CHAPTER III

MATERIALS AND METHODS

Instruments, materials, chemical reagents and media

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

Methods

1. Isolation of acetic acid bacteria

Acetic acid bacteria were isolated from many kinds of fruits, flowers and related materials by an enrichment culture approach using Glucose-Ethanol medium, Sorbitol medium, Sucrose-Acetic acid medium and Methanol-Peptone-Yeast extract (MPY) medium (Appendex B). The enriched samples were incubated at pH 4.5 and 30°C for 3-5 days and then the cultures were streaked onto Glucose-Ethanol-Yeast extract-$CaCO_3$ (GEY- $CaCO_3$) agar plates (Yamada *et al.*, 1976; 1999). The acetic acid bacteria that formed clear zones around colonies on the agar plates were selected as acid-producing bacterial strains. They were streaked for purification on agar plate and were preserved mainly at –80°C in an ultra low temperature freezer. Sterile 20% glycerol in glucose-yeast extract-peptone was used as a cryoprotectant.

2. Identification methods

2.1 Cell morphology and cultural characteristics

The colonies grown on GEY-$CaCO_3$ agar medium (Appendix B) 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.

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.

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 C) was timed for 1 min with 1 ml of stain at ambient temperature. The slide was washed in tap water, counterstained with the Hucker modification of Gram crystal violet for 1 min, washed, blotted dry, and examined under oil immersion starting near the wax line.

2.2 Physiological and biochemical characteristics

2.2.1 Oxidation/Fermentation catabolism test The isolate were inoculated in Hugh and Leifson's medium (Appendix B) 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 one 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.

2.2.2 Catalase test Cells were transferred onto slide, and immediately covered by 3% (v/v) hydrogen peroxide. The evolution of gas bubbles indicated a positive test.

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

2.2.4 Oxidation of acetate and lactate The isolates were inoculated into oxidation of acetate and lactate test medium (Appendix B). The cultures were incubate with out shaking for 7 days at 30°C. A positive was indicated by blue color whereas a negative was by yellow color.

2.2.5 Growth in the media containing 0.3% acetic acid at pH 3.5 The isolates were inoculated into the medium containing 0.3% acetic acid at pH3.5 (Appendix B). The culture were incubated with out shaking for 7 days at 30°C.

2.2.6 Growth in the media containing 30% D-glucose The isolates were inoculated into the medium containing 30% D-glucose (Appendix B). The culture were incubated with out shaking for 7 days at 30°C.

2.2.7 Formation of water-soluble brown pigment The isolates were inoculated on the GEY-$CaCO_3$ agar medium (Appendix B). After incubating at 30°C for 7-30 days, water soluble brown to dark brown pigment were observed.

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

2.2.9 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 B). The culture were incubated with out shaking for 7 days at 30°C, and observed the results everyday. The carbon sources were used in this study are 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.

2.3 Chemotaxonomic characteristics

2.3.1 Ubiquinone analysis The ubiquinone of acetic acid bacteria isolates were extracted and quanlitative determined. The isolates were culture in GEY medium (Appendix B) with shaking condition at 30°C for 48 hours. Cells were collected by centrifugation and approximately 1 g wet cell were suspend in 100 ml of absolute ethanol: diethylether (1:3) and shaked for 20 minutes. 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.

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-10). 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) 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 0.1 x SSC. RNase A solution was added into the DNA solution, incubated at 37°C for 20 min, then extracted by 2 ml of phenol-chloroform. After centrifugation at room temperature, 12,000 rpm (9,200 g) for 10 min, the upper layer was transferred to 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 0.1 x 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 and Doty (1962).

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/litre of 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 hour, and then 10 µl of alkaline phosphatase solution (Appendix C) was added and keep at 37°C for 1 hour. DNA base composition of DNA hydrolysate was analyzed by HPLC using conditions as shown in Table 3.1

Table 3.1 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

2.4 Molecular characteristics

2.4.1 The 16S-23S rDNA internal transcribed spacer region restriction fragments length polymorphism (RFLP) analyses. (Yukphan *et al.*, 2004)

2.4.1.1 Polymerase chain reaction for restriction fragments length polymorphism (PCR-RFLP) of 16S-23S rDNA ITS regions DNA of acetic acid bacteria was extracted by the method described by Saito and Miura (1963). The extracted DNA of acetic acid bacteria was amplified with two primers. The primers contained 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). The polymerase chain reaction in this study included two steps as the first step to find optimal concentration of DNA template and optimal condition of PCR. The second step for amplification of the target DNA in large volume. All condition show and describe in Table 3.2 and Table 3.3.

(a) Optimum condition of PCR

The extracted DNA was tested for the optimal condition of PCR. The PCR reactions were carried out as described in Table 3.2.

Table 3.2 Determination of optimal condition of PCR

Reagents		Initial concentration	1 x volume
dNTP		2.0 mM.	1.00 µl.
$MgCl_2$		25.0 mM.	0.60 µl.
Primer	1522F	10.0 pmol/µl.	0.50 µl.
	38R	10.0 pmol/µl.	0.50 µl.
Taq DNA polymerase		5.0 unit/µl.	0.05 µl.
10x*Taq* buffer		10.0 x	1.00 µl.
dH_2O		-	5.85 µl.
DNA template		Undiluted, 10^{-1}, 10^{-2}	0.50 µl.
Total volume			10.00 µl.

(b) Large scale PCR (100 µ x 2 = 200 µ l)

After found optimal dilution PCR in the first step continue to large scale PCR was carried out as described in Table 3.3.

Table 3.3 Large scale PCR

Reagents		Initial concentration	1 x volume
dNTP		2.0 mM.	10.00 µl.
$MgCl_2$		25.0 mM.	6.00 µl.
Primer	1522F	10.0 pmol/µl.	2.00 µl.
	38R	10.0 pmol/µl.	2.00 µl.
Taq DNA polymerase		5.0 unit/µl.	0.50 µl.
10x*Taq* buffer		10.0 x	10.00 µl.
dH_2O		-	65.50 µl.
DNA template		Optimally diluted	4.00 µl.
Total volume			100.00 µl.

Both PCR condition used a same method as nucleotide fragment were amplified by Peltier Thermal cycle model MJ Research DYAD ALD 1244 and performed by the PCR cycling program was started with an initial denaturation of DNA at 94 °C for 5 min, continued with 30 cycle at 92 °C for 30 sec, 56 °C for 45 sec, 72 °C for 1 min and following by final extension at 72 °C for 7 min, end temperature of PCR products collected at 4 °C.

Five micro litters of PCR products were mixed with 2 microlitter of loading dye and subjected to 0.8 % agarose gel electrophoresis with submerged in 1x TAE buffer (diluted by 50 x TAE buffer). The 1-kb DNA ladder was standard marker. The electrophoresis was conducted with Ac-STEP-UP/DOWN TRANSFOMER model SD-500W. (PROTECH ELECTRIC CO., LTD) as a constant voltage of 100 V for 40 min. PCR products were staining with ethidium bromide solution, washed by distilled water and viewed with BIORAD Gel Doc UV transilumination together with photographed by camera as carried out by Molecular analyst program version V 1.5 with Windows 95 software.

2.4.1.2 Purification of PCR products with QIAquick column The PCR products of the large scale PCR were purified by QIAquick column as follows: add 900 µl of PB solution into 1.5 ml microfuge tube and add of 200 µl PCR product, mix (invert tube 3-5times), place the solution into the QIAquick column, centrifuge at 6,000 rpm for 30 sec to discard the filtrate, add 750.0 µl of PE buffer into the column, centrifuge at 8,000 rpm for 30 sec to discard the filtrate, centrifuge at 8,000 rpm for 1.0 min, move the column to the new 1.5 ml microfuge tube, pipette 40 µl EB buffer into the center of the column, incubate at room temperature for 10 min and performed at centrifuge at 14,500 rpm for 1.0 min and stored at 4 $^{\circ}$C. The purified DNA was subjected to electrophoresis same on described above.

2.4.1.3 The 16S-23S rDNA restriction pattern analyses The purified PCR products were digested by five restriction enzymes included *Hpa*II, *Hae*III, *Bsp*1286I, *Mbo*II and *Ava*II. Reaction and condition described as separated to two reactions or different for each restriction enzyme. Checks the results by agarose gel electrophoresis on described above but used ten-microlitter of RFLP products were checked by 2.5 % agarose gel electrophoresis which used 1.7 µl of 50 bp DNA ladder (mixed with 1.2 µl 6x loading dye) for marker. For the other condition of electrophoresis used the method as described above.

Table 3.4 Condition of 16S-23S rDNA ITS PCR-RFLP for restriction enzyme

Reagents	1x reaction
Restriction enzyme	1.0 µl
DNA	13.0 µl
Buffer B^{+}	2.0 µl
Nano pure water	4.0 µl
Total volume	20.0 µl

Incubated at 37 ^{o}C for overnight.

2.4.2 16S rDNA sequence and phylogenetic analysis

2.4.2.1 Isolation of DNA for amplification by PCR The bacterial isolates were harvest and suspend in 180 µl of Tris-EDTA buffer. Then, added 20 µl of 10% SDS and incubation at 50^{o}C for 10 minutes. Supernatant were extracted twice with 200 µl of Phenol:chlorofrom:isoamyl alcohol (25:24:1 v/v). DNA was precipitated with colded absolute ethanol, place at 20^{o}C for 10 minutes and collected the DNA by centrifugation at 14,500 rpm for 15 minutes. DNA pellet were rinsed with 70% and 90% ethanol and then dried up (15-30 minutes at room temperature). The dried DNA is dissolved in 50 µl milli Q water.

2.4.2.2 Amplification of 16S rDNA The 16S rDNA were amplified with primers 20F (5'-GAG TTT GAT CCT GGC TCA G-3', Position 9-27) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', Position 1509-1492) (Yamada *et al.*, 2000 and Katsura *et al.*, 2001). Position in the rDNA fragment were based on the Escherichia coli numbering system (accession number V00348; Brosius *et al.*, 1981). Amplification is carried out in 100 µl of reaction mixture containing 100 ng of genomic DNA, 2.5 U of Taq polymerase, 20 mM of each dNTP, 10 pM of each primer, 10 mM Tris-HCl and 1.5 mM $MgCl_2$. The reaction is performed for pre-denaturing at 94^{o}C for 3 min and followed by 25 PCR cycles for denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 2 min and then followed by the final extension at 72^{o}C for 3 min. The amplified DNA is purified with QIAquick PCR Purification Kit according to the manufacturer's instruction. Visualization of the purified of amplified DNA is performed by electrophoresis using 0.8% agarose in 1X TAE buffer (0.09M Tris-borate, 0.001M EDTA; pH 8.0) and strained with ethidium bromide ($8x10^{-5}$ µg/ml) and observed under UV Transilluminator.

2.4.2.3 DNA sequencing Amplified 16S rDNA were sequenced directly with an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit on an ABI PRISM model 310 Genetic Analyzer (both from Applied Biosystems). The following primers were used for sequencing: 20F, 1500R, 520F (5'-CAG CAG CCG CGG TAA TAC-3', Position 519-536), 520R (5'-GTA TTA CCG CGG CTG CTG-3', Position 536-519), 920F (5'- AAA CTC AAA TGA ATT GAC GG-3', Position 907-926) and 920R (5'- CCG TCA ATT CAT TTG AGT TT-3', Position 926-907).

2.4.2.4 Phylogenetic analysis 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 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).

3. Screening of the high acetic acid-producing strains

3.1 Culture medium and growth condition

The basal culture medium mainly used was typically Glucose-Glycerol-Yeast extract Potato (GGYP) medium (Appendex B). *A. pasteurianus* strains were inoculated in 200 ml of seed medium (GGYP medium) at 30°C and 40°C on a rotary shaker 200 rpm.

3.2 Preparation of crude enzyme ADH solution

Cells were harvested by centrifugation at 7500xg for 10 min and washed twice with cooled 0.1M potassium phosphate buffer (KPB), pH 6.0. The washed cells were resuspended in the same buffer and passed through a sonicator. Remove cells by using centrifugation at 7500xg for 10 min and then the supernatant was used as ADH assay.

3.3 Alcohol dehydrogenase assay

Alcohol dehydrogenase (ADH) was measured as described by the method of Ameyama, 1982. The reaction mixture contained enzyme solution, buffer (McIlvaine buffer; McB, pH 5.0), substrate (ethanol) 100µl, 0.1M ferricyanide solution. After 5 min, dupanol reagent 0.5 ml was added to the medium incubated 20 min. After 20 min dH_2O was added at a final volume of 3.5 ml. The reduction of the absorbance at 660 nm was followed with an UV

spectrophotometer. One unit of enzyme activity was defined as the quantity of enzymes catalyzing the oxidation of 1 μmol of the acetaldehyde or ethanol, or the reduction of 1 μmol of ferricyanide 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.

4. Acetic acid production of the selected strain

The selected strain was cultivated in GGYP medium at 30°C on a rotary shaker 200 rpm for 5 days. The biomass evolution was determined for its growth by using the turbidimetrical method (the optical density, OD) at 660 nm for every 24 h.

4.1 Effects of ethanol concentration

The effects of ethanol concentration were determined by inoculating the selected isolate (high ADH activities) in 0.5%yeast extract with different amounts of ethanol ranging from 2 to 8% (v/v).

4.2 Effects of acetic acid concentration

The effects of acetic acid concentration were determined by inoculating the selected isolate (high ADH activities) in 0.5%yeast extract and suitable ethanol (from 4.1) with different amounts of acetic acid ranging from 0.5 to 2.0% (v/v).

4.3 Effects of temperature

Temperature effects on acetic acid production were determined by incubating the selected isolate in 0.5%yeast extract and suitable ethanol and acetic acid as in 4.1, 4.2 and incubating at 30, 37 and 40°C.

Acetic acid in the culture medium produced by the selected isolate was measured by titration with 0.5N NaOH using phenolphthalein as a pH indicator and calculated the total acetic acid production as described by Helrich, 1990 (Appendex E).