CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and equipments

3.1.1 Chemicals

1. Diuron ($C_9H_{10}Cl_2N_2O$), 99.5% purity was obtained from Chem Service, U.S.A.

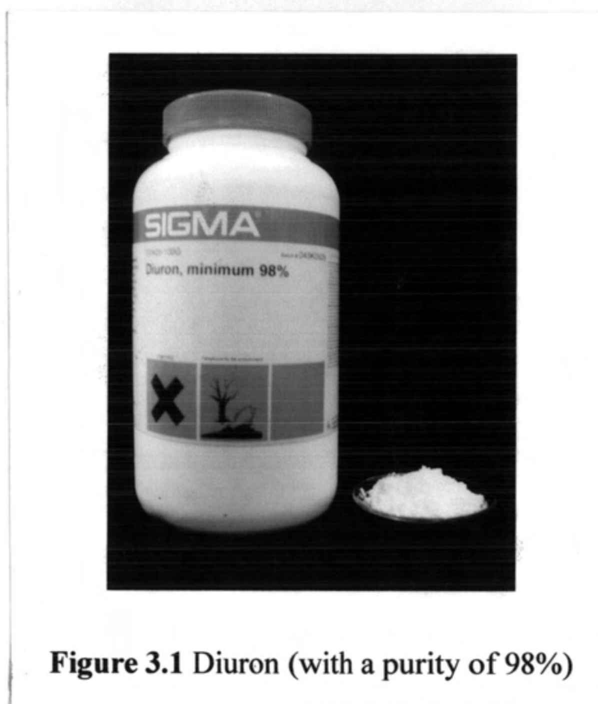

Figure 3.1 Diuron (with a purity of 98%)

2. Acetronitrile (HPLC grade), ethanol (HPLC grade), methanol (HPLC grade), acetone (Analytical grade), *n*-butanol (Analytical grade), dichloromethane (Analytical grade) and ethylacetate (Analytical grade) were obtained from Lab-Scan, Ireland.

3. Sodium chloride ($NaCl$), potassium phosphate (KH_2PO_4) and magnesium sulphate ($MgSO_4.7H_2O$) were obtained from Carlo Brba, France.

4. Agar, peptone, yeast extract and di-sodiumhydrogen phosphate anhydrous (Na_2HPO_4) were obtained from Scharlau, Spain.

5. Calcium chloride ($CaCl_2.2H_2O$) was obtained from Merck, Germany.

6. Ammonium chloride (NH_4Cl) was obtained from M&B laboratory chemicals, England.

7. Ferric ammonium citrates, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), iodonitroterazolium chloride (INF), *N,N*-dimethylformamide, acrylamide PAGE and N,N'-methylene-bis-acrylamide were obtained from Sigma, USA.

8. Glucose monohydrate was obtained from Asia pacific specialty chemicals limited, Australia.

3.1.2 Equipments

1. High performance liquid chromatography LC10ADVP, Shimazu, Japan
2. Microcentrifuge 5804R, Eppendorf, U.S.A.
3. Autoclave MLS-3020, Sanyo Electric, Japan
4. Incubator shaker, Innova4000, New Brunswick Scientific, U.S.A.
5. Electroporation, Biorad, U.S.A.
6. Gel Document, Syngene, U.S.A.
7. UV transilluminater, BioDoc-It^{TM} System, UVP, U.S.A.
8. A rotary evaporator, Buchi, rotovapor R-200, Germany
9. PROTEAN II xi cells, Biorad, U.S.A.

3.2 Culture medium

3.2.1 The mineral salt medium (MSM)

The mineral salt medium used for bacterial cultivation (Widehem *et al.*, 2002)

Media

Na_2HPO_4	4.000 g
KH_2PO_4	1.500 g
NH_4Cl	1.000 g
$MgSO_4.7H_2O$	0.200 g
$CaCl_2.2H_2O$	0.010 g
$FeSO_4.7H_2O$	0.005 g

The component was dissolved in 1 liter of distilled water and adjusted pH to 6.8-6.9 by 1 M NaOH. The mineral salt medium was autoclaved at 121°C for 15 minutes.

3.2.2 Luria bertani medium (LB)

Tryptone	10 g
Yeast extracts	5 g
NaCl	10 g

LB medium was dissolved in 1 liter of distilled water, adjusted pH to 7 and autoclaved at 121°C for 15 minutes.

3.3 Soil preparation

Soil of sample was different two types; it was collected form at depth of 2-10 cm. The first and second soil types were collected from mango orchard in Nakornayok province and cassava field in Nakhornrtchasima province, respectively. The physical and chemical properties (such as soil texture, pH, organic matter, organic carbon, nitrogen, available phosphorous, C:N ratio, moisture content and water holding capacity) of soil samples were analyzed at Department of Soil Science, Faculty of Agriculture, Kasetsart University and Soil and Water group, Agricultural Chemistry Division. Analysis method for soil properties were in Table 3.1.

The soil samples were preparated by removal the useless particle and debris. Then, the soil samples were air dried and sieved through 2 mm mesh to be suitable in the experiment. The sieved soil was kept at room temperature until used.

Table 3.1 The analysis methods for soil properties

Properties	Methods
Soil texture	Hydrometer method (Gee and Bauder, 1979)
pH	pH meter with water (1:1)
Organic matter	Wet oxidation method (McLeod, 1973)
Organic carbon	Walkley-Black method (Walkley and Black, 1934)
Nitrogen	Kjeldahl method (Jackson, 1958)
Phosphorus	Bray II method (Bray and Kurtz, 1945)
Water holding capacity	Comparison between wet weight and dried weight

3.4 Recovery of Diuron from soil

Five grams of sieved soil were spiked with diuron to give the final concentration of 500 or 1,000 ppm in 22-ml screw-capped vial. The diuron stock solution was prepared by dissolving diuron in methanol to the desired concentration. The sample vials were incubated at room temperature for 1 and 15 days before extraction. Then, diuron was extracted by adding 10 ml of each organic solvent including methanol (50%, 80% and 100% (v/v)), ethanol (50%, 80% and 100% (v/v)) and acetonitrile (50%, 80% and 100% (v/v)) to find the highest diuron recovery efficiency. After that, the sample vials were rotated by the rotator overnight at room temperature. Then, the soil suspension sample was allowed to settle before the extracted liquid solution was collected. Finally, the amount of diuron recovered from each extraction was analyzed by high performance liquid chromatography (HPLC) which was described in section 3.6.2 (Diuron analysis). All tests were carried out in triplicates.

3.5 Biodegradation of diuron by bacterial in liquid medium

Bacterial pure culture, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3, were screened from soil by Miss Hathairath T.Wattanaphon. They could produce biosurfactant when cultured in liquid mineral salt medium with 20% glucose.

Bacterial consortiums were isolated from 4 agricultural soils by Enrichment technique. In a pre-enrichment step, 10 grams of the 4 type soils were homogenized in a 250 ml Erlenmeyer flask containing 100 ml of mineral salt medium supplemented with 100 ppm diuron. Then, the flasks of pre-enrichment step were incubated at room temperature (~30°C) on a rotary shaker for seven days. In the enrichment step, 10 ml of the pre-enrichment culture were suspended in 100 ml of mineral salt medium

supplemented with the same quantity of diuron. And then, the flasks of enrichment step were incubated on a rotary shaker at the same condition of pre-enrichment step. Finally, the enrichment cultures were plated onto mineral salt medium agar supplemented with diuron in order to isolate strains able to degrade this herbicide.

3.5.1 Preparation of bacterial inoculum

Bacterial pure culture, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 were cultured in liquid mineral salt medium with 20 ppm diuron, while 4 bacterial consortiums, isolated by Enrichment technique, were cultured in liquid mineral salt medium with 10 ppm shaken at 250 rpm, 30°C for 1 day.

3.5.2 Study of diuron degradation

Ten ml of bacterial inoculum was added into 90 ml liquid mineral salt medium containing 20 ppm diuron and shaken at 250 rpm, 30°C. The sample was collected and then, the remaining diuron in liquid medium was analyzed by HPLC as described in section 3.6.2.

3.6 Biodegradation of diuron in soil

3.6.1 Microcosm description of bioremediation treatments

Three bioremediation processes including natural attenuation, biostimulation and bioaugmentation were studied to evaluate the efficiency of diuron degradation.

Bioremediation: Condition A

- Natural attenuation

Five grams of 20 or 100 ppm diuron (solubilized in methanol) spiked soil were placed in 22-ml screw-capped vial. The spiked diuron was degraded by the ability of the natural microorganisms in soil.

- Biostimulation

Five grams of 20 or 100 ppm diuron spiked soil were placed in 22-ml screw-capped vial. Then, Triton X-100 (1, 5 and 10 CMC) and *n*-butanol was added in the vial to final concentration 20.6, 103, 206 and 100 ppm, respectively. It was used as a biostimulating agent of diuron biodegradation.

- Bioaugmentation

The preparation of bacterial inoculum

Bacterial pure culture, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3, were cultured in the liquid mineral salt medium with 0.5% glucose and 20 ppm diuron and shaken at 250 rpm, 30°C for 2-3 days. The bacterial cells were harvested by centrifugation at 12,000 rpm for 10 min. Cell pellet was washed with 0.85% sodium chloride solution twice. The cell pellet was resuspended with 0.85% sodium chloride solution and diluted to OD_{600} about 1 (approximate of 10^8 CFU/g soil) before adding to the bioaugmentation treatment.

Bioaugmentation treatment

Five grams of 20 or 100 ppm diuron spiked soil were placed in 22-ml screw-capped vial. Bacterial pure culture, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3, were used for bioaugmentation. Each type of the bacterial inoculum (500 μl) were added into the vials and well mixed with the soil.

<u>Bioremediation: Condition B</u>

- Natural attenuation

Five grams of 20 or 100 ppm diuron (solubilized in methanol) spiked soil were placed in 22-ml screw-capped vial. The spiked diuron was degraded by the ability of the natural microorganisms in soil.

- Biostimulation

Five grams of 20 or 100 ppm diuron spiked soil were placed in 22-ml screw-capped vial. Then, NH_4Cl was added in the vial to final concentration 1,000 ppm. It was used as a biostimulating agent of diuron biodegradation.

- Bioaugmentation

The preparation of bacterial inoculum

Bacterial consortium A1 was cultured in the liquid mineral salt medium with 10 ppm diuron and shaken at 250 rpm, 30°C for 2-3 days. The bacterial cells were harvested by centrifugation at 12,000 rpm for 10 min. Cell pellet was washed with 0.85% sodium chloride solution twice. The cell pellet was resuspended with 0.85% sodium chloride solution and diluted to OD_{600} about 1 (approximate of 10^8 CFU/g soil) before adding to the bioaugmentation treatment.

Bioaugmentation treatment

Five grams of 20 or 100 ppm diuron spiked soil were placed in 22-ml screw-capped vial. Bacterial consortium A1 was used for bioaugmentation. The bacterial inoculum (500 µl) was added into the vials and well mixed with the soil.

The soil moisture of all treatments were adjusted to approximate 50% water holding capacity (WHC) using sterile water. The vials were incubated at room temperature (~30°C). The moisture content was maintained the reduced weight of the soil microcosm every week and more sterile water was added to compensate. All tests were carried out in triplicates

3.6.1.1 Description of control experiment

Sterilized soil was used for the control condition. The soils were sterilized by autoclaving (3 times at 121°C, 15 min). Five grams of sterilized soil were placed in 22-ml screw-capped vial and spiked with diuron to give the final concentration of 20 or 100 ppm.

3.6.1.2 Sampling time

The soil samples of each treatment were collected every week for condition A, while condition B was collected every 5 days.

3.6.1.3 Experimental flow chart

The experimental flow chart was outlined in Figure 3.2

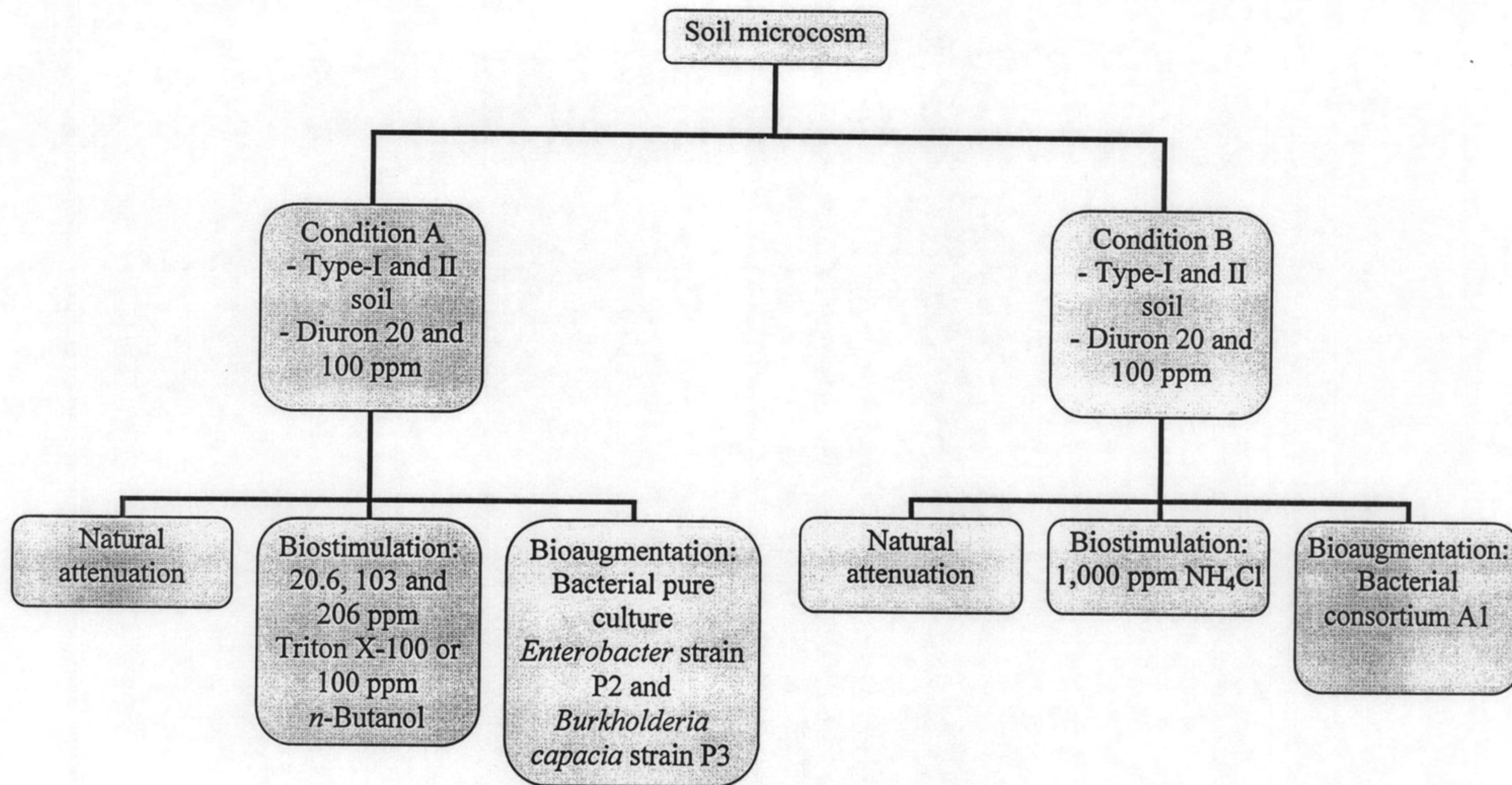

Figure 3.2 The experimental flow chart of soil microcosms.

3.6.2 Diuron analysis

3.6.2.1 Diuron extraction

Diuron was extracted from 5 g soil by adding 10 ml of 80% acetonitrile into the sample vials. The sample vials were rotated by rotator overnight at room temperature. After that, the sample vials was allowed to settle and then collected the liquid solution.

3.6.2.2 Diuron analysis by HPLC

The liquid solution was centrifuged at 12,000 rpm 10 min. The supernatant was collected and filtered with 0.45 μm diameter filter. Diuron was quantitatively analyzed by a reverse phase HPLC. The column used was C_{18} column (Inersil®, 4.6x250 mm) and compounds were detected by UV absorbance at 240 nm. The HPLC mobile phase was acetonitrile:water (80:20 v/v), with a flow rate of 1 ml/ min. With this analysis condition, diuron was eluted out at retention time of 4 min.

3.6.2.3 Diuron calibration curve

Diuron calibration curve was used for diuron analysis in soil. The standard diuron was prepared by dilution to various concentration of diuron. Then, the various concentrations of diuron were analyzed in HPLC as described in section 3.6.2. The calibration curve of diuron is shown in Appendix A.

3.6.3 Microbial analysis

3.6.3.1 Determination of the amount of diuron degrading bacteria

Plate count technique was determined for the amount of diuron degradation from bacteria. Soil samples were diluted 10-fold serial dilution with sterile

distilled water. Then, the serial dilution of cell suspension was spreaded on mineral salt medium agar plates which 20 ppm of diuon as carbon source. The plates were incubated at 30°C for 3-4 days. The diuron degrading bacteria were appearing colonized on the plate then, accounted the number of colony on agar plate.

The number of bacteria per milliliter of culture was calculated by

$$\text{Bacteria per ml of original solution (CFU g soil}^{-1}\text{)} = \frac{\text{(Number of counted bacteria) x (Dilution factor)}}{\text{Volume of added suspension to plate}}$$

3.6.3.2 Total microbial activity by dehydrogenase activity assay

Total microbial activity in soil was monitored by dehydrogenase activity assay (Alef, 1995). Soil sample (1 g) was mixed with 1.5 ml Tris buffer and 2 ml 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) solution. Next, the suspension was incubated at 40°C in the dark for 2 hours. Then, it was mixed with 10 ml extraction solution and kept in the dark. Finally, the enzymatic product (Iodonitrotetrazolium violet formazan; INF) was measured using a spectrophotometer at 464 nm. The sterile soil was used as a control.

For the calibration curve, INF standard solution (100 μg ml^{-1}) was prepared by dissolving 10 mg INF in 80 ml extractant (*N,N*-dimethylformamide:ethanol (1:1 v/v)) and bringing with the same extractant to 100 ml. INF standard solution was pipetted 0, 1, 2, 5, 7 and 10 ml into test tubes. The 13.5 ml extractant was added to each tube and mixed thoroughly. The calibration concentrations were 0, 100, 200,500, 700 and 1,000 μg INF per test (Appendix B).

3.6.3.3 Analysis of microbial community

Changes in bacterial populations were studied using 16S rDNA-SSCP. SSCP entails electrophoresis of single-strand DNA fragments of suitable size through a nondenaturing polyacrylamide gel electrophoresis, followed by visualization. This thesis focus on SSCP of the PCR fragments, although the technique is applicable to other kinds of nucleic acid fragments. Under appropriate conditions (notably low temperature and nondenaturing condition), DNA strands fold into structures that migrate according to their shape. DNA strands of different sequence generally do not assume the same shape, and so have distinct gel mobility. Recent evidence suggests that these mobility differences are based primarily on tertiary rather than secondary structure of the DNA molecules (Liu *et al.*, 1999).

3.6.3.3.1 DNA extraction from soil

The extraction method was described Lei et al., 2006. Two grams of soil sample (wet weight) were suspended in 3.6 ml of extraction buffer (0.25 M NaH_2PO_4, 0.1 M Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 1% CTAB (w/v)) in a 10 ml centrifuge tube, and 0.4 ml of 20 % (w/v) and 2 g of glass beads were added. The tube was vigorously shaken for 1 min and incubated at 68 °C for 1 h with the tube being inverted up and down every 15 min. Then the tube was centrifuge, the supernatant was transferred to fresh tube and added 300 μl of 5 M potassium acetate and 1 ml of 40% polyethylene glycol 8000 (w/v). The tube was centrifuged at 13,000 x g for 15 min at 4°C. The supernatant was removed and 1 ml of 1% CTAB (w/v), 1.4 M NaCl, 0.1 M EDTA pH 8.0 was added to the tube to suspend the pellet. The tube was incubated at 68 °C for 15 min. Then the mixture was extracted with an equal volume of chloroform. The upper

aqueous was transferred to a fresh tube and 1.0 volume at room temperature for 15 min. Then the tube was centrifuged at 13,000 x g for 15 min at 4°C. The pellet was rinsed with 500 µl of 70% ethanol (v/v) and the tube was centrifuged at 13,000 x g for 15 min at 4°C. The supernatant was removed and the pellet was allowed to dry in the air 15 min. It was dissolved in 20 µl of TE buffer pH 8.0 and the DNA solution was analyzed by 0.8% agarose gel electrophoresis. The genomic DNA was eluted by GF-1 Gel DNA recovery Kit (Vivantis, Malaysia) and amplified by PCR.

3.6.3.3.2 Polymerase chain reaction of 16S rDNA

Primer used for 16S rRNA PCR amplification were of soil bacteria were PRBA338F+CG clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and PRUN518R (5'-ATTACCGCGGCTGCTGG-3'). The expected PCR product size was 200 bp. The PCR mixtures contained 1X Taq PCR Master Mix (USB corp.), 200 ng DNA Template and 0.16 mmole of each primer. The PCR was performed with three rounds as followed.

The first round	: initial denaturation at 94 °C for 3 minutes
	; 1 cycle
The second round	: Denaturation at 94 °C for 30 seconds
	: Anealing at 55 °C for 30 seconds
	: Extension at 72 °C for 45 seconds
	; 30 cycles
The third round	: Final extension at 72 °C for 10 minutes
	; 1 cycle

The PCR product was analyzed by 1.5% agarose gel electrophoresis.

Four volume of SSCP loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol and 10 mM NaOH) and heat to 90 °C for 5 min. was added into the amplified product, then place on ice for 3 min, the exact temperature and time of denaturation were signification important. Then, the denaturated PCR products are loaded in nondenaturing polyacrylamide gel.

3.6.3.3.3 Non-denaturation Polyacrylamide gel electrophoresis

Nondenaturing polyacrylamide gel was used for size fractionation of both single and double-strand DNA. The glass plates (23x19 cm.,PROTEIN II xi cell) were cleaned and prepared as described in Appendix C. The polyacrylamide gel solution (40 ml) containing 17.5% polyacrylamide (37.5:1 acrylamide:bis-acrylamide), 1X TBE, 5% glycerol, 240 μl of 10% ammonium persulphate and 24 μl of TEMED, the prepared gel and polymerized for 4 hours or overnight. The denaturated PCR products were electrophoretically analyzed on native polyacrylamide gel at 250 volts for 16 h at 4 °C. The electrophoresed bands were visualized by silver staining.

3.6.3.3.4 Silver staining

The gel plates were carefully separated by a plastic wedge. The long glass plate with the gel was placed in a plastic tray, containing 1.5 l of the fix/stop solution, and then was well agitated for 30 min. The gel was soaked in the deionized water and shaken well for 5 min. The process was done three times. The gel was lifted out of the tray. The gel was transferred to 0.1% silver nitrate (1.5 l) and incubated with agitation at room temperature for 30 min. The gel was soaked, in 1.5 l of deionized water,

shaken with agitation and immediately placed in the tray containing 1.5 l of chilled developing solution. The step is very important that it should take no longer than 5-10 sec to soak the gel in the water and transfer it to chilled developing solution. The gel was well agitated until the first band could be seen (usually 1.5-2 min). The gel was then transferred to another tray containing 1.5 l of chilled developer and shaken until bands from every lane were observed (usually 2-3 min). One litre of the fix/stop solution was directly added to the developing solution and shaken continuously for 3 min. The stained gel was soaked in deionized water twice for 3 min each. The gel was air dried and taken photography for SSCP analysis.