials

*Isolates from Dr.Somboon Tanasupawat (51 isolates)

The all isolates were streaked for purification on GEY-$CaCO_3$ agar plate and kept in cold room at 4 ^{o}C at the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Sterile 20% glycerol in Glucose-Yeast extract-Peptone was used as a cryoprotectant.

4.2 Identification of isolates

The all isolates were general charecteristics are Gram-negative and aerobic and rod-shaped, produced catalase but not oxidase, and showed clear zones on GEY/$CaCO_3$ agar plate. Groups 1-9 (86 isolates), colonies are cream, shiny, circular, motile with peritrichous flagella; Groups 10-14 (38 isolates), colonies are white, shiny, raised circular, motile with polar flagella; Group 15 (4 isolates) colonies are brown, shiny, smooth, and raised motile with peritrichous flagella; Groups 16-18 (15 isolates) colonies are pink, shiny, smooth, and raised, motile with peritrichous flagella; Group 19 (4 isolates) colonies are brown, produce a brownish liquid pigment after 10 days and motile with peritrichous flagella.

All physiological and biochemical characteristics as showed in Table B-1 and Table B-2 (Appendix B). Summary, all of the isolates were strictly aerobic and positive for catalase, utilized glucose oxidatively. They grew at 30°C. Groups 1 to 9 and group 19 can oxidize acetate and lactate. On the other hand, groups 10 to 15 did not oxidize acetate and lactate and grew on mannitol agar but not on glutamate agar. They produced D-gluconate, 2-keto- D-gluconate, and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate. Group 16 to Group 18 can oxidize acetate and lactate to carbon dioxide and water, but the activity is not intense. Acetic acid was produced on ethanol/calcium carbonate agar. Produces 2-keto-D-gluconate and 5-keto-D-gluconate, but not 2,5-diketo-D-gluconate from D-glucose. Only Group 18 could produce levan-like mucous substance. All isolates showed major ubiquinone as Q-10 except isolates in Group 1 to Group 9 showed major ubiquinone as Q-9 therfore, they can assigned as *Acetobacter* species (Asai, 1935).

On the basis of their phenotypic and chemotaxonomic characteristics including ITS-RFLP analysis and the rDNA sequencing analysis, were identified as 4 genus; *Acetobacter* (86 isolates), *Gluconobacter* (42 isolates), *Asaia* (15 isolates), and *Gluconacetobacter* (4 isolates).

4.2.1 Isolates assigned to the genus *Acetobacter*

The eighty-six isolates assigned to the genus *Acetobacter* were divived into 9 groups; *A. pasteurianus* 27 isolates (Group 1), *A. orientalis* 13 isolates (Group 2), *A. lovaniensis* 10 isolates (Group 3), *A. indonesiensis* 13 isolates (Group 4), *A. tropicalis* 4 isolates (Group 5), *A. ghanensis* 8 isolates (Group 6), *A. orleanensis* 4 isolates (Group 7), *A. syzygii* 4 isolates (Group 8), and *Acetobacter* sp. 3 isolates (Group 9).

Group 1 composed of 27 strains G352-1, G377-2, G378-1, G378-2, G378-4, G379-2, G380-1, G375-3, G363-1, G379-6, G377-1, LG5-1, MG70-1, LG6-1, SL21-2, BB90-1, PHD-23, PHD-24, PHD-32, PHD-33, PHD-56, PHD-57, PHD-70, PHD-71, PHD-76, PHD-77 and PHD-78.

They produced acid from L-arabinose, *meso*-erythritol, D-fructose, D-galactose, D-glucose, D-mannose, D-melibiose, and D-xylose, and weakly from D-arabinose, glycerol, D-sorbitol, and sucrose. Some strains produced acid weakly from dulcitol and raffinose but none produced acid from lactose, maltose, D-mannitol, L-rhamnose, or L-sorbose. They grew on *meso*-erythritol, but not on D-arabitol, L-arabitol and *meso*-ribitol. They did not produce 2-keto-D-gluconate from D-glucose. (Appendix B). All isolates grew at 37 ^{o}C. The DNA G+C contents of LG5-1 and SL21-2 were 53.5 and 53.6 mol%. They showed almost the same phenotypic characteristics as *A. pasteurianus* TISTR 1056^{T} (Cleenwerck *et al*., 2008; Table 4.2). All of the isolates were located within the cluster of *A. pasteurianus* (Fig. 4.8) and had 99.9% pair-wise 16S rDNA sequence similarity with the type strain of *A. pasteurianus* TISTR 1056^{T}. The representative strain LG5-1 (line 16) showed restriction patterns that coincided with those of *A. pasteurianus* TISTR 1056^{T} when digested with *Taq*I and *Alu*I. In *Taq*I restriction endonuceases, This strain showed the same restriction pattern as *A. orientalis* NBRC 16606^{T} (line 14) and *A. pasteurianus* TISTR 1056^{T} (line 15) but discriminated from *A. orientalis* NBRC 16606^{T} (line 14) when digested with *Alu*I restriction endonuceases (Fig. 4.1).

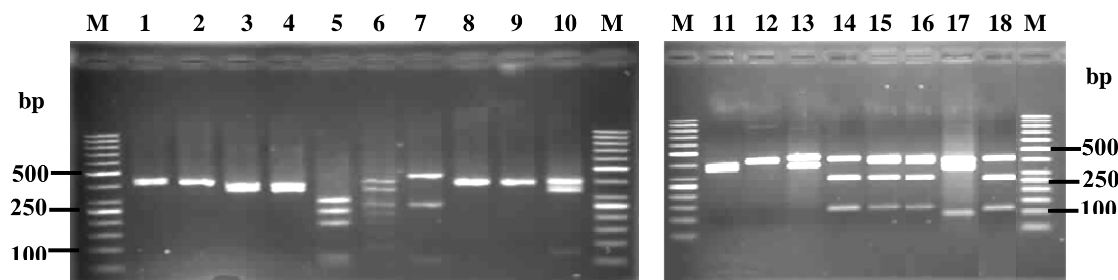
Form the data obtained above, all isolates in Group 1 were identified as *A. pasteurianus*.

Group 2 composed of 13 strains, LP120-2, KL13-3, KL13-2, MM129-2, PHD-12, PHD-34, PHD-35, PHD37, PHD-38, PHD-51, PHD-73, PHD-74 and PHD-75.

They produced acid from D-glucose, and some cases from mannose. Some isolates produced acids weakly from L-arabinose, raffinose, and xylose, but did not produce acid from D-arabinose, dulcitol, *meso*-erythritol, D-galactose, D-fructose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sorbitol, or sucrose. The isolates did not grow on *meso*-erythritol, D-arabitol, L-arabitol and *meso*-ribitol (Appendix B). All the isolates produced 2-keto-D-gluconate from D-glucose. DNA G+C content of KL13-2 and LP120-2 were 52.2 and 52.4 mol%. They showed almost the same phenotypic characteristics as *A. orientalis* NBRC 16606^T (Lisdiyanti *et al*., 2001; Table 4.2). All the isolates were located within the cluster of *A. orientalis* (Fig. 4.8) and had ranged 99.9-100% pair-wise 16S sequence similarity to the type strain of *A. orientalis*. In ITS-RFLP analysis, the representative strain PHD-12 (line 18) showed the same restriction pattern as *A. orientalis* NBRC 16606^T (line 14) and *A. pasteurianus* TISTR 1056^T (line 15) when digested with *Taq*I but discriminated from *A. pasteurianus* TISTR 1056^T (line 15) when digested with *Alu*I restriction endonuceases (Fig. 4.1).

Form the data obtained above, all isolates in Group 2 were identified as *A. orientalis*.

*(a) Taq*I



(b) ***Alu*I**

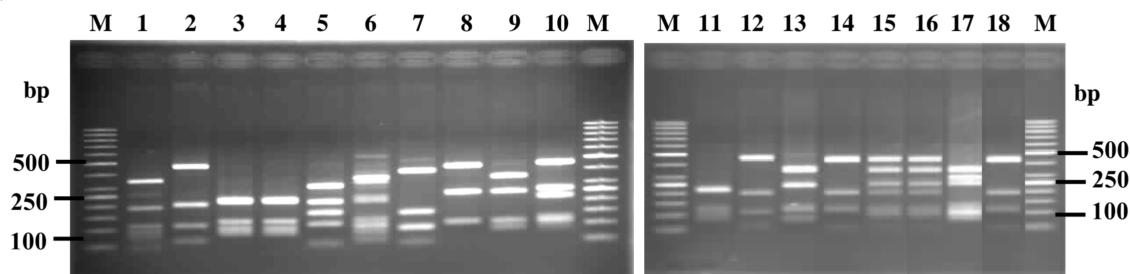


Fig. 4.1 Restriction patterns of 16S-23S rDNA region PCR products of type strains of *Acetobacter* species by digestion with *Taq*I (a) and *Alu*I (b) restriction endonuceases.

1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisae* LMG 1625^T; 3, *A. sysgii* NBRC 16604^T, 4, *A. ghanensis* LMG 23848^T; 5, *A. cibinongensis* NBRC 16605^T; 6, *A. estunensis* NBRC 13751^T; 7, *A. peroxydans* NBRC 13755^T; 8, *A. senegalensis* LMG 23690^T; 9, *A. tropicalis* NBRC 16470^T; 10, *A. indonesiensis* NBRC 16471^T; 11, *A. lovaniensis* NBRC 13753^T; 12, *A. malorum* LMG 1746^T; 13, *A. nitrogenifigens* LMG 23498^T; 14, *A. orientalis* NBRC 16606^T; 15, *A. pasteurianus* TISTR 1056^T; 16, isolate LG5-1 (Group 1); 17, *A. aceti* IFO 14818^T; 18, isolate PHD-12 (Group 2); M, 50 bp DNA markers

Group 3 contained 10 isolates included G374-3, G375-4, PHD-16, PHD-17, PHD-18, PHD-25, PHD-26, PHD-63, PHD-9 and PHD-92.

They produced acid from D-glucose, mannose, L-arabinose, raffinose, and xylose, but not produced acid from D-arabinose, dulcitol, *meso*-erythritol, D-galactose, D-fructose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sorbitol, or sucrose. The isolates did not grow on *meso*-erythritol, D-arabitol, L-arabitol and *meso*-ribitol (Appendix B). Isolates PHD-16 and PHD-17 were 57.2 and 58.3 mol%G+C. They showed almost the same phenotypic characteristics as *A. lovaniensis* (Lisdiyanti *et al*., 2000; Table 4.2). All of the isolates were located within the cluster of *A. lovaniensis* NBRC 13753^T and had 99.9% 16S rDNA sequence similarities respectively to the type strain of this species (Fig. 4.8). The representative strain PHD-16 (line 17) showed restriction patterns that coincided with those of the type strain of *A. orientalis* NBRC 16606^T when digested with *Taq*I, *Hpa*II and *Ava*II. In *Taq*I, it shown the same restriction pattern as *A. lovaniensis* NBRC 13753^T (line 11), *A. sysgii* NBRC 16604^T (line 3) and *A. ghanensis* LMG 23848^T (line 4) but discriminated from *A. ghanensis* LMG 23848^T (line 4) and *A. sysgii* NBRC 16604^T (line 3) when digestion with *Hpa*II and *Ava*II, respectively (Fig. 4.2).

Form the data obtained above, all isolates in Group 3 were identified as *A. lovaniensis*.

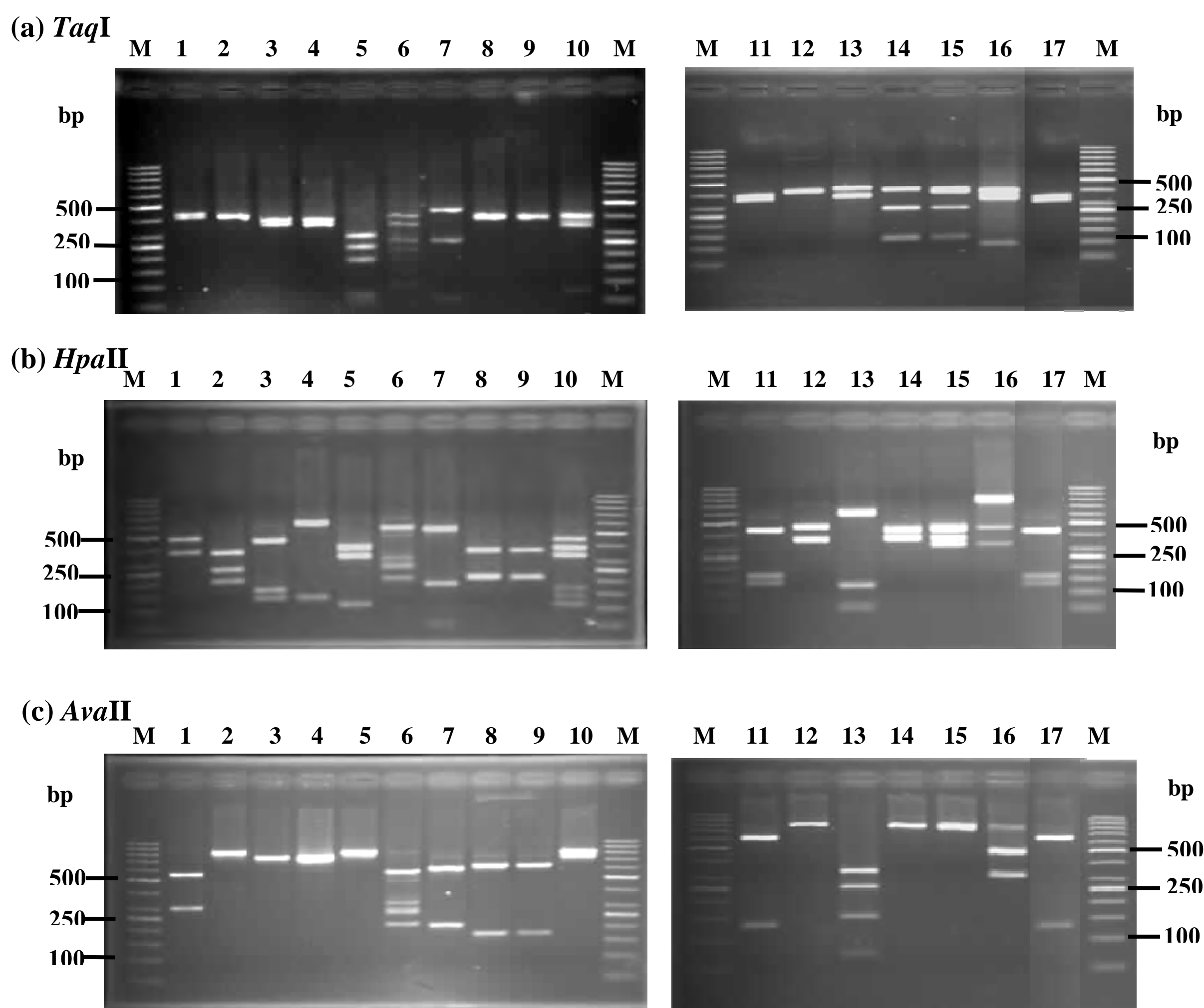


Fig. 4.2 Restriction patterns of 16S-23S rDNA ITS region PCR products of type strains of *Acetobacter* species by digestion with *Taq*I (a), *Hpa*II (b) and *Ava*II (c) restriction endonuceases.

1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisae* LMG 1625^T; 3, *A. sysgii* NBRC 16604^T, 4, *A. ghanensis* LMG 23848^T; 5, *A. cibinongensis* NBRC 16605^T; 6, *A. estunensis* NBRC 13751^T; 7, *A. peroxydans* NBRC 13755^T; 8, *A. senegalensis* LMG 23690^T; 9, *A. tropicalis* NBRC 16470^T; 10, *A. indonesiensis* NBRC 16471^T; 11, *A. lovaniensis* NBRC 13753^T; 12, *A. malorum* LMG 1746; 13, *A. nitrogenifigens* LMG 23498^T; 14, *A. orientalis* NBRC 16606^T; 15, *A. pasteurianus* TISTR 1056^T; 16, *A. aceti* IFO 14818^T; 17, isolate PHD-16 (Group 3); M, 50 bp DNA markers

Group 4 contained 13 isolates included GU1-1, GU1-3, G379-4, G380-2, G355-1, PHD-3, PHD-5, PHD-7, PHD-8, PHD-9, PHD-13, PHD-44 and PHD-45.

They produced acid from D-glucose, L-arabinose, D-xylose, D-galactose, and D-mannose. Some isolates produced acids from D-fructose, D-mannose, D-melibiose and D-xylose but none did from *meso*-erythritol, dulcitol, lactose, maltose, D-mannitol, L-rhamnose, raffinose, L-sorbose, or sucrose. The all isolates produce D-gluconate and 2-keto-D-gluconate from D-glucose, ketogenesis from glycerol (Appendix B). DNA G+C content of isolates PHD-3 and PHD-5 were 54.0 and 54.3 mol%. They showed almost the same phenotypic characteristics as *A. indonesiensis* (Lisdiyanti *et al*., 2000; Table 4.2). All of the isolates were located within the cluster of *A. indonesiensis* and had sequence similarity in a ranged from 99.9-100% between the to the type strain of this species (Fig. 4.8). They also showed restriction patterns that coincided with those of the type strain of *A. indonesiensis* when digested with *Alu*I. The representative strain GU1-3 (line 11) showed the same restriction pattern as *A. indonesiensis* NBRC 16471^T (line 10) and discriminate form other *Acetobacter* species when digestion with *Alu*I restriction endonuceases (Fig. 4.3).

Form the data obtained above, all isolates in Group 4 were identified as *A. indonesiensis.*

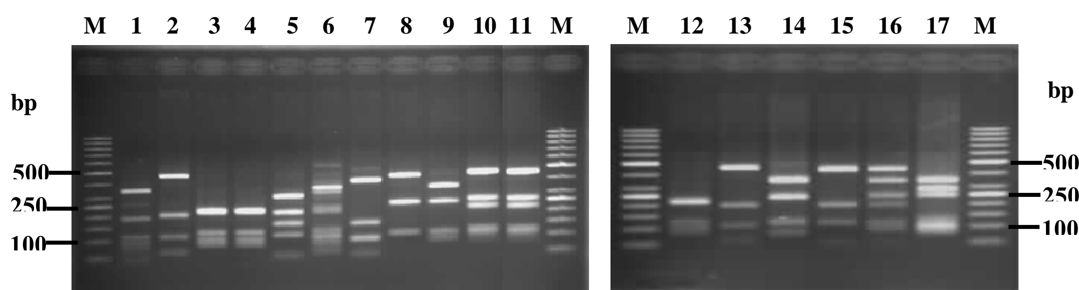


Fig. 4.3 Restriction patterns of 16S-23S rDNA ITS region PCR products of type strains of *Acetobacter* species by digestion with *Alu*I restriction endonuceases.

1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisae* LMG 1625^T; 3, *A. sysgii* NBRC 16604^T, 4, *A. ghanensis* LMG 23848^T; 5, *A. cibinongensis* NBRC 16605^T; 6, *A. estunensis* NBRC 13751^T; 7, *A. peroxydans* NBRC 13755^T; 8, *A. senegalensis* LMG 23690^T; 9, *A. tropicalis* NBRC 16470^T; 10, *A. indonesiensis* NBRC 16471^T; 11, isolate GU1-3 (Group 4); 12, *A. lovaniensis* NBRC 13753^T; 13, *A. malorum* LMG 1746; 14, *A. nitrogenifigens* LMG 23498; 15, *A. orientalis* NBRC 16606^T; 16, *A. pasteurianus* TISTR 1056^T; 17, *A. aceti* IFO 14818^T; M, 50 bp DNA markers

Group 5 composed of 4 isolates included GB23-1, PHD-4, PHD-6 and PHD-42.

The isolates produced acids from L-arabinose, D-galactose, D-glucose, and D-mannose, and to less of an extent from maltose, raffinose, and D-xylose, but not from D-arabinose, dulcitol, *meso*-erythritol, D-fructose, glycerol, lactose, D-mannitol, D-melibiose, L-rhamnose, L-sorbose, D-sorbitol, and sucrose. They did not grow on *meso*-erythritol, D-arabitol, L-arabitol or *meso*-ribitol (Appendix B). They produced D-Gluconate and 2-keto-D-gluconate from D-glucose, ketogenesis from glycerol. Isolates GB23-1 and PHD-4 were 55.5 and 56.0 mol% G+C. They showed almost the same phenotypic characteristics as *A. tropicalis* NBRC 16470^{T} (Lisdiyanti *et al*., 2000; Table 4.2). All the isolates were located within the cluster of *A. tropicalis* and showed 99.9% pair-wise 16S rDNA sequence similarity with the type strain of *A. tropicalis* (Fig. 4.8). All isolates gave restriction patterns that coincided with those of the type strain of *A. tropicalis* when digested with *Alu*I. The representative strain PHD-4 (line 11) showed the same restriction pattern as *A. tropicalis* NBRC 16470^{T} (line 9) and discriminated form other *Acetobacter* species when digestion with *Alu*I (Fig. 4.4).

Form the data obtained above, all isolates in Group 5 were identified as *A. tropicalis.*

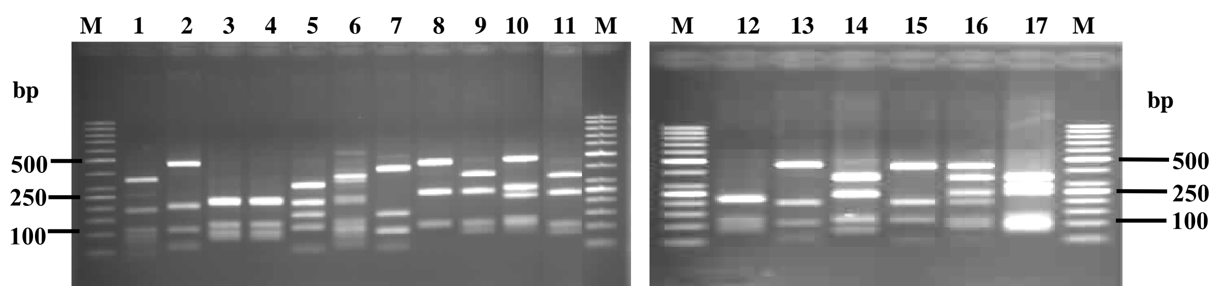


Fig. 4.4 Restriction patterns of 16S-23S rDNA ITS region PCR products of type strains of *Acetobacter* species by digestion with *Alu*I restriction endonuceases.

1, *A. orleanensis* NBRC 13752^{T}; 2, *A. cerevisae* LMG 1625^{T}; 3, *A. sysgii* NBRC 16604^{T}, 4, *A. ghanensis* LMG 23848^{T}; 5, *A. cibinongensis* NBRC 16605^{T}; 6, *A. estunensis* NBRC 13751^{T}; 7, *A. peroxydans* NBRC 13755^{T}; 8, *A. senegalensis* LMG 23690^{T}; 9, *A. tropicalis* NBRC 16470^{T}; 10, *A. indonesiensis* NBRC 16471^{T}; 11, isolate PHD-4 (Group 5); 12, *A. lovaniensis* NBRC 13753^{T}; 13, *A. malorum* LMG 1746; 14, *A. nitrogenifigens* LMG 23498^{T}; 15, *A. orientalis* NBRC 16606^{T}; 16, *A. pasteurianus* TISTR 1056^{T}; 17, *A. aceti* IFO 14818^{T}; M, 50 bp DNA markers

Group 6 composed of 8 isolates included PH108-1, TM7-1, TM7-3, PHD-14, PHD-15, PHD-61, PHD-62 and PHD-72.

The isolates produced acid from D-glucose, D-arabinose and D-sorbitol. Some isolates produced acids from L-arabinose, D-fructose, D-mannose, D-melibiose and D-xylose but none did from *meso*-erythritol, dulcitol, D-galactose, glycerol, lactose, maltose, D-mannitol, L-rhamnose, raffinose (one weakly), L-sorbose, or sucrose. They did not grow on *meso*-erythritol, D-arabitol, L-arabitol and *meso*-ribitol (one weakly). They did not produce 2-ketogluconic acid, 5-ketogluconic acid or 2,5-diketogluconic acid from glucose, were able to grow on glycerol (weakly), but not on maltose or methanol as carbon source (Appendix B). DNA G+C content of isolate TM7-1 was 57.2 mol%. They showed the same phenotypic characteristics as *A. ghanensis* (Cleenwerck et al., 2007; Table 4.2). Therefore, they were identified as *A. ghanensis*. All of the isolates were located within the cluster of *A. ghanensis* and showed 99.9% pair-wise 16S rDNA sequence similarity with the type strain of *A. ghanensis* (Fig. 4.8) and gave restriction patterns that coincided with those of the type strain of *A. ghanensis* when digested with *Taq*I, *Hpa*II and *Ava*II. The representative strain PHD-14 (line 5) showed the same restriction pattern as *A. ghanensis* LMG 23848^T (line 4), *A. sysgii* NBRC 16604^T (line 3) and *A. lovaniensis* NBRC 13753^T (line 12) when digested with *Taq*I. It can discriminate from *A. sysgii* NBRC 16604^T (line 3) and *A. lovaniensis* NBRC 13753^T (line 12) when digestion with *Hpa*II and *Ava*II, respectively (Fig. 4.5).

Form the data obtained above, all isolates in Group 6 were identified as *A. ghanensis.*

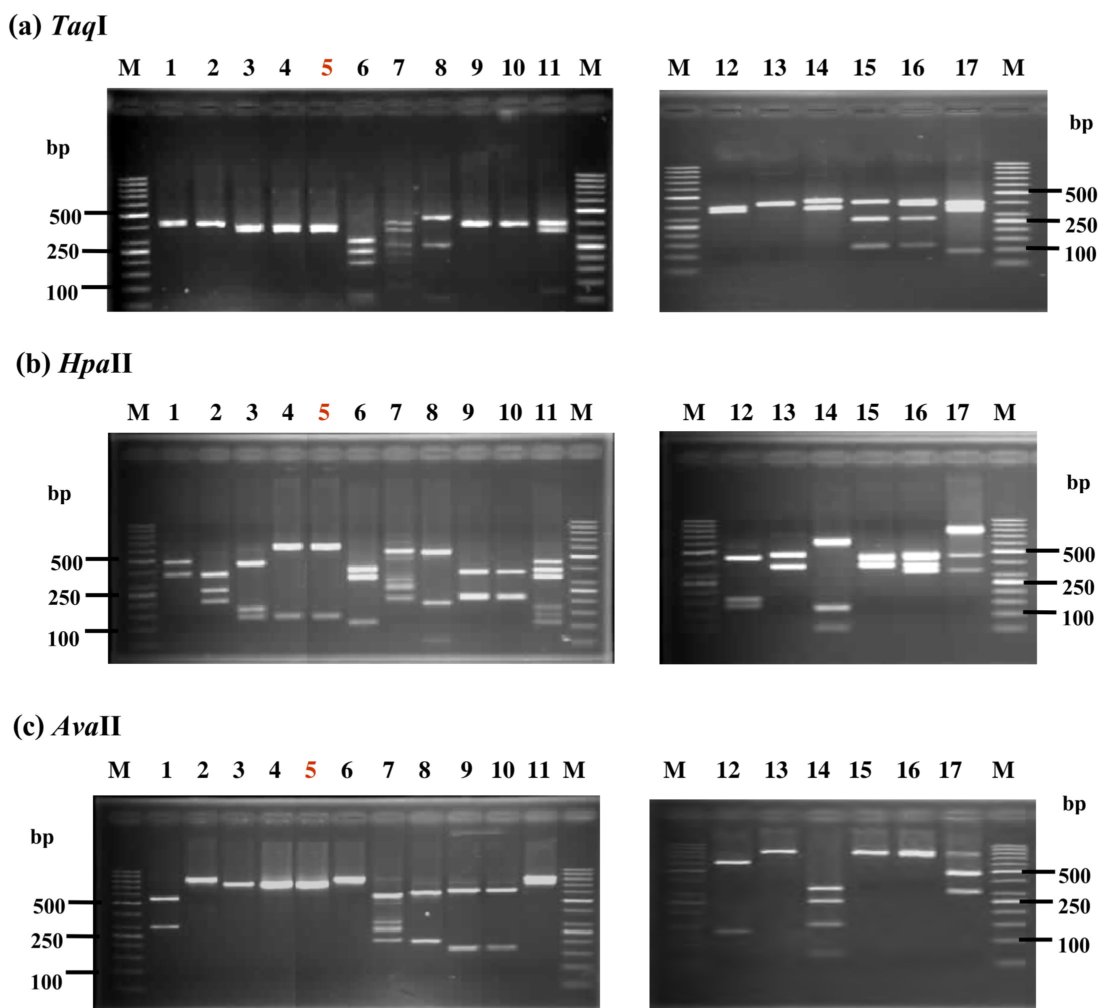


Fig. 4.5 Restriction patterns of 16S-23S rDNA ITS region PCR products of type strains of *Acetobacter* species by digestion with *Taq*I (a), *Hpa*II (b) and *Ava*II (c) restriction endonuceases.

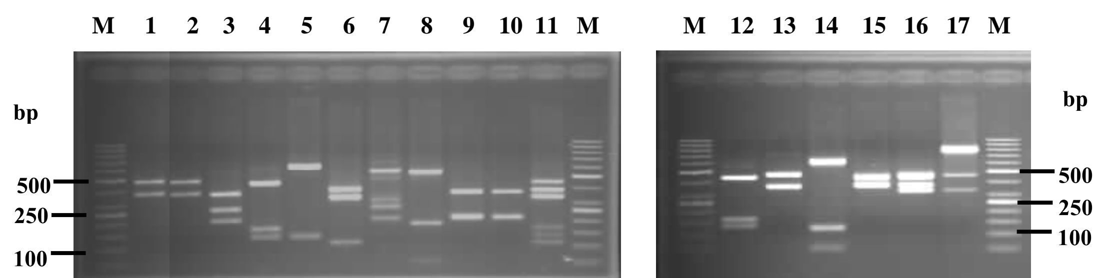
1, *A. orleanensis* NBRC 13752^T; 2, *A. cerevisae* LMG 1625^T; 3, *A. sysgii* NBRC 16604^T, 4, *A. ghanensis* LMG 23848^T; 5, isolate PHD-14 (Group 6); 6, *A. cibinongensis* NBRC 16605^T; 7, *A. estunensis* NBRC 13751^T; 8, *A. peroxydans* NBRC 13755^T; 9, *A. senegalensis* LMG 23690^T; 10, *A. tropicalis* NBRC 16470^T; 11, *A. indonesiensis* NBRC 16471^T; 12, *A. lovaniensis* NBRC 13753^T; 13, *A. malorum* LMG 1746^T; 14, *A. nitrogenifigens* LMG 23498^T; 15, *A. orientalis* NBRC 16606^T; 16, *A. pasteurianus* TISTR 1056^T; 17, *A. aceti* IFO 14818^T; M, 50 bp DNA markers

Group 7 composed of 4 isolates included PHD-84, PHD-85, PHD-86 and PHD-87.

They produced acid from D-glucose, maltose, L-arabinose but from D-arabinose, dulcitol, *meso*-erythritol, D-fructose, D-galactose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, raffinose, L-sorbose, D-sorbitol, sucrose, and D-xylose. It grew on *meso*-erythritol, but did not grow on D-arabitol, L-arabitol or *meso*-ribitol (Appendix B). They produced gluconic acid from glucose. They did not produce 5-ketogluconic acid from glucose. Isolates PHD-84 and PHD-85 were 55.8 and 56.3 mol%G+C. They showed the same phenotypic characteristics as *A. orleanensis* (Lisdiyanti *et al*., 2000; Table 4.2). In a phylogenetic tree, all of the isolates were located within the cluster of *A. orleanensis* and showed 99.9% pair-wise 16S rDNA sequence similarity with the type strain of *A. orleanensis* (Fig. 4.8). All isolates also gave restriction patterns that coincided with those of the type strain of *A. orleanensis* when digested with *Hpa*II and *Ava*II (Table 4.5 and Fig. 4.7). The representative strain PHD-85 (line 2) showed the same restriction pattern as *A. orleanensis* NBRC 13752^T (line 1), *A. malorum* LMG 1746^T (line 13) when digested with *Hpa*II and discriminate from *A. malorum* LMG 1746^T (line 13) when digestion with *Ava*II restriction endonuceas, respectively (Fig. 4.6).

Form the data obtained above, all isolates in Group 7 were identified as *A. orleanensis*.

(a) *Hpa*II



(b) *Ava*II

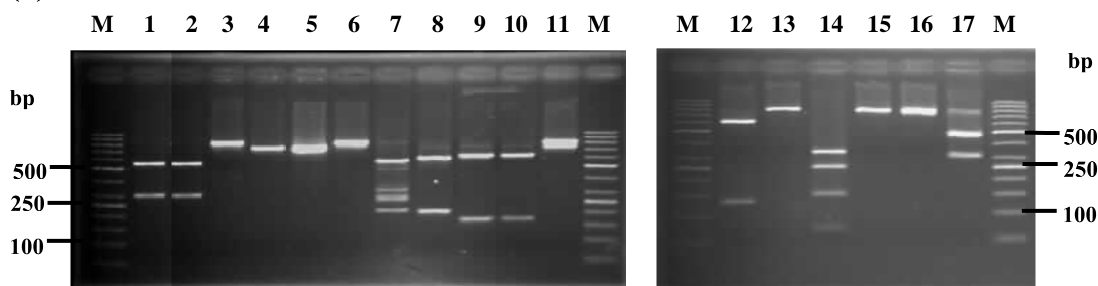


Fig. 4.6 Restriction patterns of 16S-23S rDNA ITS region PCR products of type strains of *Acetobacter* species by digestion with *Hpa*II (a) and *Ava*II (b) restriction endonuceases.

1, *A. orleanensis* NBRC 13752^T; 2, isolate PHD-85 (Group 7); 3, *A. cerevisiae* LMG 1625^T; 4, *A. sysgii* NBRC 16604^T, 5, *A. ghanensis* LMG 23848^T; 6, *A. cibinongensis* NBRC 16605^T; 7, *A. estunensis* NBRC 13751^T; 8, *A. peroxydans* NBRC 13755^T; 9, *A. senegalensis* LMG 23690^T; 10, *A. tropicalis* NBRC 16470^T; 11, *A. indonesiensis* NBRC 16471^T; 12, *A. lovaniensis* NBRC 13753^T; 13, *A. malorum* LMG 1746^T; 14, *A. nitrogenifigens* LMG 23498^T; 15, *A. orientalis* NBRC 16606^T; 16, *A. pasteurianus* TISTR 1056^T; 17, *A. aceti* IFO 14818^T; M, 50 bp DNA markers

Group 8 composed of 4 isolates included SL22-1, G379-3, G380-4 and G379-5.

The isolates produced acid from D-glucose and maltose but not from L-arabinose, D-arabinose, dulcitol, *meso*-erythritol, D-fructose, D-galactose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, raffinose, L-sorbose, D-sorbitol, sucrose, and D-xylose. It grew weakly on *meso*-erythritol, but did not grow on D-arabitol, L-arabitol or *meso*-ribitol (Appendix B). Isolate SL22-1 was 53.8 mol% G+C. They showed the same phenotypic characteristics as *A. syzygii* NBRC 16604^T (Lisdiyanti *et al*., 2001; Table 4.2). In a phylogenetic tree, all of the isolates were located within the cluster of *A. syzygii* (Fig. 4.8) and showed 99.9% pair-wise 16S rDNA sequence similarity to the type strain of *A. syzygii* and gave restriction patterns that coincided with those of the type strain of *A. orleanensis* when digested with *Taq*I and *Hpa*II. The representative strain G379-3 (line 4) showed the same restriction pattern as *A. sysgii* NBRC 16604^T (line 3), *A. ghanenesis* LMG 23848^T (line 5) and *A. lovaniensis* NBRC 13753^T (line 12) when digested with *Taq*I. It can discriminate from *A. ghanenesis* LMG 23848^T (line 5) and *A. lovaniensis* NBRC 13753^T (line 12) when digestion with *Hpa*II, respectively (Fig. 4.7).

Form the data obtained above, all isolates in Group 8 were identified as *A. syzygii*.

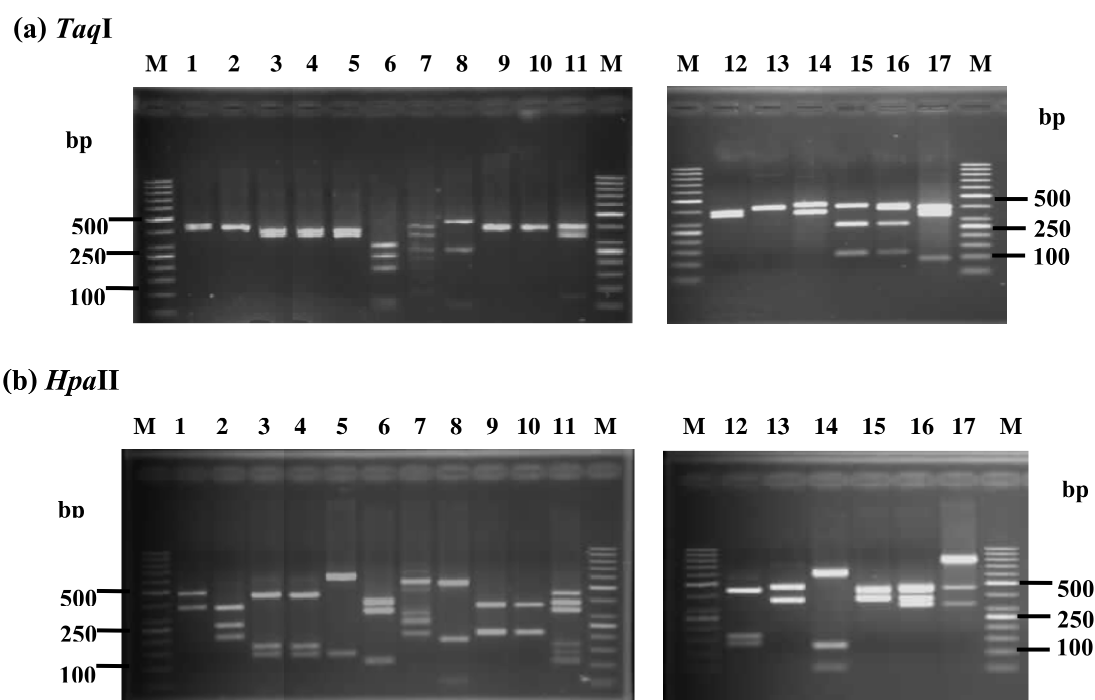


Fig. 4.7 Restriction patterns of 16S-23S rDNA ITS region PCR products of type strains of *Acetobacter* species by digestion with *Taq*I (a) and *Hpa*II (b) restriction endonuceases.

1, *A. orleanensis* NBRC 13752; 2, *A. cerevisiae* LMG 1625^{T}; 3, *A. sysgii* NBRC 16604; 4, G379-3 (Group 8); 5, *A. ghanenesis* LMG 23848; 6, *A. cibinongensis* NBRC 16605^{T}; 7, *A. estunensis* NBRC 13751; 8, *A. peroxydans* NBRC 13755; 9, *A. senegalensis* LMG 23690; 10, *A. tropicalis* NBRC 16470^{T}; 11, *A. indonesiensis* NBRC 16471^{T}; 12, *A. lovaniensis* NBRC 13753^{T}; 13, *A. malorum* LMG 1746^{T}; 14, *A. nitrogenifigens* LMG 23498^{T}; 15, *A. orientalis* NBRC 16606^{T}; 16, *A. pasteurianus* TISTR 1056^{T}; 17, *A. aceti* IFO 14818^{T}; M, 50 bp DNA markers

Table 4.2 Differential characteristics of isolates in *Acetobacter*

Characteristics	G1	*Ap*	G2	*Aori*	G3	*Al*	G4	*Ai*	G5	*At*	G6	*Ag*	G7	*Aorl*	G8	*As*	G9
Oxidation of																	
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37 ^{o}C	w(+10)	w	-(w2)	-	-(w2)	-	-	-	-	-	-	-(w2)	-	-	-	-	-
Growth at 40 ^{o}C	-(w7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on																	
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	w(-7)	-	w(-3)	-	w(-2)	-	W	-	w	-	w(-2)	w	w	-	w	w	-
Production of																	
2-keto gluconic acid	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+
5-keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Major quinone	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9	Q-9
Acid production from																	
meso-Erythritol	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	w	-	-	w(-4)	-(w2)	-	-	-	-	-	w
Raffinose	w(-2)	w	-(w5)	-	-	-	-	-	w	w	-(w1)	-	-	-	-	-	-
DNA G+C mol%	53.5	53.2-54.3*	55.2	52.0-52.8*	58.3	57.1-58.9*	54.3	54.0-54.2*	56.0	55.6-56.2*	57.2	56-9-57.3*	55.8	55.7-58.1*	53.8	54.3-55.4*	56.3

Ap, *A. pasteurianus* TISTR 1056^{T}; G1, Group 1 (27 isolates); *Aori*, *A. orientalis* NBRC 16606^{T}; G2, Group 2 (13 isolates); *Al*, *A. lovaniensis* NBRC 13753^{T}; G3, Group 3 (10 isolates); *Ai*, *A. indonesiensis* NBRC 16471^{T}; G4, Group 4 (13 isolates); *At*, *A. tropicalis* NBRC 16470^{T}; G5, Group 5 (4 isolates); *Ag*, *A. ghanensis* LMG 23848 ^{T}; G6, Group 6 (8 isolates); *Aorl*, *A. orleanensis* NBRC 13752^{T}; G7, Group 7 (4 isolates); *As*, *A. syzgii* NBRC 16604^{T}; G8, Group 8 (4 isolates); G9, Group 9 (3 isolates); *, data cite from Cleenwerck *et al* (2008).

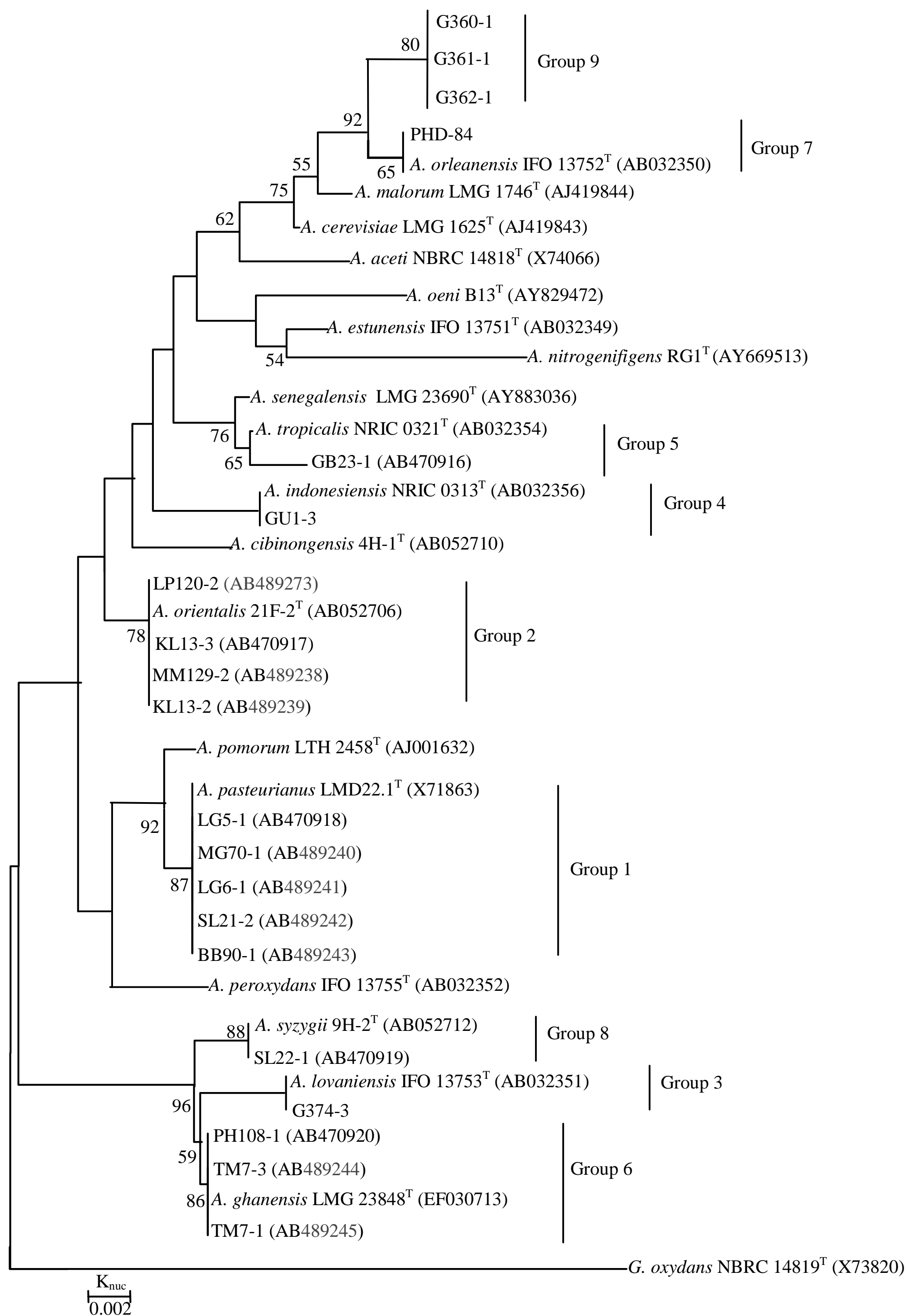


Fig. 4.8 Neighbour-joining-tree showing the phylogenetic position of Group 1 to Group 9 based on 16S rDNA sequences. Bar, 0.002 substitution per nucleotide position. Bootstrap values (%) derived from 1,000 replications.

Table 4.3 Restriction pattern type of isolates when digested with *Taq*I, *Alu*I, *Hpa*II and *Ava*II restriction endonuclease

Group/Isolate no.	Restriction pattern digested with			
	*Taq*I	*Alu*I	*Hpa*II	*Ava*II
Group 1 G377-2, G378-1, G378-2, G378-4, G379-2, G380-1, G375-3, G363-1, G379-6, G377-1, G352-1, LG5-1, MG70-1, LG6-1, SL21-2, BB90-1, PHD-23, PHD-24, PHD-32, PHD-33, PHD-56, PHD-57, PHD-70, PHD-71, PHD-76, PHD-77, PHD-78	*Aori, Ap*	*Ap*	-	-
Group 2 LP120-2, KL13-3, KL13-2, MM129-2, PHD-12, PHD-34, PHD-35, PHD-37, PHD-38, PHD-51, PHD-73, PHD-74, PHD-75	*Aori, Ap*	*Aori*	-	-
Group 3 G374-3, G375-4, PHD-16, PHD-17, PHD-18, PHD-25, PHD-26, PHD-63, PHD-91, PHD-92	*As, Ag, Al*	-	*As, Al*	*Al*
Group 4 GU1-1, GU1-3, G379-4, G380-2, G355-1, PHD-3, PHD-5, PHD-7, PHD-8, PHD-9, PHD-13, PHD-44, PHD-45	-	*Ai*	-	-
Group 5 GB23-1, PHD-4, PHD-6, PHD-42	-	*At*		
Group 6 PH108-1, TM7-1, TM7-3, PHD-14, PHD-15, PHD-61, PHD-62, PHD-72	*As, Ag, Al*	-	*Ag, Al*	*Ag*
Group 7 PHD-84, PHD-85, PHD-86, PHD-87	*Aorl, Am*	-	-	*Aorl*
Group 8 SL22-1, G379-3, G380-4, G379-5	*As, Ag, Al*		*As*	
Group 9 G360-1, G361-1, G362-1	-	-	*Aorl, As*	*A*.sp

Group 9 composed of 3 isolates included G360-1, G361-1 and G362-1.

The isolates produced acid from D-glucose, D-mannose, L-arabinose, D-xylose and maltose (weakly positive) but not from L-arabinose, D-arabinose, dulcitol, *meso*-erythritol, D-fructose, D-galactose, glycerol, lactose, maltose, D-mannitol, D-melibiose, L-rhamnose, raffinose, L-sorbose, D-sorbitol, sucrose. Growth reached to 37 ^{o}C in 2 days but weakly positive. All isolates oxidized ethanol to acetic acid and produced gluconic acid from glucose. They did not produce 5-ketogluconic acid from glucose. The DNA G+C content of isolates ranged from 56.9-57.3 mol% G+C. All strains have phenotypic characteristics similar to *A. orleanensis* (Lisdiyanti *et al*., 2000) but they are clearly distinguished from *A. orleanensis* by ITS-RFLP analysis and DNA-DNA relatedness.

In a phylogenetic tree based on 16S rDNA sequences (1,402 bp), the isolates were located in the lineage of the genus *Acetobacter* and constituted a cluster separate from the type strains of the known species, The sequence similarity were 99.7% sequence similarity to the type strains of *A. orleanensis* (Fig. 4.9).

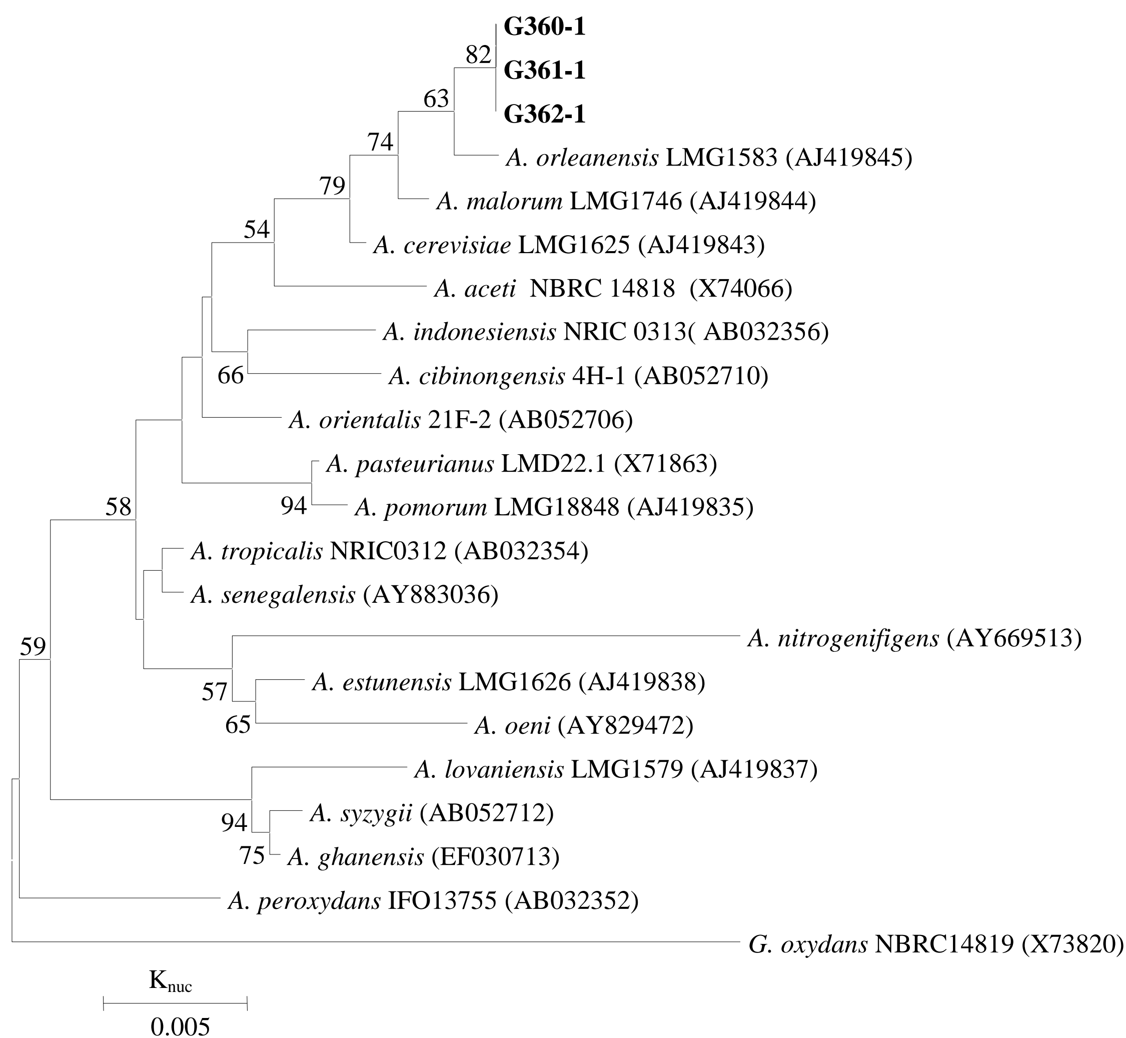


Fig. 4.9 Neighbour-joining-tree showing the phylogenetic position of isolates G360-1, G361-1 and G362-1 in Group 9 based on 16S rDNA sequences. Bar, 0.005 substitution per nucleotide position. Bootstrap values as previously.

In ITS-RFLP analysis, the representative strain G360-1 (line 2) showed the same restriction pattern forms as *A. orleanensis* NBRC 13752^T (line 1), *A. cerevisiae* LMG 1625^T (line 3), *A. malorum* LMG 1746^T (line 13) when digested with *Hpa*II but different pattern from other *Acetobacter* species when digestion with *Ava*II restriction endonuceas, respectively (Fig. 4.10).

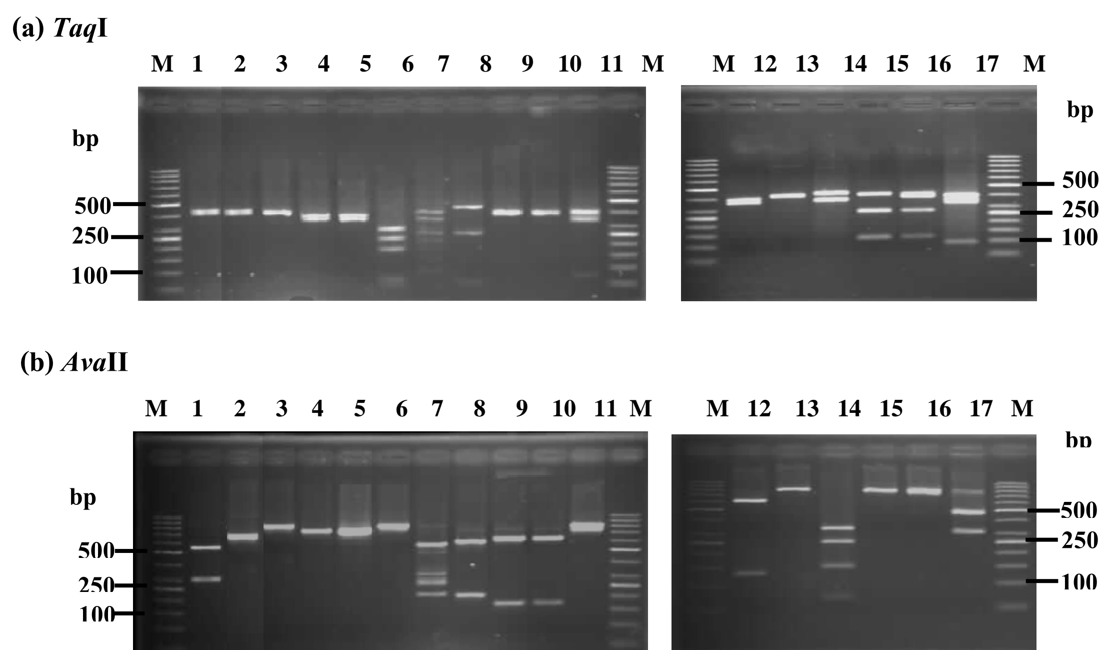


Fig. 4.10 Restriction patterns of 16S-23S rDNA region PCR products of type strains of *Acetobacter* species by digestion with *Taq*I (a) and *Ava*II (b) restriction endonuceases.

1, *A. orleanensis* NBRC 13752^T; 2, isolate G360-1; 3, *A. cerevisiae* LMG 1625^T; 4, *A. sysgii* NBRC 16604^T, 5, *A. ghanenesis* LMG 23848^T; 6, *A. cibinongensis* NBRC 16605^T; 7, *A. estunensis* NBRC 13751^T; 8, *A. peroxydans* NBRC 13755^T; 9, *A. senegalensis* LMG 23690^T; 10, *A. tropicalis* NBRC 16470^T; 11, *A. indonesiensis* NBRC 16471^T; 12, *A. lovaniensis* NBRC 13753^T; 13, *A. malorum* LMG 1746^T; 14, *A. nitrogenifigens* LMG 23498^T; 15, *A. orientalis* NBRC 16606^T; 16, *A. pasteurianus* TlSTR 1056^T; 17, *A. aceti* IFO 14818^T; M, 50 bp DNA markers

Table 4.4 DNA-DNA relatedness of strains G360-1, G361-1 and G362-1

Strain	%DNA-DNA relatedness with labeled strains			
	G360-1	NBRC 13752^T	LMG 1746^T	LMG 1625^T
G360-1	100	36	24	19
G361-1	100	38	27	22
G362-1	100	39	35	20
A. orleanensis NBRC 13752^T	38	100	42	31
A. malorum LMG 1746^T	29	39	100	22
A. cerevisiae LMG 1625^T	22	35	26	100
A. aceti NBRC 14818^T	15	22	36	43
G. oxydans NBRC 14819^T	6	5	9	5

Strain G360-1 represented levels of DNA-DNA relatedness of 100, 100, 100, 38, 29, 22, 15 and 6% respectively to strains G360-1, G361-1 and G362-1, *A. orleanensis* NBRC 13752^T, *A. malorum* LMG 1746^T, *A. cerevisiae* LMG 1625^T, *A. aceti* NBRC 14818^T *and G. oxydans* NBRC 14819^T (Table 4.4).

The data obtained above supported that strains G360-1, G361-1 and G362-1 should be proposed as a new species in the genus *Acetobacter*.

4.2.2 Isolates assigned to the genus *Gluconobacter*

The forty-two isolates that were assigned to the genus *Gluconobacter* were divided into 6 groups; *G. frateurii* 8 isolates (Group 10), *G. japonicus* 8 isolates (Group 11), *G. thailandicus* 6 isolates (Group 12), *G. oxydans* 13 isolates (Group 13), Group 14 (3 isolates), Group 15 (4 isolates).

Group 10 composed of 8 isolates included, GB222-1, GB223-3, AP59-1, AP59-2, PHD-30, PHD-31, PHD-66 and PHD-67.

The isolates produced dihydroxyacetone from glycerol and grew at 30^{c}C. Some strains did not grow at 37 ^{o}C. They produced 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose but not 2,5-diketo-D-gluconate. They produced acids from L-arabinose, D-galactose, D-glucose, *meso*-erythritol (weakly), and D-xylose (weakly) glycerol, maltose (weakly), and sucrose but not from D-arabinose, dulcitol, D-fructose, lactose, D-mannose, D-mannitol, D-melibiose, L-rhamnose, raffinose, L-sorbose, and D-sorbitol (Appendix B). The isolates grown on D-arabitol, L-arabitol and *meso*-ribitol (weakly) but not on *meso*-erythritol, being different from strains of *G. oxydans* and *G. cerinus*. Isolates GB222-1 and AP59-1 were 55.1 and 55.2 mol% G+C. They showed almost the same phenotypic characteristics as *G.*

frateurii NBRC 3264^T (Yukphan *et al*., 2004c). All the isolates were located within the cluster of *G. frateurii* (Fig. 4.11) and had 99.9% pair-wise ITS gene sequence similarity to the type strain of *G. frateurii*. All the isolates gave the same restriction patterns as the type strain of *G. frateurii* when digested with *Bst*NI, *Mbo*II and *Mbo*I. The representative strain GB222-1 showed the same restriction pattern as *G. frateurii* NBRC 3264^T (line 3) and *G. thailandicus* BCC 14116^T (lane 5) when digested with *Bst*NI but it discriminate from *G. thailandicus* BCC 14116^T (lane 5) when digestion with *Mbo*I restriction endonucease (Table 4.6, Fig. 4.12).

Form the data obtained above, all isolates in Group 10 were identified as *G. frateurii*.

<u>Group 11</u> composed of 8 isolates included, GB132-1, F143-1, FB125, GB126, GB127, GB223-2, PHD-28 and PHD-29.

They produced acids from D-glucose and *meso*-erythritol (weakly), and some cases, from L-arabinose, D-galactose, glycerol, D-mannose, D-melibiose, sucrose, and D-xylose. Some isolates produced acids weakly from D-arabinose, dulcitol, D-fructose, lactose, and D-mannitol, but none produced acids from maltose, raffinose, L-rhamnose, L-sorbose, or D-sorbitol. They grew on *meso*-erythritol, D-arabitol, L-arabitol (weakly), and *meso*-ribitol (Appendix B). The isolates produced dihydroxyacetone weakly from glycerol. All the isolates were distinguished from *G. frateurii* by the ability to grow on *meso*-erythritol and produce acid (weakly) from raffinose. Isolates GB125 and GB132-1 were 56.2 and 56.4 mol% G+C. They showed almost the same phenotypic characteristics as *G. japonicus* NBRC 3271^T (Malimas *et al*., 2009a). All isolates were located within the cluster of *G. japonicus* (Fig. 4.11) and had 99.8% pair-wise ITS gene sequence similarity with the type strain of *G. japonicus*. All the isolates gave the same restriction patterns as the type strain of *G. japonicus* when digested with *Bst*NI, *Mbo*II and *Mbo*I. The representative strain GB132-1 showed the same restriction pattern as *G. japonicus* NBRC 3271^T (line 8) and *G. oxydans* NBRC 14819^T (lane 1) when digested with *Bst*NI but it discriminate from *G. oxydans* NBRC 14819^T (lane 1) when digestion with *Mbo*II restriction endonucease (Table 4.6, Fig. 4.12).

Form the data obtained above, all isolates in Group 11 were identified as *G. japonicus*.

Group 12 composed of 6 isolates included PHD-11, PHD-21, PHD-22, PHD-36, PHD-39, and PHD-40.

All isolates produced dihydroxyacetone from glycerol. They grew on D-arabitol, L-arabitol, *meso*-ribitol, and *meso*-erythritol, but not on dulcitol, their growth in L-arabitol, and *meso*-ribitol (weakly). Acid were produced from L-arabinose, D-fructose, D-galactose, D-glucose, glycerol, D-mannitol (variable and weak), D-ribose, L-sorbose (weak), D-xylose, and ethanol. In contrast, no acid production were found from maltose, D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol and sucrose. They grew on mannitol agar but not on glutamate agar (Appendix B). They produced D-gluconate, 2-keto-D-gluconate, and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate or a water-soluble brown pigment. The isolates were growth reached to 37ºC. Isolates PHD-21 and PHD-22 were 57.2 and 58.3 mol% G+C. They showed almost the same phenotypic characteristics as *G. thailandicus* (Tanasupawat *et al*., 2004). All isolates were located within the cluster of *G. thailandicus* and had 99.9% pair-wise ITS gene sequence similarity with the type strain of *G. thailandicus* (Fig. 4.11). The representative strain PHD-11 showed the same restriction pattern as *G. thailandicus* BCC 14116^T (line 5) and *G. frateurii* NBRC 3264^T (line 3) when digested with *Bst*NI but it discriminate from *G. frateurii* NBRC 3264^T (line 3) when digestion with *Mbo*I restriction endonucease (Table 4.6, Fig. 4.12).

Form the data obtained above, all isolates in Group 12 were identified as *G. thailandicus.*

Group 13 composed of 13 isolates included PHD-10, PHD-19, PHD-20, PHD-27, PHD-41, PHD-55, PHD-59, PHD-60, PHD-81, PHD-82, PHD-43, PHD-83 and PHD-88.

They produce acid from L-arabinose, D-fructose, D-galactose, D-glucose, glycerol, D-mannitol (weak), D-ribose (weak), L-sorbose, maltose and D-xylose. In contrast, no acid production were found from D-mannose, melibiose, raffinose, L-rhamnose, D-sorbitol and sucrose.They grew on D-glucose, D-fructose, D-mannitol, D-sorbitol, glycerol, *meso*-ribitol (weakly)and maltose but not on sucrose, raffinose dulcitol, D-arabitol and L-arabitol. They grew on mannitol agar but not on glutamate agar (Appendix B). The isolates produced acetylmethyl carbinol from lactate and grew at 30°C. They produces 2-keto-D-gluconate and 5-keto-D-gluconate but not 2,5-keto-D-gluconate (Appendix B). They showed almost the same phenotypic characteristics as *G. oxydans* (Yukphan *et al*., 2004c). All of isolates were located within the cluster of *G. oxydans* NBRC 14819^T (Fig. 4.11) and had 99.9% pair-wise ITS gene sequence similarity with *G. oxydans* NBRC 14819^T. The representative strain PHD-10

showed the same restriction pattern as *G. oxydans* NBRC 14819^T (line 1), *G. roseus* NBRC 3990^T (lane 7) and *G. sphaericus* NBRC 12467^T (lane 9) when digested with *Mbo*II but it discriminate from *G. roseus* NBRC 3990^T (lane 7) and *G. sphaericus* NBRC 12467^T (lane 9) when digestion with *Mbo*I restriction endonucease (Fig. 4.12).

Form the data obtained above, all isolates in Group 13 were identified as *G. oxydans.*

Table 4.5 Differential characteristics of *Gluconobacter* isolates

Characteristics	G10	*Gf*	G11	*Gja*	G12	*Gt*	G13	*Go*	G14	G15
Oxidation of										
Acetate	-	-	-	-	-	-	-	-	-	-
Lactate	-	-	-	-	-	-	-	-	-	-
Water soluble brown pigment	-	-	-	-	-	-	-	-	-	+
Glutamate agar	-	-	-	-	-	-	-	-	-	-
Mannitol agar	+	+	+	+	+	+	+	+	+	+
Production of										
2-keto gluconate	+	+	+	+	+	+	+	+	+	+
5-keto gluconate	+	+	+	+	+	+	+	+	+	+
2,5-diketo gluconate	-	-	-	-	-	-	-	-	-	-
Major quinone	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10	Q-10
Acid production from										
meso-Erythritol	w(+2)	w	+(w3)	+	+(w2)	+	+(w4)	+	+	+
Maltose	w(-2)	w	-	-	w(-2)	w	+	+	w	+
Raffinose	+	+	+	+	w	w	w	w	+	+
DNA G+C mol%	55.2	55.1*	56.2	56.4*	57.2	55.8*	60.2	60.3*	57.2	60.4

Gf, *G. frateurii* NBRC 3264^T; G10, Group 10 (8 isolates); *Gja*, *G. japonicus* NBRC 3271^T; G11, Group 11 (8 isolates); *Gt, G. thailandicus* BCC 14116^T; G12, Group 12 (6 isolates); *Go*, *G. oxydans* NBRC 14819^T; G13, Group 13 (13 isolates); G14, Group 14 (3 isolates); G15, Group 15 (4 isolates); * data cite from Malimas *et al*., 2009b.

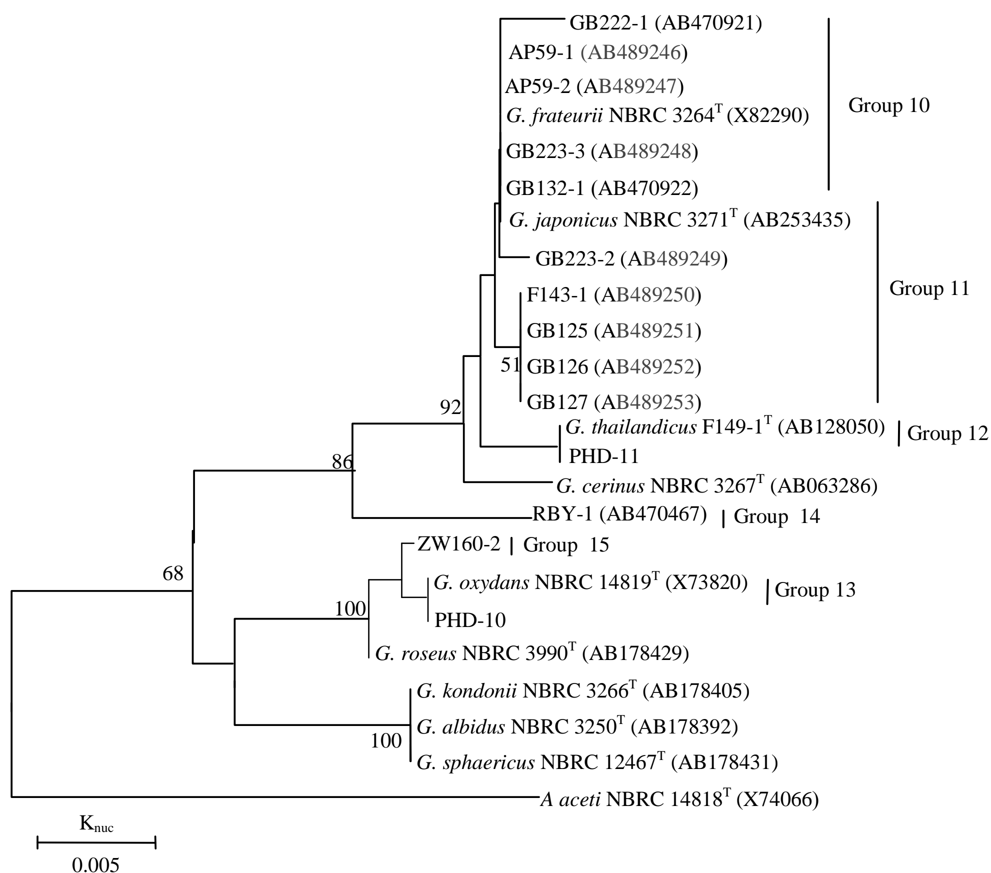


Fig. 4.11 Neighbour-joining-tree showing the phylogenetic position of Group 10 to Group 15 based on 16S rDNA sequences. Bar, 0.005 substitution per nucleotide position. Bootstrap values as previously.

Table 4.6 Restriction pattern type of isolates when digested with *Bst*NI, *Mbo*II and *Mbo*I restriction endonuclease

Groups/Isolates no.	**Restriction pattern digested with**		
	*Bst*NI	*Mbo*II	*Mbo*I
Group 10 GB222-1, GB223-3, AP59-1, AP59-2, PHD-30, PHD-31, PHD-66, PHD-67	*Gf, Gt*	-	*Gf*
Group 11 GB132-1, F143-1, GB125, GB126, GB127, GB223-2, PHD-28, PHD-29	*Go, Gj*	*Gj*	-
Group 12 PHD-11, PHD-21, PHD-22, PHD-36, PHD-39, PHD-40	*Gf, Gt*	-	*Gt*
Group 13 PHD-10, PHD-19, PHD-20, PHD-27, PHD-41, PHD-55, PHD-59, PHD-60, PHD-81, PHD-82, PHD-43, PHD-83, PHD-88	-	*Go, Gr, Gs*	*Go*
Group 14 RBY-1, PHD-1, PHD-2	*Go, Gj*	*Gj*	*G*. sp1
Group 15 ZW160-2, LC155-1, LG156-2, JJ157-2	*Go, Gr, Gs*	*G*. sp2	-

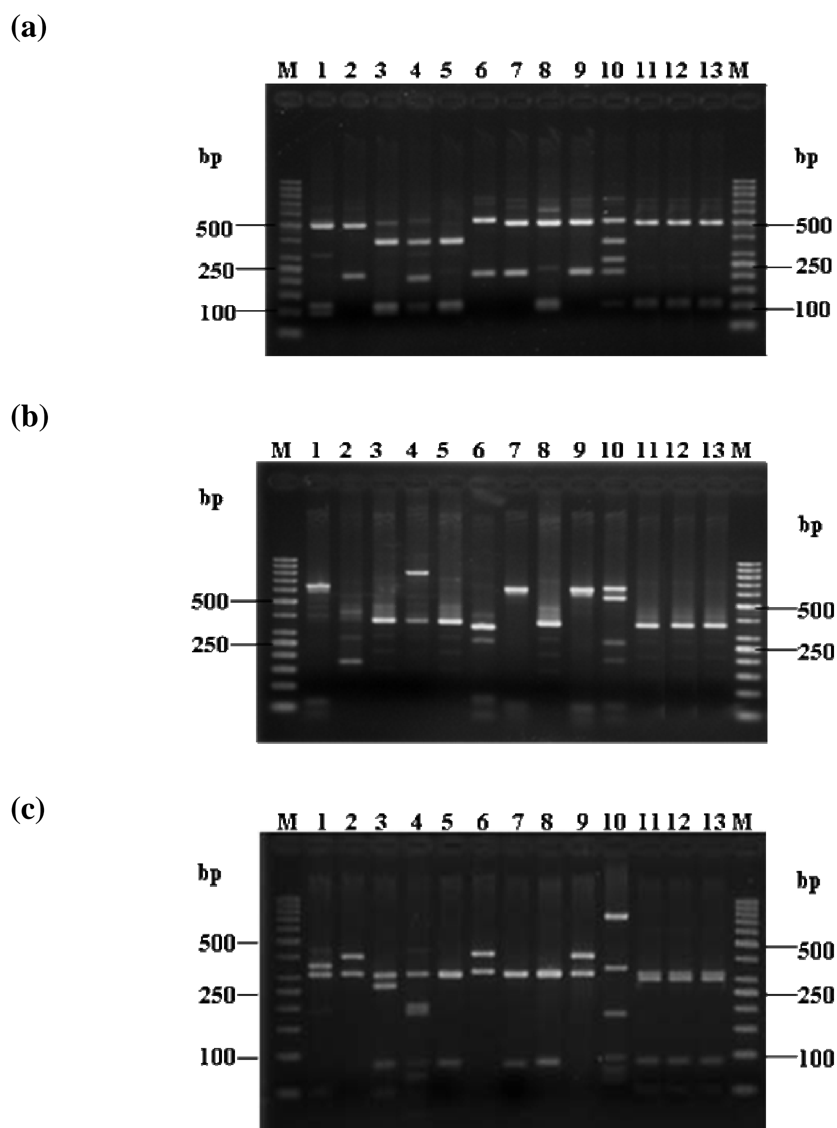


Fig. 4.12 Digestion of ITS PCR products of type strains of *Gluconobacter* with restriction endonucleases *Bst*NI (a), *Mbo*II (b) and *Mbo*I (c).

1, *G. oxydans* NBRC 14819^T; 2, *G. cerinus* NBRC 3267^T; 3, *G. frateurii* NBRC 3264^T; 4, *G. albidus* NBRC 3250^T; 5, *G. thailandicus* BCC 14116^T; 6, *G. kondonii* NBRC 3266^T; 7, *G. roseus* NBRC 3990^T; 8, *G. japonicus* NBRC 3271^T; 9, *G. sphaericus* NBRC 12467^T; 10, *G. kanchanaburiensis* BCC 15889^T; 11, *G. nephelii* strain RBY-1^T; 12, *G. nephelii* strain PHD-2; 13, *G. nephelii* strain PHD-2; M, 50-bp DNA marker.

Group 14 composed of 3 isolates included RBY-1, PHD-1, PHD-2.

All isolates motile with polar flagella (Fig. 4.13). The DNA G+C contents of the strains were 57.2–57.6 mol%. Strains RBY-1, PHD-1 and PHD-2 produced dihydroxyacetone from glycerol, differing from the type strains of *G. oxydans*, *G. albidus*, *G. cerinus*, and *G. japonicus* (weakly positive) (Table 4.5). In acid production, the three strains produced acid from *meso*-erythritol, differing from the type strains of *G. albidus* (weakly positive), *G. kanchanaburiensis* (weakly positive), *G. frateurii* (weakly positive), *G. roseus* (negative) and *G. sphaericus* (negative). The three strains produced acid from maltose weakly, but the type strains of *G. japonicus* and *G. thailandicus* did not (Malimas *et al*., 2009; Tanasupawat *et al*., 2004). The three strains grew on *meso*-erythritol weakly, but the type strains of *G. frateurii* and *G. albidus* did not. In growth on pentitols, the three strains grew on D-arabitol and L-arabitol, differing from the type strain of *G. japonicus* (weakly positive) as show in Table 4.7.

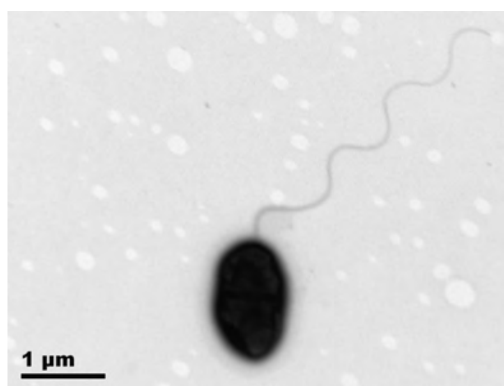


Fig. 4.13 Transmission electron micrograph of strain RBY-1^T cultivated on mannitol agar slants at 30°C for 24 h.

Table 4.7 Differentiatial characteristics of *G. nephelii* strains RBY-1, PHD-1 and PHD-2

Characteristic	RBY-1	PHD-1	PHD-2	*Gj*	*Gf*	*Gt*	*Gc*	*Go*	*Ga*	*Gko*	*Gr*	*Gs*	*Gkn*	*Gw*
Dihydroxyacetone from glycerol	+	+	+	w	+	+	w	w	w	+	+	+	+	+
Water-soluble brown pigment	-	-	-	-	-	-	-	-	-	-	-	+	+	+
2,5-diketo-D-gluconate formation	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Acid production from														
L-Sorbose	-	-	-	+	-	-	+	w	w	w	-	-	w	w
meso-Erythritol	+	+	+	+	w	+	+	+	w	+	-	-	w	-
D-Mannitol	w	w	w	+	-	w	+	w	w	+	w	w	+	+
Glycerol	w	w	w	+	w	-	+	w	+	+	w	w	w	w
Melibiose	-	-	-	+	w	-	+	+	-	+	-	+	w	w
Maltose	w	w	w	-	w	-	+	w	w	w	-	-	w	-
Growth on														
meso-Erythritol	w	w	w	+	-	w	+	w	-	+	+	-	w	w
D-Arabitol	+	+	+	w	+	+	+	-	-	w	w	w	+	+
L-Arabitol	+	+	+	w	+	w	-	-	-	-	-	-	-	-
meso-Ribitol	w	w	w	+	+	w	w	-	-	-	-	-	-	-
DNA G+C (mol%)	57.2	57.6	57.4	56.4†	55.1†	55.8†	55.9†	60.3†	60.0†	59.8†	60.5†	59.5†	59.5†	56.6†

Gj, *G. japonicus* NBRC 3271^{T}; *Gf*, *G. frateurii* NBRC 3264^{T}; *Gt*, *G. thailandicus* BCC 14116^{T}; *Gc*, *G. cerinus* NBRC 3267^{T}; *Go*, *G. oxydans* NBRC 14819^{T}; *Ga*, *G. albidus* NBRC 3250^{T}; *Gko*, *G. kondonii* NBRC 3266^{T}; *Gr*, *G. roseus* NBRC 3990^{T}; *Gs*, *G. sphaericus* NBRC 12467^{T}; *G.kn*, *G. kanchanaburiensis* BCC 15889^{T}; *Gw*, *G. wancherniae* BCC 15775^{T}. +, positive; w, weakly positive; -, negative. †Data from Yukphan *et al*. (2010)

In a phylogenetic tree based on gene ITS sequences (715 bases) the three strains constituted a cluster along with the type strain of *G. japonicus* (Fig. 4.15). The calculated pair-wise sequence similarity in ITS gene sequences of the strain was 98.1% when compare with with the type strain of *G. japonicus*. The data obtained above supported that strain RBY-1 was separated phylogenetically from the type trains of the eleven *Gluconobacter* species in ITS gene sequences (Fig. 4.14).

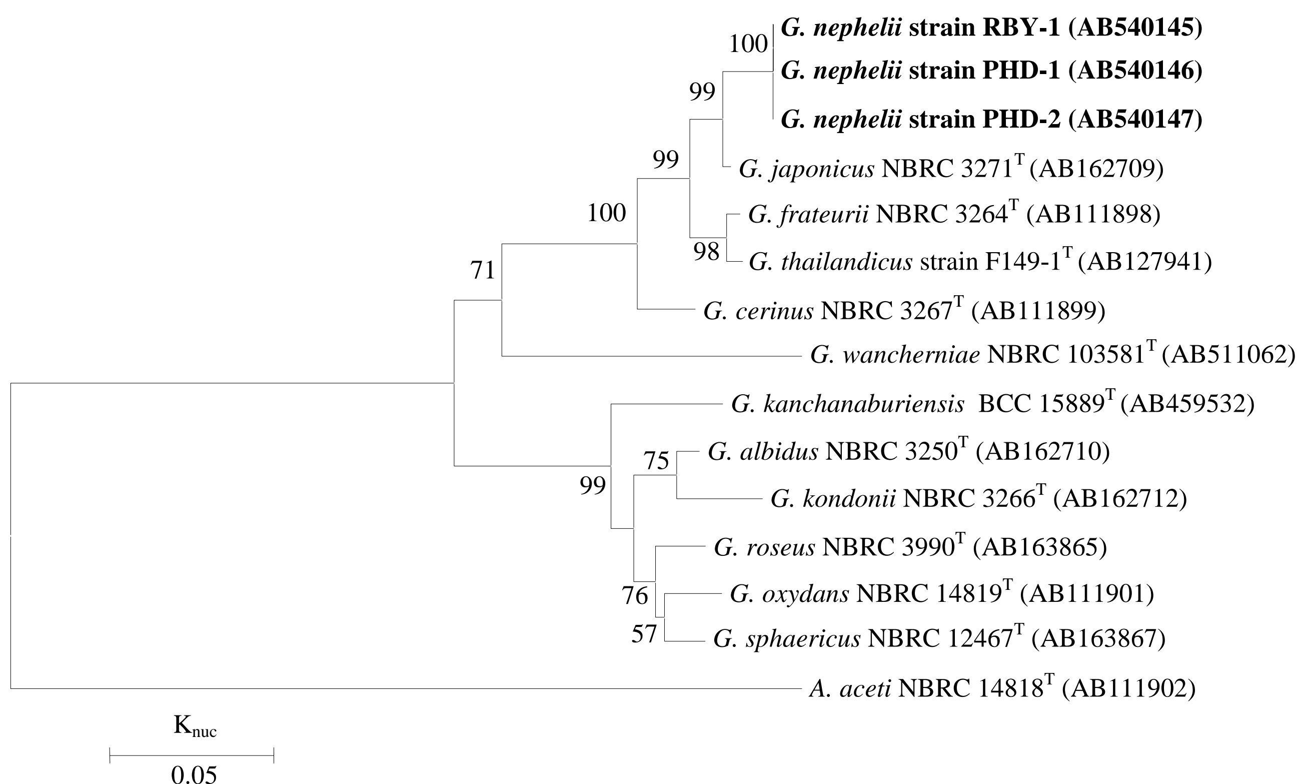


Fig. 4.14 Phylogenetic relashionships of *Gluconobacter nephelii* strains RBY-1, PHD-1 and PHD-2. The phylogenetic trees based on 16S-23S rDNA sequences were constructed by the neighbor-joining method. The type strain of *Acetobacter aceti* was used as an outgroup. Numbers at nodes indicate bootstrap percentages derived from 1000 replications.

The three strains producing restriction fragments in *Bst*NI, *Mbo*II and *Mbo*I respectively comprising 499, 111, 98 and 7 bp, 360 and 355 bp and 306 and 355 bp and 306, 302, 85, 14 and 8 bp and distinguished from the type strains of known *Gluconobacter* species by *Bst*NI digestion as well as from the type strain of *G. oxydans* by *Mbo*II and *Mbo*I digestions (Fig. 4.13). Additionally, the three strains were distinguished from the type strain of *G. japonicus* by *Mbo*I digestion.

The data obtained indicate that the three strains are discriminated from the type strains of the *Gluconobacter* species by the ITS gene restriction analysis using *Bst*NI, *Mbo*II and *Mbo*I.

Strain RBY-1 represented low level of DNA-DNA relatedness ranged from 11-34% to know *Gluconobacter* (Table 4.8). The data obtained above indicate that the three strains are genetically separated at the species level from the type strains of the eleven *Gluconobacter* species and constituted a single species.

Therefore strains RBY-1, PHD-1 and PHD-2 identified were proposed as a new species of the genus *Gluconobacter* namely, *Gluconobacter nephelii*.

Table 4.8 DNA-DNA relatedness of *Gluconobacter nephelii* strains RBY-1^T, PHD-1 and PHD-2.

Strain	%DNA-DNA relatedness with labeled strains			
	RBY-1	NBRC 3271^T	NBRC 3264^T	BCC 14116^T
RBY-1	100	38	30	25
PHD-1	100	40	33	28
PHD-2	100	42	33	26
G. japonicus NBRC 3271^T	34	100	49	57
G. frateurii NBRC 3264^T	34	46	100	41
G. thailandicus BCC 14116^T	29	53	37	100
G. cerinus NBRC 3267^T	27	30	33	42
G. albidus NBRC 3250^T	23	28	36	30
G. kondonii NBRC 3266^T	18	25	45	40
G. kanchanaburiensis NBRC 14819^T	15	22	38	39
G. roseus NBRC 3990^T	12	19	30	32
G. oxydans NBRC 14819^T	11	15	23	26
G. sphaericus NBRC 12467^T	11	11	14	19
A. aceti NBRC 14818^T	6	4	6	5

Group 15 composed of 4 isolates included ZW160-2, LC155-1, LG156-2 and JJ157-2.

All the isolates grew at pH 3.0, 7.5, and 37°C. They did not growth at pH 8.0 and 40 °C. They grew on mannitol agar but not on glutamate agar. They produced D-gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, 2,5-diketo-D-gluconate, and a water-soluble brown pigment from D-glucose. The isolates produced dihydroxyacetone from glycerol. They grew on D-arabitol and *meso*-erythritol, but not on L-arabitol, *meso*-ribitol, and dulcitol (Table 4.9). Acid was produced from L-arabinose, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol (weak), D-mannose, D-mannitol, melibiose, D-ribose, D-sorbitol, L-sorbose (weak), D-xylose, and ethanol. In contrast, no acid production was found from amygdalin, cellobiose, maltose, meletzitose, raffinose, L-rhamnose, salicin, sucrose, and trehalose. The DNA base composition of the four isolates (60.4-60.6 mol% G+C) was similar to that of the type strain of *G. oxydans* (60.3 mol% G+C), but higher than that of the type strain of *G frateurii* and *G. cerinus* (55.1 and 55.9 mol% G+C, respectively) (Table 4.9) (Yukphan *et al.*, 2010). The major ubiquinone homologue of the isolates was Q-10, as found in the type strains of *G. oxydans*, *G. cerinus*, and *G. frateurii*. The isolate ZW160-2 contained 93.4% Q-10, 6.4% Q-9, and 0.7% Q-8; isolate LC155-1 contained 93.4% Q-10 and 6.6% Q-9 whereas LG156-2 contained 93.2% Q-10, 5.9% Q-9 and 0.9% Q-8.

Table 4.9 Differential characteristics of isolates ZW160-2, LC155-1, LG156-2 and JJ157-2

Characteristic	ZW160-2	LC155-1	LG156-2	JJ157-2	*Gj*	*Gf*	*Gt*	*Gc*	*Go*	*Ga*	*Gko*	*Gr*	*Gs*	*Gkn*	*Gw*
Water-soluble brown pigment	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
2,5-diketo-D-gluconate formation	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
Growth at 37 ^{o}C	+	+	+	+	-	-	w	-	+	-	-	-	-	-	-
Acid production from															
Inositol	w	w	w	w	+	+	-	+	+	w	+	-	w	w	w
Maltose	-	-	-	-	-	w	-	+	w	w	w	-	-	w	-
D-Mannose	+	+	+	+	w	-	+	w	+	w	w	+	+	+	+
Melibiose	+	+	+	+	+	+	-	+	+	-	+	-	+	w	w
Sucrose	-	-	-	-	w	-	-	+	-	+	w	+	+	w	w
D-Sorbitol	+	+	+	+	w	+	-	-	+	+	+	+	w	w	w
Growth on															
D-Arabitol	+	+	+	+	w	+	+	-	+	-	w	w	w	+	+
L-Arabitol	-	-	-	-	w	w	w	-	-	-	-	-	-	-	-
meso-Ribitol	-	-	-	-	+	w	w	w	-	-	-	-	-	-	-
DNA G+C (mol%)	60.5	60.6	60.5	60.4	56.4[†]	55.1[†]	55.8[†]	55.9[†]	60.3[†]	60.0[†]	59.8[†]	60.5[†]	59.5[†]	59.5[†]	56.6[†]

Gj, *G. japonicus* NBRC 3271^{T}; *Gf*, *G. frateurii* NBRC 3264^{T}; *Gt*, *G. thailandicus* BCC 14116^{T}; *Gc*, *G. cerinus* NBRC 3267^{T}; *Go*, *G. oxydans* NBRC 14819^{T}; *Ga*, *G. albidus* NBRC 3250^{T}; *Gko*, *G. kondonii* NBRC 3266^{T}; *Gr*, *G. roseus* NBRC 3990^{T}; *Gs*, *G. sphaericus* NBRC 12467^{T}; *Gkn*, *G. kanchanaburiensis* BCC 15889^{T}; *Gw*, *G. wancherniae* BCC 15775^{T}. +, positive; w, weakly positive; -, negative. [†]Data from Yukphan *et al*. (2010)

The phylogenetic tree based on 16S-23S rDNA ITS region sequences showed that strain ZW160-2 was located in the sublineage of *G. oxydans* and constituted a cluster separate from the type strains of the known species, *G. cerinus*, and *G. frateurii*. Sequence similarity of ZW160-2 represented 97.3, 83.9, 81.9, and 62.4% respectively to the type strains of *G. oxydans*, *G. cerinus*, *G. frateurii*, and *A. aceti*. (Fig. 4.15)

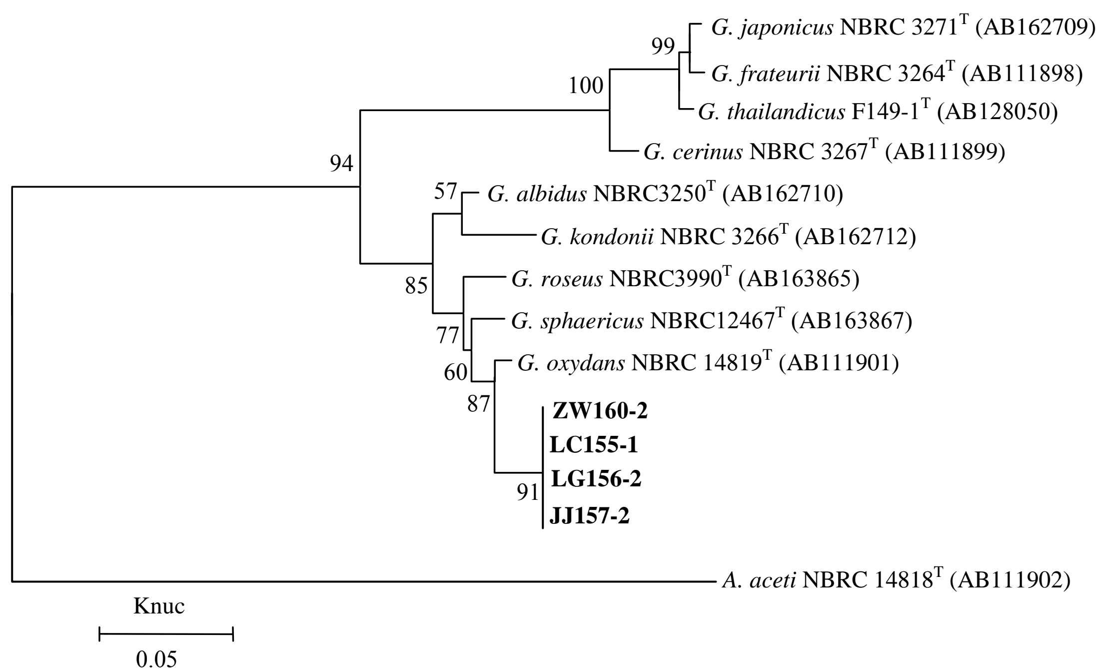
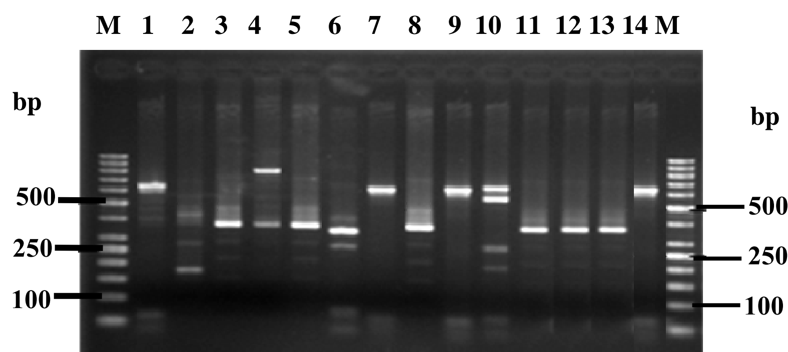


Fig. 4.15 Phylogenetic relashionships of strains ZW160-2, LC155-1, LG156-2 and JJ157-2. The phylogenetic trees based on ITS gene sequence. ITS sequences were constructed by the neighbor-joining method. The type strain of *Acetobacter aceti* was used as an outgroup. Numbers at nodes indicate bootstrap percentages derived from 1000 replications.

In the ITS-RFLP analysis, the representative strain ZW160-2 (line 14) showed the same restriction pattern forms as *G. oxydans* NBRC 14819^T (line 1), *G. roseus* NBRC 3990^T (lane 7) and *G. sphaericus* NBRC 12467 (lane 9) when digested with *Mbo*II but it discriminate from *G. oxydans* NBRC 14819^T (line 1), *G. roseus* NBRC 3990^T (lane 7) and *G. sphaericus* NBRC 12467 (lane 9) when digestion with *Bst*NI restriction endonucease (Table 4.5, Fig. 4.16).

(a) *Mbo*II



(b) *Bst*NI

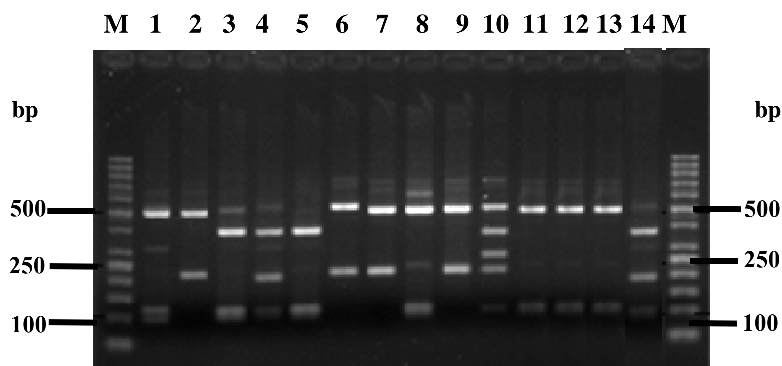


Fig. 4.16 Digestion of ITS PCR products of *Gluconobacter* ZW160-2 with restriction endonucleases *Mbo*II (a) and *Bst*NI (b).

1, *G. oxydans* NBRC 14819^T; 2, *G. cerinus* NBRC 3267^T; 3, *G. frateurii* NBRC 3264^T; 4, *G. albidus* NBRC 3250^T; 5, *G. thailandicus* BCC 14116^T; 6, *G. kondonii* NBRC 3266^T; 7, *G. roseus* NBRC 3990^T; 8, *G. japonicus* NBRC 3271^T; 9, *G. sphaericus* NBRC 12467^T; 10, *G. kanchanaburiensis* BCC 15889^T; 11, strain RBY-1^T; 12, strain PHD-2; 13, strain PHD-2; 14, isolate ZW160-2 (Group 15); M, 50-bp DNA marker.

Table 4.10 DNA-DNA relatedness of strains ZW160-2, LC155-1, LG156-2 and JJ157-2.

Strain	%DNA-DNA relatedness with labeled strains			
	ZW160-2	NBRC 14819^T	NBRC 12467^T	NBRC 3990^T
ZW160-2	100	24	26	21
LC155-1	100	28	38	54
LG156-2	100	31	41	52
JJ157-2	100	34	43	53
G. oxydans NBRC 14819^T	26	100	21	29
G. sphaericus NBRC 12467^T	22	14	100	26
G. roseus NBRC 3990^T	19	23	35	100
G. kondonii NBRC 3266^T	19	21	38	43
G. albidus NBRC 3250^T	17	28	38	30
G. cerinus NBRC 3267^T	16	30	33	42
G. thailandicus BCC 14116^T	13	53	37	26
G. frateurii NBRC 3264^T	12	46	38	41
G. japonicus NBRC 3271^T	11	33	47	42
G. kanchanaburiensis NBRC 14819^T	11	25	36	45
A. aceti NBRC 14818^T	5	4	5	3

Strain ZW160-2 represented levels of DNA-DNA relatedness of 100, 100, 100, 100, 26, 22, 20, 19, 17, 16, 13, 12, 11, 11 and 5% respectively to strains ZW160-2, LC155-1, LG156-2, JJ157-2, *G. oxydans* NBRC 14819^T, *G. sphaericus* NBRC 12467^T, *G. roseus* NBRC 3990^T, *G. kondonii* NBRC 3266^T, *G. albidus* NBRC 3250^T, *G. cerinus* NBRC 3267^T, *G. thailandicus* BCC 14116^T, *G. frateurii* NBRC 3264^T, *G. japonicus* NBRC 3271^T, *G. kanchanaburiensis* BCC 15889^T, and *A. aceti* NBRC 14818^T (Table 4.10).

The data obtained above supported that the four strains ZW160-2, LC155-1, LG156-2 and JJ157-2 should be proposed as a new species of the genus *Gluconobacter*.

4.2.3 Isolates assigned to the genus *Asaia*

In *Asaia* species were divived 3 groups; *As. bogorensis* 8 isolates (Group 16), *As. siamensis* 5 isolates (Group 17), *Asaia* sp. 2 isolates (Group 18).

Table 4.11 Differential characteristics of isolates in *Asaia* species

Characteristic	G16	*Asb*	G17	*Ass*	*Ask*	*Asl*	GB23-2	GB23-3
Oxidation of acetate and lactate	w	w	w	w	w	w	w	w
Growth on								
30% Glucose	+	+	+	+	+	+	+	+
0.35% acetic acid	w	w	w	w	w	-	w	w
L-Rhamnose	+(w6)	+	w	w	w	+	w	w
D-Mannitol	+(w3)	+	+(w5)	+	w	+	w	+
D-Sorbitol	+(w3)	+	+(w6)	+	w	+	w	w
Dulcitol	w	w	-	-	w	+	-	-
Maltose	w	w	w(-2)	w	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Acid production from								
L-Rhamnose	+(w4)	+	w	w	+	+	-	-
D-Mannitol	+(w6)	+	+	+	w	w	w	w
D-Sorbitol	w	w	+(w4)	+	w	w	w	w
Dulcitol	w	w	-	-	w	w	-	-
Sucrose	-	-	-	-	-	-	-	w
Raffinose	w	w	-	-	-	-	-	-
Ethanol	-	-	w	w	-	w	+	+
16S rDNA gene restriction pattern type with[a]								
*Hpy*AV	*Ab*	*Ab*	*Ab*	*Ab*	*Ab*	*Ab*	*Asp*	*Asp*
DNA G+C content (mol%)	60.1	60.2*	59.4	59.3*	60.3*	60.8*	59.7	59.8

+, positive; w, weakly positive; -, negative. [a]For detailed information of restriction fragments, see the references (Malimas *et al*., 2008a; Yukphan *et al*., 2004).

*The data were cited from the references (Katsura *et al*., 2001; Malimas *et al*., 2008a;Yamada *et al*., 2000; Yukphan *et al*., 2004).

Asb, *Asaia bogorensis* BCC 12264^T; G16, Group 16 (8 isolates); *Ass*, *Asaia siamensis* BCC 12268^T; G17, Group 17 (5 isolates); *Ask*, *Asaia krungthepensis* BCC 12978^T; *Asl*, *Asaia lannensis* BCC 15733^T

Group 16 composed of 8 isolates included PHD-46, PHD-47, PHD52, PHD-64, PHD-65, PHD-68, PHD-79 and PHD-80.

All isolates grew on glutamate agar and mannitol agar, but did not grow at the expense of methanol. They did not produce water-soluble brown pigment. All isolates did not produce acetic acid from ethanol. They produced dihydroxyacetone from glycerol was weakly. The isolates produced 2-keto-D-gluconate and 5-keto-D-gluconate, but not 2,5-diketo-D-gluconate. All the isolates produced acid from D-glucose, D-mannose, D-fructose, L-sorbose, D-xylose, L-arabinose, D-ribose, dulcitol, *myo*-inositol, ribitol, D-arabitol, xylitol, *meso*-erythritol, glycerol, melibiose and sucrose, but not from lactose. They produced acid from D-mannitol and D-sorbitol differed among strains (Appendix B). The G+C content of isolate PHD-46 was 60.1 mol%. They showed the same phenotypic characteristics as *As. bogorensis* (Yamada *et al*., 2000). All of the isolates were located within the cluster of *As. bogorensis* and showed 100% pair-wise 16S rDNA sequence similarity with the type strain of *As. bogorensis* (Fig. 4.17). All isolates also gave restriction patterns that coincided with those of the type strain of *As. bogorensis* when digested with *Sty*I, *Bso*JI, *Sna*BI, and *Hpa*II (Fig. 4.19).

From data obtained above, all isolated in Group 16 identified as *As. bogorensis.*

Group 17 composed of 5 isolates included PHD-48, PHD-49, PHD-50, PHD-58 and PHD-69.

They grew on glutamate agar and mannitol agar and produced acetic acid from ethanol. They did not growth on methanol. They produced dihydroxyacetone from glycerol, 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but not 2,5-diketo-D-gluconate, and produced a water-soluble brown pigment. They produced acid is from D-glucose, D-mannose, D-fructose, L-sorbose, D-xylose, L-arabinose, D-ribose, D-mannitol, D-sorbitol, *myo*-inositol, ribitol, D-arabitol, xylitol, *meso*-erythritol, glycerol, melibiose and sucrose, but not from lactose and dulcitol (Appendix B). The isolates had a major ubiquinone as Q-10. The DNA G+C content of isolate PHD-48 was 59.4 mol%. They showed the same phenotypic characteristics as *As. siamensis* (Katsura *et al*., 2001). All of the isolates were located within the cluster of *As. siamensis* and showed 100% pair-wise 16S rDNA sequence similarity with the type strain of *As. siamensis* (Fig. 4.17). All isolates also gave restriction patterns that coincided with those of the type strain of *As. siamensis* when digested with *Sty*I, *Bso*JI, *Sna*BI, and *Hpa*II (Fig. 4.19).

From data obtained above, all isolated in Group 16 identified as *As. siamensis*.

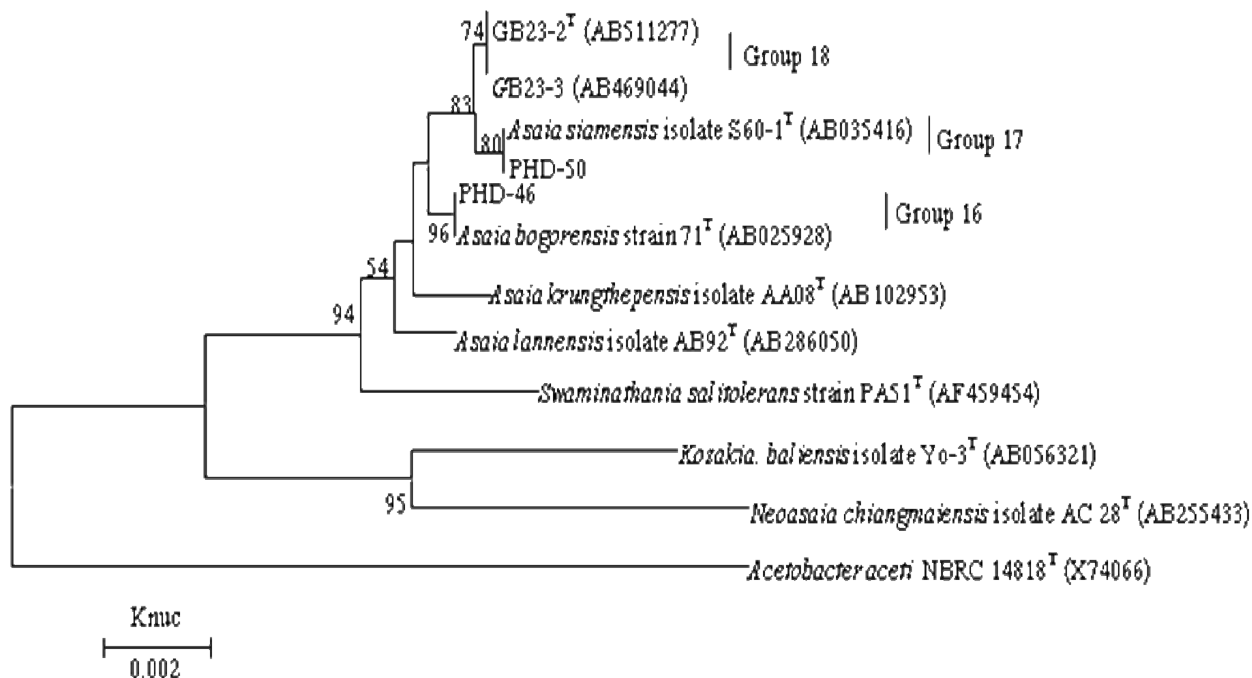


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Fig. 4.17 Neighbour-joining-tree showing the phylogenetic position of Group 16 to Group 18 based on 16S rDNA sequences. Bar, 0.002 substitution per nucleotide position. Bootstrap values as previously.

Group 18 composed of 2 isolates included GB23-2 and GB23-3.

They grew on glutamate agar and mannitol agar. They produced acetic acid on ethanol/calcium carbonate agar, 2-keto-D-gluconate and 5-keto-D-gluconate but not 2,5-diketo-D-gluconate from D-glucose. They produced dihydroxyacetone from glycerol, but the activity is not intense. They produced acid from D-glucose, D-mannose, D-galactose, D-xylose, D-arabinose, L-arabinose, D-fructose, L-sorbose, D-mannitol (weakly positive), D-sorbitol (weakly positive), D-arabitol, L-arabitol, *meso*-ribitol, *meso*-erythritol, glycerol, melibiose, and ethanol but did not from L-rhamnose, dulcitol, sucrose (weakly positive in isolate GB23-3), and raffinose. They grew on D-glucose, D-mannose, D-galactose, D-xylose, D-arabinose, L-arabinose (weakly positive), L-rhamnose (weakly positive), D-fructose, L-sorbose, D-mannitol (weakly positive in isolate GB23-2^T), D-sorbitol (weakly positive), D-arabitol, L-arabitol, *meso*-ribitol, *meso*-erythritol, glycerol, melibiose, sucrose, and ethanol but did not on dulcitol, raffinose, maltose, and lactose. DNA base composition is 59.7–59.8 mol%.

A phylogenetic tree, isolates GB23-2 and GB23-3 were located within the lineage of the genus *Asaia* and formed an independent cluster from the type strains of the four known *Asaia* species (Fig. 4.18). The calculated pair-wise sequence similarities of isolate GB23-2 were 99.7, 99.9, 99.4, and 99.3% respectively to the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, and *Asaia lannensis*. The data obtained suggested that isolates GB23-2 and GB23-3 constitute a new taxon within the genus *Asaia*.

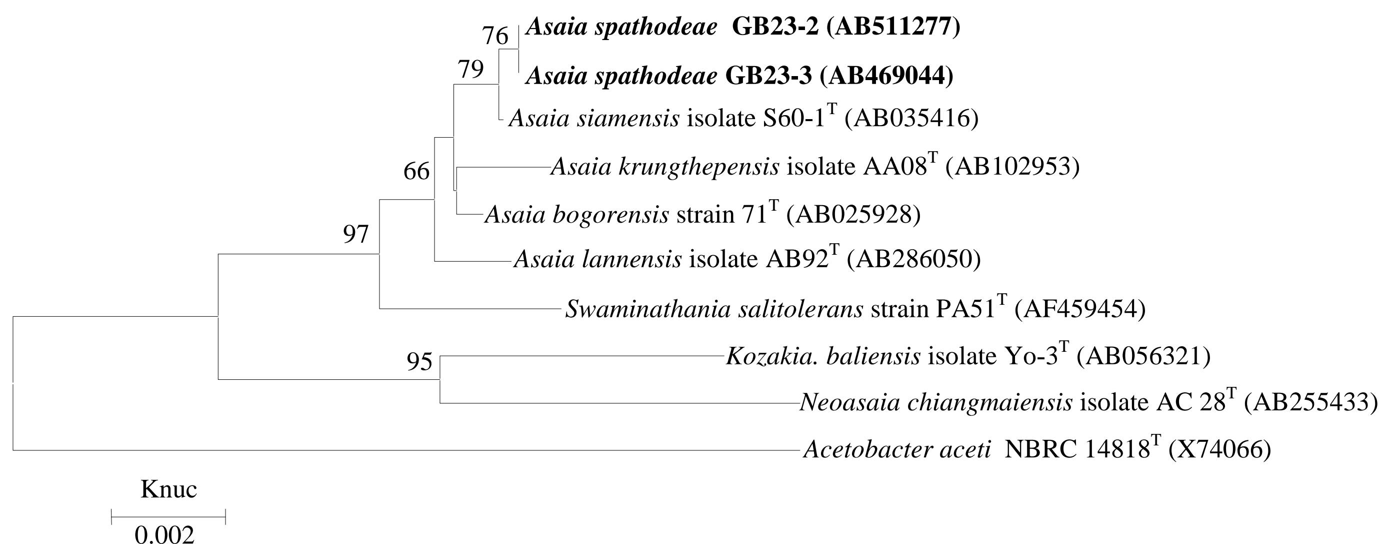

Fig. 4.18 Phylogenetic relationships of *Asaia spathodeae* isolates GB23-2 and GB23-3 based on 16S rDNA gene sequences. The phylogenetic tree was constructed by the neighbor-joining method. Numbers at nodes indicate bootstrap percentages derived from 1,000 replications.

The two isolates produced restriction fragments comprised of: 1) 790, 327, 214, and 83 bp in *Sty*I digestion; 2) 738, 205, 172, 148, 123, 91, 87, 87, 55, 29, 28, 24, 16, and 11 bp in *Bsa*JI digestion; 3) no restriction fragment in *Sna*BI digestion; 4) 445, 421, 216, 210, 58, 53, 15, and 11 bp in *Hpa*II digestion; 5) 454, 350, 190, 196, 169, 152, and 99 bp in *Hpy*AV digestion. On the other hand, the type strains of the four *Asaia* species produced almost identical restriction fragments comprised of 454, 295, 190, 166, 151, 99, and 55 bp in *Hpy*AV digestion.

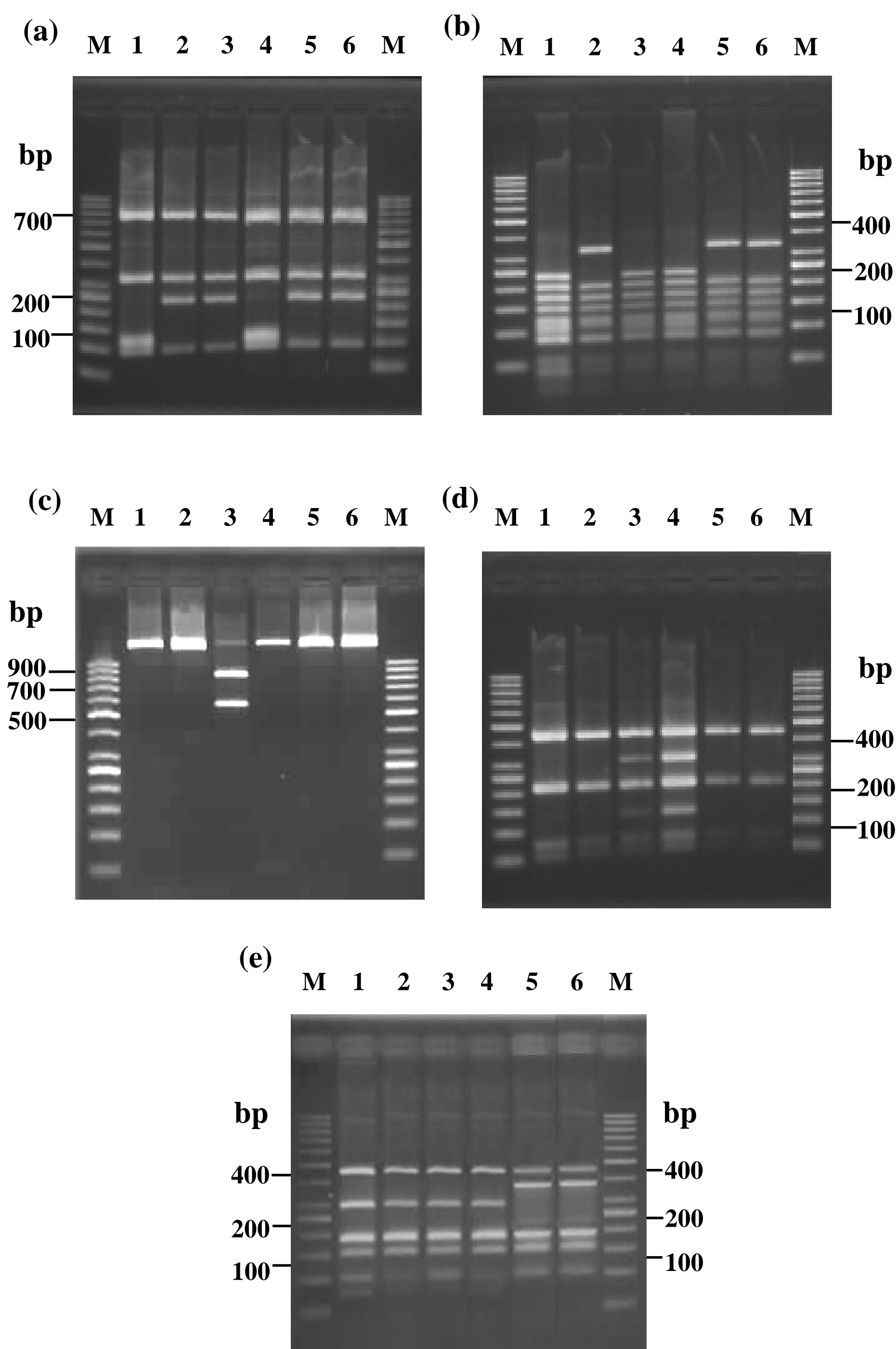

Fig. 4.19 Restriction of 16S rDNA gene PCR products of *Asaia spathodeae* isolates GB23-2 and GB23-3 by digestion with five restriction endonucleases.

The restriction patterns were obtained by digestion with *Sty*I (a), *Bsa*JI (b), *Sna*BI (c), *Hpa*II (d), and *Hpy*AV (e). Lanes: 1, *Asaia bogorensis* BCC 12264^T; 2, *Asaia siamensis* BCC 12268^T; 3, *Asaia krungthepensis* BCC 12978^T; 4, *Asaia lannensis* BCC 15733^T; 5, *Asaia spathodeae* isolate GB23-2^T; 6, *Asaia spathodeae* isolate GB23-3; M, 50-bp DNA marker.

Isolates GB23-2 and GB23-3 were hybridized with DNAs from test strains, levels of DNA-DNA hybridization were 100, 100, 27, 38, 44, 36, and 5% and 100, 100, 31, 40, 48, 40, and 6% respectively with isolates GB23-2 and GB23-3 and the type strains of *Asaia bogorensis*, *Asaia siamensis*, *Asaia krungthepensis*, *Asaia lannensis*, and *Acetobacter aceti* (Table 4.12).

Table 4.12 DNA base composition and DNA-DNA hybridization of *Asaia* sp. GB23-2 and GB23-3.

	DNA-DNA relatedness (%) of						
Labeled DNA from	GB23-2	GB23-3	BCC 12264^T	BCC 12978^T	BCC 12268^T	BCC 15733^T	NBRC 14818^T
GB23-2	100	100	27	38	43	36	5
GB23-3	100	100	31	40	48	40	6
As. bogorensis BCC 12264^T	21	28	100	56	59	46	8
As. krungthepensis BCC 12978^T	35	43	27	100	39	23	5
As. siamensis BCC 12268^T	41	53	22	38	100	34	6
As. lannensis BCC 15733^T	31	41	30	36	23	100	5

In this isolates was proposed, the name of *Asaia spathodeae* sp. nov. to accommodate the two isolates as the fifth species of the genus *Asaia*.

4.2.4 Isolates assigned to the genus *Gluconacetobacter*

Group 19 composed of 4 isolates included PHD-53, PHD-54, PHD-89 and PHD-90.

They were gram-negative, obligate aerobes, motile and non motile rods. Formed orange, round, mucous, smooth and convex colonies. They were oxidize acetate and lactate. All the isolates grew in the presence of 0.35% acetic acid. They grew on glutamate agar.They produced acid from glycerol, L-arabinone, D-glucose, glycerol, D-mannose and sorbitol *meso*-erythritol but not on ducitol (Appendix B). The DNA G+C content of isolates PHD-53 and PHD-89 were 62.3 and 64.3 mol% respectively. They showed the same phenotypic characteristics as *Ga. liquefaciens* so they were identified as *Ga. liquefaciens* (Franke *et al.*, 1999).

The calculated pair-wise sequence similaritie of the strains were 99.9% pair-wise 16S rDNA sequences with the type strain of *G. liquefaciens* in for 1412 bases (Fig. 4.20).

Table 4.13 Characteristics of isolates in *Gluconacetobacter* species

Characteristic	G19 (4 isolates)	*Ga.l*	*Ga.s*
Oxidation of acetate and lactate	+	+	+
Growth on			
Glutamate agar	+	+	+
Mannitol agar	+	+	+
30% Glucose	-	-	+
Ketogenesis from glycerol	+	+	-
Acid production from			
D-Mannitol	-	-	-
D-Sorbitol	-	-	-
Dulcitol	-	-	-
DNA G+C content (mol%)	62.3-64.3	62-65*	62-67*

*Cited from Franke *et al* (1999). G19, Group 19; Ga.*s, Ga. sacchari* SRI 1794; *Ga.l, Ga. liquefaciens* IFO 12388

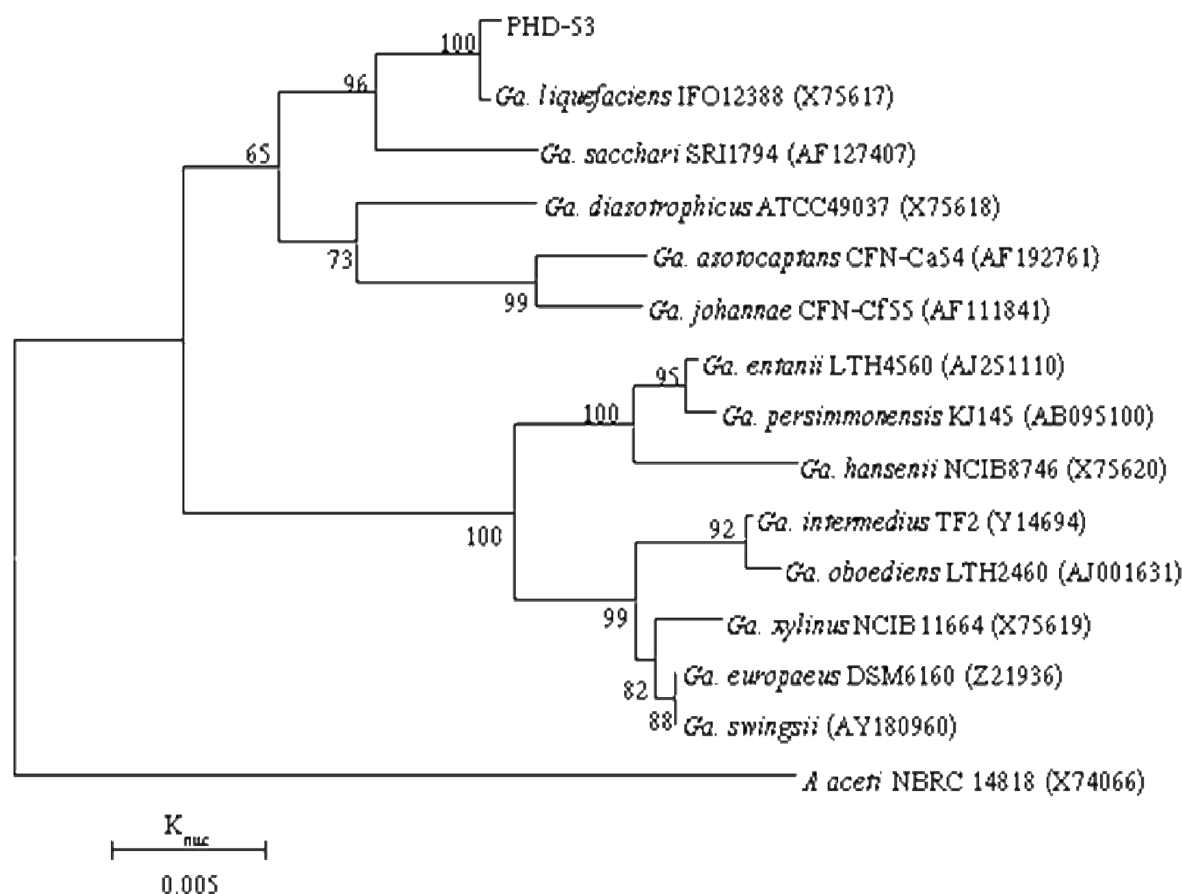

Fig. 4.20 Neighbour-joining-tree showing the phylogenetic position of Group 19 based on 16S rDNA sequences. Bar, 0.005 substitution per nucleotide position. Bootstrap values Bootstrap values as previously.

4.3 Oxidative products of *Acetobacter* and *Gluconobacter*

4.3.1 Acetic acid production

4.3.1.1 ADH activity assay:

The eighty-six isolates of *Acetobacter* showed ADH activity ranged from 2.05 to 7.52 unit/mg at 30°C. The isolate PHD-23 showed the highest ADH activity, 7.52 unit/mg at 30°C (Fig. 4.21). Therefore, *A. pasteurianus* isolate PHD-23 was selected for optimization in acetic acid production.

ADH activity

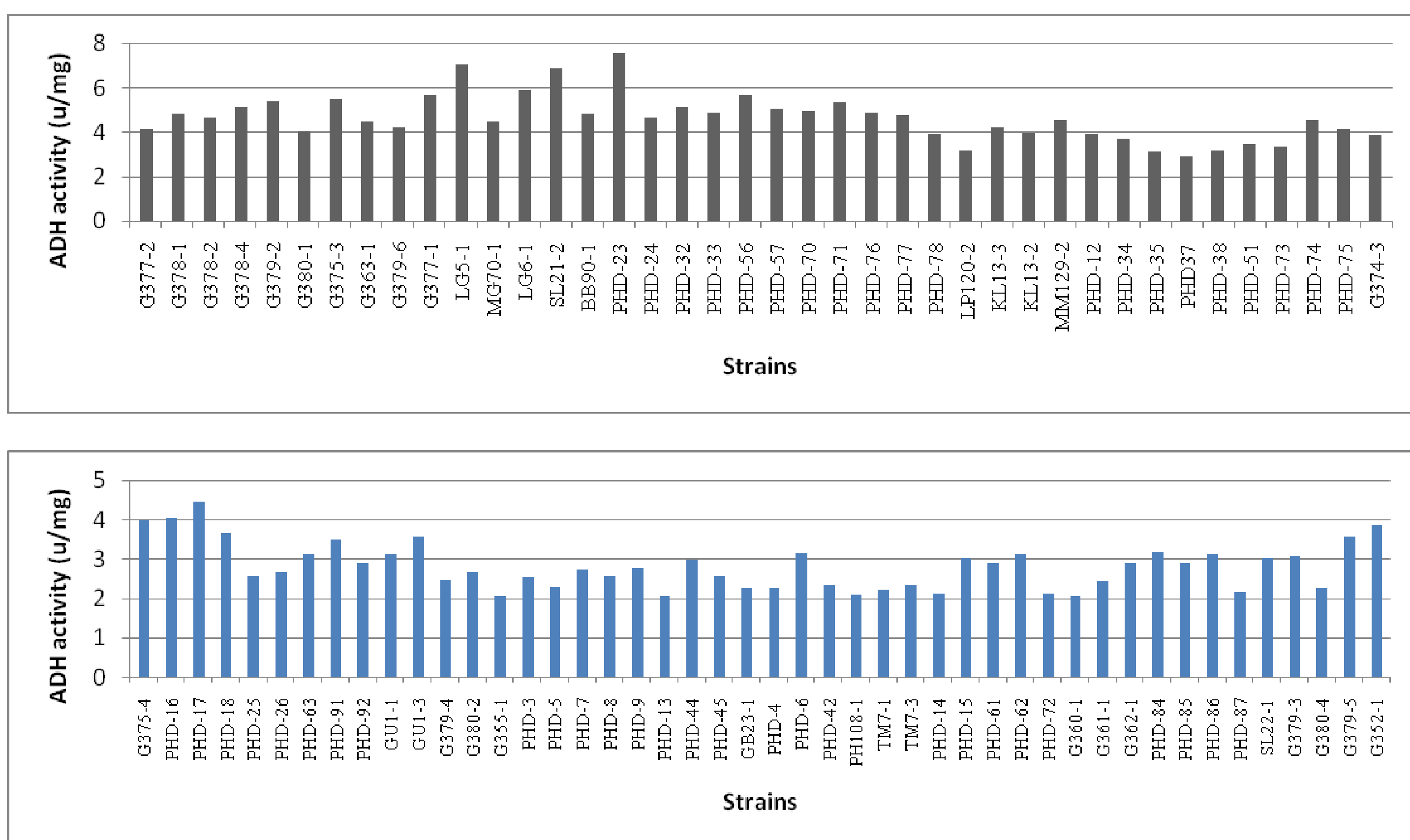

Fig. 4.21 Alcohol dehydrogenase (ADH) activity of isolated assigned to *Acetobacter*.

4.3.1.2 Effect of ethanol and initial acetic acid concentration acetic acid production

Effects of initial ethanol concentrations

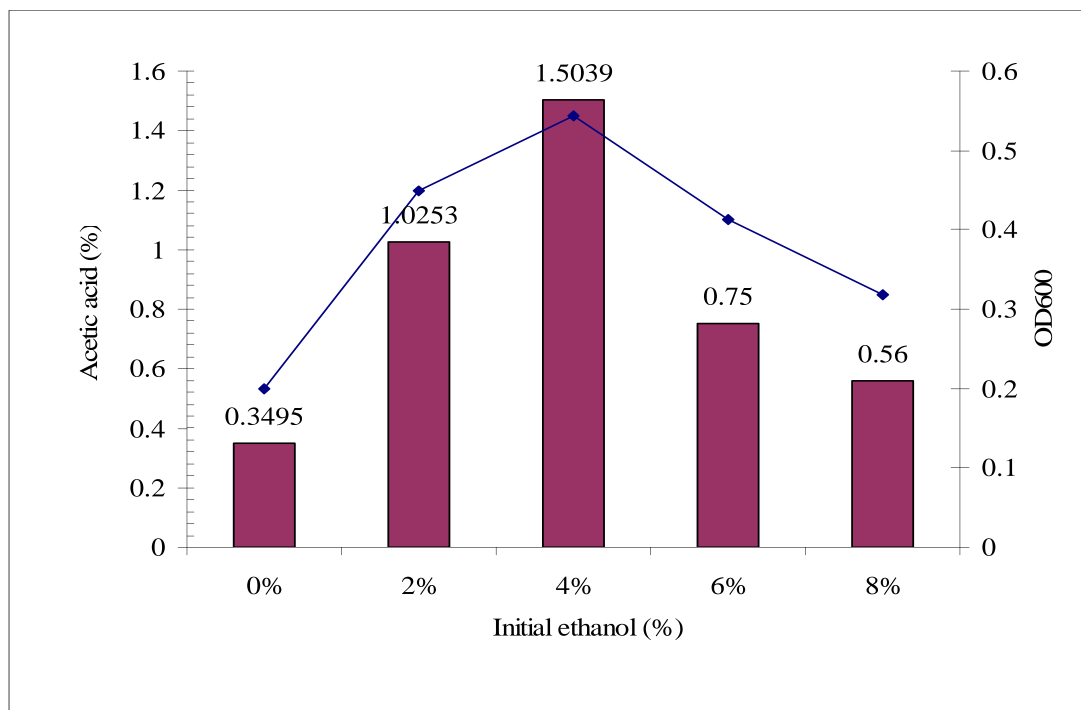

Fig. 4.22 Effect of initial ethanol concentrations on acetic acid production from *A. pasteurianus* isolate PHD-23 at 0, 2, 4, 6, 8, 10, 12% ethanol. Symbols: acetic acid (coloumn), growth (line).

(The medium composed of 0.3% yeast extract and various concentrations of ethanol were added initially as indicated, incubated at 30º C, 200 rpm for 3 days.)

The optimum ethanol concentration for acetic acid production was 4%. At the peak at 4% ethanol, *A. pasteurianus* isolate PHD-23 produced about 1.5% acetic acid. When ethanol was added higher 4% up to 8%, low acetic acid were observed (Fig. 4.22) and this strain still growth at ethanol concentration up to 8%. On the other hand, *A. lovaniensis* SKU 1108 and SKU 1112 have an ethanol tolerance up to 9% and no appreciable lag period but when ethanol was fed to 10%, a fairly long lag time was observed (Saeki *et al*., 1997).

Effects of initial acetic acid concentration

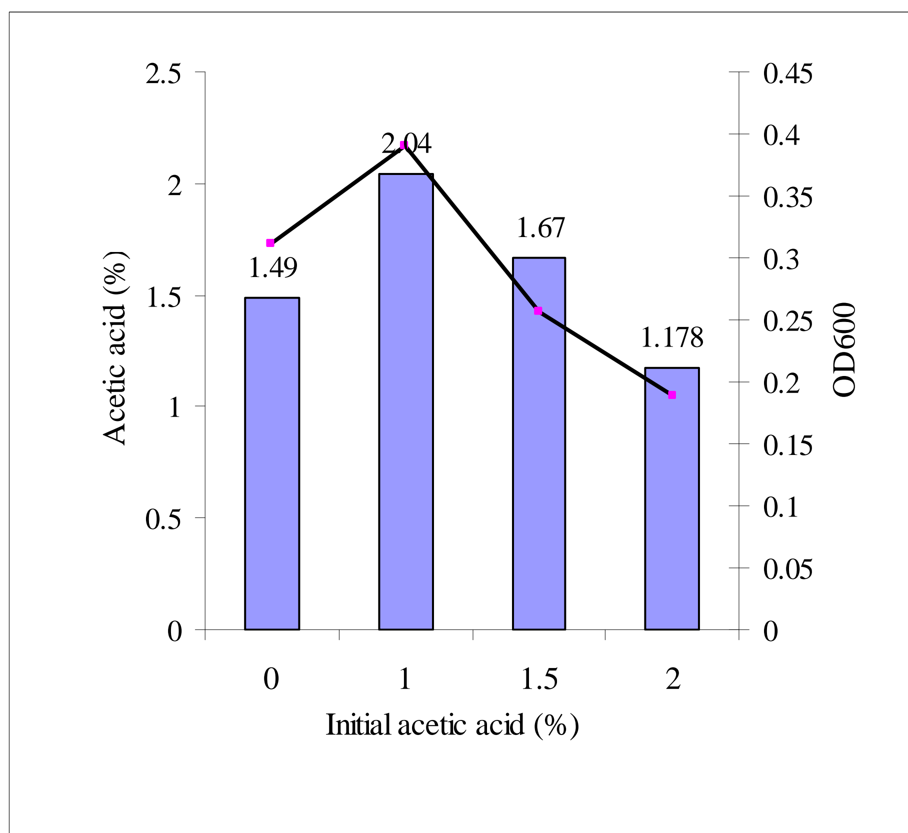

Fig. 4.23 Effect of initial acetic acid concentration on acetic acid production from *A. pasteurianus* isolate PHD-23 at 0, 1, 1.5, 2, 2.5, 3% acetic acid. Symbols: acetic acid (coloumn), growth (line).

(The medium composed of 0.3% yeast extract, 4% ethanol and various concentrations of acetic acid were added initially as indicated, incubated at 30º C, 200 rpm for 3 days.)

The effect of initial acetic acid concentration on acetic acid production from *A. pasteurianus* isolated PHD-23 at 0-2% as indicated and ethanol was added initially to 4% to all cultures found that *A. pasteurianus* isolate PHD-23 still oxidized ethanol, and acetic acid was accumulated when the initial concentrations of acetic acid were less than 2% (Fig. 4.23). From the data suggested that *A. pasteurianus* isolate PHD-23 could produce the highest acetic acid concentration grown in the medium with out addition of acetic acid, growth this strain was decreased when acetic acid increased and found that it showed no growth when acetic acid was added at 2.5 and 3%. On the other hand, *A. lovaniensis* SKU 1108 and SKU 1112 still oxidized ethanol, and acetic acid was accumulated when the initial concentrations of acetic acid were less than 4% (Saeki *et al*., 1997).

4.3.2 Dihydroxyacetone production (DHA) by *Gluconobacter* strains

4.3.2.1 Preliminary screening for DHA production

Forty-two isolates of *Gluconobacter* were tested for their ability to produce DHA. Initial screening was tested based on the diphenylamine method. DHA was observed by the blue color development. The sugar quantity can be estimated based on the standard graph of a series of known concentration of the DHA. It was found that 42 isolates were produced large accumulations of DHA ranged from 20.05 to 42.52 g/l at 30°C for 4 days (Appendix D). The *G. oxydans* isolate PHD-27 showed the highest DHA production reached to 42.52 g/l (Fig 4.24) and was selected for futher experiment.

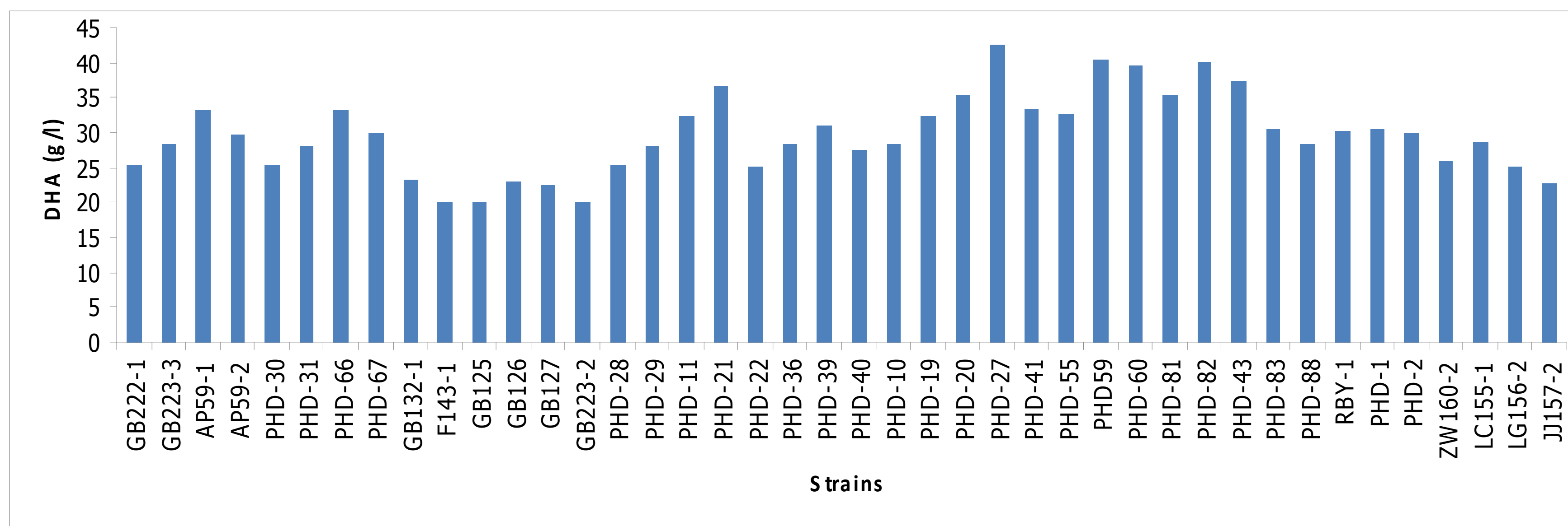

Fig. 4.24 Preliminary screening for DHA production from strains assigned to *Gluconobacter*

4.3.2.2 DHA production

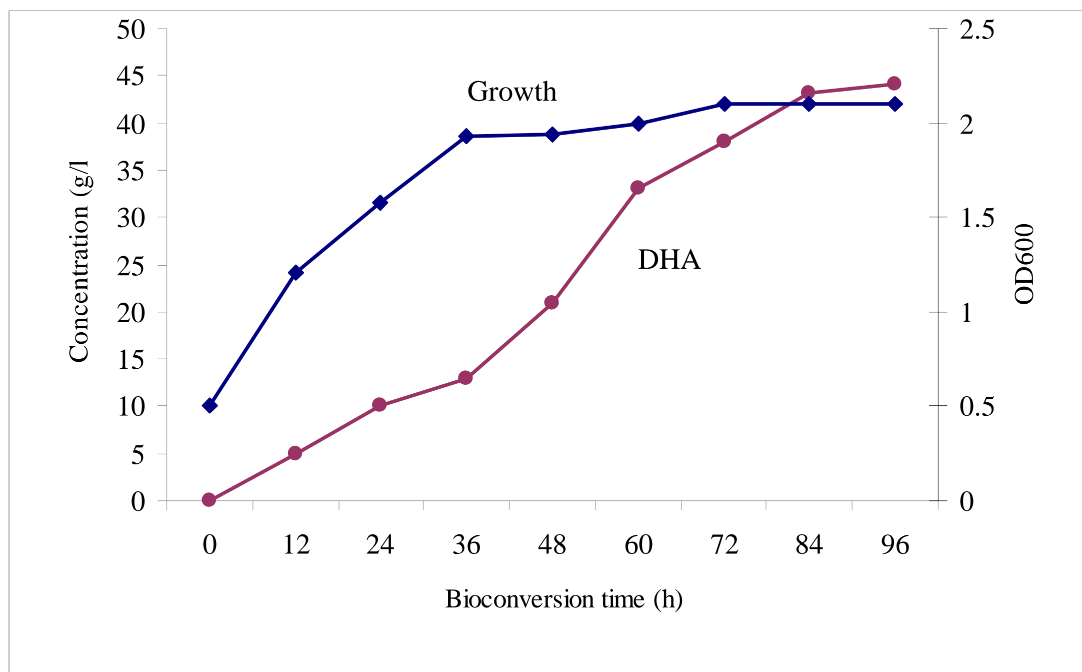

Fig. 4.25 Time-course of DHA production and growth for the *G. oxydans* isolate PHD-27 (The medium composed of 5% glycerol and 1% yeast extract, incubated at 30º C, 200 rpm for 4 days.)

The *G. oxydans* isolate PHD-27 produced the DHA reached the maximum to 44.08 g/l at 30°C by conversion time of 84 h and generated DHA at a rate of 0.52 g/l/h at 30°C. In the growth period, *G. oxydans* isolate PHD-27 showed good growth without lag phase and produced rapid DHA after late log phase (Fig. 4.25).

The final DHA concentration by *G. oxydans* that was improved by genetic engineering in shake-flask experiments reached 30 g/l while the wild type produced 18–25 g/l of DHA from 50 g/l glycerol (Gatgens *et al.*, 2007). These data suggested that the DHA production from *G. oxydans* isolate PHD-27 higher than *G. oxydans* was improved by genetic engineering and wild type (Gatgens *et al.*, 2007).

Lili *et al*. (2006) reported *Acetobacter* sp. produced the DHA concentration reached the maximum of 26.12 g/l at by co000000nversion time of 96 h with residual glycerol of 8.7 g/l. The data supported that *Acetobacter* has lower ability DHA production than *Gluconobacter*.

4.3.3 L-sorbose production by *Gluconobacter* strains

4.3.3.1 Preliminary screening for L-sorbose production

Forty-two isolates of *Gluconobacter* were tested for their ability to produce L-sorbose. Initial screening was tested based on the resorcinol method. This assay is rapid and simple in determining the presence of ketoses as observed by the cherry-red colour development. The sugar quantity can be estimated based on the standard graph of a series of known concentration of the L-sorbose. It was found that 42 isolates were produced large accumulations of L-sorbose ranged from 19.99 to 48.39 g/l at 30°C for 48 h (Fig 4.26). In the pH of the culture broth came down from 6.3 to 4.7 (data not show).

Moonmangmee *et al*. (2000) isolated and screened thermotolerant *Gluconobacter* for D-fructose and L-sorbose production. In the part of L-sorbose production, fermentation efficiency and fermentation rate of the strain CHM54 was quite high and rapidly oxidized D-sorbitol to L-sorbose at almose 100% within 24 h at 30°C. The efficiency of L-sorbose fermentation by the strain CHM54 at 37°C was superior to that observed at 30°C. Therefore, in the present work are interested in screening thermotolerant *Gluconobacter* for L-sorbose production.

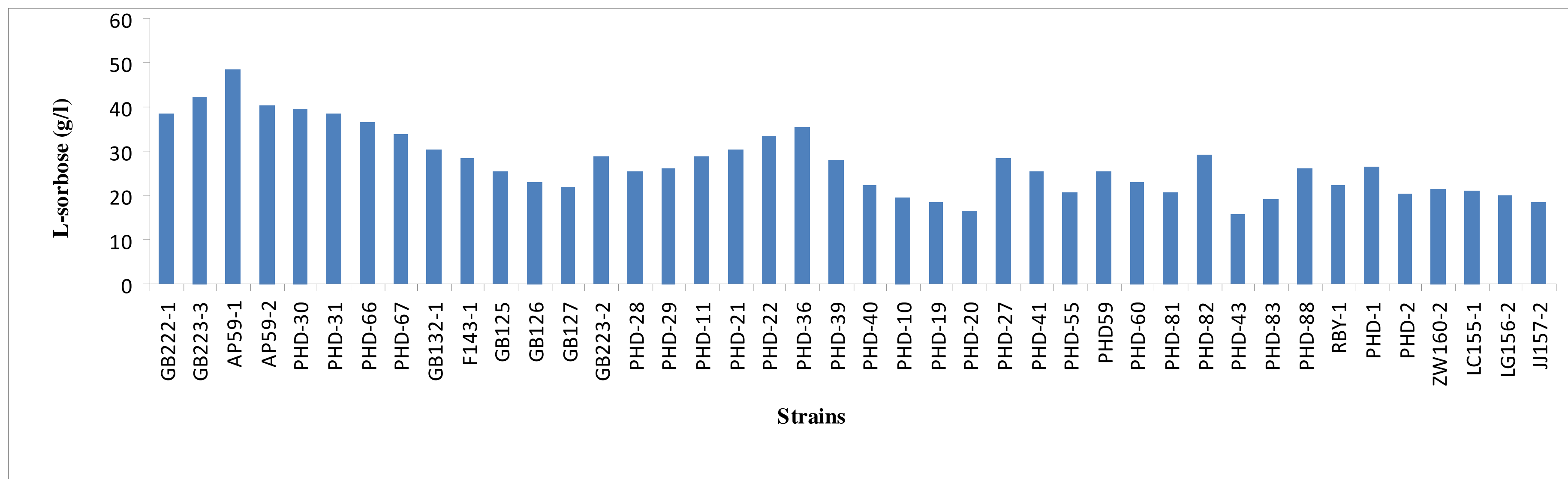

Fig. 4.26 Preliminary screening for L-sorbose production from strains assigned to *Gluconobacter*

CHAPTER V

CONCLUSION

One hundred and forty-seven isolates of AAB were isolated from fruits, flower and other materials collected in Thailand. All isolates were Gram-negative, aerobic, catalase positive, and oxidase-negative rod-shaped bacteria. They produced clear zones on the GEY-$CaCO_3$ agar plates and grew on the GEY broth at pH 3.5. The strains of *Acetobacter* contained Q-9 as the major quinone while the other genus contained Q-10. The DNA G+C contents ranged from 52.2-58.3 mol% in *Acetobacter*, 55.1-60.4 mol% in *Gluconobacter*, 59.7-60.1 mol% in *Asaia*, and 62.3-64.3 mol% in *Gluconacetobacter*. On the basis of their phenotypic and chemotaxonomic characteristics, RFLP-ITS analysis and the rDNA sequencing analysis, they were divided into 4 genus and 19 groups. (1) *Acetobacter* (86 isolates), (2) *Gluconobacter* (42 isolates), (3) *Asaia* (15 isolates), and (4) *Gluconacetobacter* (4 isolates).

Acetobacter isolates were identified as *A. pasteurianus* (Group 1, 26 isolates), *A. orientalis* (Group 2, 13 isolates), *A. lovaniensis* (Group 3, 10 isolates), *A. indonesiensis* (Group 4, 13 isolates), *A. tropicalis* (Group 5, 4 isolates), *A. ghanensis* (Group 6, 8 isolates), *A. orleanensis* (Group 7, 4 isolates), *A. syzygii* (Group 8, 4 isolates), and *Acetobacter* sp. (Group 9, 3 isolates). The isolates G360-1, G361-1 and G362-1 (Group 9) were constituted a cluster separate from *A. orleanensis* NBRC 13752^T and showed 99.7% sequence similarity to *A. orleanensis* NBRC 13752^T and discriminated from known *Acetobacter* species when digestion with *Hpa*II and *Ava*II and low DNA-DNA relatedness (15-38%). Therefore, they should be proposed as a new species in the genus *Acetobacter*.

Gluconobacter isolates were identified as *G. frateurii* (Group 10, 8 isolates), *G. japonicus* (Group 11, 8 isolates), *G. thailandicus* (Group 12, 6 isolates), *G. oxydans* (Group 13, 13 isolates), *Gluconobacter* sp. (Group 14, 3 isolates) and *Gluconobacter* sp. (Group 15, 4 isolates). The isolates RBY-1, PHD-1 and PHD-2 (Group 14) showed 98.1% ITS genes sequence similarity to *G. japonicus* NBRC 3271^T and discriminated from known *Gluconobacter* species when digestion with *Taq*I, *Alu*I, *Hpa*II and *Ava*II and low DNA-DNA relatedness (11-38%). Therefore, the name *Gluconobacter nephelii* sp. nov. was proposed for them. The isolates ZW160-2, LC155-1, LG156-2 and JJ157-2 (Group 15) showed 97.3% ITS genes sequence similarity to *G. oxydans* NBRC 14818^T and discriminated from known *Gluconobacter* when digestion with *Taq*I, *Alu*I, *Hpa*II and *Ava*II and low DNA-DNA

relatedness (11-26%). Therefore, they should be proposed as a new species in the genus *Gluconobacter*.

Asaia isolates were identified as *As. bogorensis* (Group 16, 8 isolates), *As. siamensis* (Group 17, 5 isolates), *As. sphathodeae* (Group 18, 8 isolates). The isolates GB23-2 and GB23-3 (Group 18) were 99.9% 16S rDNA gene sequence similarity to *As. siamensis* NBRC 16457^T and discriminated from other *Asaia* species when digestion with *Sty*I, *Bsa*JI, *Sna*BI, *Hpa*II and *Hpy*AV and low DNA-DNA relatedness (21-43%). Therefore, the name *Asaia spathodeae* sp. nov. was proposed for them.

Gluconacetobacter isolates were identified as *Ga. liquefaciens* (Group 19, 4 isolates). The isolates PHD-53, PHD-54, PHD-89 and PHD-90 were 99.9% 16S rDNA gene sequences.

Eighty-six isolates of *Acetobacter* were screened for ADH activity and they exhibited ADH activity ranged from 2.05 to 7.52 unit/mg at 30°C. The isolate PHD-23 showed the highest ADH activity and was selected for further study. Effect of ethanol and initial acetic acid concentration acetic acid production revealed that this isolate could produce highest acetic acid when the medium composed of ethanol 4% and with out the addition of acetic acid.

Forty-two isolates of *Gluconobacter* were screened for DHA and L-sorbose production, the isolates could produce DHA ranged from 20.05 to 42.52 g/l at 30°C. *G. oxydans* PHD-27 showed the highest DHA production. Isolate PHD-27 could produce the DHA with the maximum of 44.08 g/l at 30°C by conversion time of 84 h and generated DHA at a rate of 0.52 g/l/h at 30°C. The tested isolates produced L-sorbose ranged from 19.99 to 48.39 g/l at 30°C and *G. frateurii* AP59-1 produced the largest amount of L-sorbose.

The identification, distributioned, ADH activity, DHA and L-sorbose production of all isolates were summaried in Appendix B-5.

In Thailand, the diversity and distribution of acetic acid bacteria have been reported however the screening of oxidative products should be intensive further studies.

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APPENDICES

APPENDIX A

CULTURE MEDIA AND REAGENTS FOR IDENTIFICATION

All media were dispensed and steriled in autoclave for 15 min at 15 lb. pressure (121 ºC) except for acid from carbon sources test which was sterilized at 10 lb for (110 ºC) 10 min.

1. Enrichment Media

1.1 Glucose-Ethanol medium

Glucose	1.5%
Ethanol	0.5%
Peptone	0.3%
Yeast Extract	0.3%
Acetic acid	0.35%

(Adjust pH 3.5 with HCl)

1.2 Sorbitol medium

Sorbitol	2.0%
Peptone	0.3%
Yeast extract	0.3%

(Adjust pH 3.5 with HCl)

1.3 Sucrose-Acetic acid medium

Sucrose	2.0%
Peptone	0.3%
Yeast extract	0.3%
Acetic acid	0.35%

(Adjust pH 3.5 with HCl)

1.4 Methanol-Peptone-Yeast extract (MPY) medium

Methanol	0.8%	(added after autoclaving)
Peptone	0.3%	
Yeast extract	0.3%	

(Adjust pH 4.0 with HCl)

2. Glucose-Ethanol-Yeast extract-$CaCO_3$ (GEY- $CaCO_3$) agar plate

Glucose	2.0%
Ethanol	0.5%
Peptone	0.3%
Yeast Extract	0.3%
$CaCO_3$	0.7%
Agar	1.5%

3. Cryoprotectant for preservation (20% glycerol)

Glucose	2.5%
Glycerol	20%
Peptone	0.5%
Yeast Extract	0.3%

4. Growth in test media (4 kinds)

4.1 Glucose-ethanol with 0.3% acetic acid medium

Glucose	1.5%
Ethanol	0.5%
Peptone	0.3%
Yeast Extract	0.3%
Acetic acid	0.3%

(Adjust pH 3.5 with HCl)

4.2 Glucose-ethanol without acetic acid medium

Glucose	1.5%
Ethanol	0.5%
Peptone	0.3%
Yeast Extract	0.3%

(Adjust pH 3.5 with HCl)

4.3 Sorbitol medium

Sorbitol	2.0%
Peptone	0.3%
Yeast extract	0.3%

(Adjust pH 3.5 with HCl)

4.4 Sucrose with 0.3% acetic acid medium

Sucrose	2.0%
Peptone	0.3%
Yeast extract	0.3%
Acetic acid	0.3%

(Adjust pH 3.5 with HCl)

5. Oxidation/fermentation medium

Glucose	10%
Peptone	2.0%
NaCl	5%
K_2HPO_4	0.3%
Bromthymol blue	0.03%
Agar	3%

(Adjust pH 7.1, autoclave at 110°C, 10 min)

6. Acetate/Lactate medium

Peptone	0.2%
Yeast extract	0.2%
Sodium acetate or Ca-DL-lactate	0.2%
Bromthymol blue	0.002%

(Adjust pH 6.4)

7. Acid production medium

Yeast extract	0.5%
Bromocresol purple	0.2%
Carbon source	1.0%

(Adjust pH 6.4, autoclave at 110°C, 10 min)

8. Glucose-Glycerol-Yeast extract Potato (GGYP) medium

Potato	10%
Glucose	0.5%
Glycerol	1.0%
Yeast extract	1.0%
Peptone	1.0%

9. Glucose-Glycerol-Yeast extract (GGY) medium

Glucose	2.0	%
Glycerol	1.0	%
Yeast extract	0.5	%
Agar	1.5	%

10. Medium for ketogenesis in glycerol

Yeast extract	3	%
Glycerol	3	%
Agar	1.5	%

11. Detection reagent for TLC

O-phenylenediamine	0.5	g
Water	3.75	ml
HCl	0.81	ml
Ethanol	25	ml

12. Flagella staining

Basic fuchisin	0.5	g
Tannic acid	0.2	g
Aluminium sulfate	0.5	g

Solvent was composed of a mixture 95% ethanol, 0.5 ml of glycerol, and 7.5 ml of tris(hydroxymethyl)aminomethane(tris)buffer.

APPENDIX B

PHYSIOLOGICAL, BIOCHEMICAL CHARACTERISTICS AND OXIDATIVE PRODUCTIONS

Table B-1 Physiological and biochemical characteristic of 147 isolates

Group	Group 1												
Characteristics	G377-2	G378-1	G378-2	G378-4	G379-2	G380-1	G375-3	G363-1	G379-6	G377-1	LG5-1	MG70-1	LG6-1
Oxidation of													
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	-	-	-	-	vw	-	-	-	-	vw	-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	+	+	-	+	+	+	+	+	-	-	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 1												
Characteristics	SL21-2	BB90-1	PHD-23	PHD-24	PHD-32	PHD-33	PHD-56	PHD-57	PHD-70	PHD-71	PHD-76	PHD-77	PHD-78
Oxidation of													
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	-	-	vw	-	-	-	-	-	vw	-	-	vw	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	-	+	+	-	+	-	-	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 2												
Characteristics	LP120-2	KL13-3	KL13-2	MM129-2	PHD-12	PHD-34	PHD-35	PHD37	PHD-38	PHD-51	PHD-73	PHD-74	PHD-75
Oxidation of													
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	-	-	vw	-	-	vw	vw	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 3										Group 4		
Characteristics	G374-3	G375-4	PHD-16	PHD-17	PHD-18	PHD-25	PHD-26	PHD-63	PHD-91	PHD-92	GU1-1	GU1-3	G379-4
Oxidation of													
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	-	-	-	-	vw	vw	-	-	-	-	vw	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 4										Group 5		
Characteristics	G380-2	G355-1	PHD-3	PHD-5	PHD-7	PHD-8	PHD-9	PHD-13	PHD-44	PHD-45	GB23-1	PHD-4	PHD-6
Oxidation of													
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	vw	-	-	-	vw	vw	-	-	-	-	vw	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 5	Group 6								Group 7			
Characteristics	PHD-42	PH108-1	TM7-1	TM7-3	PHD-14	PHD-15	PHD-61	PHD-62	PHD-72	PHD-84	PHD-85	PHD-86	PHD-87
Oxidation of													
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	-	vw	vw	-	-	-	-	vw	vw	-	-	vw	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	-	-	-	-	-	-	-	-	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 8				Group 9			Group 10				
Characteristics	PHD-85	PHD-86	PHD-87	SL22-1	G360-1	G361-1	G362-1	GB222-1	GB223-3	AP59-1	AP59-2	PHD-30
Oxidation of												
Acetate	+	+	+	+	+	+	+	-	-	-	-	-
Lactate	+	+	+	+	+	+	+	-	-	-	-	-
Growth on												
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid												
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	-	vw	-	vw	vw	vw	-	+	+	w	+	+
Methanol	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose												
2-Keto gluconic acid	+	+	+	-	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	-	-	-	-	-	-	-	+	+	+	+	+

+, positive; - negative; w, weak; vw, very weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 10			Group 11								Group 12	
Characteristics	PHD-31	PHD-66	PHD-67	GB132-1	F143-1	GB125	GB126	GB127	GB223-2	PHD-28	PHD-29	PHD-11	PHD-21
Oxidation of													
Acetate	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactate	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	+	+	+	w	w	+	+	+	w	w	+	+	+
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+

+, positive; - negative; w, weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 12				Group 13								
Characteristics	PHD-22	PHD-36	PHD-39	PHD-40	PHD-10	PHD-19	PHD-20	PHD-27	PHD-41	PHD-55	PHD-59	PHD-60	PHD-81
Oxidation of													
Acetate	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactate	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on													
30% Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid													
Glutamate agar	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol agar	+	+	+	w	w	+	+	+	+	+	w	+	+
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose													
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+

+, positive; - negative; w, weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 13				Group 14			Group 15			
Characteristics	PHD-82	PHD-43	PHD-83	PHD-88	RBY-1	PHD-1	PHD-2	ZW160-2	LC155-1	LG156-2	JJ157-2
Oxidation of											
Acetate	-	-	-	-	-	-	-	-	-	-	-
Lactate	-	-	-	-	-	-	-	-	-	-	-
Growth on											
30% Glucose	-	-	-	-	-	-	-	-	-	-	-
0.35% acetic acid											
Glutamate agar	-	-	-	-	-	+	+	-	-	-	-
Mannitol agar	+	w	+	+	+	+	+	+	+	+	+
Methanol	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	+	+	+	+
Production of polysaccharide	-	-	-	-	-	-	-	+	+	+	+
Dihydroxyacetone from glycerol	+	+	+	+	+	+	+	+	+	+	+
Production of ketogluconic acid from Glucose											
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	+	+	+	+
5-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+

+, positive; - negative; w, weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 16								Group 17			
Characteristics	PHD-46	PHD-47	PHD-52	PHD-64	PHD-65	PHD-68	PHD-79	PHD-80	PHD-48	PHD-50	PHD-58	PHD-69
Oxidation of												
Acetate	w	w	w	w	w	w	w	w	w	w	w	w
Lactate	w	w	w	w	w	w	w	w	w	w	w	w
Growth on												
30% Glucose	+	+	+	+	+	+	+	+	+	+	+	+
0.35% acetic acid												
Glutamate agar	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol agar	+	+	+	w	+	w	+	+	+	+	w	w
Methanol	-	-	-	-	-	-	-	-	-	-	-	-
Water soluble pigment production	-	-	-	-	-	-	-	-	-	-	-	-
Production of polysaccharide	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroxyacetone from glycerol	w	w	w	w	w	w	w	w	w	w	w	w
Production of ketogluconic acid from Glucose												
2-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	-	-	-	-	-	-	-	-	-	-
5-Keto gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+

+, positive; - negative; w, weak

Table B-1 Physiological and biochemical characteristic of 147 isolates (continued)

Group	Group 18		Group 19			
Characteristics	GB23-2	GB23-3	PHD-53	PHD-54	PHD-89	PHD-90
Oxidation of						
Acetate	w	w	+	+	+	+
Lactate	w	w	+	+	+	+
Growth on						
30% Glucose	+	+	-	-	-	-
0.35% acetic acid						
Glutamate agar	+	+	+	+	+	+
Mannitol agar	+	+	+	+	+	+
Methanol	-	-	-	-	-	-
Water soluble pigment production	-	-	+	+	+	+
Production of polysaccharide	-	-	+	+	+	+
Dihydroxyacetone from glycerol	w	w	+	+	+	+
Production of ketogluconic acid from Glucose						
2-Keto gluconic acid	+	+	+	+	+	+
2,5 di-Ketogluconic acid	-	-	+	+	+	+
5-Keto gluconic acid	+	+	+	+	+	+

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates

Group	Group 1												
Carbon sources	G377-2	G378-1	G378-2	G378-4	G379-2	G380-1	G375-3	G363-1	G379-6	G377-1	LG5-1	MG70-1	LG6-1
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	w	+	w	-	+	w	w	-	+	+	+	-
D-Galactose	+	+	w	+	w	+	+	+	+	w	+	w	w
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabinose	w	w	+	w	+	+	w	w	+	+	w	w	w
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Rhamnose	-	-	-	-	-	-	-	w	w	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	w	+	+	-	+	+	-	-	+	w	w	w	w
Dulcitol	w	w	w	-	-	w	w	-	-	-	w	w	-
meso-Erythritol	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	w	+	w	-	w	+	+	+	w	w	-	+	+
Raffinose	w	+	w	w	w	-	-	w	w	-	w	-	-
Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 1												
Carbon sources	SL21-2	BB90-1	PHD-23	PHD-24	PHD-32	PHD-33	PHD-56	PHD-57	PHD-70	PHD-71	PHD-76	PHD-77	PHD-78
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	-	+	w	-	+	w	w	-	-	-	-	-
D-Galactose	+	+	w	+	w	w	+	+	+	w	+	w	w
D-Fructose	+	+	+	+	+	-	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	w	+	-	-	+	-	-	+	w	w	+	+
D-Arabinose	-	-	+	w	+	+	-	-	+	+	-	-	w
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Rhamnose	-	-	-	-	-	-	-	w	w	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	w	+	+	-	+	+	-	-	+	w	-	w	-
Dulcitol	-	-	w	-	-	w	w	-	-	-	w	w	-
meso-Erythritol	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	w	+	w	-	w	+	+	+	w	w	-	+	+
Raffinose	w	+	w	-	-	-	-	w	w	-	w	-	-
Ethanol	+	+	+	+	w	+	w	w	+	+	w	+	+
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 2												
Carbon sources	LP120-2	KL13-3	KL13-2	MM129-2	PHD-12	PHD-34	PHD-35	PHD37	PHD-38	PHD-51	PHD-73	PHD-74	PHD-75
D-Glucose	+	+	+	+	+	w	+	+	+	+	w	+	+
D-Mannose	+	+	w	+	+	+	+	+	w	+	w	+	+
D-Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Fructose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	w	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	w	w	-	-	w	-	-	w	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	w	-	-	w	-	-	-	-	-
meso-Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	w	w	+	+	+	w	w	w	+	+	w	w	+
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 3										Group 4		
Carbon sources	G374-3	G375-4	PHD-16	PHD-17	PHD-18	PHD-25	PHD-26	PHD-63	PHD-91	PHD-92	GU1-1	GU1-3	G379-4
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	w	+	-	w	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	-	+	+	-	-	-	-	-	-	+
L-Sorbose	+	+	+	+	+	w	+	+	+	+	w	+	+
D-Xylose	-	+	w	+	+	-	-	+	w	+	w	+	+
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	w	w	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	+	+	-	-	-	+	+	-	-	-	-
meso-Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	w	-	-	w	-	-	-
Maltose	+	+	+	+	+	w	+	+	+	+	w	+	+
Lactose	+	+	w	+	+	+	+	+	w	+	w	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	w	w	-	-	-	-	w	w
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	w	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	w	w	-	-	w	-	-	w	-	-	-
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 4											Group 5	
Carbon sources	G380-2	G355-1	PHD-3	PHD-5	PHD-7	PHD-8	PHD-9	PHD-13	PHD-44	PHD-45	GB23-1	PHD-4	PHD-6
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	w	-	w	-	-	w	-	-	-	-
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 5	Group 6								Group 7			
Carbon sources	PHD-42	PH108-1	TM7-1	TM7-3	PHD-14	PHD-15	PHD-61	PHD-62	PHD-72	PHD-84	PHD-85	PHD-86	PHD-87
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	w	+	w	+	+	+	+	+
D-Galactose	+	+	+	w	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	w	w	-	-	-	-	w	w	w	-	-	-	-
D-Arabinose	-	-	-	w	-	-	-	-	-	w	-	w	-
L-Arabinose	-	-	-	-	-	-	-	-	-	w	-	-	-
L-Rhamnose	-	-	w	-	-	w	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Erythritol											-	-	-
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	w	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	w	-	-	-	-	-	-
Ethanol	-	w	w	w	+	-	-	w	w	+	-	w	w
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Ribitol	-	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation and various carbon sources of 147 isolates (continued)

Group	Group 8				Group 9			Group 10					
Carbon sources	SL22-1	G379-3	G380-4	G379-5	G360-1	G361-1	G362-1	GB222-1	GB223-3	AP59-1	AP59-2	PHD-30	PHD-31
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	-	-	-	-	-	-
D-Galactose	+	+	+	+	w	w	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	-	-	-	-	-	-	-	+	+	+	+	+	+
D-Arabinose	w	-	-	-	-	-	-	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	+	+	+	+	+	+
L-Rhamnose	-	-	-	-	-	w	-	+	+	+	+	+	+
D-Mannitol	-	-	-	-	-	-	-	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	-	+	+	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-
meso-Erythritol	-	-	-	-				+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	-	-	-	-	-	-	-	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	w	-	-	-	-	-	w	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	+	+	+	+	+	+
Ethanol	+	w	+	+	+	-	+	-	w	w	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	+	+	+	+	+	+
meso-Ribitol	-	-	-	-	-	-	-	+	+	+	+	+	+

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 10		Group 11								Group 12		
Carbon sources	PHD-66	PHD-67	GB132-1	F143-1	GB125	GB126	GB127	GB223-2	PHD-28	PHD-29	PHD-11	PHD-21	PHD-22
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	-	-	+	+	+	+	+	w	w	+	+	w	+
D-Galactose	w	w	+	+	+	w	w	+	+	+	+	w	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Arabinose	w	w	+	+	+	-	-	-	-	+	+	+	-
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	-
L-Rhamnose	+	+	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	+	+	+	+	+	+	+	+	+	w	w	+	+
D-Sorbitol	+	+	+	+	-	-	-	-	+	+	+	w	+
Dulcitol	+	-	w	w	w	+	+	+	w	w	+	w	w
meso-Erythritol	+	+	+	+	+	+	+	+	+	+	+	w	-
Glycerol	+	+	+	+	+	+	+	+	+	w	w	+	-
Maltose	+	+	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	+	+	-	-	-	-	-	+	+	+	-
Melibiose	-	-	+	+	+	+	+	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	-
Raffinose	+	+	-	-	-	-	-	-	-	-	-	-	-
Ethanol	w	w	+	w	w	-	-	w	w	w	+	-	-
D-Arabitol	-	-	+	+	+	+	+	+	+	+	+	+	-
L-Arabitol	+	+	+	+	+	+	-	-	-	-	-	+	-
meso-Ribitol	+	+	+	+	+	+	+	+	+	-	-	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 12			Group 13									
Carbon sources	PHD-36	PHD-39	PHD-40	PHD-10	PHD-19	PHD-20	PHD-27	PHD-41	PHD-55	PHD-59	PHD-60	PHD-81	PHD-82
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	-	+	+	-	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	w	w	-	w	w	-	w	w	-	w	w	-	-
D-Xylose	+	+	-	-	-	-	w	-	-	+	+	-	-
D-Arabinose	+	+	-	w	+	-	-	w	-	w	w	-	-
L-Arabinose	w	w	-	w	+	-	w	w	-	+	+	-	-
L-Rhamnose	w	w	-	w	w	-	w	w	-	w	w	-	-
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	w	w	w	+	+	w	w	w	w	w	w	w	w
meso-Erythritol	w	w	-	+	+	-	+	+	-	w	+	-	-
Glycerol	+	+	-	w	w	-	+	+	-	+	+	-	-
Maltose	+	+	-	w	w	-	+	+	-	+	+	-	-
Lactose	w	w	+	+	+	-	w	w	-	+	+	+	-
Melibiose	+	+	+	+	+	-	w	w	-	+	+	+	-
Sucrose	+	+	+	+	+	-	+	+	-	w	w	+	-
Raffinose	+	+	-	+	+	-	+	+	-	w	w	-	-
Ethanol	+	+	-	+	+	-	w	w	-	-	-	-	-
D-Arabitol	+	+	-	w	w	-	+	+	-	w	w	-	-
L-Arabitol	w	w	-	-	-	-	w	w	-	w	w	-	-
meso-Ribitol	w	w	-	w	w	-	w	w	-	w	w	-	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 13			Group 14			Group 15				Group 16			
Carbon sources	PHD-43	PHD-83	PHD-88	RBY-1	PHD-1	PHD-2	ZW160-2	LC155-1	LG156-2	JJ157-2	PHD-46	PHD-47	PHD-52	PHD-64
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	w	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	+	+	-	-	-	-	w	w	w	w	+	+	-	+
D-Xylose	+	+	-	+	+	+	+	+	+	+	+	+	+	w
D-Arabinose	+	+	-	+	+	+	-	-	-	-	+	+	+	w
L-Arabinose	+	+	-	+	+	+	+	+	+	+	+	+	-	+
L-Rhamnose	+	+	-	w	w	w	-	-	-	-	+	+	+	+
D-Mannitol	+	+	+	w	w	w	+	+	+	+	w	w	+	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	w	w	+	+
Dulcitol	w	w	w	-	-	-	-	-	-	-	+	+	w	w
meso-Erythritol	+	+	-	+	+	+	w	w	w	w	+	+	-	+
Glycerol	+	+	+	w	w	w	+	+	+	+	+	+	-	+
Maltose	+	+	+	w	w	w	-	-	-	-	+	+	+	+
Lactose	+	+	+	-	-	-	+	+	+	+	+	+	+	+
Melibiose	+	w	+	-	-	-	+	+	+	+	+	+	-	+
Sucrose	+	w	+	+	+	+	-	-	-	-	-	-	-	-
Raffinose	w	+	+	+	+	+	-	-	-	-	-	+	+	-
Ethanol	w	+	-	+	+	+	+	+	+	+	-	-	-	-
D-Arabitol	+	+	-	w	w	w	+	+	+	+	-	+	+	-
L-Arabitol	+	+	-	w	w	w	+	+	+	+	-	+	+	-
meso-Ribitol		+	+	w	w	w	w	w	w	w	-	+	+	-

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 16				Group 17					Group 18	
Carbon sources	PHD-65	PHD-68	PHD-79	PHD-80	PHD-48	PHD-49	PHD-50	PHD-69	PHD-58	GB23-2	GB23-3
D-Glucose	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	w	w	w	+	+	+	+	w	w	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	w	+	+	+	+
L-Sorbose	+	-	+	+	-	+	w	w	w	+	+
D-Xylose	+	-	+	+	-	+	w	w	+	+	+
D-Arabinose	+	-	+	+	-	+	+	w	w	+	+
L-Arabinose	+	-	+	+	-	+	+	-	w	+	+
L-Rhamnose	+	+	+	+	-	+	+	-	-	-	-
D-Mannitol	+	+	w	+	+	w	+	w	w	w	w
D-Sorbitol	+	+	w	+	+	w	+	w	+	w	w
Dulcitol	+	w	+	+	w	+	w	+	+	-	-
meso-Erythritol	+	-	+	+	-	+	+	w	+	+	+
Glycerol	+	-	+	+	-	+	+	-	-	+	+
Maltose	+	-	w	+	-	+	+	w	w	-	-
Lactose	+	-	w	+	-	+	+	+	+	-	-
Melibiose	+	+	+	+	-	+	+	+	-	+	+
Sucrose	-	+	-	-	-	+	+	+	+	-	w
Raffinose	+	+	-	+	+	-	+	+	+	-	-
Ethanol	-	-	-	-	w	w	w	w	w	+	+
D-Arabitol	+	+	-	+	+	-	+	w	-	+	+
L-Arabitol	+	+	-	+	+	-	+	w	-	+	+
meso-Ribitol	+	+	-	+	+	-	+	w	w	+	+

+, positive; - negative; w, weak

Table B-2 Acid formation from various carbon sources of 147 isolates (continued)

Group	Group 19			
Carbon sources	PHD-53	PHD-54	PHD-89	PHD-90
D-Glucose	+	+	+	w
D-Mannose	+	+	+	+
D-Galactose	+	w	w	+
D-Fructose	+	+	+	+
L-Sorbose	+	w	+	w
D-Xylose	+	+	+	w
D-Arabinose	-	+	+	w
L-Arabinose	-	w	+	w
L-Rhamnose	+	w	+	w
D-Mannitol	-	-	-	-
D-Sorbitol	-	-	-	-
Dulcitol	-	-	-	-
meso-Erythritol	+	w	+	w
Glycerol	+	-	-	w
Maltose	+	+	+	w
Lactose	+	+	w	+
Melibiose	+	w	w	+
Sucrose	+	w	+	+
Raffinose	+	w	+	w
Ethanol	+	-	-	w
D-Arabitol	+	+	+	w
L-Arabitol	+	+	+	w
meso-Ribitol	+	w	+	w

+, positive; - negative; w, weak

Table B-3 Acetic acid production when the medium was added by various ethanol concentrations at 30ºC for 3 days.

Ethanol (%)	OD_{600}	Acetic acid (%)
0	0.2	0.35
2	0.448	1.03
4	0.543	1.5
6	0.412	0.75
8	0.319	0.56
10	0.086	0.48
12	0.041	0.33

Table B-4 Acetic acid production when the medium was added by various acetic acid concentrations at 30ºC for 3 days.

Ethanol (%)	OD_{600}	Acetic acid (%)
0	0.312	1.49
1	0.39	2.04
1.5	0.257	1.67
2	0.189	1.178
2.5	0.051	0.05
3	0.085	0.03

Table B-5 Identification, ADH activity, DHA and L-sorbose production

Isolate no.	Source	Location	Identification	ADH activity (unit/mg)	Production of	
					DHA (g/l)	L-sorbose (g/l)
PHD-14	Jujube[2]	Trad	*A. ghanensis*	2.12	-	-
PHD-15	Kaffir lime[2]	Changmai	*A. ghanensis*	3.02	-	-
PH108-1	Peach[2]	Pathumthani	*A. ghanensis*	2.08	-	-
PHD-61	Pineapple[2]	Chantaburi	*A. ghanensis*	2.88	-	-
PHD-62	Pineapple[2]	Chantaburi	*A. ghanensis*	3.12	-	-
PHD-72	Rose apple[2]	Ubon	*A. ghanensis*	2.11	-	-
TM7-1*	Tomato[3]	Bangkok	*A. ghanensis*	2.22	-	-
TM7-3*	Tomato[3]	Bangkok	*A. ghanensis*	2.34	-	-
GU1-1*	Fermented starch[3]	Uttaradit	*A. indonesiensis*	3.12	-	-
GU1-3*	Fermented starch[3]	Uttaradit	*A. indonesiensis*	3.56	-	-
G379-4*	Fermented starch[3]	Uttaradit	*A. indonesiensis*	2.46	-	-
G380-2*	Fermented starch[3]	Uttaradit	*A. indonesiensis*	2.66	-	-
PHD-3	Guava[2]	Kanchanaburi	*A. indonesiensis*	2.05	-	-
PHD-5	Guava[2]	Kanchanaburi	*A. indonesiensis*	2.54	-	-
PHD-7	Guava[2]	Ubon	*A. indonesiensis*	2.28	-	-
PHD-8	Hog Plum[2]	Nongkhai	*A. indonesiensis*	2.72	-	-
PHD-9	Ixoria / Ixora[1]	Rayong	*A. indonesiensis*	2.58	-	-
PHD-13	Jujube[2]	Trad	*A. indonesiensis*	2.75	-	-
G355-1*	Khao-mak (sweeted rice)[3]	Nakonpathom	*A. indonesiensis*	2.05	-	-
PHD-44	Musk-melon[2]	Saraburi	*A. indonesiensis*	2.99	-	-
PHD-45	Musk-melon[2]	Saraburi	*A. indonesiensis*	2.56	-	-
G375-4*	Fermented starch[3]	Uttaradit	*A. lovaniensis*	3.87	-	-
G374-3*	Fermented starch[3]	Uttaradit	*A. lovaniensis*	3.96	-	-
PHD-16	Kaffir lime[2]	Changmai	*A. lovaniensis*	4.05	-	-
PHD-17	Kaffir lime[2]	Saraburi	*A. lovaniensis*	4.45	-	-
PHD-18	Kaffir lime[2]	Saraburi	*A. lovaniensis*	3.66	-	-
PHD-25	Longan[2]	Rayong	*A. lovaniensis*	2.58	-	-
PHD-26	Longan[2]	Rayong	*A. lovaniensis*	2.65	-	-
PHD-63	Pineapple[2]	Chantaburi	*A. lovaniensis*	3.12	-	-
PHD-91	Tamarind[2]	Chantaburi	*A. lovaniensis*	3.48	-	-
PHD-92	Tamarind[2]	Chantaburi	*A. lovaniensis*	2.89	-	-
PHD-12	Jujube[2]	Trad	*A. orientalis*	3.16	-	-
KL13-2*	Kaffir lime[2]	Bangkok	*A. orientalis*	3.98	-	-
KL13-3*	Kaffir lime[2]	Bangkok	*A. orientalis*	4.21	-	-
LP120-2*	Loog-pang khaomak[3]	Pathumthani	*A. orientalis*	4.55	-	-
PHD-34	Mango[2]	Bangkok	*A. orientalis*	3.89	-	-
PHD-35	Mango[2]	Bangkok	*A. orientalis*	3.66	-	-
PHD-37	Mango[2]	Nontaburi	*A. orientalis*	3.12	-	-
PHD-38	Mango[2]	Nontaburi	*A. orientalis*	2.89	-	-
MM129-2*	Musk-melon[2]	Bangkok	*A. orientalis*	3.15	-	-
PHD-51	Orange[2]	Khon Kaen	*A. orientalis*	3.45	-	-
PHD-73	Rumbutan[2]	Khon Kaen	*A. orientalis*	3.33	-	-
PHD-74	Rumbutan[2]	Khon Kaen	*A. orientalis*	4.56	-	-

[1]Flower, [2]Fruit, [3]Other materials

*Isolates from Dr.Somboon Tanasupawat (51 isolates)

Table B-5 Identification, ADH activity, DHA and L-sorbose production (continued)

Isolate no.	Source	Location	Identification	ADH activity (unit/mg)	Production of DHA (g/l)	Production of L-sorbose (g/l)
PHD-75	Rumbutan[2]	Khon Kaen	*A. orientalis*	4.12	-	-
PHD-84	Star fruit[2]	Chantaburi	*A. orleanensis*	3.16	-	-
PHD-85	Star fruit[2]	Chantaburi	*A. orleanensis*	2.88	-	-
PHD-86	Strawberry[2]	Trad	*A. orleanensis*	3.11	-	-
PHD-87	Strawberry[2]	Trad	*A. orleanensis*	2.14	-	-
BB90-1*	Banana[2]	Bangkok	*A. pasteurianus*	4.85	-	-
G375-3*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	4.23	-	-
G377-1*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	4.48	-	-
G377-2*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	5.53	-	-
G378-1*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	5.66	-	-
G378-2*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	4.12	-	-
G378-4*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	4.83	-	-
G379-2*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	4.67	-	-
G380-1*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	5.12	-	-
G363-1*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	5.39	-	-
G379-6*	Fermented starch[3]	Uttaradit	*A. pasteurianus*	4.22	-	-
G352-1*	Khao-mak (sweeted rice) [3]	Nakonpathom	*A. pasteurianus*	4.02	-	-
PHD-23	Little Yellow Star[1]	Rayong	*A. pasteurianus*	7.01	-	-
PHD-24	Little Yellow Star[1]	Rayong	*A. pasteurianus*	5.89	-	-
LG5-1*	Longan[2]	Bangkok	*A. pasteurianus*	4.48	-	-
LG6-1*	Longan[2]	Bangkok	*A. pasteurianus*	7.52	-	-
PHD-32	Mango[2]	Khon Kaen	*A. pasteurianus*	4.63	-	-
PHD-33	Mango[2]	Khon Kaen	*A. pasteurianus*	5.08	-	-
MG70-1*	Mango[2]	Bangkok	*A. pasteurianus*	4.88	-	-
PHD-56	Peach[2]	Bangkok	*A. pasteurianus*	5.68	-	-
PHD-57	Peach[2]	Bangkok	*A. pasteurianus*	5.06	-	-
PHD-70	Red Grape[2]	Rayong	*A. pasteurianus*	4.96	-	-
PHD-71	Red Grape[2]	Rayong	*A. pasteurianus*	5.33	-	-
PHD-76	Salas[2]	Rayong	*A. pasteurianus*	4.89	-	-
PHD-77	Salas[2]	Rayong	*A. pasteurianus*	4.75	-	-
PHD-78	Salas[2]	Rayong	*A. pasteurianus*	3.89	-	-
SL21-2*	Sapodilla[2]	Bangkok	*A. pasteurianus*	6.89	-	-
G379-3*	Fermented starch[3]	Uttaradit	*A. syzygii*	3.08	-	-
G380-4*	Fermented starch[3]	Uttaradit	*A. syzygii*	2.24	-	-
G379-5*	Fermented starch[3]	Uttaradit	*A. syzygii*	3.56	-	-
SL22-1*	Sapodilla[2]	Bangkok	*A. syzygii*	3.02	-	-
PHD-4	Guava[2]	Kanchanaburi	*A. tropicalis*	2.24	-	-
PHD-6	Guava[2]	Ubon	*A. tropicalis*	3.14	-	-
PHD-42	Musk-melon[2]	Bangkok	*A. tropicalis*	2.34	-	-
GB23-1*	*Spathodea campanulata*[1]	Bangkok	*A. tropicalis*	2.24	-	-

[1]Flower, [2]Fruit, [3]Other materials

*Isolates from Dr.Somboon Tanasupawat (51 isolates)

Table B-5 Identification, ADH activity, DHA and L-sorbose production (continued)

Isolate no.	Source	Location	Identification	ADH activity (unit/mg)	Production of	
					DHA (g/l)	L-sorbose (g/l)
G360-1*	Fermented starch[3]	Uttaradit	*Acetobacter* sp. nov.	2.06	-	-
G361-1*	Fermented starch[3]	Uttaradit	*Acetobacter* sp. nov.	2.44	-	-
G362-1*	Fermented starch[3]	Uttaradit	*Acetobacter* sp. nov.	2.89	-	-
PHD-46	Night Jasmine[1]	Rayong	*As. bogorensis*	-	-	-
PHD-47	Night Jasmine[1]	Rayong	*As. bogorensis*	-	-	-
PHD-52	Pagoda flower[1]	Rayong	*As. bogorensis*	-	-	-
PHD-64	Plumeria flower[1]	Chonburi	*As. bogorensis*	-	-	-
PHD-65	Plumeria flower[1]	Chonburi	*As. bogorensis*	-	-	-
PHD-68	Quassia[1]	Rayong	*As. bogorensis*	-	-	-
PHD-79	Sapodilla[2]	Chantaburi	*As. bogorensis*	-	-	-
PHD-80	Sapodilla[2]	Chantaburi	*As. bogorensis*	-	-	-
PHD-48	Night Jasmine[1]	Saraburi	*As. siamensis*	-	-	-
PHD-49	Orange[2]	Khon Kaen	*As. siamensis*	-	-	-
PHD-50	Orange[2]	Khon Kaen	*As. siamensis*	-	-	-
PHD-58	Periwinkle[1]	Rayong	*As. siamensis*	-	-	-
PHD-69	Quassia[1]	Rayong	*As. siamensis*	-	-	-
GB23-3	*Spathodea campanulata*[1]	Bangkok	*As. spathodeae* sp.nov.	-	-	-
GB23-2	*Spathodea campanulata*[1]	Bangkok	*As. spathodeae* sp.nov.	-	-	-
AP59-1*	Apple[2]	Bangkok	*G. frateurii*	-	33.24	48.39
AP59-2*	Apple[2]	Bangkok	*G. frateurii*	-	29.65	40.53
GB222-1*	Guava[2]	Bangkok	*G. frateurii*	-	25.41	38.59
PHD-30	Longan[2]	Trad	*G. frateurii*	-	25.43	39.68
PHD-31	Longan[2]	Trad	*G. frateurii*	-	28.02	38.54
PHD-66	Pum melo[2]	Ubon	*G. frateurii*	-	33.12	36.58
PHD-67	Pum melo[2]	Ubon	*G. frateurii*	-	30.08	33.89
GB223-3*	Sugar apple[2]	Bangkok	*G. frateurii*	-	28.35	42.45
F143-1	*Canna indica*[1]	Bangkok	*G. japonicus*	-	20.19	28.52
PHD-28	Longan[2]	Changmai	*G. japonicus*	-	25.33	25.44
PHD-29	Longan[2]	Changmai	*G. japonicus*	-	28.22	26.31
GB132-1*	Manila tamarind[2]	Bangkok	*G. japonicus*	-	23.22	30.52
GB125*	Plum mango[2]	Nakonpathom	*G. japonicus*	-	19.99	25.22
GB126*	Plum mango[2]	Nakonpathom	*G. japonicus*	-	23.12	23.21
GB127*	Plum mango[2]	Nakonpathom	*G. japonicus*	-	22.44	22.11
GB223-2*	Sugar apple[2]	Bangkok	*G. japonicus*	-	20.05	20.05
PHD-1	Litchi[2]	Roie	*G. nephelii* sp.nov.	-	30.41	26.53
PHD-2	Litchi[2]	Roie	*G. nephelii* sp.nov.	-	29.89	20.31
RBY-1	Rambutan[2]	Bangkok	*G. nephelii* sp.nov.	-	30.28	22.48
PHD-10	Jackfruit[2]	Nongkhai	*G. oxydans*	-	28.45	19.56
PHD-19	Kaffir lime[2]	Khon Kaen	*G. oxydans*	-	32.32	18.45

[1]Flower, [2]Fruit, [3]Other materials

*Isolates from Dr.Somboon Tanasupawat (51 isolates)

Table B-5 Identification, ADH activity, DHA and L-sorbose production (continued)

Isolate no.	Source	Location	Identification	ADH activity (unit/mg)	Production of DHA (g/l)	Production of L-sorbose (g/l)
PHD-20	Kaffir lime[2]	Khon Kaen	*G. oxydans*	-	35.44	16.44
PHD-27	Longan[2]	Changmai	*G. oxydans*	-	42.52	28.28
PHD-41	Mangosteen[2]	Trad	*G. oxydans*	-	33.56	25.33
PHD-43	Musk-melon[2]	Bangkok	*G. oxydans*	-	37.58	15.77
PHD-55	Papaya[3]	Phuket	*G. oxydans*	-	32.78	20.89
PHD-59	Petunia[1]	Chonburi	*G. oxydans*	-	40.33	25.56
PHD-60	Petunia[1]	Chonburi	*G. oxydans*	-	39.69	23.12
PHD-81	Sapodilla[2]	Trad	*G. oxydans*	-	35.36	20.96
PHD-82	Sapodilla[2]	Trad	*G. oxydans*	-	40.12	29.36
PHD-88	Strawberry[2]	Trad	*G. oxydans*	-	28.42	26.33
PHD-11	Jackfruit[2]	Nongkhai	*G. thailandicus*	-	32.44	28.96
PHD-21	Langsat[2]	Chantaburi	*G. thailandicus*	-	36.58	30.28
PHD-22	Langsat[2]	Chantaburi	*G. thailandicus*	-	25.24	33.58
PHD-36	Mango[2]	Bangkok	*G. thailandicus*	-	28.32	35.42
PHD-39	Mangosteen[2]	Trad	*G. thailandicus*	-	31.2	28.19
PHD-40	Mangosteen[2]	Trad	*G. thailandicus*	-	27.65	22.14
PHD-53	Palm juice[2]	Nongkhai	*Ga. liquefaciens*	-	-	-
PHD-54	Palm juice[2]	Nongkhai	*Ga. liquefaciens*	-	-	-
PHD-89	Sugar apple[2]	Chantaburi	*Ga. liquefaciens*	-	-	-
PHD-90	Sugar apple[2]	Chantaburi	*Ga. liquefaciens*	-	-	-
JJ157-2*	Jujube[2]	Bangkok	*Gluconobacter* sp.nov.	-	22.86	18.46
LC155-1*	Lichi[2]	Bangkok	*Gluconobacter* sp.nov.	-	28.71	20.98
LG156-2*	Longan[2]	Bangkok	*Gluconobacter* sp.nov.	-	25.12	19.88
ZW160-2*	Rakam (*Zalacca wallichiana*)[2]	Bangkok	*Gluconobacter* sp.nov.	-	25.98	21.35

[1]Flower, [2]Fruit, [3]Other materials

*Isolates from Dr.Somboon Tanasupawat (51 isolates)

Table B-6 L-sorbose and DHA production from *Gluconobacter* sp. (continued)

	Production of	
Strains	L-sorbose(g/l)	DHA (g/l)
PHD-88	26.33	28.42
RBY-1	22.48	30.28
PHD-1	26.53	30.41
PHD-2	20.31	29.89
ZW160-2	21.35	25.98
LC155-1	20.98	28.71
LG156-2	19.88	25.12
JJ157-2	18.46	22.86

Table B-7 DHA production from *G. oxydans* isolate PHD-80.

Culture periods (h)	DHA (g/l)	Optical Density at 600 nm
0	0	0.5
12	5	1.21
24	10	1.58
36	13	1.93
48	21	1.94
60	33	2
72	38	2.1
84	43.2	2.1
96	44.08	2.1

APPENDIX C

REAGENTS FOR DNA EXTRACTION AND PURIFICATION DETERMINATION OF DNA BASE COMPOSITION, DNA-DNA HYBRIDIZATION AND DNA SEQUENCING

1. DNA extraction and DNA base composition

1.1 Saline –EDTA

NaCl	8.76	g
EDTA	37.22	g

NaCl and EDTA were dissolved in 1 L ultra pure water and adjusted the pH 8.0 by adding N HCl and then steriled by autoclaving at 121 ºC, 15 pounds/inch pressure,for 15 min.

1.2 10% (W/V) SDS

Sodium dodecyl sulphate	10	g
Distilled water	90	ml

Dissolved and made up to 100 ml with distilled water.

1.3 Phenol: Chloroform (1:1, v/v)

Crystalline phenol was liquidified in water bath at 65°C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle.

1.4 20X SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	L

Adjusted pH to 7.0 and steriled by autoclaveing at 121 ºC 15 lb/ $inch^2$ pressure, for 15 minutes. Note: To prepare 0.1× SSC and 0.2× SSC, the 20× SSC were diluted at 200 and 100 times, respectively before used.

1.5 RNase A solution

RNase A	20	mg
0.15MNaCl	10	ml

Dissolved 20 mg of RNase A in 10 ml 0.15 M NaCl and heated at 95°C for 5-10 min. Kept in -20°C.

1.6 0.1M Tris-HCl (pH 8.0)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	ml

Dissolved and adjusted to pH 8.0 by adding 0.1 N HCl. Made to 100 ml with distilled water.

1.7 RNase T1 solution

RNase T1	80	ul
0.1 M Tris-HCl (pH 7.5)	10	ml

Mixed 80 µl of RNase T1 in 10 ml of 0.1 M Tris-HCl (pH 7.5) and heated at 95°C for 5 min. Kept in -20°C.

1.8 Nuclease P1 solution

Nuclease P1	0.1	mg
40 mM CH_3COONa + 12 mM $ZnSO_4$ (pH 5.3)	1	ml

Dissolved and stored at 4°C.

1.9 Alkaline phosphatase solution

Alkaline phosphatase	2.4	units
0.1 M Tris-HCl (pH 8.1)	1	ml

1.10 Proteinase K

Proteinase K (Sigma)	4	mg
50 mM Tris-HCl (pH 7.5)	1	ml

(Use freshly prepared solution)

1.11 3M Sodium acetate pH 5.2

To 800 ml of distilled water, 408.1 g of sodium acetate was added and adjusted the pH to 5.2 with glacial acetic acid. The volume was adjusted to 1 litre. The solution was sterilized by autoclaving for 15 minutes at 15 lb/in^2.

1.12 TE buffer

1M Tris-Cl (pH 8.0)	4	ml
1 m M Na_2-EDTA (pH 8.0)	1.6	ml
dH_2O	394.4	ml

2. DNA-DNA hybridization

2.1 Phosphate-buffer saline (PBS)

NaCl	8.00	g
KCl	0.20	g
KH_2PO_4	0.12	g
Na_2HPO_4(anhydrous)	0.91	g
Distilled water	1	L

Steriled by autoclaveing at 121 ºC, 15pounds/inch 2 pressure, for 15 minutes

2.2 20X SSC (20 x standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	L

Adjusted pH to 7.0 and steriled by autoclaveing at 121 ºC 15 lb/ $inch^2$ pressure, for 15 minutes

2.3 100X Denhardt solution

Bovine serum albmin (Fraction V)	2	g
Polyvinylpyrrolidone	2	g
Ficoll400	2	ml

Dissolve in 100 ml ultra pure water and was stored at 4 ºC until used.

2.4 Salmon sperm

Salmon sperm DNA	10	mg/ml

Salmon sperm DNA 10 mg was dissolved in 10 Mm Tris + EDTA buffer pH 7.6 volume 1 ml, boiled for 10 min and then immediately cooled in ice. Sonicated salmon sperm DNA solution foe 3 min and was store at 4 ºC until used.

2.5 Prehybridization solution

100x Denhardt solution	2	ml
10 mg/ml Salmon sperm DNA	1	ml
20x SSC	10	ml
Formamide	50	ml
Distilled water	34	ml

All of ingredients were dissolved in ultra pure water steriled and kept at 4 ºC

2.6 Hybridization solution

Prehybridization	100	ml
Dextran sulfate	5	g

Dissolved dextran sulfate in Prehybridization solution and keep at 4 ºC

2.7 Solution 1

Bovine serum albumin (Fraction V)	0.25	g
Triton X – 100	50	µl
PBS	50	ml

All of ingredients were mixed and keep at 4 ºC

2.8 Solution 2

Strepavidin –POD conjugate	1	µl
Solution1	4	ml

Dissolved Strepavidin- POD conjugate in solution 1 before used. The solution 2 was freshly prepared.

2.9 Solution 3

3,3',5,5' Tetramenthylbenzidine (TMB)

(10 mg/ml in DMFO) 100 ml

0.3% H_2O_2 100 ml

0.1 M citric + 0.2 M Na_2HPO_4 buffer pH 6.2 in 10% DMFO 5 ml

All of ingredients were mixed and used. The solution 3 was freshly

3. HPLC conditions for DNA base composition analysis

Table C-1 HPLC conditions for DNA base composition analysis

Detector wave length	270 nm
Column	Nakarai Cosmosil packed column $5C_{18}$ (150x4.6 mm)
Column temperature	Room temperature
Eluent	0.2 M $NH_4H_2PO_4$: acetonitrile (20:1, v/v)
Flow rate	1 ml/min
Sample	5-10 µl

4. Primers for DNA amplification and sequencing

For 16S rRNA gene sequencing

20F (5'-GAG TTT GAT CCT GGC TCA G'-3)

1500R (5'-GTT ACC TTG TTA CGA CTT'-3)

520F (5'-CAG CAG CCG CGG TAA TAC-3')

520R (5'-GTA TTA CCG CGG CTG CTG-3')

920F (5'-AAA CTC AAA TGA ATT GAC GG-3')

920R (5'-CCG TCA ATT CAT TTG AGT TT-3')

For 16S-23S rRNA gene sequencing

1522F (5' TGC GG(C/T) TGG ATC ACC TCC T 3')

38R (5' GTG CC(A/T) AGG CAT CCA CCG 3')

Talaf (5' AGA GCA CCT GCT TTG CAA 3')

Talar (5' ACC CCC TGC TTG CAA A 3')

APPENDIX D

REAGENTS AND STANDARD ASSAY FOR OXIDATIVE PRODUCTIONS

1. Determination of protein (Lowry *et al*., 1951)

The protein and soluble peptide content was measured by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as standard.

1.1 Reagents

A: 2% sodium carbonate in 0.1N NaOH

B: 0.5% $CuSO_4$.$5H_2O$ in 1% sodium citrate

C: 1 N Folin-Ciocalteu's phenol reagent (2N Folin Phenol was diluted with distilled water to the final concentration in 1N, the solution should be freshly prepared before use.)

D: 1 ml Reagent B + 50 ml Reagent A (or similar ratio) Make up immediately before use.

1.2 Procedure

1.2.1. Place 0.1 ml of proper dilution of culture broth (for protein determination) or clear supernatant of reaction mixture (for soluble peptide determination)

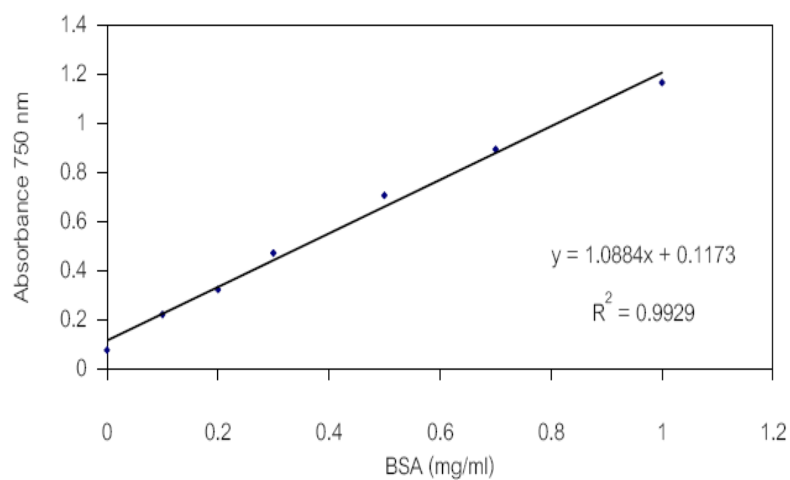
1.2.2. Add 1 ml of Reagent D into the tube and vortex immediately. Incubate at room temperature for 10 min3. After the 10 min incubation, add 0.1 ml of Reagent C to sample and vortex immediately. Incubate 30 min at room temperature.

1.2.3 Absorbance (OD) of samples was measured at 750 nm. Concentrations of the samples were compared to the standard curve for determination of values. Distilled water was used instead of sample as a blank.

1.3 Preparation of standard curve of BSA

Standards of 0, 0.1, 0.2, 0.3, 0.5, 0.7 and 1.0 mg/ml were prepared from BSA. The reactions were carried out with the same procedure as described previously. Absorbances were plotted against concentrations of standards.

Standard curve of Bovine serum albumin (BSA)

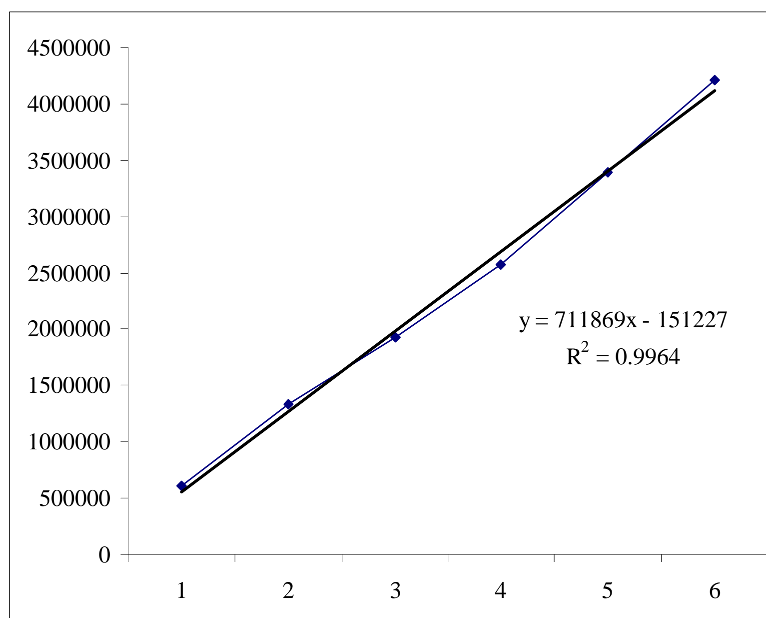


2. Acetic acid and ethanol analysis by Gas Chromatography (GC)

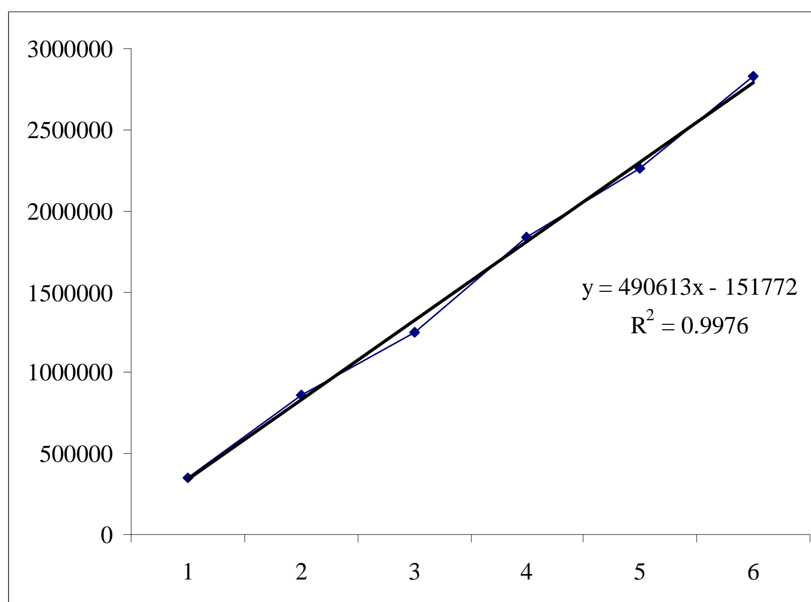
2.1 Gas Chromatography (GC)

Detector	Flame Ionization Detector (FID)
Flaming gas	H_2 and N_2
Injection vol.	2 µl

Standard curve of Ethanol



Standard curve of acetic acid



3. Dihydroxyacetone (DHA) assay

3.1 Preparation of diphenylamine

Dissolved diphenylamine 0.6 g in 6 ml of 98% sulfuric acid and added solution into 54 ml of conc. 99% acetic acid, mixed well. Keep solution in dark bottle.

3.2 Methods assay for standard curve of Dihydroxyacetone (DHA)

0, 10, 20, 30, 40, 50 µl of 0.1M DHA

↓

Added dH_2O until final vol. 500 µl

↓

Added diphenylamine 2.5 µl, Mix

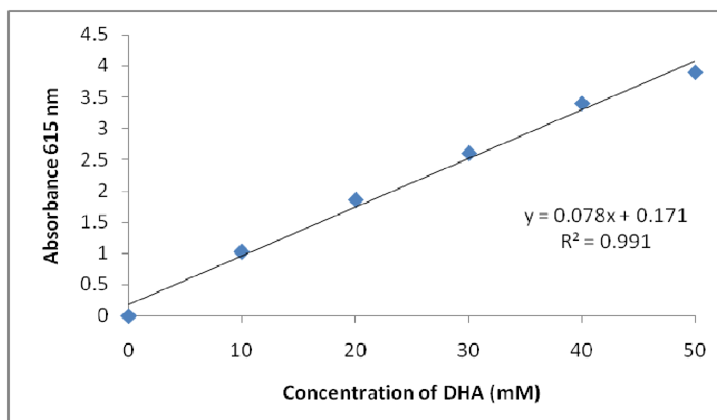
↓ Incubated 80◦C in water bath, 20 min

Stoped reaction with ice box, 5 min

↓

OD_{615}

Standard curve of Dihydroxyacetone (DHA)



4. L-Sorbose assay

4.1 Preparation of resorcinol

Dissolved resorcinol 0.5 g in 100 ml of dH_2O and added 3.5 ml of solution into 12 ml of conc. HCl and then added 19.5 ml dH_2O, mixed well. Keep solution in dark bottle.

4.2 Methods assay for standard curve of L-Sorbose

0, 25, 50, 75, 100, 125, 150 µl of 0.1M L-sorbose

↓

Added dH_2O until final vol. 500 µl

↓

Added resorcinol 2.5 ml, Mix

↓

Incubated in water bath 80°C, 10 min

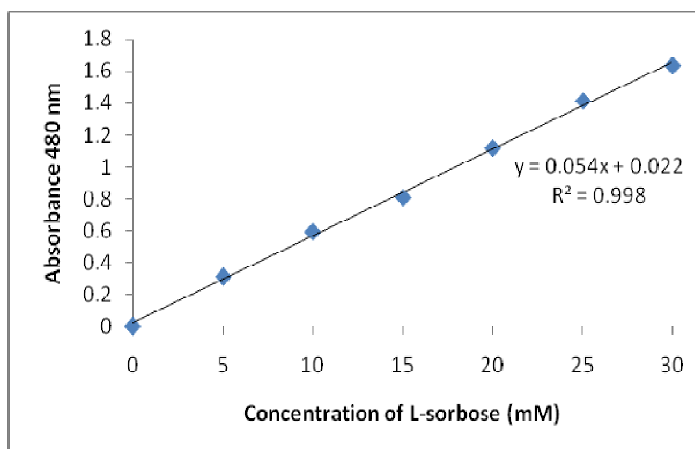
↓

Stoped reaction in ice bath 5 min

↓

OD 480 nm

Standard curve of L-Sorbose



5. Potato medium

D-glucose	5	g
Yeast extract	10	g
Polypeptone	10	g
Glycerol	20	g
Potato extract	100	ml

(filled to 1 liter with tap water)

6. 1M Ethanol

Absolute ethanol	2.916	ml
dH_2O	47.08	ml

7. McIlvaine buffer; McB, pH 5.0

50 mM Citric acid	9.7	ml
100 mM $Na_2HPO_4.12H_2O$	10.3	ml

8. 0.1M Ferricyanide solution

Potassium hexacyanoferrate III	3.29	g
dH_2O	100	ml

9. Dupanol reagent

$Fe_2(SO_4)_3.H_2O$	5	g
SDS	3	g
85% phosphoric acid	93	ml

(fill up to 1L with DW)

10. YE medium

Yeast extract	0.3	%
Ethanol	0-12	%

11. YEA medium

Yeast extract	0.3	%
Ethanol	4	%
Acetic acid	1-3	%

12. DHA production medium

Glycerol	5	%
Yeast extract	1	%
	(pH 5.0)	

13. L-sorbose production medium (Moonmangmee *et al*., 2000)

D-sorbitol	5	%
Peptone	0.3	%
Yeast extract	0.3	%
	(pH 5.0)	

APPENDIX E

NUCLEOTIDE SEQUENCES OF REPRESENTATIVE STRAINS

1. 16S rDNA sequence

1.1 GB23-1 (AB470916)

TGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCATGCTTAACTCATGCAAGTCGCACGAAGGTTTC
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CA

1.2 KL13-3 (AB470917)

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1.3 LG5-1 (AB470918)

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1.4 SL22-1 (AB470919)

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1.5 PH108-1 (AB470920)

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1.6 GB222-1 (AB470921)

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1.7 GB132-1 (AB470922)

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1.8 G374-3

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2. 16S-23S rDNA sequence

2.1 AP59-1 (AB489246)

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2.2 GB125 (AB489251)

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2.3 PHD-11

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GGCGTTCGGTGGATGCCTTGGCAC

2.4 PHD-10

TGCGGCTGGATCACCTCCTTTCAAGGATATTTCCTGACACGTTGGGGAATTCCGAAATAAAAGTCCTTG
GCCAGATTGGCTGAGGCGCCAGACAATCCTGGATGATTGGCCTCAGGGCGCCGTCAACATATCCCTTCC
TATAATTTTCCCGGGCTAGTAGCTCAGTTGGTTAGAGCACACGCTTGATAAGCGTGGGGTCGGAGGTTC
AAGTCCTCCCTGGCCCACCAGAGATTGGGGGCATAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGG
GGGTCGTCGGTTCGAACCCGTCTGCCTCCACCAGTCACGGTTGAGATTTCGGTCTCCTCTGAGTGGTGT
AGAGCTCAAGATCTGGTCCGTAAGGGAAAATCATAGGAAACGGGACGTCTGTTTCCTGATCCTGTCTCC
ACGAAGTTGGATGACGCGTCAGGGATGTTGGGTCTTTTAGAGTGTGAATAGGTTGGTGCGCAGTCTGCG
TGTGCCATTTCCGGGTTGGTCTGACCCGTTGCTGTGGAGCTTAGGCTTCATTGCAATGCTAAGGAAATG
GGGAAGCATTCAGACAGCGTGAAGAAAAGTGTGACTGACACCGCATGAGTGCGGAAGTCGGCAGCATCT
TTGTGGTGTGGCTGGTTTCTGTGCATGTGTCTTCATCCTCGGGTGAAGAATGGTGTGAGAATGAGAAGG
GCGTTCGGTGGATGCCTTGGCAC

2.5 RBY-1 (AB540145)

TGCGGCTGGATCACCTCCTTTCAAGGATGTTTCCCAATACGTTGGAGAGCTCCGAAATAAAAGTCCTTG
GTTGTATCAGCCAAGGCGCCAGACGATCCTGGAACCTGGAGTTCAAGGCGCCGTCAACATATCCCTTCC
TATATTTTCCCGGGGTAGGTAGTCAGTTGGTTAGAGCACACGCTTGATAAGCGTGGGKTCGGAGGTTCA
AGTCCTCCCTGGCCCCCCAGAGTTTGGGGGCATAGCTCAGCTGGGAGAGCACCTGCTTTGCAAGCAGGG
GGTCGTCGGTTCGAACCCGTCTGCCTCCACCACCTTTGGTTGAGATTTCGGTCTCCTGAAGGTGGTGAA
GAGCTCAAAATCTGGTCACTGGGAAAATCATAGGAAATGGGAAGTCTGTTTCCTGATCCTGTCACCGCA
TAGTCGGATGAAGCGTCAGGGATGTTGGGTCTTTTAGAGTGTGAATAGGTTGGTGCACTGTCTGCGTGT
ACCGTTCTCGGGTTGGTCTGACCCGTCTGCTGTCTGGCTTTGTCAGGTAGCGGCGTTAAGAGAACGGAT
GAAGCATTCAGACGGTGACGAAAAGAGTGTGACTGACACTGTCGTGGACAGAAGTCGGTCCATACCTCT
CGGGGTGTGGCTGGTTTCTGTGCATGTGTTTCATCAGCAATGATGATTTGATGGTGTGAGAATGAGAAG
GGCGTTCGGTGGATCCCTTGGCACA

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