CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Culture medium

Mineral Salt Medium (MSM) contained

- Di-potassium hydrogen orthophosphate (K_2HPO_4: Scharlau, Spain; BDH Laboratory, England)
- Di-sodium hydrogen orthophosphate (Na_2HPO_4: Scharlau, Spain; Merck, Germany)
- Potassium nitrate (KNO_3: Merck, Germany; Univar, Australia)
- Calcium chloride ($CaCl_2.7H_2O$: Merck, Germany)
- Magnesium sulfate ($MgSO_4.7H_2O$: BDH Laboratory, England; Baker Analyzed, USA)
- Ferrous sulfate Heptahydrate ($FeSO_4.7H_2O$: Redel-de-Haën, Germany)
- Manganese chloride Tetrahydrate ($MnCl_2.4H_2O$: Carlo Erba, Italy)
- Zinc chloride ($ZnCl_2$: Fluka, Switzerland)
- Cobalt chloride ($CoCl_2.6H_2O$: Carlo Erba, Italy)
- Nickel (II) chloride Hexahydrate ($NiCl_2.6H_2O$: Ajax Finechem, Australia)
- Copper (II) chloride Dihydrate ($CuCl_2.2H_2O$: Merck, Germany)
- Boric acid (H_3BO_3: Merck, Germany)
- Sodium molybdate Dihydrate ($NaMoO_4.2H_2O$: Fluka, Switzerland)
- Sodium selenite Pentahydrate ($NaSeO_3.5H_2O$: Fluka, Switzerland)
- Sodium tungstate Dihydrate ($NaWO_4.2H_2O$: Fluka, Switzerland)
- Sulfuric acid (H_2SO_4: Merck, Germany)

2.1.2 Biosensor

- Phenol (C_6H_5OH: Merck, Germany)
- Pyrocatechol ($C_6H_6O_2$: Fluka, Switzerland)
- 2-Monochlorophenol (: Fluka, Switzerland)
- 4-Monochlorophenol (: Fluka, Switzerland)
- 2,4-Dichlorophenol ($C_6H_4Cl_2O$: Aldrich, Sweden; Fluka, Switzerland)
- Pentachlorophenol ($C_6H_6OCl_5$: Aldrich, USA)
- 2,4,6-Trichlorophenol ($C_6H_3Cl_3O$: Aldrich, Sweden; Fluka, Switzerland)
- Sodium acetate ($CH_3COONa._3H_2O$: Carlo Erba, Italy; Merck, Germany)
- D(+)-Glucose Monohydrate ($C_6H_{12}O_6.H_2O$: Riedel-de Haën, France; Merck, USA)
- Sucrose ($C_{12}H_{22}O_{11}$: BDH Laboratory, England; Merck, Germany)
- Citric acid ($C_6H_8O_7$: Merck, Germany)
- Benzene (C_6H_6: Merck, Germany)
- Benzoic acid (C_6H_5COOH: Carlo Erba, Italy)
- Hydrochloric acid (HCl: Scharlau, Spain)
- Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$: Fluka, Switzerland)
- Potassium di-hydrogen orthophosphate (KH_2PO_4: Merck, Germany)
- Sodium alginate (Fluka, Switzerland)

2.1.3 Gas chromatography/mass spectrometry

- Dichloromethane (CH_2Cl_2: Merck, Germany)

2.2 Equipments

2.2.1 Biosensor

- Peristaltic pump (Minipulse3, Gilson, France)
- Peristaltic pump (U4-MIDI; ALITEA®AB, Stockholm, Sweden)
- Sample injector (VICI; Valco, Switzerland)
- Sample injector (VICI; Valco, USA)
- Potentiostat (Zäta-Elektronik; Höör, Sweden)
- Biological oxygen monitoring (YSI Model 5300, Yellow Spring Instrument Company, USA)
- Oxygen electrode (YSI Model 5331, Yellow Spring Instrument Company, USA)
- Chart recorder (Kipp & Zonen, Holland)
- Chart recorder (Ross recorder model 202, USA)
- Plastic syringe (Nipro, Thailand)
- Rotary shaker (SK 101, Thailand)
- UV-Vis Spectrophotometer (Specord S 100, Analytikjena, Germany)
- Varian model 3350 Gas Chromatograph with Porapak Q HayeSep Q 80/100 mesh column (Walnut Creek, USA)
- Microliter pipeter (Effpendorf, Germany)

2.2.2 Gas chromatography/mass spectrometry

- HP-5890 Gas chromatograph with HP-5973 mass selective detector (Agilent Technologies, USA)

2.3 Principle of cell-based biosensor for 2,4-dichlorophenol

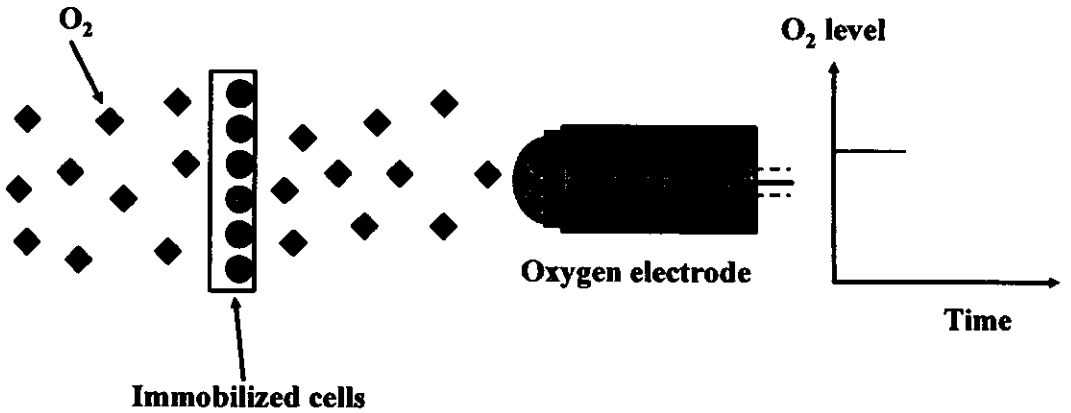
To construct a cell-based biosensor for monitoring 2,4-dichlorophenol, the mixed culture microbes used for 2,4-dichlorophenol biodegradation was immobilized by entrapment and used as a biological sensing element in the biosensor system. When there was no analyte, a part of dissolved oxygen was consumed by the immobilized mixed culture microbes and detected by the oxygen electrode. The steady state transducer output signal at the beginning represented the amount of dissolved oxygen remained in the solution after endogenous respiration of the

immobilized mixed culture microbes without the analyte (Figure 11a). When 2,4-dichlorophenol (the analyte) was injected into the sensor system, it was assimilated by the immobilized mixed culture microbes, resulting in an increase of bacterial respiration rate and oxygen consumption. Therefore, less oxygen could be detected by oxygen electrode, resulting in a decrease in the output sensor signal. The sensor signal should be proportional to the concentration of 2,4-dichlorophenol to be measured (Figure 11b). In this work mixed culture microbes from two sources were investigated, *i.e.* Sweden and Thailand.

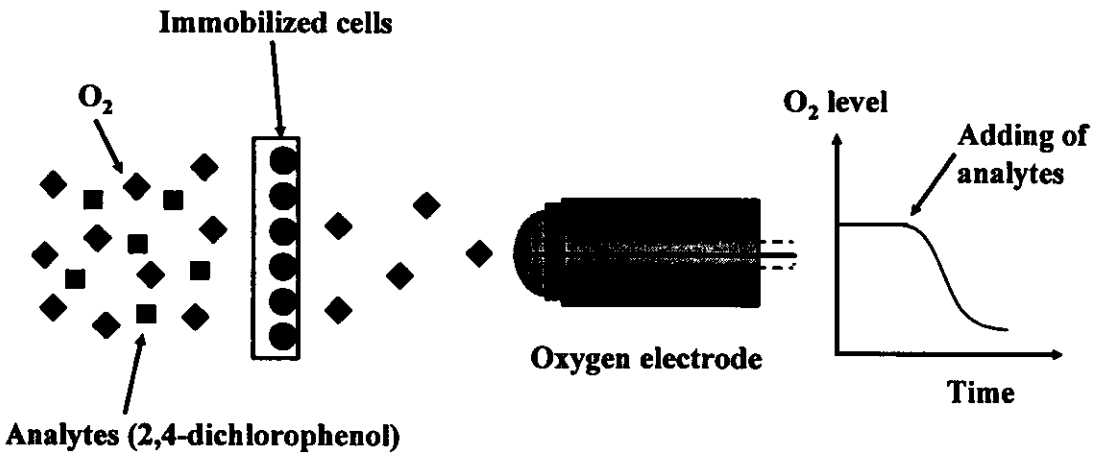
2.4 Swedish mixed-culture bacteria

2.4.1 Culture medium

The mixed culture bacteria was inoculated into a mineral salt medium (MSM) containing 2,4-dichlorophenol as a carbon source and energy. The MSM composed of (mg l^{-1}): K_2HPO_4 4000, Na_2HPO_4 5200, KNO_3 3000, $CaCl_2.7H_2O$ 10, $MgSO_4.7H_2O$ 500, $FeSO_4.7H_2O$ 10, $MnCl_2.4H_2O$ 5.5, $ZnCl_2$ 0.68, $CoCl_2.6H_2O$ 1.2, $NiCl_2.6H_2O$ 1.2, $CuCl_2.2H_2O$ 0.85, H_3BO_3 0.0031, $NaMo_4.2H_2O$ 0.012, $NaSeO_3.5H_2O$ 0.013, and $NaWO_4.2H_2O$ 0.0165. The medium's pH was adjusted to 7.00 to 7.20 using 10% H_2SO_4 (Guieysse *et al.*, 2001).



(a)



(b)

Figure 11 Schematic diagram of microbial sensor of the respiration-activity measurement type

(a) Without analytes

(b) With analytes

2.4.2 Biodegradation of 2,4-dichlorophenol

The study of biodegradation of chlorophenols by mixed culture bacteria was carried out by Zilouei and co-workers in Lund, Sweden (Zilouei *et al.*, 2006). Pentachlorophenol-contaminated soil (Ljungby, Sweden) and pesticide-contaminated soil (Sweden) were mixed with sewage sludge and inoculated into MSM liquid growth medium containing 50 mg l^{-1} each of 2-monochlorophenol, 4-monochlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol as sole sources of carbon and energy. The culture was incubated at room temperature (23 ± 1°C). When chlorophenols were completely degraded by the mixed culture bacteria, it was then inoculated into a bioreactor (Zilouei *et al.*, 2006).

To construct a biosensor for monitoring 2,4-dichlorophenol, the culture from such a bioreactor needed to be acclimated to 2,4-dichlorophenol.

2.4.2.1 Culture conditions

A preliminary test was carried out to evaluate the maximum concentration of 2,4-dichlorophenol for the cultivation. A mixed culture bacteria taken from an aerobic biofilm reactor used for chlorophenols biodegradation that was able to biodegrade 2,4-dichlorophenol as a carbon source and energy was used as inoculum. Five milliliters of inoculum was inoculated into five 120 ml serum flasks, each of which contained 65 ml MSM liquid growth medium supplied with 2,4-dichlorophenol at 20.0, 50.0, 80.0, 110.0 and 150.0 mg l^{-1}. The flasks were then sealed with rubber septa to allow gas sampling from the flasks' headspaces and agitated at 120 rpm on a rotary shaker in the dark at room temperature (23 ± 1°C).

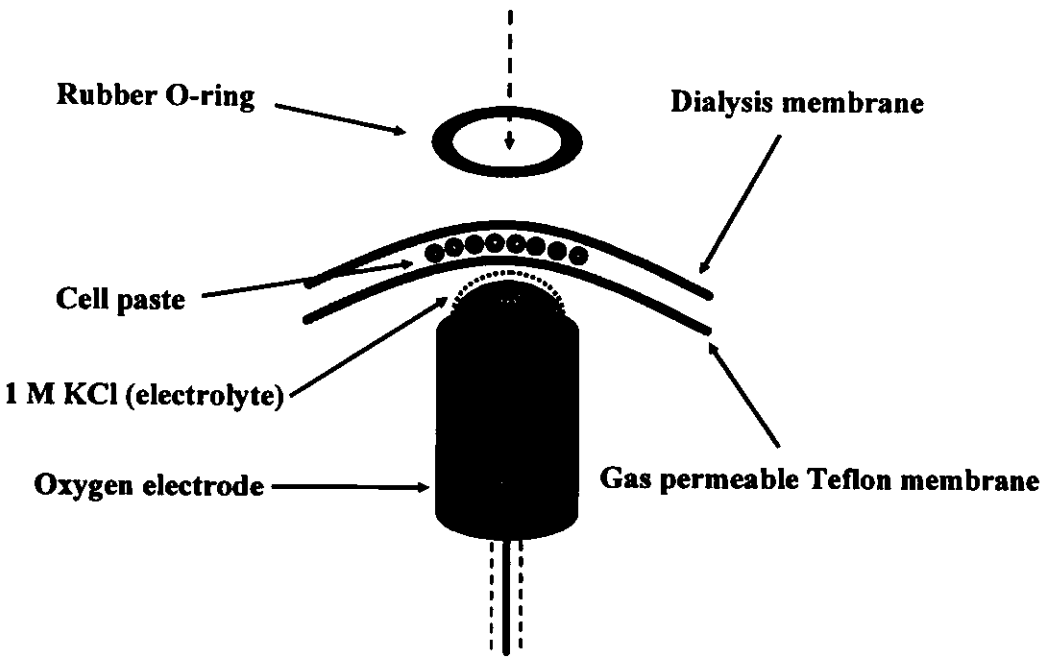
Since the metabolism of mixed culture bacteria by using 2,4-dichlorophenol consumed oxygen, which is the terminal electron acceptor, the reduction of amount of oxygen from each flask' headspace can indicate the microbial activity. The reduction of oxygen was analyzed by injecting gas samples from the flasks' headspace into a gas chromatograph (Varian model 3350, Walnut Creek, USA using Porapak Q HayeSep Q 80/100 mesh column and Molesieve 5A 45/60 column, Hayes Separations Inc. USA) with a thermal conductivity detector. The temperatures of column, injector and detector were 70, 110 and 150 °C, respectively. Helium was used as carrier gas (Björnsson *et al.*, 2001).

2.4.2.2 Preparation of mixed culture bacteria for immobilization

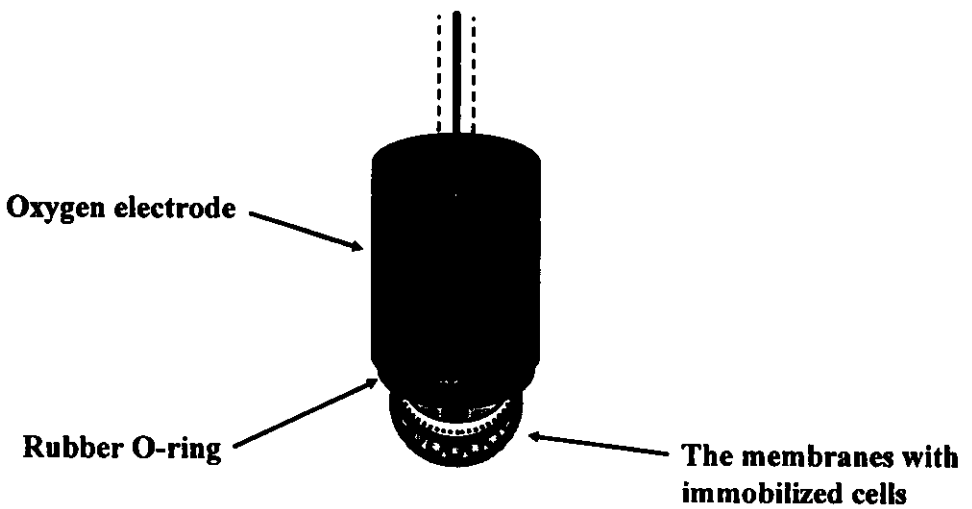
The mixed culture bacteria were taken from an aerobic bioreactor used for chlorophenols biodegradation. It was first enriched in 500 ml MSM liquid growth medium supplied with 50 mg l^{-1} of 2,4-dichlorophenol (best condition obtained from the above experiment) in 1000 ml conical flasks. The culture flasks were agitated at 120 rpm on a rotary shaker for 10 days in the dark at room temperature (23 ± 1°C). When the cell population reached the log phase (10 days) (the phase where the number of cells increase exponentially), it was used for biosensor preparation.

2.4.3 Immobilization of mixed culture bacteria on a Clark type oxygen electrode

Seventy-five milliliters of microbial culture in the log phase was harvested by centrifugation for 5 minutes at room temperature (23 ± 1°C). After discharging the supernatant, the cells were washed once with 5 ml of 100 mM potassium phosphate buffer (KH_2PO_4-K_2HPO_4, pH 7.50) for 2-3 minutes before being centrifuged again under the same condition. The supernatant was discarded, and the cells were incubated by suspending the cell paste in 20 ml potassium phosphate buffer and gently stirred with a magnetic stirrer for 12 h for stabilization. The suspended cells were then centrifuged for 5 min, and the supernatant was discarded. The cell pellet, 0.700 g, was placed on a Teflon membrane (1.5 cm × 1.5 cm PTFE, thickness 10 μm, Goodfellow, Cambridge, UK). A few drops of 100 mM potassium phosphate buffer was added before placing a dialysis membrane (Spectra/por® 2-dialysis membrane, MWCO 12,000-14,000, Spectrum Laboratories, Inc., Houston, TX) to cover the cell preparation in such a way that no air bubbles were trapped. A few drops of 1M potassium chloride were placed on the surface of a Clark type oxygen electrode to act as an electrolyte (Figure 12a). The membranes, with microbial cells in between, were placed on top with the Teflon side facing the electrode and were fixed on the surface of the electrode by means of a rubber O-ring (Figure 12b) (Liu *et al.*, 2000).



(a)



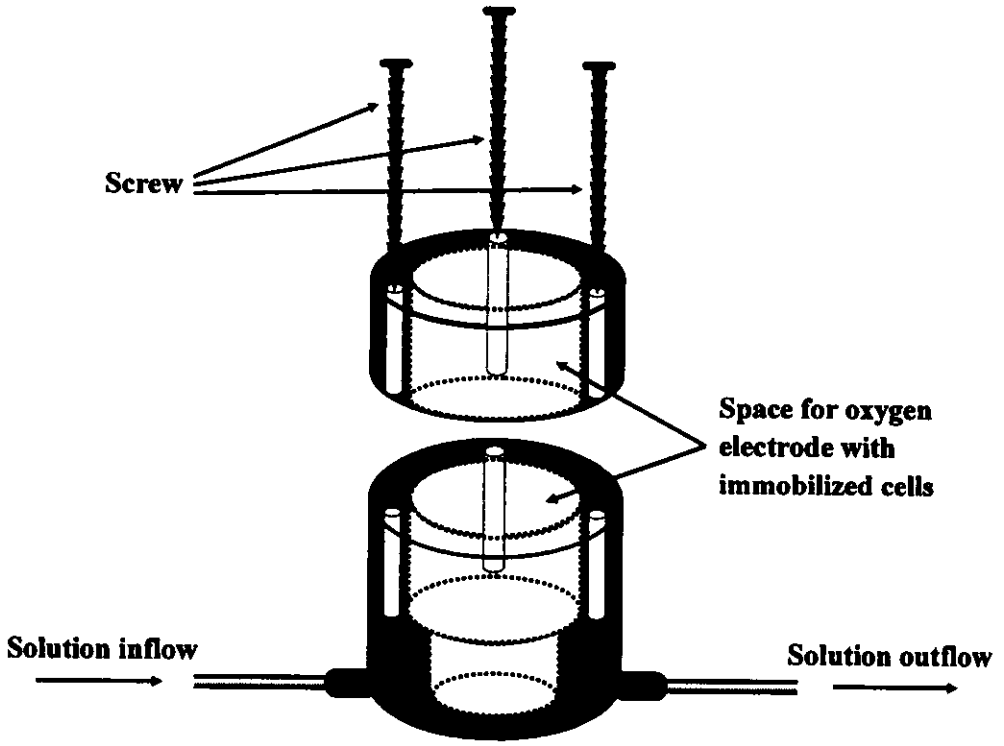
(b)

Figure 12 An immobilized mixed culture bacteria in combination with a Clark type oxygen electrode.

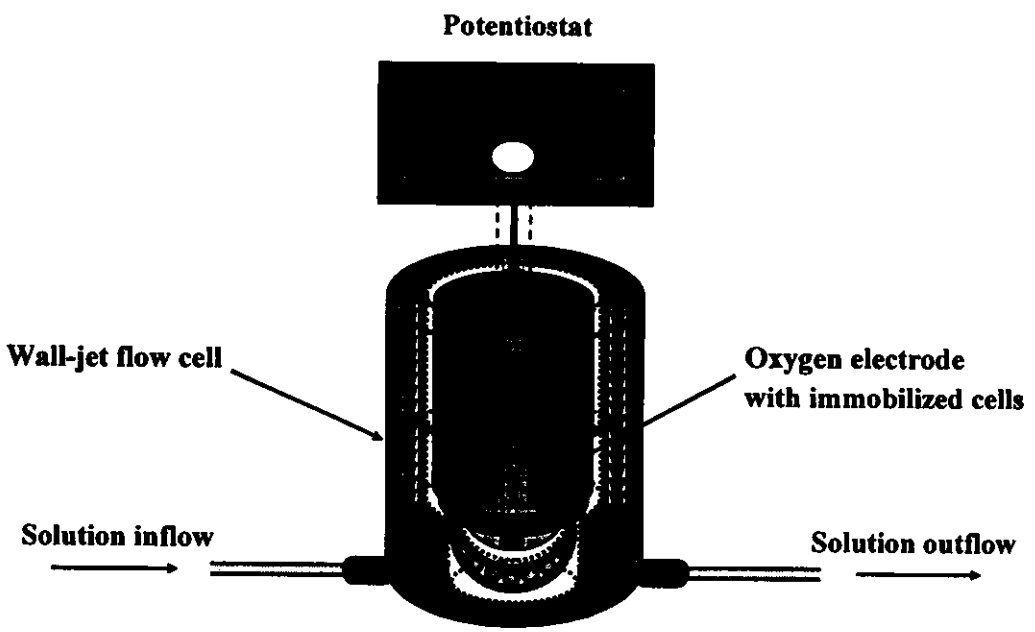
(a) The composition of synthetic microbial membranes

(b) The oxygen electrode covered with synthetic microbial membranes

A Clark type oxygen electrode with immobilized cells was used as a transducer for monitoring of 2,4-dichlorophenol in biosensor system. It was placed in the wall-jet flow cell with a modified cover to hold the sensor (Figure 13) and incorporated in a Flow Injection (FI) system. A hundred millimolar of potassium phosphate buffer (pH 7.50) was used as carrier solution. Before the analysis of 2,4-dichlorophenol, 100 mM of potassium phosphate buffer was passed through the flow cell for 12h to stabilize the sensor. 2,4-Dichlorophenol was then injected as a sample to analyze in a biosensor system.



(a)



(b)

Figure 13 Schematic diagram of the wall-jet flow cell

(a) Without the electrode

(b) With the immobilized cells oxygen electrode

2.4.4 Instrumentations

In this work a flow injection biosensor system was applied to analyze 2,4-dichlorophenol. A Clark type oxygen electrode with immobilized cells was used as a transducer. The principle of an oxygen electrode is a small electrochemical cell consists of two or three electrodes, which are usually combined in a single body. A constant potential is applied to obtain a current response that is related to the concentration of the target analyte. This is accomplished by monitoring the transfer electron(s) during the redox process of the analyte (Wang, 2000).

Clark type oxygen electrode measures the concentration of dissolved oxygen in the solution. The electrode consisted of Pt-cathode and Ag-anode encased in an epoxy block. Both electrodes are contained in a single body. The entire tip of electrode was covered with the synthetic microbial membrane, which contained 1M KCl solution act as electrolyte. Oxygen can be reduced at a cathode according to the following reaction.

$$O_2 + 2H_2O + 4\bar{e} \rightleftharpoons 4OH^-$$

One molar KCl allows the current to flow and silver chloride is formed on the silver anode.

$$Ag^o\,(metal) + Cl^- \rightleftharpoons AgCl + \bar{e}$$

The current depends on the rate of diffusion of oxygen to the cathode and this rate is proportional to dissolved oxygen concentration. The electrode was inserted in a flow cell and connected to a potentiostat. The data was recorded on the chart recorder.

A flow injection biosensor system (Figure 14) consists of

a) A peristaltic pump (U4-MIDI; ALITEA®AB, Stockholm, Sweden), and equipment to aerate the buffer in buffer reservoir, where steady flow rate of the solution was controlled.

b) An injection valve (VICI; Valco, Europe, Schenkon, Switzerland) and an injection loop (Valco), where a specific volume of the sample was injected into the biosensor system through the sample carrier buffer.

c) A detection unit consist of a wall-jet flow cell and an immobilized cells Clark type oxygen electrode, where the sample was utilized by the immobilized cells resulting in an increase of oxygen consumption. The decrease of oxygen concentration in the solution could be detected by the electrode. The detection unit was integrated to the potentiostat (Zäta-Elektronik) where a potential of -600 mV *vs.* Ag/AgCl was applied to Pt-working electrode. The output was recorded on the chart recorder (Kipp & Zonen, Holland).

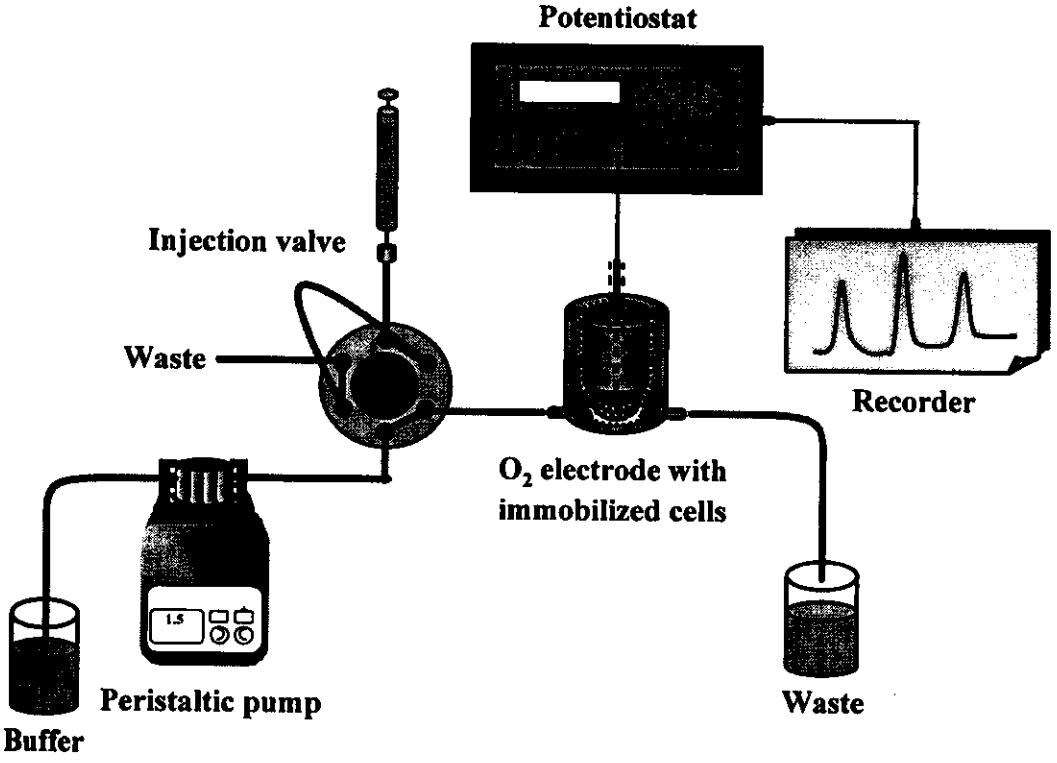


Figure 14 Schematic diagram showing the microbial biosensor by entrapment of mixed culture bacteria on a Clark-type oxygen electrode

2.4.5 Data analysis

A typical recorder output of the flow injection system has the form of a peak (Figure 15), the height (H), width (W), or area (A) of which related to the concentration of the analyte. The time span between the sample injection and the peak maximum (S), which yields the analytical readout as peak height, is the residence time. The time used from peak height until the response come to the baseline is washout time and the time used between injection of sample and a 95% decrease of the flow response, is the analysis time (Jurkiewicz *et al.*, 1998). In this work the peak area was chosen to interpret raw data. For each concentration the average of three injections was obtained.

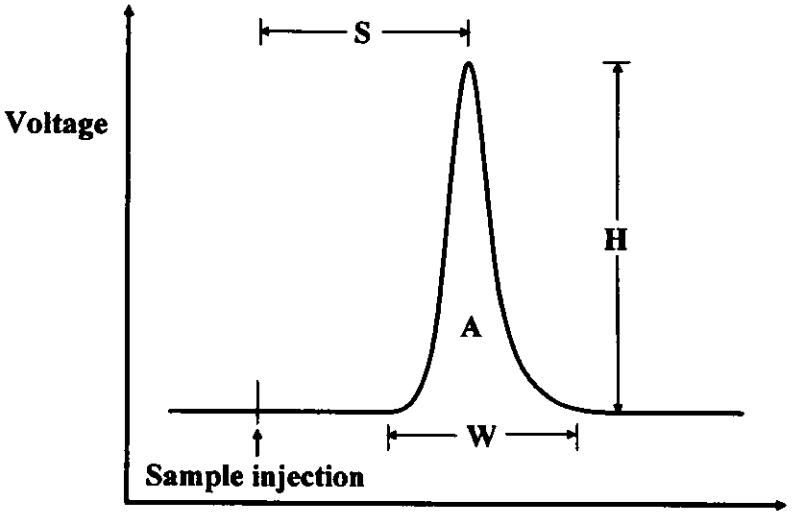
2.4.6 Optimization of operational conditions

In this part the affected parameters were evaluated to obtain the efficient performance of the flow injection biosensor system. Carrier flow rate, sample volume, buffer pH and buffer concentration need to be optimized to yield the highest sensitivity. The measurements were done in triplicate by varying only one parameter and kept other parameters constant. The concentration of standard 2,4-dichlorophenol solution used to test the system were 0.05, 0.10, 0.20, 0.30 and 0.40 mM. They were prepared in 100 mM potassium phosphate buffer, pH 7.50 (except when the buffer pH or concentration was tested). When an optimum condition was obtained, it was used to optimize the next parameter.

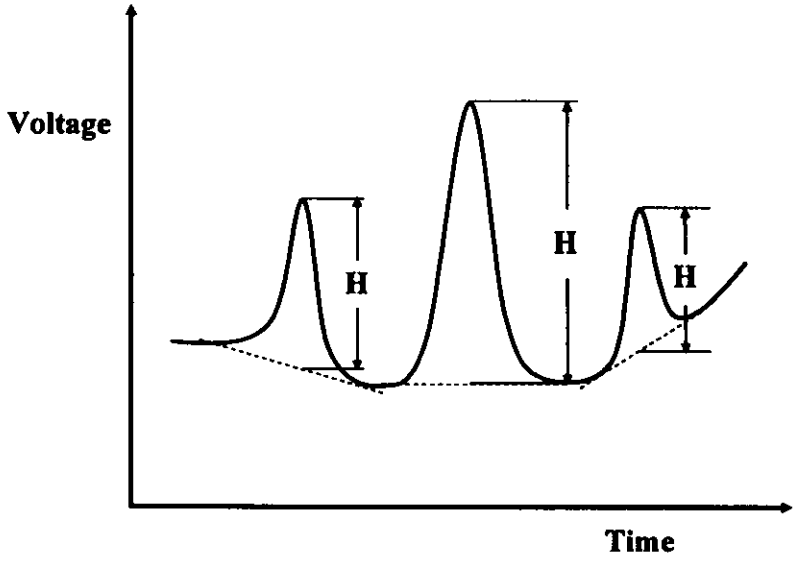
2.4.6.1 Flow rate

In a flow system, the flow rate of the carrier solution passing through the sensor is the main factor affecting the dispersion of the analyte molecules. Increasing the flow rate can reduce dispersion effects and the response time but decrease the yield of the reaction and thereby the sensor response. (Fang, 1993). Therefore, the optimization of flow rate needs to be studied.

The effect of flow rate on the sensor response was studied by performing the experiments at 0.10, 0.20 and 0.30 ml min^{-1}.



(a)



(b)

Figure 15 Flow injection signal:

(a) A typical response of immobilized cells to 2,4-dichlorophenol measured as a direct current voltage signal that is related to 2,4-dichlorophenol concentration.

(b) Peak height measurement

(S-residence time, A-peak area, H-peak height and W-peak width)

2.4.6.2 Sample volume

The response of the biosensor is directed by the amount of oxygen consumed by the mixed culture bacteria, which is itself correlated to the amount of 2,4-dichlorophenol injected. The amount of 2,4-dichlorophenol then depends on the sample volume and the concentration in the sample. By increasing the sample volume, the reaction time in the reaction flow cell of the analyte was prolonged. Thereby, larger sample volume normally produces higher sensor response (Liu *et al.*, 2004). Therefore, to improve the sensor sensitivity and detection limit, the optimization of sample volume was investigated. The influence of sample volume was tested by injecting 10 µl, 25 µl, 50 µl and 100 µl of standard 2,4-dichlorophenol.

2.4.6.3 Buffer pH

In general, the optimum pH for growth of bacteria is around 7, although some may be obligately acidophilic and thrive at pH below 2. Bacterial growth generally results in a decrease of the pH of the medium by the release of acidic metabolites. Conversely, some microorganisms can increase the pH of their surrounding environment. The pH affects the activity of microbial enzymes and the ionization of chemicals and, thus, plays a role in the transport of nutrients and toxic chemicals into the cell (Bitton, 1994).

Catalytic activities of the enzymes involve in 2,4-dichlorophenol catabolism are a function of the pH. This parameter can potentially affect the sensor response. Therefore, it is necessary to determine the optimum pH of potassium phosphate buffer solution that will result in the maximum sensitivity of the microbial biosensor. The effect of buffer pH was studied by varying the pH of 100 mM of potassium phosphate buffer used as carrier solution. The study values were 6.50, 7.00, 7.50 and 8.00.

2.4.6.4 Buffer concentration

The effect of buffer concentration has to be examined because buffer species can exert effects on biological system in three main ways. They may specifically stimulate or depress enzyme activity. They may interfere or react with substrates, inhibitor or cofactor. They may exert effects because of their ionic strength

(Perrin and Dempsey, 1974). However, at a low buffer concentration, the system is low ionic strength. Therefore, the optimum concentration of working buffer was also investigated by testing four different potassium phosphate buffer concentrations, 10, 30, 50 and 100 mM.

The optimized parameters and values are summarized in Table 1.

Table 1 Assayed and optimized values of the operational conditions of microbial biosensor (Swedish mixed culture bacteria).

Conditions	Optimized values
1. Flow rate (ml min^{-1})	0.10, 0.20 and 0.30
2. Sample volume (μl)	10, 25, 50 and 100
3. Buffer pH	6.50, 7.00, 7.50 and 8.00
4. Buffer concentration (mM) (Potassium phosphate buffer)	10, 30, 50 and 100

2.4.7 Linearity

Setting the flow rate, sample volume, buffer pH and buffer concentration at optimum conditions, standard solutions of 2,4-dichlorophenol from 0.01 mM to 0.80 mM were injected into the sensor system. The linearity of the response of immobilized mixed culture bacteria on a Clark type oxygen electrode was evaluated from the calibration curve.

2.4.8 Stability

The stability of the sensor related to the stability of the immobilized cells. In this work, the mixture of different type of microorganisms was used, therefore, complex patterns of the mixed culture behaviour must be taken into consideration. The possible patterns of behaviour of two species in a homogeneous culture might include: (1) the influence of the same/different growth-limiting substrate concentration(s) on the specific growth rate of each species, which represents the rate of growth per unit amount of biomass, (2) product of one species is substrate for the others, (3) inhibitory product of one species is the limiting substrate

of the other species. In heterogeneous microbial culture, more complex effects can occur. Consequently, the constituent of microbial mixed cultures may vary with time, which results in unstable sensor performance of the multi-strains based sensor after a certain period of operation (Liu and Mattiasson, 2002). Furthermore, during sensor storage, the immobilized cells would start to lyse and the lysis rate might vary in different microbial strains. The oxygen consumption for various strains could also be different even for assimilation of the same organic substrate. These can result in a decrease of sensor sensitivity and poor stability of sensor response (Liu *et al.*, 2000). Therefore, a stable sensor performance over a desired operational period is essential for a reliable biosensor system.

Under optimum conditions, standard solutions of 2,4-dichlorophenol, 0.02, 0.05, 0.10, 0.15, 0.20 and 0.30 mM, were injected into the sensor system everyday. The operational stability of the immobilized mixed culture bacteria was investigated by evaluating the sensitivity of the same electrode to 2,4-dichlorophenol.

2.4.9 Response characteristic for other compounds

A common metabolic step of aerobic organisms consists of the consumption of oxygen and addition of a hydroxyl group to the phenol structure. The catechol type compound generated is a common metabolic step for biodegradation of phenolic compounds and has also been shown for biodegradation of benzene rings as well as biphenyls. Substitution of two hydroxyl groups, ortho to each other, allows enzymatic ring fission to occur, yielding organic acids (Autenrieth *et al.*, 1991). These reasons can indicate that mixed culture microbes can also assimilate other aromatic compounds. Moreover, glucose is commonly reported to be the prefer substrate. When glucose present in the mixture, it was always consumed first by mixed culture microbes (Autenrieth *et al.*, 1991). The selectivity of microbial biosensors is known to be generally rather low. Therefore, microbial biosensor seemed important to characterize the substrate selectivity of sensor. (Skládal *et al.*, 2002).

The response characteristic of the sensor to different phenolic compounds and generic substrates was studied. This was done by injecting 100 µl of solution containing 0.10 mM of the target substance and 0.10 mM of 2,4-dichlorophenol. The sensor response from the mixture of target substance and 2,4-

dichlorophenol was compared to the sensor response of 2,4-dichlorophenol and calculated as the increase (%) of sensor response. The target substances in this study were phenol, 2-monochlorophenol, 4-monochlorophenol, 2,4,6-trichlorophenol, glucose, sucrose, sodium acetate and citric acid.

2.5 Thai mixed-culture microbes

2.5.1 Biodegradation of 2,4-dichlorophenol

In this study, the activated sludge from wastewater treatment ponds of Songklanagarind Hospital, Prince of Songkla University, was collected to study the biodegradation of 2,4-dichlorophenol (Figure 16). The sludge was acclimated by using 2,4-dichlorophenol as a sole source of carbon and energy. After the culture reached the exponential growth phase, it was used as an inoculum for biosensor preparation.

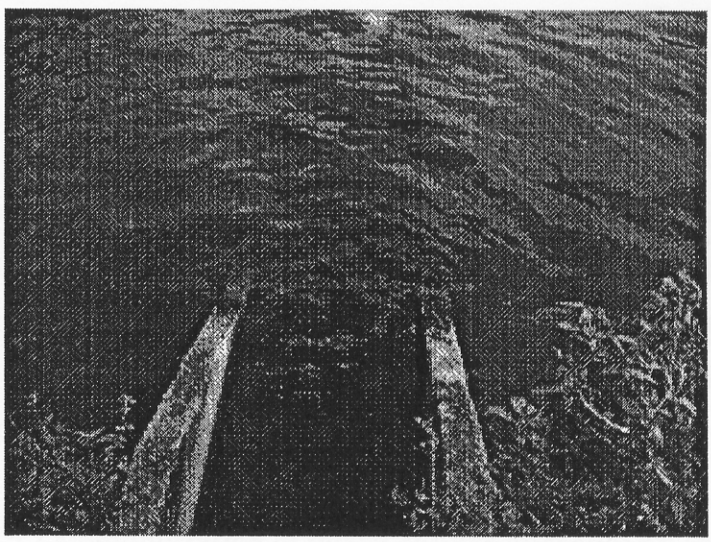


Figure 16 Picture showing the sampling site from wastewater treatment ponds of Songklanagarind Hospital, Prince of Songkla University

2.5.1.1 Culture conditions

The study was carried out to evaluate the maximum concentration of 2,4-dichlorophenol for the cultivation. After first acclimation of the activated sludge with 30.0 mg l^{-1} of 2,4-dichlorophenol, the mixed culture microbes was taken to be enriched and study the microbial growth kinetics at the concentration of 20.0, 30.0,

40.0, 50.0 and 60.0 mg l^{-1}. Twenty milliliters of culture was inoculated into MSM liquid growth medium supplied with 2,4-dichlorophenol in 250 ml conical flasks. The flasks were then agitated at 170 rpm on a rotary shaker at room temperature (25 ± 2°C). During the cultivation, the conical flasks were kept in the dark by aluminum foil wrapping to prevent the photodegradation of 2,4-dichlorophenol.

The culture was evaluated for the microbial growth kinetics in aerobic condition. Growth of microbial population was defined as a decrease in 2,4-dichlorophenol concentration, assimilated by mixed culture microbes. The decrease can be determined by UV spectrophotometry technique. The absorbance at 285 nm of two milliliters of culture samples were measured by a spectrophotometer (Specord S 100, Analytikjena, Germany). The maximum concentration of 2,4-dichlorophenol that could be assimilated by the mixed culture bacteria, was determined and used for further cultivation for biosensor preparation.

2.5.1.2 Preparation of mixed culture microbes for immobilization

The mixed culture microbes were taken from the first enrichment culture used for 2,4-dichlorophenol biodegradation. Twenty milliliters of the culture was inoculated into 50 ml of MSM growth medium supplied with 60.0 mg l^{-1} of 2,4-dichlorophenol (best condition obtained from the above experiment) and agitated at 170 rpm at room temperature (25 ± 2°C). To prevent the photodegradation of 2,4-dichlorophenol, the culture flasks were wrapped by aluminum foil. When the culture reached the log phase (4 days), it was harvested.

2.5.2 Immobilization of mixed culture microbes by entrapment

Entrapment of cells in alginate is one of the simplest, cheapest, non-toxic and the most frequently used method of immobilization. Sodium alginate and calcium chloride were used to prepare the alginate beads containing the whole cells.

Sodium alginate solution (3% w/v) was prepared by dissolve 0.3 g sodium alginate in 5 ml distilled water. The contents were stirred vigorously for 20 minutes to obtain thick uniform slurry without any undissolved lumps. A hundred milliliter of mixed culture microbes was harvested by centrifugation at 6000 rpm for 10 minutes. The solution on the top part was discarded and the remaining 5 ml of cell

solution was mixed with alginate slurry and stirred for 5 minutes to obtain a uniform mixture. The slurry was drawn into a syringe and added drop-wise into 250 ml of 0.2 M $CaCl_2$ solution in a suction flask, connected to a suction pump (Figure 17) and 1-2 mm diameter of alginate gel beads with immobilized cells were obtained. The alginate beads in 0.2 M $CaCl_2$ were gently stirred for 10 minutes and kept for curing at 4°C for 1 hour. The cured beads were washed with 100 mM tris-HCl containing 10 mM $CaCl_2$ (pH 6.50) 3-4 times. When the beads were not used, they were preserved in the same buffer in a refrigerator (Adinarayana *et al.*, 2004).

To construct a biosensor for monitoring 2,4-dichlorophenol, 5.20 g of the immobilized cells in alginate beads was packed in a small plastic column (3.5 cm × 0.5 cm). Membranes were used to cover the ends of the reaction column to retain the beads (Figure 18). The packed column was then placed in a flow injection system to determine 2,4-dichlorophenol by measuring the amount of oxygen in the solution, which changed with the microbial activity. A hundred millimolar of tris-HCl buffer containing 10 mM $CaCl_2$ (pH 6.50) was used as carrier solution. The buffer was passed through the column for 12 hours for stabilization, before 2,4-dichlorophenol was analyzed.

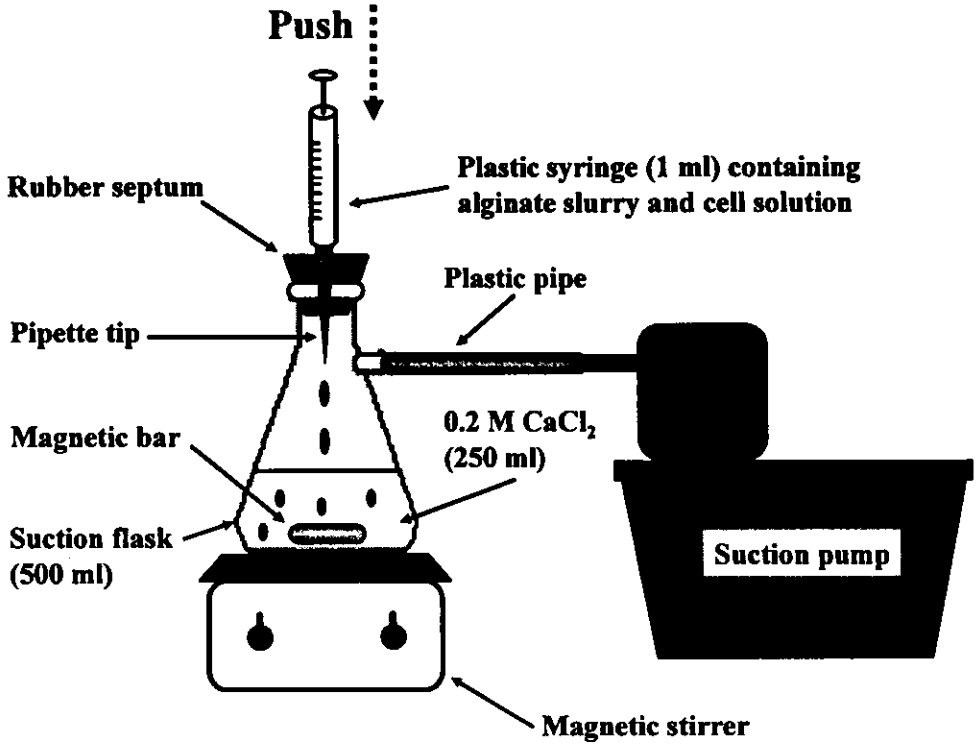


Figure 17 Schematic diagram showing the equipment set up for immobilization

2.5.3 Instrumentation

The sensor for 2,4-dichlorophenol used a Clark type oxygen electrode (YSI Model 5331 Oxygen electrode, Yellow Spring Instrument Company, USA) as a transducer connected to a monitor (YSI Model 5300 Biological Oxygen Monitoring) (Figure 19). The electrode consisted of a Pt-cathode, 0.63 cm in diameter, and Ag-anode encased in an epoxy block. Both electrodes are contained in a single body. The entire tip of electrode was covered with a gas-permeable membrane, which contained 1.5 M KCl solution.

The flow injection biosensor system (Figure 20) consists of

a) A peristaltic pump (Minipulse3, Gilson, France) and equipment to aerate the buffer in buffer reservoir, where steady flow rate of the solution was controlled.

b) An injection valve (VICI; Valco, USA), where a specific volume of sample was injected into the biosensor system through the sample carrier buffer.

c) A reaction unit, an immobilized cells reactor, where the analyte was assimilated by the mixed culture bacteria resulting in the change of oxygen in the solution.

d) A detection unit consists of an oxygen electrode inserted into a flow cell in the middle of a water insulator jacket and connected to an oxygen monitor (Figure 21). The electrode monitored the change in oxygen of the solution due to the microbial activity and recorded the output signal on a chart recorder (Ross recorder model 202, USA). The water insulator jacket was used to control the temperature of the oxygen electrode.

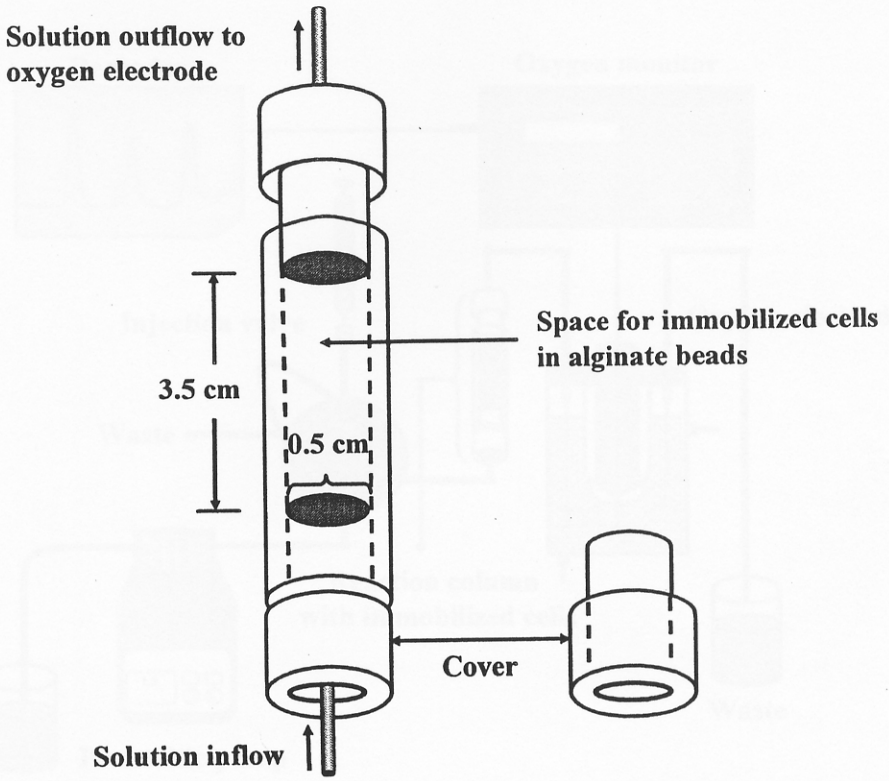


Figure 18 Schematic diagram of the column

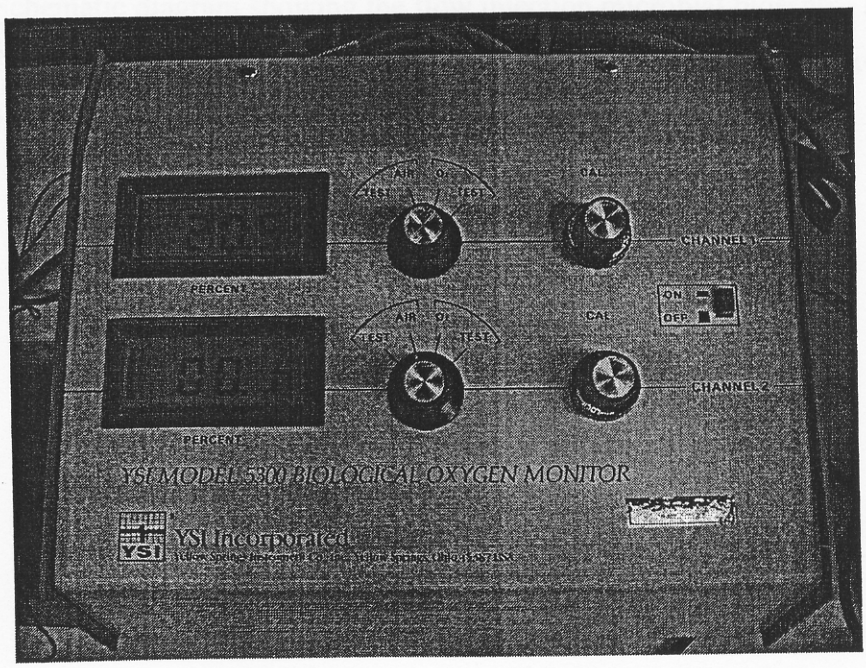


Figure 19 Picture showing the YSI oxygen monitor

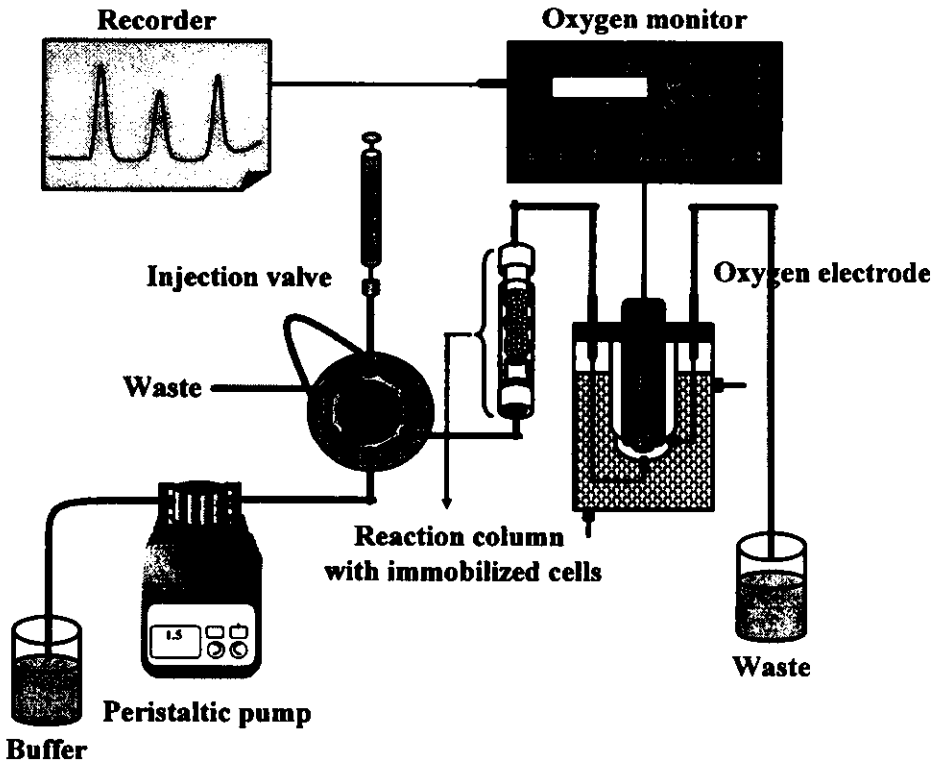


Figure 20 Schematic diagram showing the flow injection microbial biosensor system. The entrapped mixed culture microbes in alginate beads were packed in a plastic reaction column.

2.5.4 Data analysis

In this work peak height was chosen to interpret the raw data since it was easily identified and directly related to the detector response. The peak height value (H) was measured as the distance from baseline to peak maximum (Figure 15 (a)). Baseline drift can be corrected by interpolating the baseline between the start and finish of the peak (Figure 15 (b)) (Snyder and Kirkland, 1979). For each concentration the average of three injections was obtained.

2.5.5 Optimization of operational conditions

The affecting parameters of the microbial flow injection biosensor for 2,4-dichlorophenol were studied to obtain a good performance system. Carrier flow rate, sample volume, buffer pH and buffer concentration were optimized. The measurements were done in triplicate by varying only one parameter and kept other parameters constant. The standard 2,4-dichlorophenol solutions were prepared in 100 mM Tris-HCl buffer containing 10 mM $CaCl_2$, pH 6.50 (except when the buffer pH or concentration was tested). When an optimum condition was obtained, it was used to optimize the next parameter.

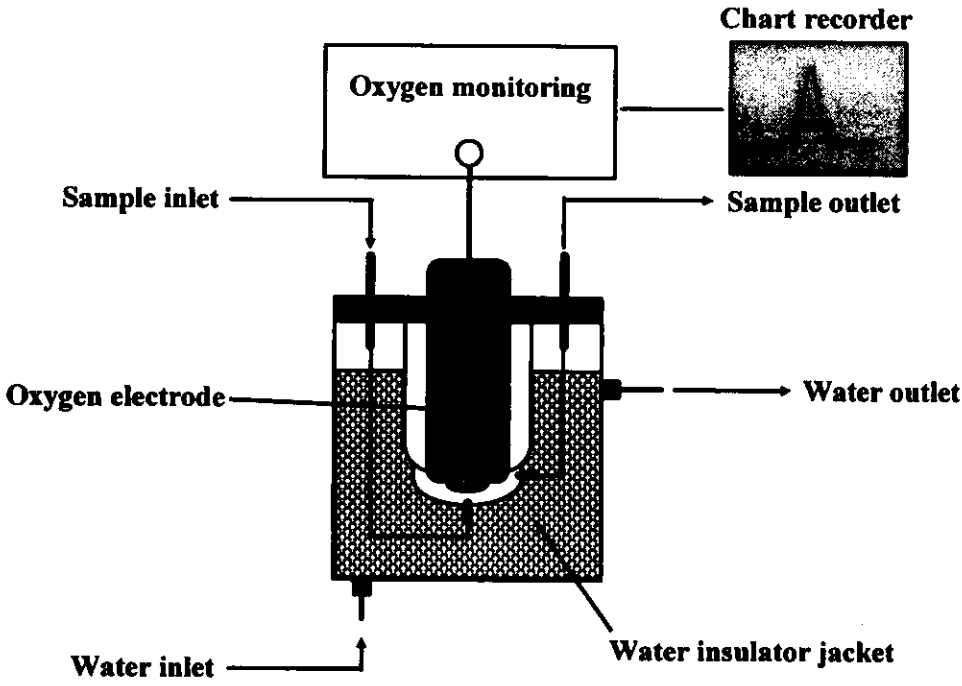


Figure 21 Schematic diagram of the microbial biosensor detection unit

2.5.5.1 Flow rate

In a flow injection system, the flow rate of the carrier solution passing through the reaction column and the detector was investigated at 0.10, 0.25 and 0.50 ml min^{-1}. A hundred millimolar of tris-HCl buffer (pH 6.50) containing 10 mM $CaCl_2$ was used as a carrier stream.

2.5.5.2 Sample volume

The optimization of sample volume was studied to improve the limit of detection of the sensor. The influence of sample volume was tested by injecting 300, 400 and 500 µl of 0.50-30.0 mg l^{-1} standard 2,4-dichlorophenol.

2.5.5.3 Buffer pH

Since the catalytic activities of the enzymes in the cell involve in 2,4-dichlorophenol catabolism are a function of pH, the optimization of buffer pH was evaluated by varying the pH of 100 mM of tris-HCl buffer (containing 10 mM $CaCl_2$)

from 6.50, 7.00 and 7.50. Standard 2,4-dichlorophenol solutions, 0.30-5.00 mg l^{-1}, were injected into the sensor system.

2.5.5.4 Buffer concentration

The effect of working buffer concentration was tested by varying the concentration of tris-HCl buffer, pH 6.50, containing 10 mM $CaCl_2$ from 50, 100, 150, 200 and 250 mM. Five mg l^{-1} of 2,4-dichlorophenol was injected into the sensor system.

The optimized parameters and values are summarized in Table 2.

Table 2 Assayed and optimized values of the operational conditions of microbial biosensor (Thai mixed culture microbes).

Conditions	Optimized values
1. Flow rate (ml min^{-1})	0.10, 0.25 and 0.50
2. Sample volume (µl)	300, 400 and 500
3. Buffer pH	6.50, 7.00 and 7.50
4. Buffer concentration (mM) (Tris-HCl buffer containing 10 mM $CaCl_2$)	50, 100, 150, 200 and 250

2.5.6 Linearity

Setting the flow rate, sample volume, buffer pH and buffer concentration at optimum conditions, standard solutions 2,4-dichlorophenol from 0.001 to 65.0 mg l^{-1} were injected into the sensor system. The linearity of the response of immobilized cells in a reaction column was investigated from the calibration curve.

2.5.7 Stability

The operational stability of the immobilized mixed culture microbes was studied by evaluating the sensitivity of the same reaction column to 2,4-dichlorophenol. Under optimum conditions, standard solutions of 1.0, 10.0, 20.0 and 30.0 mg l^{-1} 2,4-dichlorophenol were injected into the sensor system for 5 days.

2.5.8 Response characteristic for other compounds

The response characteristic of the sensor to different phenolic compounds, aromatic compounds, intermediate products and generic substrates was studied. This was done by injecting 500 µl of solution containing 9.0 mg l^{-1} of 2,4-dichlorophenol and 9.0 mg l^{-1} of target substances. The mixture of target substances with 2,4-dichlorophenol in the sample at 9.0 mg l^{-1} were also injected into the sensor system. The sensor response from 2,4-dichlorophenol and target substances were combined and compared to sensor response of the mixture.

2.5.9 Repeatability

The repeatability of microbial biosensor for 2,4-dichlorophenol was tested on one packed reaction column of immobilized cells in alginate beads (5.20 g) by injecting 500 µl of 7.0 mg l^{-1} 2,4-dichlorophenol for eight times. The average value, standard deviation (S.D.) and relative standard deviation (%RSD) were calculated.

2.5.10 Reproducibility

The mixed culture microbes from same batch culture was divided into 4 packed columns (5.20 g each). The reproducibility of the columns for 2,4-dichlorophenol was examined. Measurements were performed by injecting 500 µl of 7.0 mg l^{-1} 2,4-dichlorophenol into the sensor system. The average sensor response and standard deviation (S.D.) of each packed reaction column were calculated.

2.5.11 Determination of chlorophenols in wastewater

To demonstrate the use of the developed microbial biosensor system, it was tested using wastewater sample obtained from wastewater treatment pond of Songklanagarind Hospital, Prince of Songkla University (Figure 16). One water sample was collected from the pond prior to the aeration treatment. The physical and chemical properties of the water sample, *i.e.* pH, dissolve oxygen, conductivity and temperature were measured. Water sample (1000 ml) was collected in a clean PTFE bottle and analyzed immediately by the cell-based biosensor system and gas chromatography/mass spectrometry (GC/MS).

2.5.11.1 Cell-based biosensor system

Before the measuring of chlorophenols by microbial biosensor system, the wastewater sample was filtered through a 0.22 μm nylon membrane and then diluted 100 times with 100 mM tris-HCl buffer (pH 6.50) containing 10 mM $CaCl_2$.

To calibrate the sensor system, standard 2,4-dichlorophenol solution, 1.0, 10.0, 20.0 and 30.0 mg l^{-1} prepared in 100 mM tris-HCl buffer, pH 6.50, containing 10 mM $CaCl_2$ were injected. The flow rate was 0.10 ml min^{-1} and the sample volume was 500 μl. The calibration curve was prepared by plotting the sensor response vs. corresponding 2,4-dichlorophenol concentration. The sample solution was then injected into the sensor system. The experiments were done in triplicate and the average sensor response and standard deviation (S.D.) were calculated.

2.5.11.2 Gas chromatography/mass spectrometry

The wastewater sample was filtered through a 0.22 μm nylon membrane and extracted by ultrasonic extraction for 20 minutes. Dichloromethane (methylene chloride, CH_2Cl) was used as a solvent. The ratio between solvent to sample was 1: 2 (2 ml of sample and 1 ml of solvent).

A gas chromatograph model HP-5890 with HP-5973 mass selective detector (Agilent Technologies, USA) was used. Separation of chlorophenols was carried out using a capillary column HP-5, (30 m × 0.25 mm I.D.) with 0.25 μm film thickness. The injector and detector temperatures were set at 250 and 280 °C, respectively. Helium was used as a carrier gas with the flow rate of 1 ml min^{-1}.

The oven temperature was programmed as follows: 55 °C for 3 min, at 20 °C min^{-1} to 230 °C, 5 min hold at 230 °C. An aliquot of 2 µl from each sample was introduced into GC/MS as splitless injection. The analysis was carried out on SIM mode (Selective Ion Monitoring). The retention time was 9.46 min and *m/z* were 63 and 162 (Bagheri and Saraji, 2003).