CHAPTER 2. MATERIALS AND METHODS

2.1. Apparatus

(i) HPLC System

(a) Autosampler, Waters 600E, Waters Chromatography Div., Milford, USA.

(b) Multisovent Delivery System, Waters 600E, Waters Chromatography Div., Milford,USA.

(c) Tunable UV-Visible Absorbance Detector, Waters 486, Waters Chromatography Div., Milford, USA.

(d) Chromatography Data Workstation Maxima 820, NEC Powermate SX/16 and NEC Pinwriter P6200, NEC Technologies Inc., Boxborough, USA.

(c) Guard column, Guard PAK guard column holder (Part No.88141, Waters Chromatography Div., Milford, USA), fitted with Guard PAK C18 μBondapak pricolumn inserts (Part No. 88070, Waters Chromatography Div., Milford, USA)

(d) Analytical column, Spher180 RB ODS. 2; 5 μ; 250 mm x 4.6 mm. B Fitting.

(ii) Spectrophotometer, UV - 2100, Shimadzu Corporation, Japan.

(iii) pH Meter, pHM61, Radiometer A/S, Copenhagen, Denmark.

(iv) Rotatory Shaker, Thermolyne, ROSI 1000 Reciprocating/Orbital Shaker Incubator, Fuji Electric, Japan.

(v) Analytical Balance, Sartorius Automatic Balance, max. 600 gms., Germany.

(vi) Hot Air Sterilizer, Heraeus, temp. 0 - 250 ° C

(vii) Automatic High Pressure Steam Sterilizer. Tomy, Seiko Co. Ltd., Japan.

(viii) Gallenhamp Incubator, Model 1H - 102, England.

(ix) Sonicator, BRANSONIC, Ultrasonic Cleaner, Model 2210E -DTH. Branson Ultrasonics Corporation, USA.

(X) Colony Counter, Darkfield QUEBEC, American Optical Company.

(xi) Solution Mixer, VORTEX GENIE 2, Scientific Industries Inc., USA.

(xii) Hot Oven, EYELA Magentic Stirrer RCH - 3L, Japan.

(xiii) Filter Apparatus, Millipore Corporation, equiped with 1 litre of ground joint flask, 250 ml funnel, and tabulated base.

(ivx) Filter paper, pore size 0.5 μM, Millipore Corporation, Bedford, MA 01730.

(xv) Centrifuge Machine.

2.2. Chemicals

(i) Carbaryl (1-Naphthyl methylcarbamate), 99.9%, The Promochem Group, Institute of Organic Industrial Chemistry, Warsaw, Poland.

(ii) Carbofuran, 99.0%, The Promochem Group, Institute of Organic Industrial Chemistry, Warsaw, Poland.

(iii) Carbosulfan, 94.0%, The Promochem Group, Institut of Organic Industrial Chemistry, Warsaw, Poland.

(iv) 1-Naphthol, 99.9%, The Promochem Group, Institute of Organic Industrial Chemistry, Warsaw, Poland.

(v) Acetonitrile, HPLC grade, 99.9%, J.T. Baker Inc., USA.

(vi) Ammonium acetate, 98%, E. Merck.

(vii) Magnesium chloride hexahydrate, 98%, E. Merck.

(viii) di - Sodium hydrogen phosphate, 99%, E. Merck.

(ix) di - Ammonium hydrogen phosphate, 99.1%, J.T. Baker.

(x) Nutrient broth, dehydrated, Difco Laboratories, Detroit, USA.

(xi) Potassium dihydrogen orthophosphate, 99.5%, BDH Chemical Ltd., Poole, England.

(xii) Ferrous sulphate, 99%, Hopkin and Williams Ltd., Essex, England.

(xiii) Calcium chloride, granular, 99%, Carolina Biological Supply Company, North Carolina, USA.

(xiv) Manganous sulphate, monohydrate, 99%, Ajax Chemicals, Sydney, Australia.

(xv) Bacto peptone, Difco Laboratories, Detroit, USA.

2.3. Methods

2.3.1. Preparation of Minimum Mineral (MM) Media

Bacterial degradation of carbaryl was done in Minimum Minerals (MM) Media. This media contains only the inorganic salts where carbaryl acted as the sole source of carbon and nitrogen (Steven et al., 1993). The composition of MM is,

Potassium dihydrogen phosphate (KH_2PO_4)	2.27 g
di-Sodium hydrogen phosphate (Na_2HPO_4)	2.13 g
Sodium chloride (NaCl)	1.00 g
Magnesium chloride ($MgCl_2.6H_2O$)	1.06 g
Calcium chloride ($CaCl_2.2H_2O$)	0.100 g
Manganese sulphate ($MnSO_4.H_2O$)	0.020 g
Ferrous sulphate ($FeSO_4.7H_2O$)	0.020 g
Distilled water	1000 ml

All of these inorganic minerals were dissolved in distilled water and autoclaved at 121 °C 15 lbs/m^2 for 15 minutes. Carbaryl was sterilized with millipore filter paper and added to the minimum minerals media as a final concentration of 10 ppm. 100 ml or 50 ml of meida was put in 250 ml/ 125 ml volumetric flask.

2.3.2. Bacterial Culture

Pure bacterial culture (Isolate-5) was obtained from the microbiology Section, Department of Biology, Chiang Mai University. It was isolated from the agricultural field of the Royal Pang Da Agricultural Station, Sameong District, Chiang Mai, Thailand (Zhang, 1995). The stock culture was subcultured on nutrient agar slant every 15 days.

2.3.3. Biodegradation of Carbaryl

The selected bacterial strain of isolate-5 was cultured in nutrient agar slant and three days old slant culture was washed with sterile water. One ml of the bacterial suspension was added to 100 ml MM media and placed on a rotatory shaker at 200 rpm. 10 ml of the sample was withdrawn from the flask at fixed intervals, and absorbance was measured to record the bacterial growth. Biodegradation of carbaryl was measured by HPLC. Before injection the sample was centrifuged at 11,000 rpm for 10 minutes at 4 °C and the supernatant was transferred to a vial for HPLC injection.

2.3.3.1. Temperature

Temperature effect on biodegradation of carbaryl was done at four temperatures viz. 32 , 34 , 37 and 41 °C at a fixed pH of 6.8.

2.3.3.2. pH

The eight pH levels tested in this biodegradation i.e. 6.0, 6.5, 6.8, 7.0, 7.2 and 7.5, 8.0 and 8.5. The temperature was control for this study at 34 °C.

2.3.3.3. Nutrients

Biodegradation of carbaryl was tested in the presence of nutrient broth, yeast-extract and vitamins (e.g. vit. B_1, vit. B_6 and nicotinamide). 20 mg of each vitamin was added separately to each 100 ml of MM. 300 mg of yeast-extract was added to the 100 ml of MM media for studying the effect on biodegradation whereas in nutrient broth medium there was no MM was added. The concentration of carbaryl in all of these media was 10.0 ppm. The pH and temperature for these experiments were adjusted to 6.8 and 34 °C, respectively.

2.3.3.4. Enrichment

Bacterial strain was enriched in MM medium for 5-days with 20.0 ppm of carbaryl at PH 6.8, 34 °C and then transferred to the nutrient agar plate and subsequently to the agar slant.

2.3.3.5. Cross-feeding

Bacterial cross-feeding study with selected strain was done in the presence of two other carbamate insecticides viz. carbofuran and carbosulfan, and also in the presence of the intermediate product of carbaryl, 1-naphthol. This study was also done at pH 6.8 at 34 °C.

During this study 10 ppm of carbaryl and also 10 ppm of these three chemicals (e.g. carbofuran, carbosulfan and naphthol) was added separately to 100 ml MM. Degradation and growth rate was measured at each interval.

2.3.3.6. Bacterial Mutation

Mutation of selected bacterial strain was done by UV-irradiation. The bacteria was cultured in 100 ml nutrient broth. After optimum growth, 25 ml of this culture was centrifuged at 4000 rpm about 10 minutes at 4 °C. The supernatant was discarded and the residue was washed with 25 ml of 0.85% NaCl and centrifuged again. The residue was washed and centrifuged. After centrifugation, the supernatant was discarded carefully and the final residue was diluted to 25 ml with the same NaCl solution. A half dilution of this final bacterial suspension was done by adding 25 ml of sterilized water to 25 ml bacterial suspension. 1 ml of the diluted suspension was serially diluted to 10^{-3}, 10^{-4} , and 10^{-5} and spreaded on agar plates for viable count. From resting 49 ml of bacterial suspension, 10 ml each was transferred to four sterile petridishes for exposing the bacterial cells with UV for 10 seconds, 30 seconds, 1 and 2 minutes. Each petridish was placed on a magnetic stirrer for equally exposing UV to each cell. The intensity of the lamp was 15 Watts whereas the distance between the lamp and the petridish was 40 cm. A serial dilution of 10^{-3}, 10^{-4} , and 10^{-5} of UV-exposed bacterial cells was prepared and spreaded on nutrient agar plates. Five replicates were prepared for each dilution. After 48 hour incubation, the bacterial colony was counted on both UV and non-UV plates.

Biodegradation of carbaryl was tested with UV-exposed bacteria as previously scribed.

2.4. Bacterial Identification Test

The identification of this selected bacterial strain of isolate-5 was done by several physical and biochemical tests. The bacteria was stained by both gram-staining and flagella-staining methods. In addition, the motility of the bacteria was observed by the hanging drop technique. The following biochemical tests were done for identification: oxidase, citrate, urea, nitrate reduction, gelatin liquifaction, and sugar utilization (details in Appendix-A).

2.5. DNA Isolation and Preparation

1.5 ml of selected bacterial strain culture (A_{600} 1.6) was pipetted to eppendarf tube and centrifuged at 13,000 rpm for 2 ninutes. 300 µl of TE buffer (Tris-EDTA) was added to each tube and shaken well. Later 300 µl of lysis buffer was added (20% SDS and 2M NaOH, 1:1) and shaken well, and incubated at 60 °C for 15 minutes. After incubation, 1 mg/ml of lysozyme buffer was added to each tube and shaken well, and again incubated in a waterbath at 37 °C for 1 hour. Later, 300 µl of precipitation buffer (2.55 M KAc, pH 4.8) was added and incubated at 4 °C for 1 hour. After incubation, the sample was centrifuzed for 10 minutes at 13,000 rpm and the supernatent transferred to new tubes. Equal amounts of isopropanol was added to the tube and kept at - 20.0 °C overnight for precipitation. Later, it was centrifuged at 13,000 rpm for 15 minutes to precipitate DNA then washed with 70% ethanol (50 µ l/tube). After centrifugation at 12,000 rpm for 2 minutes it was air dried until a semisolid formed, and dissolved again with TE buffer (20 µl/tube) and kept at - 20.0 °C. Finally, the DNA was analyzed agarose gel electrophoresis stained by ethidium bromide (Marmur, 1961). The flow-chart of DNA isolation has been presented in Appendix-B.

2.6. HPLC Analysis of Carbaryl

HPLC Model

Waters 717 (Autosampler)

Waters 600E (System Controller)

waters 486 (Tunable Absorbance Detector)

Data Works Station: Maxima 820

HPLC Parameters

Column: Waters μBondapak RP C18

Column Width: 4.6 mm

Column length: 250 mm

Injection volume: 20 μl

Mobile phase

Solvent A: 0.005M NH_4-acetate buffer

Solvent B: Acetonitrile (ACN)

Isocratic analysis: 1:1 (v:v)

Flow rate: 1.0 ml/min

Degassing: Helium sparge (20 ml/min)

Column temperature: 25 °C

Detection Parameters

Wavelength of analysis: 220 nm

Wavelength of confirmation: 270 nm

Statistical Parameters

Detection limit: 1 ng/ml (signal-noise ratio 2:1)

Lower limit of determination

Water: 0.008 μg carbaryl/l water

Soil: 1.6 μg carbaryl/kg soil

Plants: 2.0 μg carbaryl/kg plant material

Recovery: >90%

2.7. Preparation of Mobile Phase:

0.0795 gm of 97% CH_3COONH_4 was weighed and dissolved in 1000 ml deionized water. 500 ml of this solution was mixed with 500 ml acetonitrile. The solvent was then passed through 0.45 μm filter paper by force of a vacuum pump and degassed in an ultrasonic bath for 30 minutes. It was stored at 4 °C when not used.

2.8. Preparation of Standard Curve:

For the preparation of calibration curve, 50 mg of carbaryl was dissolved in 50 ml volumetric flask with acetonitrile. The concentration of this solution was 1000 mg/l. From this stock solution, 100.0, 10.0, 5.0, 2.0, and 1.0 mg/l were prepared in the following ways:

Stock solution: 1000 mg/l

100 mg/l: 1 ml from 1000 mg/l diluted to 10 ml with ACN

10 mg/l: 2.5 ml from 100 mg/l diluted to 25 ml with ACN

5 mg/l: 5 ml from 10 mg/l diluted to 10 ml with ACN

2 mg/l: 2 ml from 10 mg/l diluted to 10 ml with ACN

1 mg/l: 1 ml from 10 mg/l diluted to 10 ml with ACN

The final concentrations of 1.0, 5.0, and 10.0 mg/l were used for calibration curves (Appendix-C).

2.9. Confirmation Analysis:

Confirmation of peaks of carbaryl was done by injecting the sample and standard at two different wave lengths (220 and 270 nm). The peak area ratio of sample and standard was calculated in these two wave lengths.

3.0. Recovery Analysis:

For recovery analysis, 100 ml of fresh minimum minerals media was spiked with 1 ml of 1000 mg/l carbaryl. The carbaryl concentration in MM was 10 mg/l. After mixing 10 ml of solution was collected and centrifuged at 11,000 rpm at 4 °C for 10 minutes for injection to HPLC.