CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

A large variety of chemicals are commercially produced and newly synthesized every year. During their manufacture and use, these xenobiotics are often discharged into the environment. Many of them degrade slowly and exert toxic effects on plants and animal, thus, causing large scale environmental degradation. One such group of xenobiotics is chlorophenols (Annachhatre and Gheewala, 1996; Farrell and Quilty, 2002), which is an important class of pollutants.

Chlorophenols are synthetic organic compounds, obtained from industrial processes by chlorinating phenol or hydrolyzing chlorobenzenes (Czaplicka, 2004). They are widely used in industry (Armenante *et al.*, 1999). The fate of chlorinated phenolic compounds in the environment is of great importance as they are toxic, recalcitrant and bioaccumulating (Jianlong and Yi, 1999). The recalcitrance of chlorophenols results from the carbon-halogen bond, which is cleaved with great difficulty, and the stability of their aromatic structure, resulting in their accumulation in nature (Farrell and Quilty, 2002). Because of improper treatment of these compounds, they have widely contaminated soil and groundwater and their toxicity has seriously affected living organisms (Kim *et al.*, 2002).

Because of their high toxicity, carcinogenicity and persistence, some chlorophenols (2-chlorophenol, 2,4-dichlorophenol, 4-chloro-3-methylphenol, 2,4,6-trichlorophenol and pentachlorophenol (PCP)) have been designated as priority environmental pollutants by the United States Environmental Agency (EPA, 1999). Furthermore, the International Agency for Research on Cancers (IARC) classified five chlorophenols (PCP, 2,3,4,6-tetrachlorophenol, 2,4,6-trichlorophenol, 2,4,5-trichlorophenol and 2,4-dichlorophenol) as belonging to the 2B group of possible human carcinogens (Czaplicka, 2003). Therefore, determination of chlorophenolic compounds is required.

Current analytical methods for the detection and quantification of chlorophenols are usually based on analytical separation techniques. The detection of chlorinated phenol is usually performed by means of chromatography and spectrometry. However, these techniques do not easily allow continuous on-site monitoring, are expensive, time consuming, need skilled operators, and sometimes require preconcentration steps. The application of biosensor is favorable due to some generally claimed advantages such as the relatively low-cost of realization, sometimes the good storage stability, the potential for miniaturization, and for the construction of simple portable device for fast screening purposes and in-field/on-site monitoring (Dzyadevych *et al.*, 2002; Timur *et al.*, 2003).

In this work, amperometric biosensor using mixed culture bacterial cells was developed for the detection of chlorophenols. The mixed culture bacteria, which is able to degrade chlorophenols, was used as a biological sensing element. The measurement was based on the respiratory activity of the cells. The cells were acclimated in the presence of chlorophenols as a sole source of carbon and energy and 2,4-dichlorophenol, which was classified as priority pollutant (US EPA) and carcinogen (IARC), was chosen to represent other chlorophenols in this study.

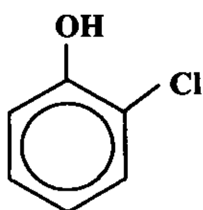
1.2 Review of literature

1.2.1 Chlorophenols

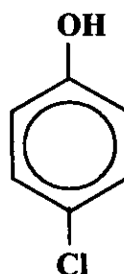
The large scale synthesis of industrially important hydrocarbons and chlorinated organic has occurred only during the past few decades. Synthetic hydrocarbons, chlorinated organics and many of their end-products represent one class of xenobiotic and persistent compounds. Many of these organic chemicals exhibit not only acute toxicity but also chronic toxic effects including mutagenic, carcinogenic, and teratogenic manifestations. Synthetic organic compounds are commonly found in industrial wastewater streams and can produce toxic upsets in wastewater treatment units. The resulting decrease in treatment efficiency may allow substantial amounts of these compounds to be discharged in effluents or concentrated in generated sludge. Chlorophenols is a group of the main products from such industry (Autenrieth *et al.*, 1991).

For the family of chlorophenols their basic structures consist of the benzene ring, -OH group and atom of chlorines. Together with 19 main compounds, chloroderivatives of methyl- and ethyl-phenol are also considered as chlorophenols. Only eight of chlorophenols have been used by the industry, *i.e.*, monochlorophenols, 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,4,5-trichlorophenol, 2,3,4,5-tetrachlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol (Figure 1). All chlorophenols, except 2-chlorophenol, are solids, with their melting points between 33 and 191 °C. In general, these compounds dissolve weakly in water, but well in organic solvents. Their water solubility decreases with increasing number of chlorine atoms in a molecule (Czaplicka, 2004).

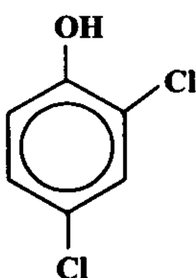
Chlorophenols can be generated from various industrial processes. Monochlorophenols can be formed during wastewater chlorination, and as a result of the breakdown of pesticides and chlorinated aromatic compounds (Armenante *et al.*, 1999). They are formed during the bleaching of pulp with chlorine. Microbial degradation of herbicides, especially of 2,4-dichlorophenoxyacetic acid or 2,4-D (Autenrieth *et al.*, 1991) (Figure 2), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and pesticides, yields numerous chlorophenols as intermediate metabolites of their decomposition. Chlorophenols with one chlorine atom have been used in production of plant protecting agent or as antiseptics (Czaplicka, 2004). Aqueous effluents from industrial operations such as polymeric resin production, oil refining, coking plants, textile and pharmaceutical also contain chlorophenolic compounds (Annachhatre and Gheewala, 1996; Jianlong and Yi, 1999). Other source of chlorophenols in the environment is the natural reactions of humic acid chlorination (Czaplicka, 2004).



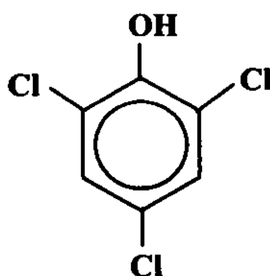
2-chlorophenol



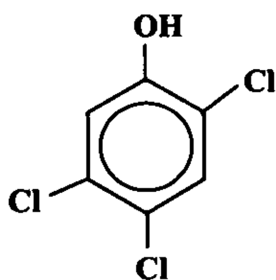
4-chlorophenol



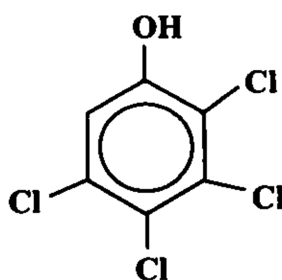
2,4-dichlorophenol



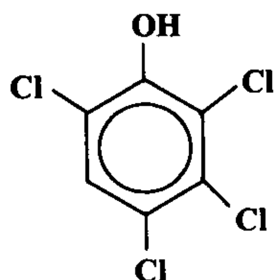
2,4,6-trichlorophenol



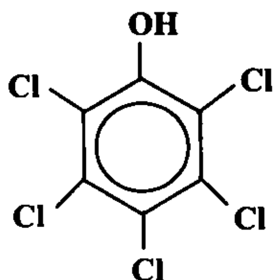
2,4,5-trichlorophenol



2,3,4,5-tetrachlorophenol



2,3,4,6-tetrachlorophenol



Pentachlorophenol

Figure 1 Structure of chlorophenols used in industry

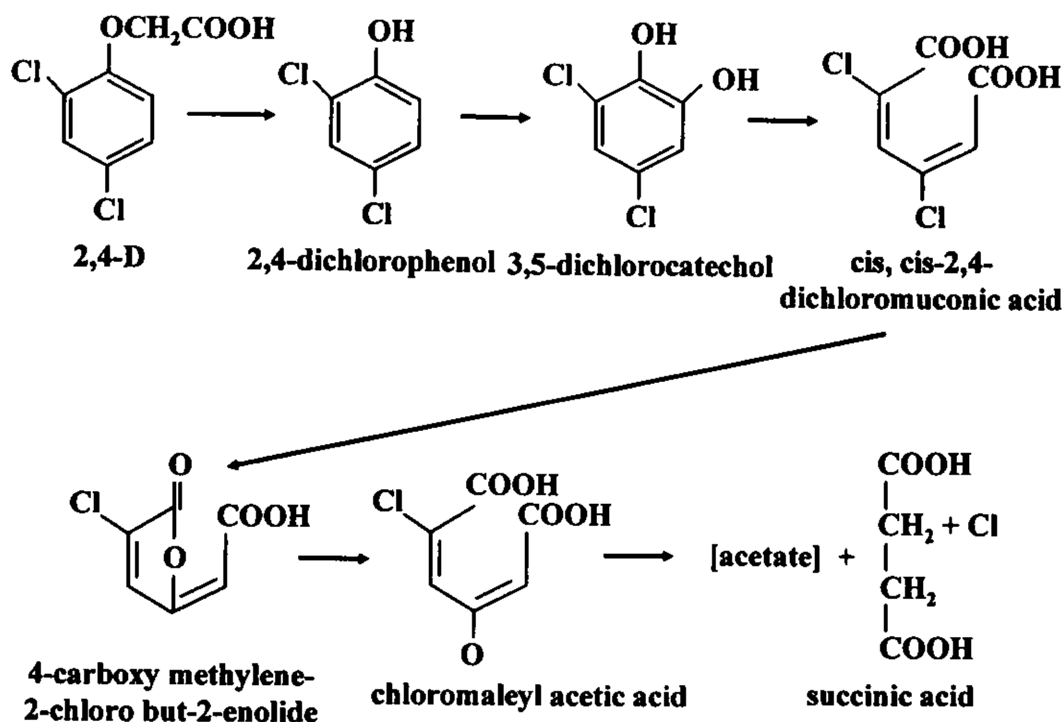


Figure 2 Degradative pathway of 2,4-D and 2,4-dichlorophenol (Modified from Autenrieth *et al.*, 1991)

Chlorophenols are one of the major pollutants, which have high toxicity to human when present above concentration limits. Toxicity of chlorophenols depends on the degree of chlorination and the position of chlorine atoms relative to the hydroxyl group. It decreases with the number of chlorine substituents. These facts may suggest that pentachlorophenol (PCP) is more toxic than other chlorophenols. It was also observed that chlorophenols with the chlorine atom at position 2- are less toxic than other chlorophenols. If chlorine atoms are substituented at 3-, 4- and 5-positions, toxicity of chlorophenols increases (Czaplicka, 2004). Possible routes of human exposure to chlorophenols are inhalation, ingestion, eye and dermal contact. The effect of chlorophenols to human exposure depends on exposure dose, duration, personal traits and habit and interactions with other chemicals present. The exposure to 2,4-dichlorophenol (DCP) can cause death, respiratory failure, bone marrow atrophy, and skin damage in animals. 2,4-dichlorophenol can also confer a distasteful taste and odor of food and water (Yee and Wood, 1997).

Since the basic structure of chlorophenols is benzene ring, it is more stable than single-chain (aliphatic) compounds. They require larger amount of energy to break apart a cyclic structure (Autenrieth *et al.*, 1991). This reason may be the cause of the persistent of these compounds in the environment. Because of their recalcitrance, bioaccumulation in the environment, toxic and health risk, this increase concern has led to the development of appropriate methods for their monitoring (Antonopoulos *et al.*, 2001).

1.2.2 Determination methods

Several analytical procedures are used to quantitate phenolic compounds. A standard colorimetric procedure based on the reaction of phenolic compounds with 4-aminoantipyrine (4-AAP) is frequently used for determination of total phenolic compounds in water samples (EPA method 9065). This method works well for the parent phenol but it cannot detect *para*-substituted phenols since they do not react with 4-aminoantipyrine (Satienperakul *et al.*, 2003). To overcome this problem other techniques based on chromatographic method were developed.

1.2.2.1 Gas chromatography

Gas chromatography (GC) in conjunction with various detection devices has been developed for monitoring phenolic compounds. The most commonly used detection device among gas chromatography detector is mass spectrometry (MS). The approach has the advantage of high sensitivity and selectivity, and the existence of mass spectra libraries for screening of unknown samples (Puig and Barceló, 1996). Flame ionization detection (FID) (Faraji, 2005) and electron capture detection (ECD) (Bagheri and Saraji, 2001) have also been investigated for the capability to determine phenolic compounds with derivatization step. In general, phenolic compounds are amenable to gas chromatography without derivatization. However, at lower concentration, peak tailing and discrimination in the injector of capillary column might occur (Bagheri and Saraji, 2001). To avoid this drawback, several derivatization reactions have been proposed to transform phenolic compounds to less polar compounds (Rodríguez *et al.*, 2000) by using pentaflourobenzoyl, heptaflourobutyric anhydride, diazomethand and acetic anhydride as derivatizing

agents. Some of the derivatizing agent (diazomethane) has potential hazards associated with its use (carcinogenic and explosive) (Puig and Barceló, 1996). Therefore, phenol acetylation with acetic anhydride in presence of carbonate or hydrogencarbonate, is one of the most studied derivatization procedures (Bagheri and Saraji, 2001).

To determine phenolic compounds at low concentration, it is necessary to apply a preconcentration step prior to the chromatographic analysis, and is usually based on liquid-liquid extraction (LLE). However, there is a general trend to change these procedures to liquid-solid extraction (LSE) to avoid the use of large amounts of toxic organic solvents (Puig and Barceló, 1996). Nowadays, solid-phase extraction (SPE) is the most common technique for sample enrichment because of its advantages over liquid-liquid extraction. Solid-phase extraction is well adapted to the handling of large water samples. The analytes are retained on the sorbent while the water passes through, and are later recovered by elution with a small volume of organic solvent (Hennion, 1991). Silica sorbent, polymeric materials and carbon are normally used as solid-phase extraction sorbent for phenolic compounds (Rodríguez *et al.*, 2000).

Chlorophenols determination by using solid-phase extraction couple to gas chromatography has been proposed. The off-line preconcentration of sixteen chlorophenols were developed by using graphite carbon black and cross-linked styrene-divinylbenzene as SPE sorbents. Acetylation procedure was performed separately from the SPE extraction. The developed method showed good detection limit and recoveries. However, extraction with cross-linked styrene-divinylbenzene has some advantages over graphite carbon black. In fact cross-linked styrene-divinylbenzene can preconcentrate larger sample volume in a shorter time and use smaller amounts of acetylating reagents (Rodríguez *et al.*, 1997). Recently, solid-phase analytical derivatizations have been reported to simplify the derivatization step by applying a derivatization on an SPE cartridge. Chlorophenols were desorbed from SPE cartridge after derivatization with pentafluoropyridine and introduced to gas chromatography-mass spectrometry. By applying this method, the matrix problem can be removed and total analytical procedure is quite simple (Kojima *et al.*, 2004).

Although gas chromatographic methods have the advantage of high sensitivity, the techniques required many steps, *i.e.* derivatization and preconcentration.

1.2.2.2 Liquid chromatography

Liquid chromatography (LC) has also been frequently used for the analysis of phenolic compounds, unlike gas chromatography no derivatization of compounds is needed (Peñalver *et al.*, 2002). Liquid chromatography can be used on-line by coupling it with liquid-solid extraction. C_{18} and C_8 columns are commonly applied in the separation step for the analysis of phenolic compounds. The most common detector is Ultraviolet (UV) detector and detection is usually carried out at 280 nm, except for nitrophenols and pentachlorophenol, which show a better signal at 310 nm. Diode array detectors (DAD) are recommended because even though little decrease in sensitivity occurs, spectral libraries can be used for confirmation purposes (Puig and Barceló, 1996).

To achieve required concentrations, the sample preparation step is still needed for the determination by liquid chromatography. Solid-phase extraction and solid-phase microextraction (SPME) are widely used to replace liquid-liquid extraction (Rodríguez *et al.*, 2000). The use of an off-line solid-phase extraction coupled to liquid chromatography for determination of phenolic compounds is still an often used technique as it requires simple instrumentation (Pocurull *et al.*, 1996). However, when comparing with off-line mode, on-line solid-phase extraction coupled to high performance liquid chromatography (HPLC) system allows easy automation with high sample throughout and good reproducibility, and the whole analysis can be completed within a shorter time (Fan *et al.*, 2003).

More recently, solid-phase microextraction has been used to determine phenolic compounds. In this extraction method, sorbent-coated silica fibers are used to extract analytes from aqueous sample. In water samples, the fiber is usually immersed to extract the analytes and the fibers are then directly transferred to the injector of a chromatograph, where the analytes are desorbed and subsequently separated and quantified. Solid-phase microextraction coupled to high performance liquid chromatography, using diode array as a detector was evaluated for the analysis

of priority phenolic compounds in water samples. Polydimethylsiloxane-divinylbenzene (PDMS-DVB) and Carbowax-templated resin (CW-TPR) were used as SPME fibers, and the desorption was carried out in static mode to prevent chromatographic peak tailing. However, the detection limits attained with diode array detection were not low enough for the analysis of phenolic compounds in natural waters at the levels established in legislation, and more sensitive detection system must be used (González-Toledo *et al.*, 2001). Thereby, solid-phase microextraction coupled to high-performance liquid chromatography with ultraviolet and electrochemical detection (ED) has been developed and applied for the determination of 11 phenolic compounds listed as priority pollutants by the US EPA. Polyacrylate fibers were used to extract analytes from the aqueous samples. The limit of detection of the method reached the phenolic compounds permitted concentrations and can be applied to analyze the samples in natural waters (Peñalver *et al.*, 2002).

For chlorophenols the detection by liquid chromatography using solid-phase extraction has been investigated. The off-line solid-phase extraction with Oasis and Speedisks of styrene-divinylbenzene SPE sorbent were evaluated. The latter had the advantage that no filtration was needed before extraction and permitted the extraction of 17 chlorophenols from real samples. Moreover, it offered a high efficiency for determination of chlorophenols from industrial effluents (Lacorte *et al.*, 1999). Due to the advantages of on-line solid-phase extraction over off-line, the on-line solid-phase extraction coupled with high performance liquid chromatography was developed to determine chlorophenols in water. A pyrrole-based conductive polymer was used in place of silica-based materials as new sorbent to solve pH instability problem. This SPE sorbent can reach low detection limit and the stability against pH is a clear advantage (Bagheri *et al.*, 2004).

1.2.2.3 Capillary electrophoresis

The other approach based on chromatographic method for monitoring phenolic compounds is capillary electrophoresis (CE). The application of capillary electrophoresis, mainly capillary zone electrophoresis (CZE), has grown in the environmental field mainly because it is fast, has high resolution, and is suitable in the analysis of polar and ionic compounds (Puig and Barceló, 1996). Capillary zone

electrophoresis is based on differences in the electrophoretic mobilities (determined by size and charge) of charged analytes in an electric field (Carabias-Martínez *et al.*, 2000). Capillary zone electrophoresis was applied to analyze phenolic compounds in natural water. Good separation of phenolic compounds has been achieved with UV detection. It can rapidly separate and sensitively detect 11 priority phenolic compounds. The sample preparation by applying solid-phase extraction, which used a cross-linked styrene-divinylbenzene copolymer as SPE sorbent, was carried out to lower limit of detection (Martínez *et al.*, 1996). Nevertheless, most of the instruments, which provide with a UV absorbance detector, the light path is limited to the inner diameter of the capillary. According to Beer's Law, it is not very attractive for sensitive detection. Therefore, the study of different internal diameter of the capillary was investigated. Moreover, the amount of samples was increased by using a longer capillary. The internal diameter in the detector (light path) has been widened to 1.5 times of the capillary. The signals were shown to be enhanced. This preconcentration system is less labour intensive and less time-consuming (Martínez *et al.*, 1997).

Although chromatographic methods can be used to quantitate specific phenolic compounds, they are expensive instruments, time-consuming, need skilled operator and required sample preparation step, which is complicate (Dzyadevych *et al.*, 2002). Biological techniques, mainly biosensor, has grown in the last few years due to the need to develop fast, simple and cost effective technologies suitable for application in the field and also as a screening method prior to chromatographic analysis (Barcélo and Puig, 1996; Timur *et al.*, 2003).

1.2.3 Biosensor

A biosensor can be defined as a device incorporating biological sensing element connected to a transducer which converts an observed change (physical or chemical) into a measurable signal (Figure 3). The biological sensing elements can be enzymes, tissue materials, microorganisms, antigens, antibodies, nucleic acid and receptors (Eggins, 1996). Biosensors are versatile analytical tools applied more and more in different field, such as medicine, food quality and safety control, and environmental pollutant monitoring (Castillo *et al.*, 2004). Biosensors offer some advantages over classical analytical techniques in term of selectivity,

sensitivity, short assay time and reduce cost of analysis. This makes them promising for use in monitoring and screening of environmental pollutants for the future (Nistor and Emnéus, 1999).

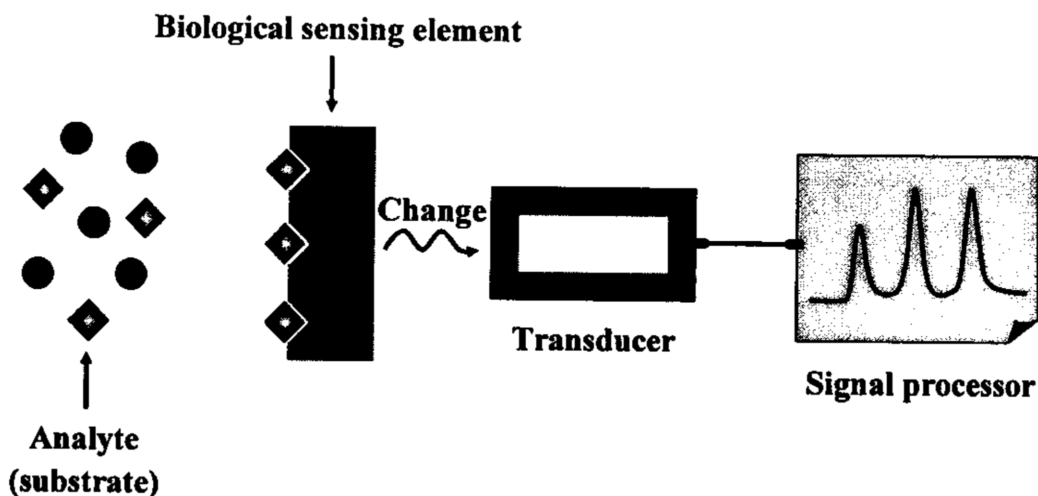


Figure 3 Schematic layout of a biosensor

1.2.3.1 Enzymatic biosensor for phenolic compounds

Purified enzymes have been most commonly used in the construction of biosensors due to their high specific activities. Several biosensors based on enzyme were investigated for the determination of phenolic compounds. Among the enzymes, tyrosinase and laccase are two groups of phenol oxidase that catalyze the transformation of a large number of phenolic and non-phenolic aromatic compounds (Durán *et al.*, 2002). Another group of enzyme is peroxidase. The mechanisms of peroxidase-catalyzed reactions have been widely studied using horseradish peroxidase (HRT) (Ruzgas *et al.*, 1995).

Tyrosinase

Numerous biosensors have been proposed for the detection of phenolic compounds based primarily on the phenol oxidase, tyrosinase. Tyrosinase is widely distributed throughout the phylogenetic scale from bacteria to mammals and even present different characteristics in different organs of the same organisms, such as in

Chlorophenols can also be determined by using tyrosinase to directly detect *o*-quinone. 4-Monochlorophenol is most widely investigated because it could not be detected by the standard method of 4-aminoantipyrene. Amperometric biosensors with (Rajesh *et al.*, 2004; Rajesh *et al.*, 2004) and without (Campuzano *et al.*, 2003) a mediator were developed. In mediated biosensors, 4-monochlorophenol can be oxidized at low potential to prevent electrode fouling. 4-Monochlorophenol tends to give higher sensitivity than phenol. Furthermore, the decreasing of concentration of protons during the conversion of 4-monochlorophenol to quinone can cause pH change. So, the measurement based on potentiometric pH-sensitive field-effect transistor can be used (Dzyadevych *et al.*, 2002; Anh *et al.*, 2002). Other chlorophenols, *i.e.* 4-chloro-3methylphenol, 3-monochlorophenol (Campuzano *et al.*, 2003; Serra *et al.*, 2002) and 2-monochlorophenol (Li *et al.*, 1998), can also be catalyzed by tyrosinase and determined the liberation of *o*-quinone. The response for chlorophenol with a chloro atom in *meta*-position was remarkably less sensitive than phenol and 4-monochlorophenol (Campuzano *et al.*, 2003), whereas the one with one *ortho*-position occupied gave no response at the tyrosinase electrode (Li *et al.*, 1998).

Although, tyrosinase-based electrochemical biosensors have been widely used, they still suffer from low enzyme stability and significant inhibition of the enzyme by reaction products (Freire *et al.*, 2002).

Laccase

Laccase is an oxidoreductase able to catalyze the oxidation of various aromatic compounds (particularly phenols) with the concomitant reduction of oxygen to water (Durán *et al.*, 2002). Laccase belongs to the so-called blue-copper family of oxidases. It is glycoprotein, which is ubiquitous in nature. It has been reported in higher plants and virtually every fungus that has been examined for them (Riva, 2006). Four Copper ions at the active site of laccase involved in a coordinated oxygen reduction. Copper sites have historically divided into three classes, copper type 1 or blue copper, type 2 or normal copper, type 3 or coupled binuclear copper center based on their spectroscopic features, which reflect the geometric and electron structure of the active site. This enzyme couples the oxidation of the substrate, which takes place

at type 1 copper site with the reduction of dioxygen to water, taking place at the type 2 and type 3 trinuclear copper cluster site.

The reaction of laccase on biosensor can be described when it was immobilized on the surface of the electrode. The laccase molecules at the surface of the electrode are oxidized by oxygen and then re-reduced by phenolic compounds, acting as electron donors for the oxidized form of the enzyme. In this reaction, phenolic compounds are converted into quinone and/or phenoxy radicals. These products can be reduced at the surface of the electrode at potential below 0 V versus saturated calomel electrode (SCE), giving a reduction current which is proportional to the phenolic compounds concentration (Jarosz-Wilkolazka *et al.*, 2005). Laccase is more stable than tyrosinase, because it is not susceptible to product inhibition. Furthermore, it can detect wide range of phenolic compounds (Freire *et al.*, 2002).

In the case where the substrates of interest cannot be oxidized directly by laccase, either because they are too large to penetrate into the enzyme active site or because they have a particularly high redox potential, e.g. lignin. It is possible to overcome this limitation with the addition of mediators, which are suitable compounds that act as intermediate substrates for the laccase, whose oxidized radical forms are able to interact with the bulky or high redox-potential substrate targets (Figure 6) (Riva, 2006).

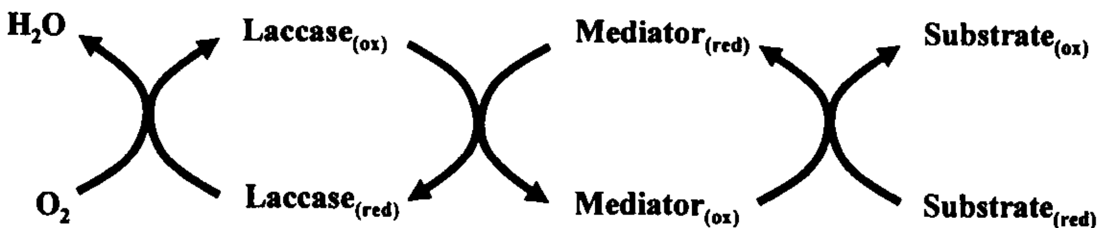


Figure 6 Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the presence of mediators (Modified from Riva, 2006)

Using laccase as a biological sensing element, the conversion of phenol to quinone or free radical can be measured in an amperometric biosensor system. A flow injection system was incorporated with a laccase based biosensor

where cathodic transient current was observed when phenol was injected (Jarosz-Wilkolazka *et al.*, 2005). Moreover, the integration of a dialysis membrane sampling in the system could protect the biosensor surface from fouling (Freire *et al.*, 2002). Besides the measurement of quinone liberation, consumed oxygen in the catalytic reaction can also be detected (Timur *et al.*, 2004; Roy *et al.*, 2005).

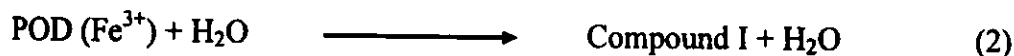
For chlorophenols, 4-Monochlorophenol has been used as a representative in the study of biosensors using laccase. The sensors were integrated into flow injection system and measured quinone products. Sensitivity of 4-monochlorophenol was not different from phenol, but phenol gave higher concentration of substrate at half reaction rate of enzyme (K_m) than 4-monochlorophenol (Jarosz-Wilkolazka *et al.*, 2005; Freire *et al.*, 2002). When compare to other phenolic compounds (catechol and L-DOPA) (Timur *et al.*, 2004), phenol also could not provide higher sensitivity

Peroxidase

Another group of enzyme is peroxidases (PODs). It represents a class of enzymes that catalyze the reduction of hydrogen peroxide or organic peroxides by suitable hydrogen donors RH_2 (reaction 1).

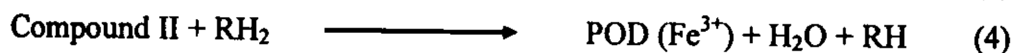
$$H_2O_2 + 2RH_2 \xrightarrow{\text{Peroxidase}} 2H_2O + 2RH \qquad (1)$$

The catalytic mechanism implies three main steps, depicted by following reactions:



$$POD\ (Fe^{3+}) + H_2O \longrightarrow \text{Compound I} + H_2O \qquad (2)$$

$$\text{Compound I} + RH_2 \longrightarrow \text{Compound II} + RH \qquad (3)$$



$$\text{Compound II} + RH_2 \longrightarrow POD\ (Fe^{3+}) + H_2O + RH \qquad (4)$$

Where compound I and II represent two reaction intermediates. If peroxidase molecules are immobilized on the surface of the electrode, compound I and compound II can be reduced back electrochemically in two ways, by a direct or mediated electron transfer. Direct electron transfer means that the oxidized forms of the enzyme are reduced back by taking electrons from the electrode surface. By

mediated electron transfer the enzyme takes the electrons from donors such as phenolic compounds, aromatic amine and ferrocenes, then the oxidized substrates are reduced back to their initial form electrochemically. The reduction current is proportional to the concentration of donor, which makes it possible to construct and use peroxidase-modified electrodes to detect phenolic compounds (Figure 7) (Lindgren *et al.*, 1997).

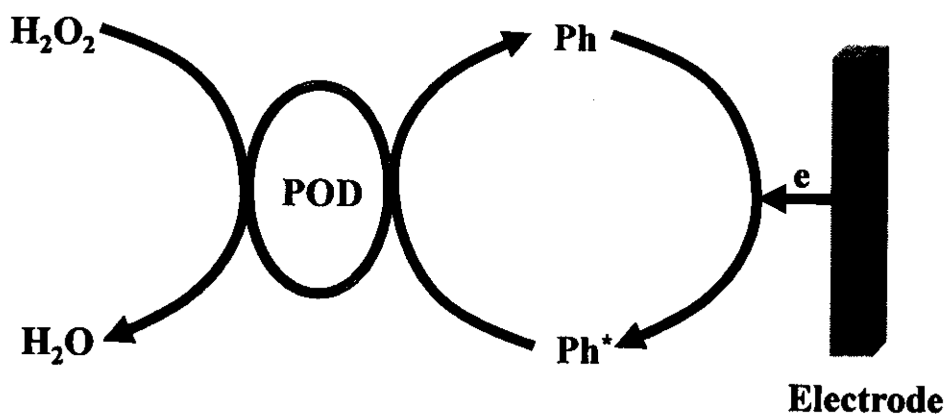


Figure 7 Mediated amperometric biosensor for phenol (Ph) with immobilized peroxidase (POD). POD is reduced by hydrogen peroxide (H_2O_2) in the present of a mediator Ph. The enzymatically formed radical species (Ph^*) is then reduced by the electrode regenerating the mediator (Ph) (Modified from Nistor and Emnéus, 1999).

By using this principle, biosensors for phenol were constructed. Horseradish peroxidase was normally immobilized on the surface of the electrode and phenoxy radicals formed during the enzymatic oxidation could be measured at -50 mV *vs.* Ag/AgCl (Lindgren *et al.*, 1997). To improve the sensitivity of the sensor for phenol, silica gel immobilization method with modification of titanium oxide was introduced (Rosatto *et al.*, 1999). Moreover, the immobilization procedure based on layer-by-layer self-assembly of horseradish peroxidase was proposed to reduce the amount of enzyme (Yang *et al.*, 2006). These systems were applied to monitor 4-monochlorophenol, 2,4-dichlorophenol, 4-chloro-3methylphenol (Lindgren *et al.*, 1997; Rosatto *et al.*, 1999) and 2-monochlorophenol (Yang *et al.*, 2006). 4-Chloro-3methylphenol gave lower response than phenol, whereas it showed higher response

than 4-monochlorophenol and 2,4-dichlorophenol. However, 2-monochlorophenol could provide higher sensor response than phenol.

In the case of horseradish peroxidase, the limitation is the necessity of hydrogen peroxide presence to complete the biocatalytic cycle (Freire *et al.*, 2002). Therefore, the use of bi-enzyme amperometric biosensor, *i.e.* tyrosinase and horseradish peroxidase was developed. In this study, there is no requirement of extraneous hydrogen peroxide. It involved the reduction of dissolved oxygen to hydrogen peroxide at the surface of electrode (Figure 9) (Chang *et al.*, 2002).

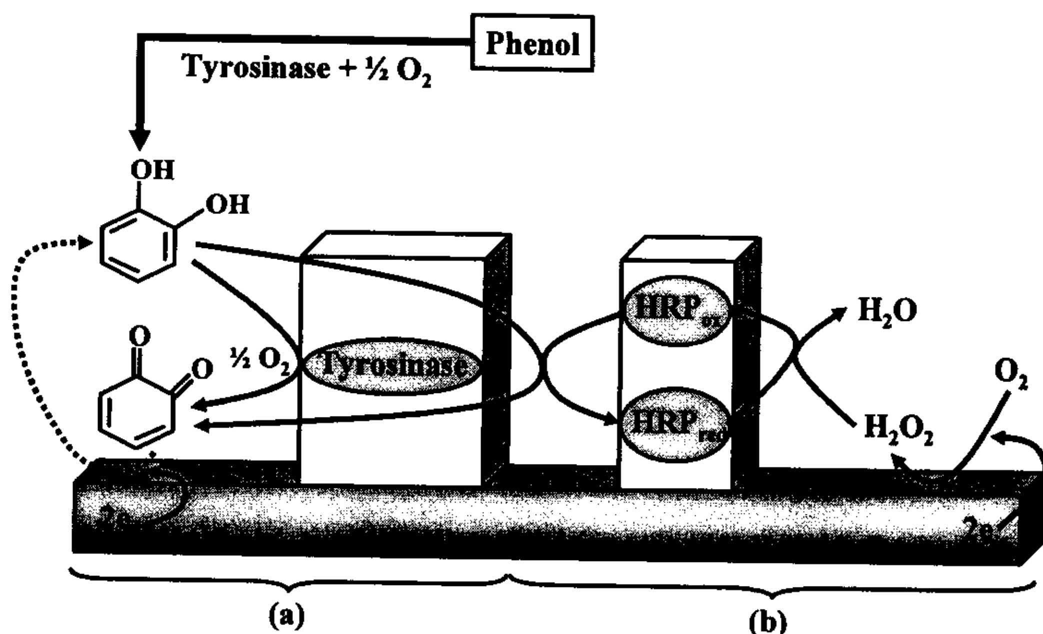


Figure 8 Schematic representation of phenolic compounds detection using tyrosinase electrode (a) and hydrogen peroxide-horseradish peroxidase-screen printed electrode (b). (Modified from Chang *et al.*, 2002)

Although enzyme based biosensors have shown to be the promising tool for phenolic compounds determination, they are usually inhibited by enzyme reaction products and/or need co-enzyme or co-substrate. Moreover, the use of enzymes as biological sensing element tends to provide short operational stability. Over 90% of the enzymes known to date are intracellular, and the operations to extract and purify enzymes are expensive. Some of these enzymes are also unstable.

In this respect, the utilization of whole cell as source of intracellular enzymes has been shown to be a better alternative method (D' Souza, 2001).

1.2.4 Cell-based biosensor for phenolic compounds

Microorganisms play an important part in many biotechnological processes in industry, in field such as brewing, pharmaceutical synthesis, food manufacture, waste water treatment and energy production. Many biosensors based on microorganisms immobilized on a transducer have been developed to assist with the monitoring of these and other processes (Eggins, 1996).

Microbial biosensors are devices incorporating a microorganism sensing element that can specifically recognize species of interest either intimately connected to, or integrated within, a suitable transducing system. The transducer is used to convert the biochemical signal into an electronic signal that can be suitably processed and outputted. In this way, the concentration of the species of interest is related to the assimilation capacity of the microorganisms, which is measured as a change in its respiration activity or as a change in its metabolic activity. The transducer can take many forms, the most important of which are potentiometric electrodes, amperometric electrode, thermistor, and photodetectors (Karube and Nakanishi, 1994).

1.2.4.1 Bioluminescent detection

Using photodetectors, microbial biosensors based on light emission from luminescent bacteria are being applied as a sensitive, rapid and non-invasive assay in several biological systems. Bioluminescent bacteria are found in nature, their habitat ranging from marine to terrestrial environments. Bioluminescent whole cell biosensor have also been developed using genetically engineered microorganism (GEM) for the monitoring of organic and pesticide contamination. The microorganisms used in this biosensors are typically produced with a constructed plasmid in which genes that code for luciferase are placed under the control of a promoter that recognize the analyte of interest (D' Souza, 2001). Typically, two types of promoters are employed. The first is the stress promoters, with the *lux* gene products functioning as reporters of transcription from these promoters. In this case,

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the presence of sublethal concentrations of many toxic chemicals leads to transcription of the *lux* genes through induction of the stress promoter, and this increase production of *lux* gene products is further expressed as a higher light intensity. The other type of commonly used promoter is the constitutive promoter. Using this system, the toxicity of chemicals can be evaluated through the bioluminescent reaction as it reduces in intensive when the cells experience a toxic or lethal condition (Choi and Gu, 2002).

The studies of bioluminescent bacteria biosensor were carried out for several chlorophenols, such as 2,4-dichlorophenol (Choi and Gu., 2002; Sinclair *et al.*, 1999), 2-chlorophenol, 4-chlorophenol, 2,4,5-trichlorophenol and pentachlorophenol by using luminometer as a transducer (Hyung and Gu., 2002). The presence of 2-Monochlorophenol, 4-monochlorophenol and 2,4-dichlorophenol can result in significant bioluminescent response, whereas 2,4,5-trichlorophenol and pentachlorophenol could not induce any sensor response. Increasing concentration of chlorophenols caused a decrease in light output, because of the toxic effects.

1.2.4.2 Respiration activity detection

Other detection principle uses the fact that microorganisms can assimilate organic compounds, resulting in a change in respiration activity, and can produce electroactive metabolites (Eggins, 1996). Thereby, the use of microbial biosensors to determine the substances is based on the presence of specific enzyme systems in microorganisms which transform certain chemical compounds (Timur *et al.*, 2003).

In the case of respiration activity measurement, changes (normally, increase) in respiration activity of microorganisms caused by assimilation can be detected by an oxygen electrode. Substrate concentrations are then estimated from these changes (Karube and Nakanishi, 1994). Most of the microbial biosensors developed, to date, are of the respiration activity type (Timur *et al.*, 2003; Kirgöz *et al.*, 2006).

Microbial cells have a number of advantages as biological sensing elements in the fabrication of biosensors. They present ubiquitously and are able to metabolize a wide range of chemical compounds. Microorganisms have a great

capacity to adapt to adverse conditions and to develop the ability to degrade new molecules with time. Moreover, the enzymes are preserved in their natural environment to protect them from inactivation by external toxicants (D' Souza, 2001). They are more tolerant of pH and temperature changes and have longer lifetimes (Eggins, 1996). Furthermore, microbial sensors are inexpensive and easily produced, possess the sensitivity and stability comparable with those of enzyme sensors and no additional effects are needed for purification of enzyme, but usually they lack of selectivity (Skládal *et al.*, 2002).

Several microbial biosensors for the analysis of phenol have been developed, mainly by using single strain culture as biological sensing element. Several strains of *Pseudomonas* have been investigated because of their ability to biodegrade aromatic compounds and the detection principle is usually based on the monitoring of cell respiration activity. These include *Pseudomonas cepacia* immobilize in calcium alginate (Thavarungkul *et al.*, 1991), *Pseudomonas putida* DSM 50026 immobilized in a thick film (Timur *et al.*, 2003; Timur *et al.*, 2004) and a graphite epoxy composite (Kirgöz *et al.*, 2006). The ability of *Pseudomonas* was also studied by immobilizing eight strains of *Pseudomonas* in screen-printed electrodes together with a mediator (Skládal *et al.*, 2002). The other type of bacteria used in the detection of phenol was *Rhodococcus sp.*, immobilized in polyvinylalcohol and placed between membranes (Riedel *et al.*, 1993).

Before used, *Pseudomonas* cells were generally induced (adapted) to be selective to the compound of interest. *Pseudomonas putida* DSM 50026, induced with phenol was used in a biosensor system and the responses were compared to controlled cells, cultured in glucose. Amperometric detection was applied to this thick film electrode system. The biosensor systems using bacterial cells with and without adaptation had different substrate specificities. However, both adapted and non-adapted cell sensors could provide response to phenol (Timur *et al.*, 2003). To improve the system, the same microorganism was immobilized on a graphite epoxy composite electrode (GECE) to provide an economical and practical disposable biosensor. The detection limit was almost 1000 times lower when compared with the thick film microbial biosensor. The developed microbial biosensor is inexpensive and easily produced (Kirgöz *et al.*, 2006).

Besides phenols, cell based biosensor for chlorophenols have also been investigated. A biosensor using *Rhododcoccus* as biological sensing element and oxygen electrode as transducer was constructed and tested with mono-, di- and trichlorophenol. Monochlorophenol showed half the signal amplitude of phenol, whereas dichlorophenol gave lower response than monochlorophenol. Trichlorophenol provide lowest response, which was 7-11% of phenol signal (Riedel *et al.*, 1993). In another work eight strains of *Pseudomonas* was tested for their ability to detect chlorophenols. Only three strains were able to transfer electrons resulting from specific oxidation of chlorophenols to the electrode (Skládal *et al.*, 2002).

Although, most biosensors for phenolic compounds are quite sensitive and selective, they prefer to use phenol as a substrate. Therefore, microbial biosensors for other phenolic compounds, such as chlorophenols are still needed.

1.2.5 Biodegradation of chlorophenols

Microbial biosensor is based on the use of specific enzyme in microorganisms. Cells can be adapted by induction to degrade particular compounds before using them as biological sensing elements in biosensor systems. Biodegradation and biotransformation of chlorophenols has been extensively studied to obtain adapted whole cells. Chlorophenols are being degraded under aerobic and anaerobic conditions. Mono-and, to lesser extent, di-chlorophenol can be metabolized by aerobic microorganisms, but aerobic attack becomes less effective with increasing chlorine atoms (Armenante *et al.*, 1999). The aerobes are more efficient at degrading toxic compounds because they grow faster than anaerobes and usually transform organic compounds to inorganic compounds (CO_2, H_2O) (Kim *et al.*, 2002).

Aerobic degradation of chlorophenols may occur using either the *ortho*-pathway or the *meta*-pathway, following the transformation to chlorocatechol (Farrell and Quilty, 2002). In general, methyl-substituted aromatic compounds are degraded via the *meta* cleavage pathway, whereas xenobiotics like chloroaromatic compounds are mineralized via the *ortho* cleavage pathway. The study on biodegradation of chlorophenols in aerobic condition revealed that 2-chlorophenol, 2,4-dichlorophenol, 2,6-dichlorophenol and 2,4,6-trichlorophenol could be more

rapidly degraded compared to pentachlorophenol, 2,4,5-trichlorophenol, 3,4-dichlorophenol and 3-chlorophenol (Annachhatre and Gheewala, 1996).

1.2.5.1 Pure culture

Both pure and mixed culture approaches have been widely studied on the aerobic degradation of chlorophenols by immobilized cells. Pure culture biodegradation of 4-monochlorophenol was extensively studied. Undefined type of pure culture showed the ability to degrade 4-monochlorophenol. By comparing between free cells and immobilized cells, the biodegradation of 4-monochlorophenol with immobilized pure culture was faster than by free cells. The immobilized cells took 90 h to completely degrade 4-monochlorophenol, while only 20% of 4-monochlorophenol was degraded by free cells (Jianlong and Yi, 1999). The degradation of methyl- and chloroaromatic substrates in matrix is often incomplete, leading to the accumulation of dead-end metabolites like chlorochatechol, chlorinated hydroxymuconic semialdehydes, or 4-carboxymethyl-methylbut-2-en-1,4-olides. Therefore, the degradation of 4-monochlorophenol via the *meta* cleavage pathway by *Comamonas testosteroni* JH5 was studied, which showed that *C. testosteroni* JH5 was able to tolerate and degrade rather high concentration of 4-monochlorophenol by *meta* cleavage pathway. Moreover, the degradation was fast and no metabolites accumulated for a longer period of time in growth cultures or resting cells (Hollender *et al.*, 1997). 4-Monochlorophenol can also be used as a non-growth substrate, when phenol was a growth substrate. 4-Monochlorophenol can also be biodegraded by *Acetobacter* culture. However, at higher initial 4-monochlorophenol concentration, the rate of degradation decreased (Hao *et al.*, 2002). In another report a pure culture of *Penicillium camemberti* was used to biodegraded pentachlorophenol in batch flask experiments and could degraded around 86% of pentachlorophenol in 21 days (Taseli and Gokcay, 2004).

1.2.5.2 Mixed culture

Mixed culture means that several kinds of microorganisms are cultured simultaneously and is divided into undefined and defined types. The main advantage

achieved by the microbial consortium formed by mixed culture is the interaction between all the species present in the flocks (Sahinkaya and Dilek, 2006).

Many pure culture studies have shown that toxic intermediates can be accumulated during biodegradation because a single organism may not have the ability to completely mineralize the xenobiotics. On the other hand, several studies have shown that mixed bacteria culture has the ability to use chlorophenols as carbon source and energy (Sahinkaya and Dilek, 2006). The difference of structure and toxicity among chlorophenols requires that various bacteria have specific qualities to degrade each compound and a mixed culture should be applied.

The biodegradation of 4-monochlorophenols by defined types mixed culture was also investigated. The studies were based on activated sludge process, consists essentially of an aerobic treatment that oxidizes organic matter to CO_2 and H_2O, NH_4 and new cell biomass (Bitton, 1994).*Pseudumonas testosteronei* CPW301 and *Psuedomonas solanacearum* TCP114 were used (Kim *et al.*, 2002). Another application used *Pseudomonas sp.* and *Pseudomonas stutzeri* in the presence of peptone (Sahinkaya and Dilek, 2006). This defined mixed culture could treat 4-monochlorophenol completely. However, 4-monochlorophenol affected the biodegradation rate and this could be explained in term of toxicity of 4-monochlorophenol to the mixed culture (Kim *et al.*, 2002). It was also observed that decreasing peptone concentration associated with decreasing biomass concentration led to a lower degradation rate, which caused accumulation of chlorophenols within the reactor (Sahinkaya and Dilek, 2006).

Besides 4-monochlorophenol, 2-monochlorophenol was also studied. *Pseudomonas putida* CP1 was added to a commercial mixed microbial community to enhance the degradation of 2-monochlorophenol, since the use of commercial mixture led to incomplete degradation. The time required for the degradation of 2-monochlorophenol by the augmented mixed culture was 2 days (Farrell and Quilty, 2002). This is shorter than the used of *Penicillium camemberti* to degrade 2-monochlorophenol, which took 21 days to degrade 53% of 2-monochlorophenol (Taseli and Gokcay, 2004).

Other studies include the use of *Pseudomonas sp.* and *Pseudomonas stutzeri* to study the degradation of 2,4-dichlorophenol (Sahinkaya and Dilek, 2006).

Pseudumonas testosteronei CPW301 and *Psuedomonas solanacearum* TCP114 to degrade 2,4,6-trichlorophenol (Kim *et al.*, 2002). These latter microorganisms could degrade 2,4-dichlorophenol up to 110 mg l^{-1} with more than 90 % removal. In the case of 2,4,6-trichlorophenol, addition of phenol as a co-metabolite could improve degradation time to 10 h (20 mg l^{-1}). On the other hand, when 4-monochlorophenol was added, 15 h of degradation time was observed.

Biodegradation of chlorophenols by undefined mixed culture was more extensively studied. Aerobic biodegradation of chlorophenols by using activated sludge was evaluated by the acclimation of sludge into the target chlorophenols. These are monochlorophenol (Zilouei *et al.*, 2006), 2,4-dichlorophenol (Quan *et al.*, 2003; Zilouei *et al.*, 2006), 2,4,6-trichlorophenol (Puhakka *et al.*, 1992; Correa *et al.*, 2003; Zilouei *et al.*, 2006) and 2,3,4,6-tetrachlorophenol (Puhakka *et al.*, 1992). It was found that activated sludge could biodegrade 2,4-dichlorophenol and other degradable compounds such as glucose and starch (Quan *et al.*, 2003). 2,4,6-Trichlorophenol was degraded by 82% (Puhakka *et al.*, 1992) or with concentration up to 237 mg l^{-1} day (Correa *et al.*, 2003). 2,3,4,6-Tetrachlorophenol showed 71% degradation (Puhakka *et al.*, 1992). When the mixture of chlorophenols (2-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol) was studied, the best removal efficiencies were achieved at a total pollutant loading rate of 11 mg l^{-1} h^{-1} where all pollutants were degraded by more than 99% (Zilouei *et al.*, 2006).

The study of biodegradation of chlorophenols, indicated that chlorophenols could be biodegraded by both mixed and pure cultures. Therefore, the applications of biodegradation of chlorophenols to construct microbial biosensor are possible and would also be useful in many other fields.

1.2.6 Microbial growth kinetics

When a suitable growth medium is inoculated with cells, the growth of the microbial population follows the growth curve displayed in Figure 9, which shows four distinct phases.

The lag phase is a period of cell adjustment to the new environment. The duration of the lag phase depends on the cells' prior history (age, prior exposure to damaging physical or chemical agents, culture medium). For example, no lag phase

is observed when an exponentially growing culture is transferred to a similar medium with similar growth conditions. Conversely, a lag period is observed when damaged cells are introduced into the culture medium (Bitton, 1994).

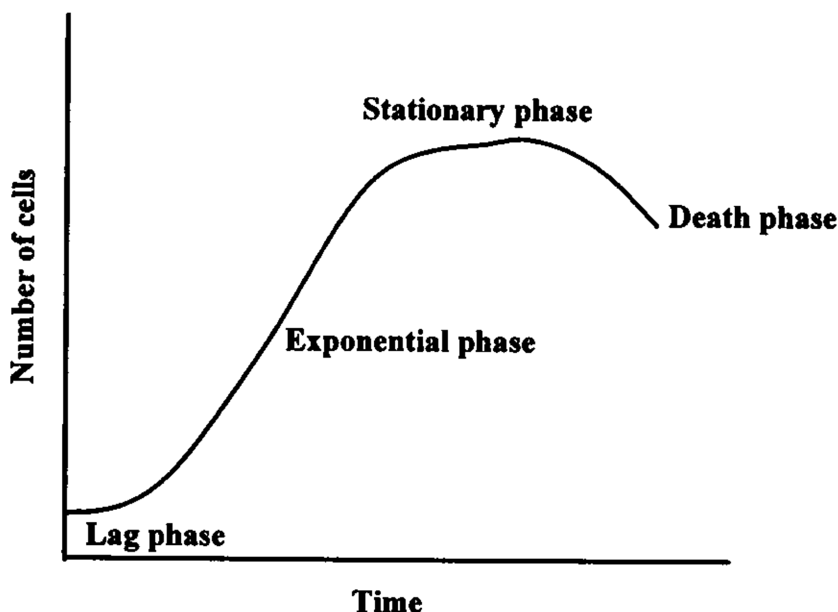

Figure 9 Microbial growth curve

The second phase is exponential growth phase (log phase). In this phase, the number of cells increases exponentially. The exponential growth rate varies with type of microorganism and growth conditions (Bitton, 1994). After the microbes were grown to the late exponential phase, the cells were generally harvested to use in biosensor (Han *et al.*, 2002). The reason is cells in the exponential growth phase are more sensitive to physical and chemical agents than those in the stationary phase (Bitton, 1994).

The third phase is stationary phase. The cell population reaches the stationary phase because microorganism cannot grow indefinitely, mainly because of the lack of nutrients and electron acceptor, and the production and accumulation of toxic metabolites. There is no net growth (cell growth is balanced by cell death or lysis) of the population during the stationary phase (Bitton, 1994).

The last phase is death phase. During this phase, the death (decay) rate of the microbial population is higher than the growth rate. Cell death may be

accompanied by cell lysis. The physical and chemical factors affect microbial growth, such as temperature, pH and oxygen level (Bitton, 1994).

1.2.7 Immobilization

The basic requirement of a biosensor is that the biological sensing element should bring the physico-chemical changes in proximity of a transducer. In this direction, immobilization technology has played a major role. Immobilization not only helps in forming the required close proximity between the biological sensing element and the transducer, but also helps in stabilizing it for reuse (D'Souza, 2001). Various methods are available for immobilization of biological component, but not always appropriate for manufacture of biosensors. The most commonly used biomaterial immobilization techniques for designing and development of specific sensors are physical adsorption (Figure 10a), covalent binding (Figure 10b), inter molecular cross-linking (Figure 10c) and entrapment (Figure 10d) (Sharma *et al.*, 2003).

Various approaches have been used for the immobilization of microorganisms. The most popular one is entrapment of cells in polymeric materials such as alginate, carrageenan or polyacrylamide. Owing to the toxicity and detrimental effect of polyacrylamide on cell viability, natural algal polysaccharides such as alginate and carrageenan have been the polymers of choice for microbial cell immobilization. Alginate reacts with most divalent cations, particularly Ca^{2+}, to form gels. However, calcium alginate beads can be destroyed if the surrounding medium contains phosphates and other calcium chelators (Bitton, 1994).

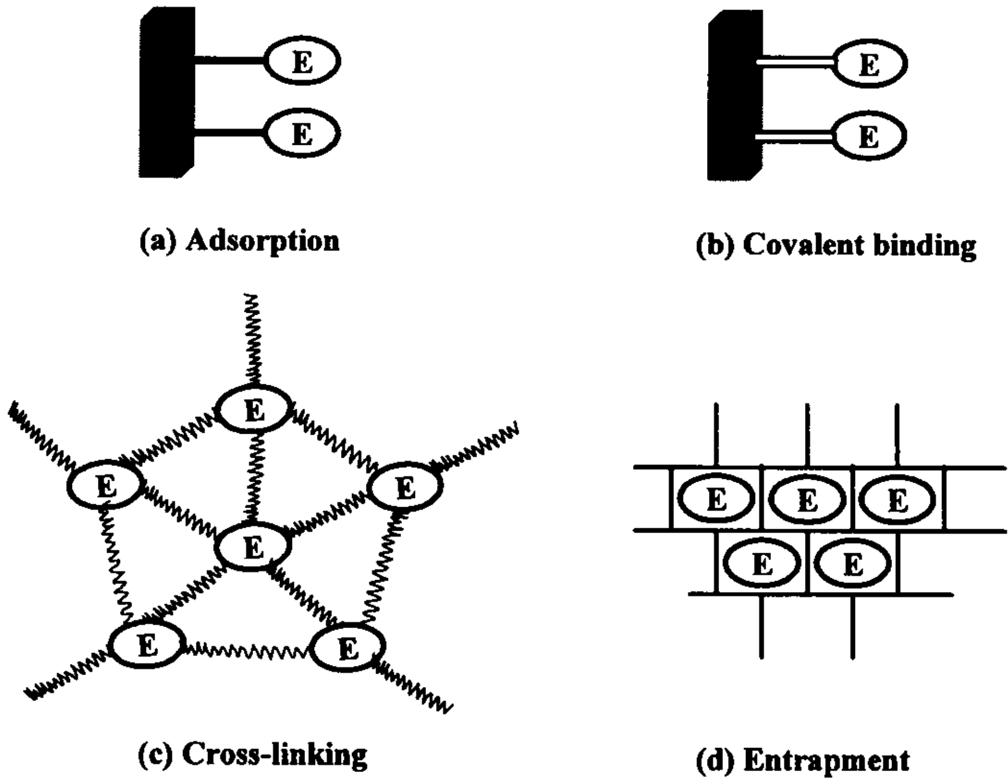

Figure 10 Immobilization methods for biological components (Modified from Sharma *et al.*, 2003)

In this work, 2,4-dichlorophenol was selected as a carbon source for the study of microbial biosensor. Two types of mixed culture microbial cells were used, *i.e.* Swedish and Thai microbial cells.

1.3 Objectives

To develop a flow injection biosensor for monitoring chlorophenols using suitable cultivated microorganisms capable of degrading chlorophenols

1.4 Benefits

It is expected that the proposed flow injection cell-based biosensor for monitoring chlorophenols will provide an alternative screening technique for chlorophenols in the environment.

1.5 Outline of the research

1.Cultivation of mixed culture microbes by using 2,4-dichlorophenol as a carbon source and study microbial growth.

2.Immobilize microbial cells by mean of membrane and gel entrapment.

3.Optimize the operating conditions of a flow injection cell-based biosensor for chlorophenols, such as flow rate, sample volume, *etc.*

4.Test the cell-based biosensor system by determining chlorophenols in wastewater and compare the results with gas chromatography/mass spectrometry.