CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Equipment	Company	Country
Amicon Ultra-4, Ultracel-50k	Millipore	Ireland
Autoclave, MLS-3020	Sanyo Electric	Japan
Biological safety cabinet, Forma Class II A2	Thermo Electron Corporation	USA
Casting frame, Mini PROTEAN®	Bio-Rad Laboratories	USA
Cell disrupter, FRENCH PRESS	Thermo Electron Corporation	USA
Concentrator, Centrivap	Labconco	USA
Conductivity meter, CDM 83	Radiometer	Denmark
Controlled environment incubator shaker, Psycrotherm™	New Brunswick Scientific	USA
Electrophoresis power supply, EC135-90	E-C Apparatus Corporation	USA
Fraction collector, Redi Frac	Pharmacia LKB	Sweden
Glass plates, Mini PROTEAN®	Bio-Rad Laboratories	USA
Gyrotary water bath shaker, G76D	New Brunswick Scientific	USA
Incubator shaker, Innova 4000	New Brunswick Scientific	USA
Lyophilyzer, Flexi-Dry μP	FTS Systems	USA
Peristaltic pump, P-1	Pharmacia Biotech	Sweden
Refrigerated centrifuge, 5804R	Eppendorf	USA

Equipment	Company	Country
Refrigerator centrifuge, Avanti™ J-30I	Beckman Coulter	USA
Spectrophotometer DU 650	Beckman	USA
Spectrophotometer DU 800	Beckman Coulter	USA
Ultracentrifuge, Optima™ L-100 XP	Beckman Coulter	USA
Ultrasonic	Banderlin	Germany

3.2 Chemicals

Chemical	Company	Country
2,6-Dichlorophenol-indophenol sodium salt dehydrate (DCIP)	Merck	Germany
Acrylamide	Merck	Germany
Agar Agar	Scharlau Microbiology	Spain
Ammonium persulphate	Merck	Germany
Ammonium sulfate	Merck	Germany
Aquasorb	BML	Thailand
Boric acid	Merck	Germany
Bovine serum albumin (BSA) (A-4503)	Sigma	USA
Bromophenol blue	BDH	England
Butyl-Toyopearl	TOSOH Corporation	Japan
Calcium chloride	Merck	Germany
Cobalt chloride	Fluka	Switzerland
Coomassie Blue G-250	Sigma	USA

Chemical	Company	Country
Coomassie Blue R-250	Sigma	USA
Copper sulphate	Carlo Erba	France
DEAE-650M Toyopearl	TOSOH Corporation	Japan
Di-potassium hydrogen phosphate	Riedel	Germany
Di-sodium carbonate	Fluka	Switzerland
Di-sodium hydrogen phosphate	Fluka	Switzerland
Ethelenediaminetetraacetic acid (EDTA)	Fluka	Switzerland
Ferrous sulphate	BDH	England
Flavin adenine dinucleotide (FAD)	Sigma	USA
Folin-Ciocalteu's reagent UN3264 Code no. 463562	Carlo Erba Reagenti	France
Glucose	Fluka	Switzerland
Glucose-6-phosphate	Sigma	USA
Glycerol	Univar	Australia
Glycine	Scharlau Microbiology	Spain
Magnesium sulphate	Carlo Erba	France
Manganese sulphate	Merck	Germany
Molybdan VI oxide	Merck	Germany
Mydol10	Kao	Japan
β-Nicotinamide adenine dinucleotide (β-NAD)	Sigma	USA
β-Nicotinamide adenine dinucleotide phosphate (β-NADP)	Sigma	USA

Chemical	**Company**	**Country**
β-Nicotinamide adenine dinucleotide reduced form (β-NADH)	Sigma	USA
Nitroblue tetrazolium (NBT)	Sigma	USA
N,N,N',N'-Tetramethylene ethylene diamine (TEMED)	BDH	England
N,N'-methyl-bis-acrylamide	Merck	Germany
Nutrient Broth	Difco	USA
Ovalbumin from chicken egg (A-5503)	Sigma	USA
Phenazine methosulfate (PMS)	Wako	Japan
Phenylmethylsulfonyl fluoride (PMSF)	Sigma	USA
Phenyl-Sepharose CL-4B	Sigma-Aldrich	Germany
p-Nitrophenyl phosphate	Sigma	USA
Polyethylene glycol 2000	Aldrich	USA
Polyethylene glycol 4000	Fluka	Switzerland
Polyethylene glycol 6000	Fluka	Switzerland
Polypropylene glycol 725	Aldrich	USA
Polypropylene glycol 1000	Aldrich	USA
Polypropylene glycol 2000	Aldrich	USA
Potassium cyanide	Sigma	USA
Potassium dihydrogen phosphate	Carlo Erba	France
Potassium sodium tartrate	Carlo Erba	France

Chemical	Company	Country
Protein molecular weight marker #SM 0431 lot. 1811	Fermentas	Canada
Sodium chloride	BDH	England
Sodium dihydrogen phosphate	Carlo Erba	France
Sodium hydroxide	Merck	Germany
Sodium laulyl sulphate (SDS)	Sigma	USA
holo-Transferrin from human (T-0665)	Sigma	USA
Tris (hydroxymethyl) aminomethane	USB	USA
Trypsin inhibitor type II-T from turkey egg white (T-4385)	Sigma	USA
Yeast extract	Scharlau Microbiology	Spain
Zinc sulphate	Merck	Germany
β-Mercaptoethanol	Sigma	USA

The other common chemicals were reagent grade from BDH, Carlo Erba, Fluka, Merck and Sigma.

3.3 Microorganism

Pseudomonas sp. PE-2, the microorganism used in this research, was a kind gift from Professor Fusako Kawai (Research Institute for Bioresources, Okayama University, Japan).

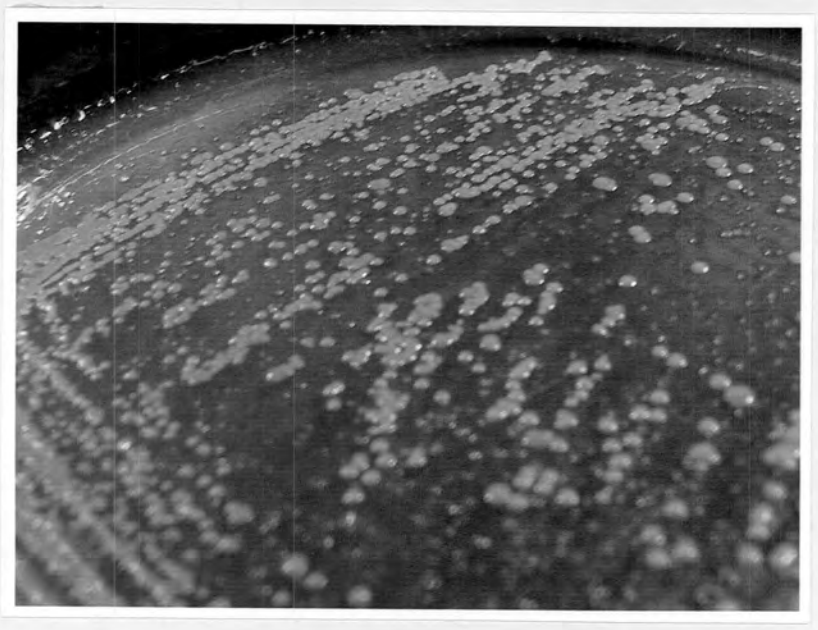

Figure 3.1 Colonies of *Pseudomonas* sp. PE-2 on 0.7% (v/v) PPG 1000 basal medium agar plate (5 days growth at 30°C)

3.4 Research methodology

The research methodology can be summarized as followed:

3.4.1 Cell growth conditions and stock cell maintenance

Cell growth conditions and stock cell maintenance of *Pseudomonas* sp. PE-2 in the laboratory can be described as followed.

3.4.1.1 Preparation of growth media

Pseudomonas sp. PE-2 which cultivated at 30°C was grown in a basal medium and a nutrient broth.

3.4.1.1.1 The basal medium

The basal medium was comprised of media and mineral solutions (Tachibana *et al.*, 2002).

I. Media

0.2% (w/v) $(NH_4)_2SO_4$

0.2% (w/v) K_2HPO_4

0.1% (w/v) NaH_2PO_4

0.02% (w/v) $MgSO_4.7H_2O$

0.02% (w/v) yeast extract

The component was dissolved in 1 liter of distilled water and the pH was adjusted to 7.2. Carbon source was one of the following compounds and was prepared as a stock solution with the concentration of 10% (v/v): PEG 2000, PEG 4000, PPG 1000 or PEG 2000, was added to the final concentration (0.7%, 1.5% or 3% (v/v)) as the sole source of carbon and as the inducer for PEG-DH and/or PPG-DH activities.

II. Mineral solutions

The following solutions were prepared as followed:

Calcium	15 mg/ml $CaCl_2$
Iron	15 mg/ml $FeSO_4.7H_2O$
Trace elements	7.8 mg/l $CuSO_4.5H_2O$
	10 mg/l H_3BO_3
	10 mg/l $MnSO_4.5H_2O$
	70 mg/l $ZnSO_4$
	10 mg/l MoO_3

One milliliter of each solution was supplemented into one liter of the basal medium and autoclaved at 121°C for 15 minutes.

3.4.1.1.2 Nutrient broth

Nutrient broth was prepared by dissolving 8g of nutrient broth powder (Difco Bacto, USA) in 1 liter of distilled water and the pH was adjusted to 7.2. The nutrient broth was also supplemented with one milliliter of each mineral solution (Material and Method 3.4.1.1.1) and autoclaved at 121°C for 15 minutes.

3.4.1.2 Preparation of cell growth on solid media

The nutrient broth agar plate and basal medium agar plate could be prepared the same method as described in section 3.4.1.1.1 and 3.4.1.1.2. Then, 1.5% (w/v) agar was added to the final concentration into the medium. The medium agar was autoclaved at 121°C for 15 minutes. Then, the warm medium agar had been aseptically poured into the autoclaved glass petri plates. The medium agar plates were left at the room temperature for 20-30 minutes. Next, *Pseudomonas* sp. PE-2 was

steaked using a loopful of cells onto the medium agar plate under sterile techniques. *Pseudomonas* sp. PE-2 was always maintained in the laboratory on three types of solid medium: nutrient broth agar plate, 0.7% (v/v) PEG 4000 basal medium agar plate and 0.7% (v/v) PPG 1000 basal medium agar plate at 4°C until used. Cells were subcultured every 30 days to fresh medium agar plates.

3.4.1.3 Preparation of stock cell culture

Pseudomonas sp. PE-2 was routinely grown on nutrient broth (section 3.4.1.1.1) at 30°C for 1 day, 0.7% PEG 2000 basal medium at 30°C for 3 days and 0.7% PPG 1000 basal medium at 30°C for 7 days (section 3.4.1.1.2). The cell culture 0.9 ml was taken into the micro-tube (1.5 ml) containing 0.1 ml autoclaved glycerol (final concentration of 10% (v/v) glycerol) as the cell stock. The stock culture was stored at -80°C.

3.4.2 Preparation of the cell-free extract from *Pseudomonas* sp. PE-2

Cells were harvested by centrifugation (12,000 rpm (22,095xg), 4°C for 20 minutes), washed twice with 50 mM Tris-HCl buffer, pH 8.0. The cell paste was homogeneously resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) (1 g wet weight cells with 5 ml buffer). Then, cell suspension was passed thrice through a French pressure cell (Thermo Electron Corporation, USA) at 20,000 psi, and centrifuged at 12,000 rpm (22,095xg), 4°C for 30 minutes to remove cell debris. The resultant supernatant was used as the cell-free extract. All procedures were performed at 4°C.

3.4.3 Enzymatic assays for PEG dehydrogenase and PPG dehydrogenase

The enzymatic assay was followed from Tachibana *et al.*, 2002 and can be described as followed.

PEG-DH and PPG-DH activities were spectrophotometrically examined at 30°C by measuring the initial rate of 2,6-dichloroindophenol (DCIP) (ε = 16,100 M^{-1} cm^{-1}) reduction at 600 nm using a phenazine methosulfate (PMS) as an electron mediator (Tachibana *et al.*, 2002). The reaction mixture consisted of 0.1 M Tris-HCl buffer (pH 8.0), 1 mM KCN, 2 mM $CaCl_2$, 0.1 mM DCIP, 0.1 mM PMS, 10 mM PEG or PPG and the enzyme solution in a total volume of 1 ml. The preparation of stock solutions and details of the assay was described in Appendix A.

The absorbance change with various electron acceptors was measured at 340 nm for Nicotinamide adenine dinucleotide (NAD) (ε = 6,200 M^{-1} cm^{-1}) or 446 nm for Flavin adenine dinucleotide (FAD) (ε = 11,300 M^{-1} cm^{-1}). Moreover, NAD and FAD could be used as cofactor for the enzymes.

In some cases, 20 μM final concentration of pyrroloquinoline quinine (PQQ) which was cofactor of the enzymes and 2 mM $CaCl_2$ were necessary incubated with the enzymes at 4°C before the enzymatic assay 30 minutes for recovery the enzyme activities.

One unit of enzyme activity was defined as the amount of enzyme that catalyzed oxidation of 1 μmol of substrate per minute under the applied conditions. Specific activity was defined as unit activity per mg protein.

3.4.4 Determination of protein concentration

Protein concentration was determined by a modified Lowry method where 0.5% sodium dodecyl sulfate was included (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard protein. The protein standard curve was shown in Appendix B.

Reagents

Solution A: 2% sodium carbonate in 0.1 M sodium hydroxide containing 0.5% sodium laulyl sulphate (SDS)

Solution B: 0.5% copper sulfate in 1% potassium sodium tartrate

Solution C: Phenol reagent (Folin-Ciocalteu's reagent)

After preparation of sample (0.4 ml), 2 ml of mixed solution A and B (A:B, 50:1) were added and rapidly mixed. The mixture was incubated at 30°C for 10 minutes. Subsequently, 0.2 ml of solution C was added, rapidly mixed and incubated at 30°C for 30 minutes. Finally, to determine the quantity of protein, the absorbance of clear blue-color solution was measured at 750 nm at room temperature. The reagent preparation was presented in Appendix C.

Protein molecular weight marker include proteins of the molecular weight of β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa), REase Bsp98I (25 kDa), β-galactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa) (Fermentas, USA).

3.4.5 Polyacrylamide gel electrophoresis (PAGE) and detection methods

Two types of PAGE, a non-denaturing and a denaturing gel, were employed for analysis of the purity and activity of the partially purified proteins.

3.4.5.1 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

Discontinuous PAGE was performed according to Bollag and Edelstein (1993) on slab gels (10 x 8 x 0.75 cm) of 7.5% (w/v) separating and 5.0% (w/v) stacking gels. The Tris-glycine buffer, pH 8.3 was used as electrode buffer (Appendix D). The enzyme (30 μg protein) was mixed with 5x sample buffer (composition and preparation was shown in Appendix D) by ratio 5:1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant current of 10 mA per slab gel at room temperature in a Mini-Gel Electrophoresis unit (Bio-Rad Laboratories, USA). A native-PAGE was always subject to both protein staining (section 3.4.5.3.1) and activity staining (section 3.4.5.3.2).

3.4.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The denaturing gel was performed according to Bollag and Edelstein (1993). The gel was prepared with 0.1% (w/v) SDS in 10% (w/v) separating and 5.0% (w/v) stacking gels, while Tris-glycine buffer pH 8.3 containing 0.1% SDS was used as electrode buffer (Appendix D). Samples to be analyzed were treated with sample buffer (Appendix D) and boiled for 5 minutes prior to gel application. The electrophoresis was performed at constant current of 10 mA per a slab gel, at room temperature on a Mini-Gel Electrophoresis unit from cathode towards anode.

3.4.5.3 Detection of proteins on polyacrylamide gel

After electrophoresis, proteins in either non-denaturing or denaturing gel were visualized by Coomassie blue staining. For non-denaturing gel, determination of PEG-DH and PPG-DH activities on the gel was also undertaken.

3.4.5.3.1 Coomassie blue staining

Gels were stained with 0.1% (w/v) of Coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid for at least 30 minutes. The slab gels were destained with a destaining solution (10% methanol and 10% acetic acid) for 1-2 hours several times until the gel background was clear.

3.4.5.3.2 Enzyme activity staining

(slightly modified from the method of Vangnai and Arp, 2001)

The non-denaturing gel was incubated for PEG-DH activity or PPG-DH activity for 5 minutes in the dark with a reaction mixture (5 ml) containing 26.4 mM nitroblue tetrazolium (NBT), together with 0.7 mM PMS and 1mM NAD^+. Either PEG 600 (for PEG-DH activity) or PPG 725 (for PPG-DH activity) was then added to a final concentration of 10 mM and incubated with gentle rocking for another at least 2 hours to develop the color. The reaction was stopped by rinsing the gel with distilled water.

In some cases, the direct combination of PEG or PPG substrate into the gel was necessary. Either PEG or PPG to the final concentration of 10 mM was combined to the separating gel mixture before gel setting. Then, the regular electrophoresis was carried out as described in section 3.4.5.1.

3.4.6 Experimental approaches

3.4.6.1 Induction of PEG deydrogenase and/or PPG dehydrogenase using various concentrations of either PEG or PPG

Pseudomonas sp. PE-2 was grown on basal medium agar plate containing 0.7% PEG 4000. A loopful of bacteria was placed into a test tube containing 5 ml of either nutrient broth or basal medium. The preparation of nutrient broth and basal medium were described in section 3.4.1.1.1 and 3.4.1.1.2, respectively. Carbon source added in the basal medium included PEG (molecular weight of 2000 and 4000) or PPG (molecular weight of 1000 and 2000) at various concentrations (0.7%, 1.5% or 3.0% (v/v)). The test tube was placed on the rotary shaker at 270 rpm, 30°C for 1 day for growth in nutrient broth and 3 days for growth in basal medium as the starting cell culture.

After that, the inoculum was transferred to a 500-ml Erlenmeyer flask containing 200-ml of medium as indicated in each experiment. Cells were grown with reciprocal shaking at 270 rpm, 30°C for 10 days. The culture was withdrawn at intervals of 24 hours in order to determine cell growth rate and activities of PEG/PPG-degrading enzymes. Growth of the bacterium was monitored via the spectrophotometer as culture turbidity (OD_{610}). PEG-DH and PPG-DH activities were assayed with the cell-free extract as described in section 3.4.2 and 3.4.3.

3.4.6.2 Cellular localization of PEG/PPG-degrading enzymes from *Pseudomonas* sp. PE-2

The determination protocol for cellular localization of the two enzymes can be briefly summarized as shown in Figure 3.2 and described below.

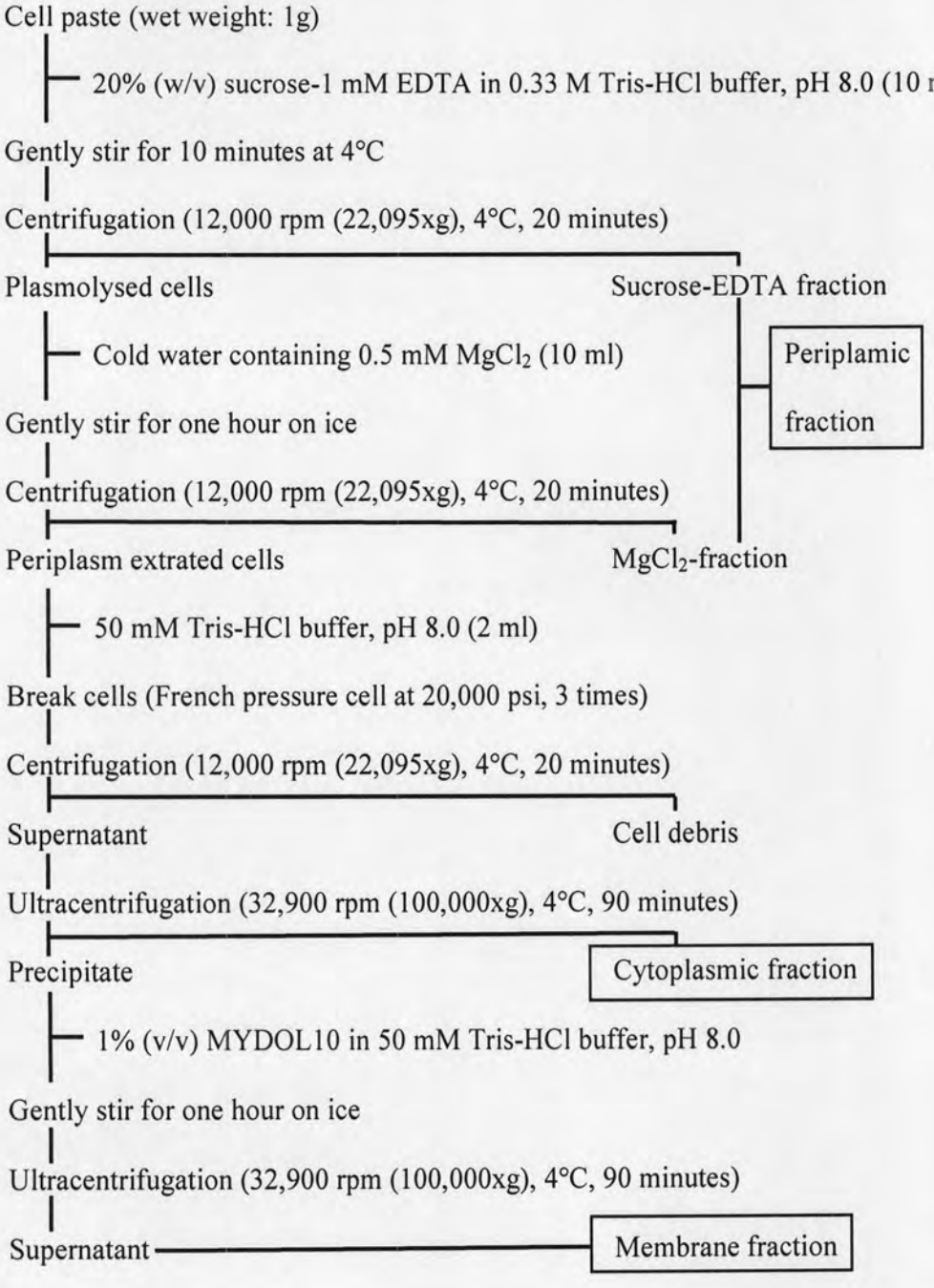

Figure 3.2 The flow chart of cell fractions preparation

3.4.6.2.1 Separation of periplasmic, membrane and cytoplasmic fractions

To examine the localization of enzymes, cell fractions were prepared by the osmotic shock method (Anraku and Heppel, 1967). The washed cells (1g wet weight) resuspended in 10 ml of 0.33 M Tris-HCl buffer, pH 8.0, containing 20% (w/v) sucrose and 1 mM EDTA were gently stirred for 10 minutes at 4°C to cause plasmolysis. The mixture was then centrifuged at 12,000 rpm (22,095xg), 4°C for 20 minutes to obtain the plasmolysis cells. The supernatant was designated as the sucrose-EDTA fraction. The plasmolyzed cells were resuspended in 10 ml of cold water containing 0.5 mM $MgCl_2$ and gently stirred for one hour on ice to extract periplasmic protein. The mixture was centrifuged at 12,000 rpm (22,095xg), 4°C for 20 minutes to remove the osmotically shocked cells. The resultant supernatant was used as the $MgCl_2$-fraction. The sucrose-EDTA and the $MgCl_2$-fractions were combined and used as the periplasmic fraction. The osmotically shocked cells were resuspended in 2 ml of 50 mM Tris-HCl buffer, pH 8.0, passed thrice through a French pressure cell at 20,000 psi, and centrifuged at 12,000 rpm (22,095xg), 4°C for 30 minutes to remove cell debris. The resultant supernatant was ultracentrifuged at 32,900 rpm (100,000xg), 4°C for 90 minutes. The resultant supernatant was used as the cytoplasmic fraction. The precipitate was resuspended in 50 mM Tris-HCl buffer, pH 8.0 with 1% (v/v) MYDOL10 and gently stirred for one hour on ice. Then, the suspension was centrifuged at 32,900 rpm (100,000xg), 4°C for 90 minutes. The resultant supernatant was used as the membrane fraction.

The PEG-DH and PPG-DH activities in each fraction were examined by the method described in section 3.4.3. The separation efficiency and purity of each cellular fraction were determined by measuring the activity of the marker enzymes: alkaline phosphatase, NADH oxidase and glucose-6-phosphate dehydrogenase.

3.4.6.2.2 Enzymatic assays for the marker enzyme in cellular fraction

3.4.6.2.2.1 Alkaline phosphatase

Alkaline phosphatase as a periplasmic marker enzyme was assayed by the method of Garen and Levinthal (1960). The assay mixture consisted of 0.1 M Tris-HCl buffer (pH 8.0), 2 mM $MgCl_2$, 20 mM *p*-nitrophenyl phosphate and the enzyme solution in a total volume of 1 ml. The hydrolysis of *p*-nitrophenol phosphate resulting in an increase of the absorbance at 405 nm was measured at 30°C ($\varepsilon_{p\text{-nitrophenol}} = 18{,}800\ M^{-1}\ cm^{-1}$).

3.4.6.2.2.2 NADH oxidase

NADH oxidase as a marker enzyme in the membrane fraction was measured according to the method of Cheng *et al.* (1970). The assay mixture consisted of 50 mM Tris-HCl buffer (pH 7.6), 0.4 mM NADH (prepared in 50 mM Tris-HCl buffer, pH 7.6) and the enzyme solution in a total volume of 1 ml. The oxidation of NADH was measured at 30°C by detecting the decrease in absorbance at 340 nm ($\varepsilon_{NADH} = 6{,}200\ M^{-1}\ cm^{-1}$).

3.4.6.2.2.3 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase as a marker enzyme in the cytoplasmic fraction was assayed by the method of Bergmeyer *et al.* (1974). The assay mixture consisted of 86 mM Tris-HCl buffer (pH 7.6), 6.9 mM $MgCl_2$, 1 mM glucose-6-phosphate, 0.39 mM $NADP^+$ and the enzyme solution in a total volume of 1 ml. The reduction of $NADP^+$ was measured at 30°C from the increase in absorbance at 340 nm (ε_{NADPH} = 6,220 M^{-1} cm^{-1}).

In all cases, one unit of enzyme activity was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of substrate per min.

3.4.6.3 Growth conditions for PEG dehydrogenase induction and preparation of cell-free extract for partial purification of PEG dehydrogenase from *Pseudomonas* sp. PE-2

Pseudomonas sp. PE-2 was cultivated in 200 ml of a basal medium containing 3% (v/v) PEG 4000 and grown on rotary shaker at 270 rpm, 30°C for 3 days. Cells were harvested by centrifugation (12,000 rpm (22,095xg), 4°C for 20 minutes), washed twice with 50 mM Tris-HCl buffer, pH 8.0 and stored at -20°C until use. The cell-free extract was prepared as described in section 3.4.2. The PEG-DH activity and protein concentration were determined by the methods described in section 3.4.3 using PEG 2000 as a substrate and section 3.4.4, respectively.

3.4.6.3.1 Partial purification of PEG dehydrogenase

PEG-DH was purified using ammonium sulfate precipitation and column chromatography techniques. Various types of column chromatography were used: CM-Cellulose, DEAE-Cellulose, DEAE-650M Toyopearl, Phenyl-Sepharose CL-4B and Butyl-Toyopearl.

3.4.6.3.1.1 Ammonium sulfate precipitation

The precipitation of cell-free extract was performed by slowly adding solid ammonium sulfate powder to various saturation ranges (0-15%, 15-30%, 30-45%, 45-60% and 60-80%). After 2 hours of gently stirring, the supernatant was collected by centrifugation at 12,000 rpm (22,095xg), 4°C for 30 minutes and then brought to the next concentration ammonium sulfate saturation. The solution was left for 2 hours at 4°C with continuous stirring and subsequently centrifuged at 12,000 rpm (22,095xg), 4°C for 30 minutes. The protein precipitated in each step was dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. The protein solution was dialyzed against 100x volume of 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF at 4°C at least 3 hours before the determination of PEG-DH activity (section 3.4.3 using PEG 2000 as a substrate) and protein concentration (section 3.4.4) were carried out, respectively. The details of all attempts and the successful range of saturation (30-45%) are described in the results section 4.3.1.1.

3.4.6.3.1.2 CM-Cellulose column chromatography

CM-Cellulose was activated by washing with 0.5 M sodium hydroxide for 2-3 times before rewashing with deionized water until the pH was reached 7.0. The active resin was resuspended in 50 mM acetate buffer, pH 5.5, acetate buffer, pH 6.0, potassium phosphate buffer, pH 7.0, Tris-HCl buffer, pH 8.0 or Tris-HCl buffer, pH 9.0, containing 2 mM $CaCl_2$ and 1 mM PMSF and packed into a 2.5 x 15 cm glass column. Equilibration of the column with the buffer for 5-10 column volumes at flow rate of 60 ml/h was controlled by peristaltic pump (Pharmacia Biotech, Sweden).

The dialyzed protein solution from section 3.4.6.3.1.1 was applied onto the CM-Cellulose column. The unbound proteins were eluted from the column with the buffer as described above. The bound proteins were then eluted from the column with a linear gradient of 0 M to 1 M of sodium chloride in the buffer, at a flow rate of 60 ml/h respectively. Fractions of 5 ml were collected using a fraction collector (Pharmacia LKB, Sweden). The protein elution profile was monitored by measuring the absorbance at 280 nm using a spectrophotometer (DU series 800, Beckman Coulter, USA). The enzyme activity of each fraction was determined as described in section 3.4.3 using PEG 2000 as a substrate. The active fractions were pooled, concentrated using Aquasorb (BML, Thailand) and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. All operations were performed at 4°C.

3.4.6.3.1.3 DEAE-Cellulose column chromatography

DEAE-Cellulose was activated by washing with 0.5 M sodium hydroxide for 2-3 times before rewashing with deionized water until the pH was reached 7.0. The active resin was resuspended in 50 mM acetate buffer, pH 5.5, acetate buffer, pH 6.0, potassium phosphate buffer, pH 7.0, Tris-HCl buffer, pH 8.0 or Tris-HCl buffer, pH 9.0, containing 2 mM $CaCl_2$ and 1 mM PMSF and packed into a 2.5 x 15 cm glass column. Equilibration of the column with the buffer for 5-10 column volumes at flow rate of 60 ml/h was controlled by peristaltic pump (Pharmacia Biotech, Sweden).

The dialyzed protein solution from section 3.4.6.3.1.1 was applied onto the DEAE-Cellulose column. The unbound proteins were eluted from the column with the buffer as described above. The bound proteins were then eluted from the column with a linear gradient of 0 M to 1 M of sodium chloride in the buffer, at a flow rate of 60 ml/h respectively. Fractions of 5 ml were collected using a fraction collector (Pharmacia LKB, Sweden). The protein elution profile was monitored by measuring the absorbance at 280 nm using a spectrophotometer (DU series 800, Beckman Coulter, USA). The enzyme activity of each fraction was determined as described in section 3.4.3 using PEG 2000 as a substrate. The active fractions were pooled, concentrated using Aquasorb (BML, Thailand) and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. All operations were performed at 4°C.

3.4.6.3.1.4 DEAE-650M Toyopearl column chromatography

DEAE-650M Toyopearl was activated by washing with 0.5 M sodium hydroxide for 2-3 times before rewashing with deionized water until the pH was reached 7.0. The active resin was resuspended in 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF and packed into a 2.5 x 14 cm glass column. Equilibration of the column with the same buffer for 5-10 column volumes at flow rate of 60 ml/h was controlled by peristaltic pump (Pharmacia Biotech, Sweden).

The dialyzed protein solution from section 3.4.6.3.1.1 was applied onto the DEAE-650M Toyopearl column. The unbound proteins were eluted from the column with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF. The bound proteins were then eluted from the column with both a linear gradient (of 0 M to 1 M) and a stepwise elution (of 0.2 M and 0.5 M) of sodium chloride in the same buffer, at a flow rate of 60 ml/h respectively. Fractions of 5 ml were collected using a fraction collector (Pharmacia LKB, Sweden). The protein elution profile was monitored by measuring the absorbance at 280 nm using a spectrophotometer (DU series 800, Beckman Coulter, USA). The enzyme activity of each fraction was determined as described in section 3.4.3 using PEG 2000 as a substrate. The active fractions were pooled, concentrated using Ultracel-50k (Amicon Ultra-4, Millipore, Ireland) and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. All operations were performed at 4°C.

3.4.6.3.1.5 Phenyl-Sepharose CL-4B column chromatography

Phenyl-Sepharose CL-4B column was activated by washing with 0.5 M sodium hydroxide for 2-3 times before rewashing with deionized water until the pH was reached 7.0. The active resin was resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF and packed into a 2.5 x 8 cm glass column. Equilibration of the column with the same buffer for 5-10 column volumes at flow rate of 30 ml/h was performed.

The pooled enzyme fraction from section 3.5.6.3.1.4 was dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF for at least 3 hours. The protein solution was then applied onto the equilibrated column at flow rate of 30 ml/h. The loaded column were washed with 10 column volumes of the same buffer and then eluted with both a linear gradient (of 1 M to 0 M) and a stepwise elution (of 0.2 M and 0 M) of ammonium sulfate in the same buffer, respectively. Sample fractions of 3 ml were collected. The protein elution profile as well as, the enzyme activity profile was generated as previously described in section 3.4.6.3.1.4. The active fractions were pooled, concentrated using Ultracel-50k and dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. The dialyzed enzyme was collected in aliquots and kept in 4°C (1 day) or -20°C (long term) for further characterization. All operations were performed at 4°C.

3.4.6.4 Growth conditions for PPG dehydrogenase induction and preparation of cell-free extract for partial purification of PPG dehydrogenase from *Pseudomonas* sp. PE-2

Pseudomonas sp. PE-2 was cultivated in 200 ml of a basal medium containing 1.5% (v/v) PPG 1000 and grown on rotary shaker at 270 rpm, 30°C for 7 days. Cells were harvested by centrifugation (12,000 rpm (22,095xg), 4°C for 20 minutes), washed twice with 50 mM Tris-HCl buffer, pH 8.0 and stored at -20°C until use. The cell-free extract was prepared as described in section 3.4.2. The PPG-DH activity and protein concentration were determined by the methods described in section 3.4.3 using PPG 1000 as a substrate and section 3.4.4, respectively.

3.4.6.4.1 Partial purification of PPG dehydrogenase

PPG-DH was purified using ammonium sulfate precipitation and column chromatography techniques various types of column chromatographic methods were used: CM-Cellulose, DEAE-Cellulose, DEAE-650M Toyopearl, Phenyl-Sepharose CL-4B and Butyl-Toyopearl.

3.4.6.4.1.1 Ammonium sulfate precipitation

The condition for preliminary ammonium sulfate precipitation of PPG-DH was the same as PEG-DH as described in section 3.4.6.3.1.1.

Solid ammonium sulfate powder was slowly added to cell-free extract to reach 60% saturation. Afterward, the supernatant was collected by centrifugation at 12,000 rpm (22,095xg), 4°C for 30 minutes. Solid ammonium sulfate powder was further added to the supernatant to give 80% saturation. The pellet was collected by centrifugation at 12,000 rpm (22,095xg), 4°C for 30 minutes, dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. The protein solution was dialyzed against 100x volume of 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF at least 3 hours before the determination of PPG-DH activity (section 3.4.3 using PPG 1000 as a substrate) and protein concentration (section 3.4.4) were carried out, respectively. The details of all attempts and the successful range of saturation (60-80%) are described in the results section 4.3.1.1.

3.4.6.4.1.2 CM-Cellulose column chromatography

The conditions of CM-Cellulose column chromatography for partially purified PPG-DH were the same as described in section 3.4.6.3.1.2.

3.4.6.4.1.3 DEAE-Cellulose column chromatography

The conditions of DEAE-Cellulose column chromatography for partially purified PPG-DH were the same as described in section 3.4.6.3.1.3.

3.4.6.4.1.4 DEAE-650M Toyopearl column chromatography

DEAE-650M Toyopearl column (2.5 x 14 cm) was washed with 2 column volumes of 0.5 M sodium hydroxide followed with 10 column volumes of distilled water. The column was equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF.

The dialyzed protein solution from section 3.4.6.4.1.1 was applied onto the DEAE-650M Toyopearl column. The unbound proteins were eluted from the column with 50 mM Tris-HCl buffer, pH 8.5, containing 2 mM $CaCl_2$ and 1 mM PMSF. The bound proteins were then eluted from the column with both a linear gradient (of 0 M to 1 M) and a stepwise elution (of 0.2 M and 0.5 M) of sodium chloride in the same buffer, at a flow rate of 60 ml/h respectively. Fractions of 5 ml were collected using a fraction collector. The protein elution profile was monitored by measuring the absorbance at 280 nm using a spectrophotometer. The enzyme activity of each fraction was determined as described in section 3.4.3 using PPG 1000 as a substrate. The active fractions were pooled, concentrated using Ultracel-50k and then dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. All operations were performed at 4°C.

3.4.6.4.1.5 Butyl-Toyopearl column chromatography

Butyl-Toyopearl column was activated by washing with 0.5 M sodium hydroxide for 2-3 times before rewashing with deionized water until the pH was reached 7.0. The active resin was resuspended in 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF and packed into a 2.5 x 3 cm glass column. Equilibration of the column with the same buffer for 5-10 column volumes at flow rate of 30 ml/h was performed.

The pooled enzyme fraction from section 3.4.6.4.1.4 was dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 1 M ammonium sulfate, 2 mM $CaCl_2$ and 1 mM PMSF for at least 3 hours. The protein solution was then applied onto the equilibrated column at flow rate of 30 ml/h. The loaded column were washed with 10 column volumes of the same buffer and then eluted with both a linear gradient (of 1 M to 0 M) and a stepwise elution (of 0.6 M and 0 M) of ammonium sulfate in the same buffer, respectively. Fractions of 3 ml were collected. The protein elution profile was monitored and enzyme activity as previously described in section 3.4.6.4.1.2. The active fractions were pooled, concentrated using Ultracel-50k and dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 1 mM PMSF. The dialyzed enzyme was collected in aliquots and kept in 4°C (1 day) or -20°C (long term) for further characterization. All operations were performed at 4°C.

3.4.6.5 Characterization of partially purified PEG dehydrogenase and PPG dehydrogenase

3.4.6.5.1 Molecular weight determination of native PEG dehydrogenase and PPG dehydrogenase by polyacrylamide gel electrophoresis (PAGE)

For molecular weight determination using nondenaturing gel electrophoresis, the protein sample must be run under a variety of acrylamide concentrations, often ranging from 4% to 12% (see section 3.4.5.1). The accumulated information from these conditions serves to reduce the effect due to protein charge. Protein mobilities are calculated as the R_f value (distance of protein migration divided by distance of migration of the dye front). A semilogarithmic plot of the R_f relative to the acrylamide gel concentration should provide a line with a slope characteristic for protein of a specific molecular weight. Protein of known molecular weight should be electrophoresed under the same conditions, and the slopes generated from these experiments define a linear relationship with the molecular weight. The molecular weight of the unknown protein of interest may be extrapolated from the data with the molecular weight standards (Hedrick and Smith, 1968).

3.4.6.5.2 Effect of pH on PEG dehydrogenase and PPG dehydrogenase activities

The partially purified PEG-DH and PPG-DH were assayed as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively), but in various pH conditions at room temperature. The 0.1 M of acetate or phosphate or Tris-HCl or glycine-NaOH was used as reaction buffers for pH 4-5.5, 5.5-8.0, 8.0-9.0 and 9.0-11.0, respectively. The result was expressed as the percentage of the relative activity as the activity obtained in Tris-HCl buffer, pH 8.0, was used as 100% activity.

3.4.6.5.3 Effect of temperature on PEG dehydrogenase and PPG dehydrogenase activities

The partially purified PEG-DH and PPG-DH were assayed by the method as described in section section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively) in Tris-HCl buffer, pH 8.0 at 15, 20, 25, 30, 40, 50 and 60°C for 3 minutes. The result was expressed as the percentage of the relative activity as the activity obtained at 25°C was used as 100% activity.

3.4.6.5.4 pH Stability of PEG dehydrogenase and PPG dehydrogenase activities

The partially purified PEG-DH and PPG-DH were incubated at 4°C for 30 minutes in 50 mM buffers at various pH values (the total volume of 1 ml). An aliquot of the enzyme solution was withdrawn at interval and the remaining activity was assayed as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). The 50 mM of acetate or phosphate or Tris-HCl or glycine-NaOH was used as a buffer for pH 4-5.5, 5.5-8.0, 8.0-9.0 and 9.0-11.0, respectively. The result was expressed as the percentage of the relative activity as the activity incubated in Tris-HCl buffer, pH 8.0, was used as 100% activity.

3.4.6.5.5 Temperature stability of PEG dehydrogenase and PPG dehydrogenase activities

The thermostability of the enzyme was investigated in the range of 25-60°C. The partially purified PEG-DH and PPG-DH in 50 mM Tris-HCl buffer, pH 8.0 were preincubated at temperature 25, 30, 40, 50 and 60°C for 30 minutes, then the residual activity was assayed as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). The result was expressed as the percentage of the relative activity as the activity preincubated at 25°C was used as 100% activity.

3.4.6.5.6 Effect of various electron acceptors on PEG dehydrogenase and PPG dehydrogenase activities

Various types of electron acceptors (DCIP, NAD and FAD) were added to reaction mixture. The enzyme activity was assayed by the method as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). The result was expressed as fold of the specific activity, as the activity obtained from PMS plus DCIP was used as 1 fold.

3.4.6.5.7 Effect of divalent metal ions on PEG dehydrogenase and PPG dehydrogenase activities

Various concentrations (2 mM, 5 mM and 10 mM) of various types of divalent metal ions ($CaCl_2$, $CoCl_2$, $CuSO_4$, $HgCl_2$, $MgCl_2$, $MnCl_2$ and $NiCl_2$) were added to reaction mixture. The enzyme activity was assayed by the method as described in section 3.4.3 (PEG 2000 and PPG 1000 as a substrate for PEG-DH and PPG-DH, respectively). The result was expressed as the percentage of the relative activity as the activity obtained from $CaCl_2$ was used as 100% activity.

3.4.6.5.8 Substrate specificity of PEG dehydrogenase and PPG dehydrogenase activities

The partially purified PEG-DH and PPG-DH activities were determined as described in section 3.4.3 when various compounds were used as their substrate (final concentration 10 mM).

The substrate tested could be classified as:

1) primary alcohol: methanol, ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol and 1-octanol

2) secondary alcohol: 2-propanol and 2-butanol

3) diol-type compound: ethylene glycol, diethylene glycol, PEG 600, PEG 2000, PEG 4000, PEG 6000, PEG 8000, PPG 725, PPG 1000 and PPG 2000

4) triol-type compound: glycerol

The result was expressed as the percentage of the relative activity in comparison with that assayed with PEG 2000 and PPG 1000 (100% activity).

3.4.6.5.9 Determination of kinetic parameters of PEG dehydrogenase and PPG dehydrogenase activities

The partially purified enzymes were used to determine the enzyme kinetic parameters (K_m and V_{max}) using PEG 600, PEG 2000, PEG 4000, PEG 6000 and PEG 8000 as the substrate of PEG-DH and PPG 725, PPG 1000 and PPG 2000 as the substrate of PPG-DH. The activities were determined as described in section 3.4.3 with varying concentration of PEGs and PPGs: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 mM. Kinetic parameter, K_m and V_{max}, were obtained from the Lineweaver-Burk plot.