CHAPTER III

METHODOLOGY

3.1 Laboratory equipments, chemicals

3.1.1 Laboratory equipments

Laboratory equipments	Company	Country
Shaker innova 4000	New brunswick scientific	USA
Shaker innova 4340	New brunswick scientific	USA
Refridgerated Centrifuge Avanti^{Tm} J-301	Beckman Coulter	USA
Spectophotometer DU650	Beckman	USA
Ultrasonic	Banderlin	Germany
Autoclave NLS-3020	Sanyo Electric Co.,Ltd	Japan
Autoclave HV-110	Hirayama	Japan
Biophotometer	Eppendoff	German
Electroparation	Bio-rad	U.S.A
Refrigerated Contrifuge 5804R	Eppendoff	German
Micropitte 20,100, 200, 1000 µl	Gilson	France
High Performance Liquid Chromatographic (HPLC) LC10ADVP	Shimazu	Japan
French press Cell Disrupter	Thermo Electron Corporation	USA
ISE (Ion Selective Electrode)	The orion model 94-17B	USA

3.1.2 Laboratory chemicals

(1) Analytical grade chemicals

Chemicals	Company	Country
Tryptone	Himedia	India
Yeast extract	Scharlau Microbiology	Spain
Agar Agar	Scharlau Microbiology	Spain
Tris	USB	USA
Folin-Ciocalteu's reagent	Carlo Erba Reagenti	Italy
Albumin bovine (BSA)	Sigma	USA
KH_2PO_4	Carlo Erba Reagenti	Italy
K_2HPO_4	Riedel	Germany
NH_4Cl	May&Baker	England
$NaNO_3$	Carlo Erba Reagenti	Italy
NaCl	BDH	England
Na_2CO_3	BDH	England
NaOH	Merck	Germany
$C_4H_{10}N_2Na_2O_6.2H_2O$ (EDTA)	BDH	England
$C_{12}H_{25}O_4SNa$ (SDS)	Sigma	USA
$COOK(CHOH)_2COONa.4H_2O$	Carlo Erba Reagenti	Italy
$MgSO_4.7\ H_2O$	Carlo Erba Reagenti	Italy
$CaCl_2.2\ H_2O$	Merck	Germany
Na_2HPO_4	Fluka	Germany
H_3BO_4	Merck	Germany
$FeSO_4.7\ H_2O$	BDH	England
$ZnSO_4.7\ H_2O$	Merck	Germany

(1) Analytical grade chemicals (continue)

Chemicals	Company	Country
$MnSO_4.H_2O$	Merck	Germany
$CuSO_4.5\ H_2O$	Scharlau Microbiology	Germany
$CoCl_2.6\ H_2O$	Merck	Germany
MoO_3	Merck	Germany
$Hg(SCN)_2$	Panreac	Spain
$Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$	Fluka	Germany
Citrate	Merck	Germany
Succinic acid	Merck	Germany
Aniline	Merck	Germany

Fig 3.1 Aniline (with 99.5% purity, lot number: K28124761112, M.W. 93 and 500 ml liquid)

(2) HPLC grade chemicals

Chemicals	Company	City
2-chloroaniline (99% purity)	Chem Service, Inc.	U.S.A
3-chloroaniline (99% purity)	Chem Service, Inc.	U.S.A
4-chloroaniline (99.5% purity)	Chem Service, Inc.	U.S.A

3,4-dichloroaniline (99.5% purity)	Chem Service, Inc.	U.S.A
4-chlorocatechol (97% purity)	Aldrich	Germany

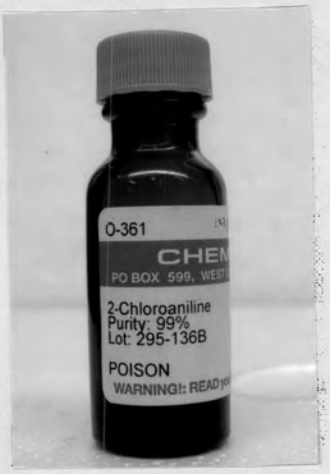

Fig 3.2 2-chloroaniline (lot number: 295-136B, M.W. 127.57 and 10g liquid)

Fig 3.3 3-chloroaniline (lot number: 322-24A, M.W. 127.57 and 5g liquid)

Fig 3.4 4-chloroaniline (lot number: 330-48A, M.W. 127.57 and 5g solid)

Fig 3.5 3,4-dichloroaniline (lot number: 287-74A, M.W.162.02 and 10g solid)

Fig 3.6 4-Chlorocatechol (CAS number 2138-22-9, M.W. 144.56 and 1g solid)

(2) HPLC grade chemicals (continue)

Chemicals	**Company**	**City**
Catechol (99.5% purity)	Aldrich	Germany
CAS No. 120-80-9		
M.W.110.11		
Methanol (99.9% purity)	Fisher Scientific	U.S.A
CAS No. 67-56-1		
M.W. 32.04		

Acetonitrile (99.9% purity) LAB-SCAN Co. Ltd., Thailand

CAS No. 75-05-8

M.W. 41.05

3.2 Culture medium

3.2.1 The mineral medium (mm)

The mineral medium was used for screening, isolation, cultivation and degradation. The mineral medium was comprised of media and trace element (Dejonghe et al., 2003).

I. Media

Na_2HPO_4	1.4196 g
KH_2PO_4	1.3609 g
$MgSO_4.7H_2O$	0.0985 g
$CaCl_2.H_2O$	0.0059 g

The component was dissolved in 1 liter of distilled water and adjusted pH to 7 by 1 N NaOH. The mineral medium was autoclaved at 121 °C for 15 minutes.

II. Trace element

H_3BO_4	0.116 g
$FeSO_4.7H_2O$	0.278 g
$ZnSO_4.7H_2O$	0.115 g
$MnSO_4.H_2O$	0.169 g
$CuSO_4.H_2O$	0.038 g
$CoCl_2.6H_2O$	0.024 g
MoO_3	0.010 g

Trace element solution was separately prepared from media as a stock solution. They were dissolved in 100 ml of distilled water and it was autoclaved at 121°C for 15 minutes.

Before using, 0.1 % (v/v) sterile trace element was supplemented in mineral medium.

3.2.2 Luria bertani medium (LB)

Tryptone	10 g
Yeast extract	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 Characteristic of 4-chloroaniline-degrading bacteria

3.3.1 Isolation of 4-chloroaniline degrading bacteria

Bacteria were isolated from twenty-one soil agricultural areas in Thailand with the history of herbicide exposure or natural area. Soil samples were collected from natural and agricultural area such as Chiangmai, Rayong, and Samutsakorn province. Subsequently, preenrichment culture techique, enrichment culture techique and isolation were used to selected the 4-chloroaniline-degradaing bacteria. First, to pre-screen bacteria from soil, preenrichment culture techique was used; then, enrichment culture techique was used to increase the quantity of 4-chloroaniline-degradaing bacteria. Preenrichment culture was prepared by adding 5 g of herbicide-contaminated soil, or natural soil in a 250-ml erlenmeyer flask that contained 100 ml of mineral medium and 25 ppm 4-chloroaniline and shaking-incubated on the rotary shaker at 150 rpm at room temperature for 5 days. Then, 5 ml of preenrichment suspension culture was then tranferred to enrichment step prepared as 250-ml erlenmeyer flask that contained 100 ml of mineral medium and 25 ppm 4-chloroaniline and shaking-incubated on the rotary shaker at 150 rpm at room temperature for 5 days. After that, a serial dilution (10^{-4} and 10^{-6}) of the enrichment culture was prepared and spreaded on to the mineral medium agar plate that contained 25 ppm 4-chloroaniline. After 5-7 days of incubation at 30°C, bacterial

colonies formed on the plate were streaked on a new mineral medium agar plate that contained 25 ppm 4-chloroaniline.

Then, growth of the isolated 4-chloroaniline-degrading bacteria were confirmed in liquid medium supplemented with 25 ppm (0.2 mM) 4-chloroaniline. Inoculum of bacteria was prepared by using a loopful of bacteria into a test tube that contained 5 ml of mineral medium, 25 ppm 4-chloroaniline and placed on the rotary shaker at 250 rpm, 30°C for 5 days. Then, the inoculum was transferred to 250-ml Erlenmeyer flask that contained 100 ml of mineral medium, 25 ppm 4-chloroaniline as substrate and again placed on the rotary shaker at 250 rpm, at 30°C for 5 days. Growth of 4-chloroaniline-degrading bacteria were monitored via the spectrophotometer as culture turbidity (OD_{590} nm). Cell growth in the presence of (0.2 mM) methanol used to dissolve 4-chloroaniline was also monitored to distinguish bacteria of which methanol can serve as a growth substrate (control).

3.3.2 Identification of 4-chloroaniline-degrading bacteria

3.3.2.1 The morphology and characteristics of colonies

(1) Morphology of bacteria

Bacterial staining was used to study shape, size of bacteria and to classify bacteria into gram positive or gram negative. Bacteria were grown in LB agar medium (as shown in APPENDIX A) for 24 hours, stained bacteria and visualized through microscopy (solution for bacterial staining and protocol as shown in APPENDIX A).

(2) Characteristics of colonies

To determine the growth of bacteria in mineral medium containing of 25 ppm 4-chloroaniline, cells were grown for 5 days ,and then the

physiological feature was observed; for example, colour, form, diameter, surface and edge.

3.3.2.2 Biochemical test

Biochemical characterizations of bacteria were analyzed by the laboratory of Institution for Scientific Research, Department of Medical Sciences, Ministry of Public Health in Thailand. Biochemical test methods are shown in APPENDIX A.

3.3.2.3 16S ribosomal DNA gene sequencing

Genomic DNA from individual bacteria strains was extracted by a standard method (Sambrook et al., 1989). The 16S rDNA gene fragment was amplified from the genomic DNA of each bacteria by polymerase chain reaction (PCR) using the bacterium-specific primers: forward primer (63f) (5′ CAGGCCTAACACATGCAAGTC 3′) and a reverse primer 1387r (5′ GGGCGGWGTGTACAAGGC 3′) (Marchesi et al., 1998). A reaction mixture (total volume of 25 µl) contained 1X Taq buffer with $(NH_4)_2SO_4$, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.4 µM of each primer, DNA sample 1µl (500 ng DNA), taq polymerase 5 units/µl (Fermentas, USA). The reaction mixture was subjected to pre-denatulation at 94°C for 3 min, 30 cycles consisting of denaturation at 95°C for 1 min, 55°C and 1 min for annealing, chain extension at 72°C for 1.3 min with an additional extension time of 72°C for 5 min. The fragments (1,300 bps) was then cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) (3,015 base pairs) and transformed into competent *Escherichia coli* DH5α cells. Plasmid DNA (DNA 650 ng/µl) from the transformant was isolated using the QIAprep Spin Miniprep kit (catalog number 27104) (Qiagen, Netherlands). The insert was then sequenced by the Macrogen Inc.

(Seoul, Korea) and compared to the most similar sequences with Clustal W in NCBI database (http://www.ncbi.nlm.nih.gov).

3.4 Degradation of 4-chloroaniline by 4-chloroaniline-degrading bacteria

3.4.1 4-Chloroaniline degradation

3.4.1.1 Preparation of inoculums of 4-chloroaniline degrading bacteria

Bacteria were grown in mineral medium agar plate containing of 0.1% yeast extract and 25 ppm 4-chloroaniline. 4-Chloroaniline was applied onto agar plate by dissolving 4-chloroaniline with methanol (stock concentration of 78 mM) and applying (spreading) 50 µl onto agar plate (20ml). A loopful of bacteria was placed into a test tube that contained 5 ml of mineral medium, 0.1% yeast extract and 25 ppm 4-chloroaniline and placed on the rotary shaker at 250 rpm, at 30°C for overnight. Then, the 5 ml inoculums was transferred to 250-ml Erlenmeyer flask that contained 100 ml of mineral medium, 0.1% yeast extract and 25 ppm 4-chloroaniline as substrate and placed on the rotary shaker at 250 rpm overnight at 30°C.

3.4.1.2 4-Chloroaniline degradation

5% Inoculum (v/v) was used to start the 100-ml bacterial culture grown in mineral medium in the presence of 25 ppm (0.2 mM) 4-chloroaniline. Cell growth was determined by optical density (OD_{590nm}) and by the increase of cell protein concentration using modified Lowry method as described in 3.4.2.3. Cells were grown at 30°C for 1-2 days. Culture medium (1 ml) was collected on 0, 2, 4, 6, 8, 10, 12 days to determine the remaining 4-chloroaniline using High Performance Liquid Chromatography (HPLC).

The amount of chloride which was released was also monitored using Ion-Selective electrode (ISE) and the colorimetric assay as described in 3.7.2.

3.4.2 Enzymatic assays for catechol 1, 2-dioxygenase and catechol 2, 3-dioxygenase activities

3.4.2.1 Preparation of bacterium inoculums for enzymatic assay

Bacteria were grown in 3 conditions of liquid medium (100 ml) as follow: 1) mineral medium containing 0.1% yeast extract, 2) mineral medium containing 0.1% yeast extract and 25 ppm 4-chloroaniline, 3) Luria Bertani medium (LB) containing of 25 ppm 4-chloroaniline. Cells were grown on rotary shaker at 250 rpm, at 30°C overnight.

3.4.2.2 Preparation of cell-free extract for enzyme assays

After 2 days of growth, cells were harvested by centrifugation at 9,000 rpm 15 min at 4°C, washed twice with 50 mM Tris-HCl buffer pH 7.8, and then resuspended with 50 mM Tris-HCl pH 7.8 (1g cells with 3 ml buffer). Then, cells were disrupted using French Pressure cell (Thermo Electron Corporation, USA). Cell pressure was used as 12,000 psi. Cell debris was removed by centrifugation at 15,000 rpm for 20 minutes to give clear crude extract. The supernatant fluid was used for enzyme assays. All procedures were performed at 4°C. The protein concentration was estimated by the method of modified Lowry method (3.4.2.3) with bovine serum albumin as standard.

3.4.2.3 Determination of protein

Modified Lowry Method (Dulley and Grieve (1975) and Lowry et al., (1951))

Reagents

A solution: 2% Na_2CO_3 in 0.1M NaOH containing 0.5% Sodium dodecyl sulfate (SDS)

B solution: 0.5% CuSO4.5H_2O in 1% Potassium Sodium tartrate

C solution: Phenol reagent

After preparation of enzyme solution (0.4 ml), 2 ml of mixed solution A +B (A: B, 50: 1) were added. Then, the mixture was incubated for 10 minute at 30°C. Subsequently, 0.2 ml of C solution was added, rapidly mixed and then, incubated for 30 min at 30°C. Finally, to determine the quantity of protein, the clear color solution was detected by measuring the optical density at 750 nm.

3.4.2.4 Enzymatic assays

Enzymatic activity was measured spectrophotometrically by monitoring the change in absorbance. The reaction was started by adding the protein followed the reaction for 40 min at 30^0C. One unit of enzyme activity was defined as the amount of enzyme that catalyzing the formation of 1 µmol product/min.

1) Catechol 1, 2–dioxygenase

Catechol 1, 2–dioxygenase was measured by following the formation of muconic acid, the *ortho*-cleavage product of catechol at 260 nm ($\varepsilon_{\text{muconic acid}}$ = 16,800 l mol^{-1} cm^{-1}) as described by Dorn and Knackmuss (1978b). The reaction mixture contained 50 mM Tris-HCl pH 7.8, 0.25 mM catechol and 1.3 mM ethylene diaminetetraacetic acid disodium salt dihydrate (EDTA).

2) Chlorocatechol 1, 2-dioxygenase

Chlorocatechol 1, 2-dioxygenase was measured by following the formation of 3-chloromuconic acid, the modified *ortho*-cleavage product of 4-chlorocatechol at 260 nm ($\varepsilon_{\text{3-chloromuconic acid}}$ = 12,400 l mol^{-1} $cm^{-1)}$ as described by Dorn and Knackmuss (1978b). The reaction mixture contained 50 mM Tris-HCl pH 7.8, 0.25 mM 4-chlorocatechol and 1.3 mM ethylene diaminetetraacetic acid disodium salt dihydrate (EDTA).

3) Catechol 2, 3–dioxygenase

Catechol 2, 3–dioxygenase I was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of catechol at 375 nm ($\varepsilon_{\text{2-hydroxymuconic semialdehyde}} = 33{,}000\ l\ mol^{-1}\ cm^{-1}$) as described by Urata et al. (2004). The reaction mixture contained 200 mM phosphate buffer pH 7.4 and 12.5 mM catechol.

4) Chlorocatechol 2, 3–dioxygenase

Chlorocatechol 2, 3–dioxygenase (Tropel, 2002) was measured by following the formation of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 4-chlorocatechol at 379 nm ($\varepsilon_{\text{5-chloro-2-hydroxymuconic semialdehyde}} = 36{,}100\ l\ mol^{-1}\ cm^{-1}$) as described by Urata et al. (2004). The reaction mixture contained 200 mM phosphate buffer pH 7.4 and 12.5 mM 4-chlorocatechol.

3.5 Optimum conditions of 4-chloroaniline degradation

According to previous method (3.4.1), 4-chloroaniline for carbon and nitrogen source, additional carbon or/and nitrogen source were supplemented as follow:

1) Carbon source: 4 mM citrate or 4 mM succinate

2) Nitrogen source: 4 mM NH_4Cl or 4 mM $NaNO_3$

3) Carbon and nitrogen source: 1mM aniline

After additional carbon and/or nitrogen source was supplemented, bacterial culture placed on rotary shaker at 250 rpm, at 30°C. Culture medium was collected on 0, 2, 4, 6, 8, 10 and 12 days. From this experiment, suitable conditions contained high percentage of total degradation or short degradation time for each 4-chloroaniline

degrading bacteria were selected and then various concentration of additional carbon or/and nitrogen source were used. Furthermore, the conditions that contained high percentage of total degradation or short degradation time for either carbon or nitrogen source were alternately mixed.

3.6 Substrate range and degradation of 4-chloroaniline at higher concentration

Types of chloroaniline such as 25 ppm (0.2 mM) 2-Chloroaniline, 25 ppm (0.2 mM) 3-chloroaniline, and 25 ppm (0.2 mM) 3,4-dichloroaniline was used to identify the range of substrate. Additionally, 50 ppm, 100 ppm, 150 ppm, and 200 ppm of 4-chloroaniline was used to monitor the effect on growth and percentage of total degradation. Bacterial culture was placed on rotary shaker at 250 rpm, at 30°C and then, culture medium was collected on 0, 2, 4, 6, 8, 10 and 12 days.

3.7 Analytical methods

3.7.1 HPLC Analysis

4-chloroaniline was examined using High Performance Liquid Chromatography (HPLC). Cell samples were harvested to remove cells by centrifugation at 12,000 rpm 10 min. Cell-free supernatant was collected mix 1:1 v/v with mobile phase, filtered with 0.45 μm syringe filter, and analyzed by reverse-phase HPLC at 40°C. The separation was performed on C18 HPLC column (Inersil ODS-3 250 x 4.6 mm) and then the compound was analyzed by UV detector at a wavelength of 240 nm. The mobile phase was acetonitrile: water: phosphoric acid mixture (70:29.75:0.25 by volume) with a flow rate 1 ml/min. Retention time of 2-chloroaniline, 3-chloroaniline 4- chloroaniline, 3,4-dichloroaniline and aniline was 4.9, 4.6, 4.4, 5.4 and 3.8 min, respectively as shown the HPLC peak in Appendix G. Another method of HPLC condition for determination of intermediate, the mobile

phase was methanol : water: mixture (70:30 by volume) with a flow rate 0.5 ml/min. The compound was analyzed by UV detector at a wavelength of 254 nm. Retention time of 4-chloroaniline was 10.9 min as shown the HPLC peak in Appendix G. This method was used by Zeyer et al. (1985) who found that the retention time of 4-chloroaniline and its intermediate (4-chlorocatechol) was 6.0 min and 3.8 min at flow rate 2.5 ml/min.

3.7.2 Analysis of Chloride ions

Chloride ion was detected via Ion-Selective electrode (ISE) (The Orion model 94-17B chloride ion electrode, USA) with chloride probe. Collected samples were harvested by centrifugation at 9,000 rpm 15 min and the supernatant was used to determine chloride.

Chloride was measured in duplicate by a colorimetric assay after 24 hour (Bergman and Sanik, 1957). After cells were removed from a 1-ml sample by centrifugation, 200 µl of 0.25 M ferric ammonium sulfate ($Fe(NH_4)(SO_4)_2.12H_2O$) in 9N HNO_3 and 200 µl of saturated mercuric thiocynate in ethanol were added and incubated for 10 min in chemical hood, and then the absorbance at 460 nm was measured (Chauhan et al., 1998). In this experiment, ferric ammonium sulfate ($Fe_2(SO_4)_3.(NH_4)_2\ SO_4.24H_2O$ in 9N HNO_3) was used.