CHAPTER II

LITERATURE REVIEWS

1. Characterization of acetic acid bacteria

Acetic acid bacteria were the strictly aerobic bacteria, gram-negative, rods, non-spore forming and smaller colonies, oxidase negative, catalase positive. 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.

The acetic acid bacteria can were found in vinegar, flowers, fruits, insects, alcohol beverages, nata de coco and fermented foods and so on. Nowadays the acetic acid bacteria were classified into six genera as followed description.

1.1 *Acetobacter*

Acetobacter strains exhibited the capability for producing acetic acid from ethanol but inhibited by high concentration of phenol. They could oxidize acetate and lactate to carbon dioxide and water and contained the major ubiquinone with nine isoprene units (Q-9) (Yamada *et al.*, 1997; Cleenwerck *et al.*, 2002). Colonies were smooth, raised, beige to brown color, and regular too wavy and smaller. They were able to produce 2-keto-D-gluconate from D-glucose but incapable to produced 5-keto-D-gluconate from D-glucose. Most members of the genus were unable to use ammonium as nitrogen source, while they use ethanol, methanol and maltose for carbon source. They grow on n-propanol with ammonium as the nitrogen source. The DNA base composition ranges from 56.0-57.6 mol % of G+C content (Cleenwerck *et al.*, 2002). The genus *Acetobacter* included many species such as *Acetobacter aceti* , *A. pasteurianus*, *A. peroxydans*, *A. alcoholophilus*,, *A. lovaniensis*, *A. estunensis*, *A. indonesiensis*, *A. tropicalis*, *A. orleanensis*, *A. cibinogensis*, *A. syzygii*, *A. orientaliss*, *A. tropicalis*, *A. malorum*, *A. cerevisiae*. Their differential characteristics are shown in Table 2.1.

Table 2.1 Differentiatial characteristics of *Acetobacter* species

Characteristics	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
Catalase	+	+	+	+	+	+	+	+	+	d	–	+	+	+
Ketogenesis from glycerol	+	nd	–	–	–	–	nd	–	d	–	–	+	–	–
Acid formation from D-glucose	+	+	+	+	–	+	+	+	+	d	–	w	+	+
Formation from D-glucose of														
2-Keto-D-gluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5-Keto-D-gluconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth in the presence of 30% glucose	–	–	nd	–	–	–	+	nd	–	–	–	+	nd	–
Growth in the presence of 10% ethanol	–	nd	–	–	–	–	nd	–	–	+	–	+	–	–
Nitrate reduction [b]	–	nd	–	–	d	d	nd	–	d	+	–	nd	–	–

Symbols: +, 90% or more of the strains positive; w, weakly positive reaction; d, 11–89% of the strains positive; –, 90% or more of the strains negative; and nd, not determined.

[a] 1., *A. aceti*; 2., *A. cerevisiae*; 3., *A. cibongensis*; 4., *A. estunensis*; 5., *A. indonesiensis*; 6., *A. lovaniensis*; 7., *A. malorum*; 8., *A. orientalis*; 9., *A. orleanensis*; 10., *A. pasteurianus*; 11, *A. peroxydans*; 12., *A. pomorum*; 13., *A. syzygii*; and 14., *A. tropicalis*.

[b] Nitrate reduction was tested in nitrate peptone according to >Franke et al. (1999).

Data from Sokollek et al. (1998b), Lisdiyanti et al. (2000), Lisdiyanti et al. (2001), and Cleenwerck et al. (2002).

1.2 *Gluconobacter*

Gluconobacter strains were incapable for completely acetate and lactate oxidation to carbon dioxide and water. They contained ubiquinone-10 (Q-10) as major ubiquinone in oxidation respiratory system (Yamada *et al.*, 1997). Even to in some species were not exhibited motility but some were motile with polar flagella. They produced water-soluble blown pigment on calcium carbonate medium plates and contained straight-chain C_{16} and C_{18} fatty acids. They were 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. They produced vinegar from ethanol and carried out by sequential membrane bound alcohol and depended on aldehyde dehydrogenase functions. 5-keto-D-gluconate was produced from D-gluconate by gluconate dehydrogenase as involved quinoneprotein glycerol dehydrogenase and produced 2-keto-D-gluconate from glucose by glucose dehydrogenase which involved flavoprotein D-gluconate dehydrogenase (Matsushita *et al.*, 2003). In addition, gluconic acid

requirement were different in some species of members in the genus. *Gluconobacter* species were very important for L-ascorbic acid or vitamin C, cost valuable sugars production and so on. *Gluconobacter* strains were mostly found in fruits and flowers. The DNA base composition ranged from 54-60 mol% of G+C content (Katsura *et al.*, 2002). The genus *Gluconobacter* included five species; *G. oxydans,* (Henneberg, 1987) *G. cerinus*, (Yamada and Akita, 1984), *G. frateurii,* (Mason and Claus, 1989) *G. thailandicus* (Tanasupawat *et al.*, 2004) and *G. albidus.* (Yukphan *et al.*, 2005). Their differential characteristics are shown in Table 2.2 (Tanasupawat *et al.*, 2004).

Table 2.2 Differential characteristics of *Gluconobacter* species

Characteristics	*G. oxydans*	*G. cerinus*	*G. frateurii*	*G. thailandicus*
Growth on *meso*-Ribitol	-	-	w	w
Growth on L-Arabitol	-	-	w	w
Acid formation from				
D-Mannose	w	+	+	-
D-Sorbitol	-	+	-	-
Maltose	+	-	-	-

1.3 *Acidomonas*

Acidomonas strains were facultatively methylotrophic bacteria. They utilizes methanol, ethanol, acetic acid, D-Glucose, glycerol and pectin as carbon and energy source. Growths on PYM medium and colonies characteristics were shiny, smooth surface, raised elevation, entire edge and white to pale-yellow colors. The major ubiquinone system was along between Q-10 with Q-9 and minor ubiquinone was Q-11 components (Yamada *et al.*, 1968). The cellular composition were a lot of straight-chain unsaturated $C_{18:1}$ fatty acid and a little straight chain saturated $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:0}$ and $C_{19:0}$ fatty acid and straight-chain unsaturated $C_{16:1}$ fatty acid and $C_{19:0}$ cyclopropane acid. In addition, they can't produce water-soluble pigment and were unable to reduce nitrate to nitrite but produce ammonia. Negative for lismus milk reaction, and Voges-Proskauer test, indole production, hydrogen sulfide formation, and gelatin and starch hydrolysis. No produce dihydroxyacetone from glycerol. Acid was produce from a D-glucose oxidatively. Utilize L-arabinose, D-xylose, D-fructose, D-galactose, maltose, sucrose, lactose, trehalose, D-sorbitol, D-mannitol, inositol, soluble starch, citric acid, lactic acid, methylamine, methane or hydrogen and some strains can utilize D-mannose weakly. They utilized ammonia,

nitrate and urea as nitrogen source. The DNA base composition ranged from 63.0-65.0 mol % of G+C content (Urakami *et al.*, 1989). This genus was included only one species, *Acidomonas methanolica* (Urakami *et al.*, 1989).

1.4 *Gluconacetobacter*

Gluconacetobacter strains were Gram-negative bacteria, 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 metabolism was oxidative, catalase-positive, oxidase-negative. Produce ubiquinone-10 for major ubiquinone system (Yamada and Kondo, 1984). Grow on AE agar medium and occurred smaller colony, round form, regular, umbonate, soft, glossy, sized 1-2 mm. Growth only on the acetic acid presented, ethanol and glucose medium by the concentration exceed about 6.0 %. Oxidize acetic acid from ethanol but dose not over-oxidization. Grow on 3 % (v/v) ethanol as medium presence of acetic acid up to 11 % (v/v) and high growth on glucose, fructose and sucrose presented in AE agar medium. Unassimilated gluconate, glycerol and lactate. No ketogluconic acid formation from glucose and no detected of formation cellulose on solid media or in broth (Schüller *et al.*, 2000). Some species were nitrogen-fixing bacteria as associated with coffee plant or living with bacterial community in rhizosphere of Chrysanthemum via plants in the nature (Fuentes *et al.*, 2001). The DNA base composition ranges from 55-63 mol % of G+C content (Schüller *et al.*, 2000). The genus *Gluconacetobacter* included *Ga. diazotrophicus*, *Ga. europaeus*, *Ga. hansenii*, *Ga. xylinus*, *Ga. liquefaciens*, *Ga. sacchari*, *Ga. intermedius*, *Ga. oboediens*, *Ga. entanii*, *Ga. azotocaptans*, and *Ga. johannae* (Yamada, 2000; Schüller *et al.*, 2000; Fuentes *et al.*, 2001).

1.5 *Asaia*

Asaia strains were Gram-negative rod bacteria, strictly aerobic, oxidase-negative and catalase-positive. Some strains were motiled by peritrichously flagella. Colonies were smaller with various characteristics such as white, yellowish, pale-pink to pink colors, shiny, smooth, raised and regular too wavy and smaller on AG medium. Oxidize acetate and lactate completely to carbon dioxide and water. Produce Q-10 for major ubiquinone in respiratory system. No produce water-soluble brown pigment. Most member were not produced acetic acid from ethanol. Growth was inhibited by 0.35 % of acetic acid and no growth on methanol. Grow on 30 °C at pH 3.0 for cultured. Assimilate ammonium sulfate for growth on vitamin-free glucose medium. Grow

on mannitol and glutamate medium. Production 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose but not produce 2,5-diketo-D-gluconate. Acid formation from D-glucose, D-mannose, D-fructose, L-sorbose and dulcitol. The DNA base composition ranges from 59.3-61.0 mol% of G+C content (Katsura *et al.*, 2001; Yukphan *et al.*, 2004). Nowadays, genus *Asaia* included *As. bogorensis*; *As. siamensis*; *As. krungthepensis* (Yamada *et al.*, 2000; Katsura *et al.*, 2001; Yukphan *et al.*, 2004).

1.6 *Kozakia*

The *Kozakia* species differentiated from other members of *Acetobacteraceae* as the capable production levan-like mucous substance from sucrose or D-fructose. *Kozakia* were Gram-negative bacteria, rod, non motile, measuring 0.6-0.8 by 2.0-3.0 μm. They were strictly aerobic, catalase-positive, oxidase-negative and major ubiquinone was Q-10. Does not produce water-soluble brown pigment from D-glucose or on calcium carbonate containing agar slant medium. They were unable to produce gelatinase, hydrogen sulfide, indols or ammonia from L-arginine and no growth on 30 % of D-glucose. They were weakly activity when oxidize acetate and lactate to carbon dioxide and water and production acetic acid from ethanol. Growth on mannitol agar but not on glutamate and not inhibited by 0.35% acetic acid at pH 3.5. No growth on 30% of D-glucose and no assimilated ammonia nitrogen on glucose medium, mannitol medium or ethanol medium without vitamins. Produce dihydroxyacetone from glycerol and produce 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose, but no produce 2,5-diketo-D-gluconate. Produce γ-pyrones from D-fructose but not from D-glucose. Acid formation produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, melibiose, raffinose, *meso*-erytritol, glycerol and ethanol but acid production from D-arabinose and sucrose was variable depending on the strain. The DNA base composition ranges from 56.8-57.2 mol% of G+C content (Lisdiyanti *et al.*, 2002). There was only one species in the genus *Kozakia*, *K. baliensis* (Lisdiyanti *et al.*, 2002).

2. Molecular Analysis

2.1 Polymerase chain reaction (PCR)

The polymerase chain reaction or PCR was based on an amplification of target DNA *in vitro*. A temperature-reswastant DNA polymerase or *Taq* polymerase, from *Thermus aquaticus* bacteria was used to DNA amplification from DNA primers. Principle of this technique including three steps for one cycle as chain reaction.

- Denaturation

Double strand DNA templates were denatured by high temperature 90-95 $^{\circ}$C to form single-stranded DNA templates.

- Annealing

At specific oligonucleotide primers were bond single-strand DNA templates at the annealing temperature, depending on melting temperature (t_m) of the oligonucleotide primers.

- Primer extension (amplification) step

DNA polymerization was performed by DNA polymerase for synthesizes new DNA molecule in a 5'⟶3' extension as using the primer to initial the reaction and exponential increase in the number of segments replicated. Amplifications by as much as million-folds can be readily achieved or about 20 cycle from theory.

Application of PCR technique included, a single-copy gene can be amplified out of a genomic sample, provided primers corresponding to know sequences of the gene were synthesized. Because of the exponential amplification, PCR was very sensitive and were detected extremely low copy number in sample. In addition, PCR technique were improved and developed to be highly sensitive and specific such as Booster PCR, Asymmetric PCR, Nested PCR, Multiplex PCR, Inverse PCR, Recombinant PCR, RT-PCR, Anchored PCR, and Expression PCR and so on. Note that, the PCR techniques were unnecessary to restrictly digest of the substrate DNA, because the primers will match to the appropriate sequence of native DNA. Furthermore, no lengthy cloning procedures were necessary, because enough DNA was amplified that a clear band on a gel was produced.

2.2 Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism analysis was method for identification and typing of microorganism by used restriction enzymes were specific locus sites to cutting nucleotide fragment for differently DNA profiles on electrophores. PCR-RFLP method were 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. After amplified DNAs following to cleaving PCR products by restriction enzyme and examine DNA fragment by agarose gel electrophoresis. This method was application for identify microorganism and useful for DNA genomic fingerprinting in plant identifications (Olive and Bean, 1999; Yukphan, 2004).

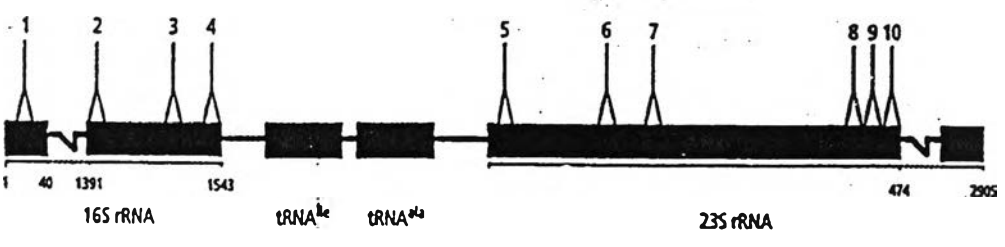

Fig. 2.1 The 16S-23S rDNA internal transcribed spacer regions

(Gürtler and Stanwasich, 1996)

The ribosomal operon was a classic marker used to trace genetic relationships and to identify strains rapidly. Of all the different regions of the ribosomal operon, the internal transcribed spacers (ITS) between 16S and 23S ribosomal DNA were frequently used as molecular markers to identify microbial species and analyze the phylogenetic relationship between strains. The ITS regions were generally found in multiple copies in most bacterial genomes. Since ITS regions hyper variable 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).

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 II and

I. 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%, rang of 4.7 mol%. Group II included of organwasms with a distinctly lower G+C content of DNA ranging from 54.2-57.6 mol%, rang of 3.4 mol%. This study proposed a new species, *Gluconobacter cerinus*, sp.nov., nom. rev, classified into Group II. Subsequently, Yamada *et al.*, (1984) were examinated of DNA homology for twenty strains of *Gluconobacter* species with other genera relation. From this study, they found differently value DNA-DNA similarity depended on relationship, also this data used for the characteristics for identification and classification in the current time.

In 2000, Ruiz *et al.* examined and identified acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. These study included *Frateuria aurantia* LMG 1558^{T}and *Escherichia coli* ATCC 11775^{T} for out-group comparisons. The amplified 16S rDNA products about 1450 bp and 16S-23S rDNA intergeic spacer PCR products about 675 and 800 bp. PCR products digestion by 4-base-cutting restriction enzymes in order to evaluate the degree of polymorphism exiting among these strains. Eigth 4-base-cutting restriction analysis of 16S rDNA PCR products was proposed as a rapid and reliable method to identify acetic acid bacteria at the level of genus and species and its applicability to identification of indigenous acetic acid bacteria was demonstrated. However, 16S-23S rDNA ITS restriction patterns of strains isolated from wine did not math those of any of the reference strains. Thus, PCR-RFLP of the 16S-23S ITS was not useful method to identification of acetic acid bacteria at the species level although it may be an adequate method to detect intraspecific differentiation. In addition when they identify acetic acid bacteria by PCR-RFLP of the gene coding for 16S rDNA. They found this examination able to rapid identification of acetic acid bacteria at the genus level after digested PCR products by *Taq*I restriction enzyme.

Trček and Teuber, 2002 reported to molecular characteristics of acetic acid bacteria isolated from spirit vinegar. They used three techniques for molecular analysis, including amplified rDNA restriction analysis (ARDRA), randomly amplified polymorphic DNA analysis (RAPD) and dot blot hybridization analysis with an WAS 1380 probe. Among 13 analyzed *Acetobacter* strains, the RAPD methods revealed three different profiles. Each of these profiles was comparable to one of the ARDRA profiles of the following reference strains thus as *A. aceti, A. hansenii* and *A. xylinus* as all were type species. RAPD profiling was more discriminatory than ARDRA profiling, being able to distinguish between isolates with identical ARDRA profiles.

In 2002, Trček and Teuber reported the genetic and restriction analysis of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria. 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.

Recently, Yukphan *et al.*, examined new approach for identification of *Gluconobaceter* Asai 1935. The 16S-23S rDNA internal transcribed spacer regions restriction and sequence analysis. This study included thirteen reference strains such as the type strains of the *Gluconobacter oxydans* NBRC 14819^T, *G. cerinus* NBRC 3267^T, *G. asaii* NBRC 3276^T, which was a junior subjective synonym of *G. cerinus*, and *G. frateurii* (IFO 3264^T) were examined for their species identification based on the sequence and the restriction analyses of the 16S-23s rDNA internal transcribed spacer regions. A phylogenetic tree constructed by the neighbor-joining method represented three clusters correspond respectively the three species, *G. oxydans*, *G. cerinus* and *G. frateurii*. 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.

2.3 DNA sequencing analysis

One of the requirements for DNA sequencing able to obtain defined fragments of DNA. Thus, there was a strong in dependence of DNA cloning and DNA sequencing technology, in as much as DNA cloning provides amplified samples of defied DNA fragments. DNA sequencing was originally performed by using radioactive labels for detection of the reaction products, and approach that was unsuitable for clinical use. Fred Sanger developed the DNA sequencing method that the sequence was then read with an automated instrument commonly used in the present, employ fluorescent nucleotides to label the DNA, Sanger method was based on DNA synthesis in the presence of dideoxynucleotides, which differ from normal deoxynucleotides in that lack of a 3'-hydroxyl group. The respective dideoxynucleotides

triphosphates (ddNTPs) can be incorporated into a growing strand, when incorporated, they terminate synthesis because lack of the 3'-hydroxy group that's necessary to bond with the next nucleotide triphosphates. Each of four reaction tubes was prepared with a single- strand DNA template for the sequence of interest, plus DNA polymerase and a section of labeled primer. Each tubes receives a small amount of a different ddNTP (ddATP, ddTTP, ddCTP, or ddGTP), together with the four normal deoxynucleotide triphosphates (dNTPs). A dideoxynucleotide will be incorporated randomly, at different sites in different syntheses in the reaction tube. Therefore, in any given tube, various truncated chain lengths will be produced, each corresponding to the point at which the respective ddNTP for that tube was incorporated and terminated strand growth. These lengths in turn were cleared indication of where the bases complementary to the ddNTPs were on the template strand. Because incorporation was random, all possible truncated fragments will be produced, corresponding to all the various positions of that particular base. Electrophoresis of the four samples in four lanes on and an acrylamide gel can visualize the fragment, where the fragments form bands. The base sequence can be determined by scanning up the gel, encompassing all four lanes, and recording whichever base occupies the terminus in the next band. Currently, during electrophoresis, these fluorescently labeled products were excited by an argon laser and automatically detected. The resulting data was stored in digital form for sequence processing into the final sequence with the aid of specialized software. There were several considerations that must be evaluated before undertaking the use of DNA sequencing for subtyping (Olive and Bean, 1999; Yukphan, 2004).

In 1995, Sievers *et al.*, studies about phylogenetic position of *Gluconobacter* species as a coherent cluster separated from all *Acetobacter* species on the based of 16S rRNA genes sequences. By phylogenetic tree relationship of 16S rRNA genes sequence of all organisms were examinations presented to differently position and closely related cluster of *Gluconobacter* with *Acetobacter*.

In 1997, Yamada *et al.*, examined for the phylogeny on based of partial sequences of 16S rRNA genes of acetic acid bacteria as the partial based sequences in positions 1220 through 1375, amount 156 bases of 16S rRNA genes of thirty-six strains of acetic acid bacteria classified in the genera *Acetobacter*, *Gluconobacter*, *Acidomonas* and *Gluconacetobacter* were compared. They reported that the *Acetobacter* species were not very distantce phylogenitically from the *Gluconobacter* species. The calculated number of base differences was seven between the type strains of *Gluconobacter oxydans* and *Acetobacter aceti*. In addition, the *Gluconacetobacter* were

very distant phylogenetically from the *Acetobacter* and *Gluconobacter* species as number of base differences was calculated to be 15-8. The *Acidomanas* species were located in phylogenetically isolated positions as 16-8 base differences.

2.4 DNA-DNA hybridization

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

Gosselé *et al.* (1983), on the basis of numerical analyses of phenotypic features and protein profiles of more than 200 strains of acetic acid bacteria, rejected the subspecies concept in classification and delineated four species in the genus *Acetobacter*: *A. aceti*, *A. hansenii*, *A. liquefaciens* and *A. pasteurianus*. These conclusive results appeared in *Bergey's Manual of Systematic Bacteriology* (De lay *et al.*, 1984). However, Yamada (1984) revived *A. xylinus* as an independent species on the basis of its ubiquinone type. Subsequently, many new species were proposed in *Acetobacter*: "*Acetobacter polyoxogenes*", *Acetobacter methanolicus*, *Acetobacter diazotrophicus*, *Acetobacter europaeus*, *Acetobacter pomorum* and *Acetobacter oboediens* and *Acetobacter intermedius*. The establisment of the genus *Gluconacetobacter* (Yamada *et al.*, 1997) and the revival of the genus *Acidomonas* (Urakami *et al.*, 1989) together with the transfer of *A. oboediens* and *A. intermedius* to the genus *Gluconacetobacter* (Yamada, 2000) decreased the number of *Acetobacter* species in 2000 to three: *A. aceti*, *A. pasteurianus* and *A. pomorum*. On the basis of a polyphasic taxonomic study including DNA-DNA hybridizations, determination of ubiquinone composition and 16S rRNA gene analysis using strains freshly isolated from Indonesian sources together with strains from culture collections, Lisdiyanti *et al.* established five new species (*A. indonesiensis*, *A. tropicalis*, *A. orientalis*, *A. syzygii* and *A. cibinongensis*), three new combinations (*A. estunensis*, *A. orleanensis* and *A. lovaniensis*), and revived *A. peroxydans* as a separate species in the genus (Lisdiyanti *et al.*, 2000;Lisdiyanti *et al.*, 2002). Cleenwerck et al. 2002 re-examined the classification of various *Acetobacter* strains, confirmed the entity of the

new combinations and new species proposed by Lisdiyanti *et al.* 2000, and additionally proposed two new species, namely *A. malorum* and *A. cerevisiae.*

In 1989, Mason and Claus reported the phenotypic characteristics correlated with DNA sequence similarity of three species of *Gluconobacter* such as *G. oxydans* (Henneberg 1897) De Ley 1961, *G. frateurii* sp. nov., and *G. asaii* sp. nov. Thirty-five strains were characteristics of the phenotypic features. The results presented for differently between three species as homology group I strains grew to optical density (OD) of only 0.5 U or less on medium containing ribitol or arabitol as the primary carbon source, and they grew to an OD only 0.5 U or less after three passages (24 h of incubation each) in nicotinate-deficient media and they identified the strains in the homology Group I as *G. oxydans* (Henneberg 1897) De Ley 1961. Homology group II strains grew to an OD of more than 1.0 U on medium containing ribitol or arabitol as the primary carbon source, and they grew to an OD of more than 1.0 U after three passages (24 h of incubation each) in nicotinate-deficient media, they identified the bacterial strains as *G. frateurii* sp.nov.,. The final group or group III was proposed as *G. asaii* sp. nov.,. The strains in this group can be grown to an OD of 0.5 U or less on medium containing ribitol or arabitol as the primary carbon source, but they grew to an OD of 1.0 U or more after three passages (24 h of incubation each) in nicotinate-deficient media.

In 1999, Tanaka *et al.* reported to reclassification of the strains with low G+C contents of DNA belonging to the genus *Gluconobacter* Asai 1935. By Three species such as *Gluconobacter cerinus*, *G. asaii* and *G. frateurii* were reported to show lower G+C contents than the *G. oxydans*. isolate 145 also showed a similar G+C content to those of the three species. They try to reclassify the three species and they conclude that *G. asaii* was conspesific and synonymous with *G. cerinus*.

In 2002 Katsura *et al.* proposed *Gluconobacter asaii* Mason and Claus 1989 was a junior subjective synonym of *Gluconobacter cerinus* Yamada and Akita 1984. This study included *Gluconobacter cerinus* and *Gluconobacter asaii* were examined for DNA base composition, DNA-DNA similarity 16S rRNA gene sequences and phenotypic characteristics thus as acid production from ethanol, growth on L-Arabitol and *meso*-Ribitol and requirement for nicotinic acid. The results showed five strains was DNA base composition ranging from 54 to 56 mol% G+C contents. They found the *Gluconobacter cerinus* IFO 3267^{T}and IAM 1832 and *G. asaii* IFO 3276^{T}and IFO 3275 showed high levels of DNA-DNA similarity (70-100%) between

each other and low values of DNA-DNA similarity (16-35%) to *G. frateurii* IFO 3264^{T}and *G. oxydans* IFO 14819^T. *Gluconobacter cerinus* IFO 3267^{T}and *G. asaii* IFO 3276^T were located at an identical position in a phylogenetic tree deduced from 16S rDNA sequences. Two *G. cerinus* strains and two *G. asaii* did not required nicotinic acid for growth and did not grow on L-arabitol or *meso*-ribitol. For *G. cerinus* IAM 1832 did not produced acid and required nicotinic acid or other growth factors. Between *G. asaii* IFO 3265 and *G. frateurii* IFO 3264^{T}showed high values DNA-DNA similarity to 97% and low similarity values (each 32%) to *G. cerinus* IFO 3267^{T}and *G. asaii* IFO 3276^T. This strain did not require nicotinic acid and grew well on L-arabitol and *meso*-ribitol. From described above they proposed reclassified *G. asaii* IFO 3265 as *G. frateurii*. In addition, the results concern to a synonymous relation ship between *G. cerinus* has priority over *G. asaii*.

3. Alcohol dehydrogenase (ADH)

Acetobacter strains was frequently used for the industrial vinegar (Saeki *et al.*, 1997). Furthermore, *Acetobacter* sp. was a strain which catalyses the biological oxidation of ethanol in acetic acid by the association of two enzymes (primary dehydrogenases) membrane bound: a quinoprotein alcohol dehydrogenase (ADH) and an aldehyde dehydrogenase (ALDH) (Matsushita *et al.*, 1994).

$$C_2H_5OH \xrightarrow{ADH} CH_3CHO + H_2O \longrightarrow \underset{\text{(hydrate acetaldehyde)}}{CH_3CH(OH)_2} \xrightarrow{ALDH} CH_3COOH + H_2O$$

These two enzymes form the enzymatic complex responsible for acetic fermentation from the genus *Acetobacter* (Matsushita *et al.*, 1994). There were also other primary dehydrogenases such as NAD-dependent. ADH located in the cytoplasm of the cells.

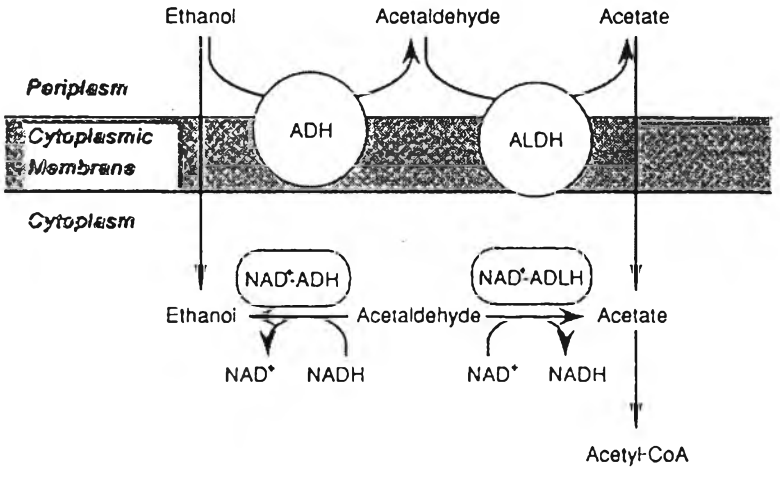

Fig. 2.2 Alcohol-oxidizing systems in acetic acid bacteria (Matsushita *et al.*, 1994)