CHAPTER 3

RESULTS AND DISCUSSION

3.1 Characteristics of biosensor response

Typical responses of the cell-based biosensors are shown in Figures 22 and 23. The amplitude of the signal (peak area or peak height), directly related to chlorophenols concentration, was measured. The response time, wash out time and analysis time were also considered.

Three signals were generally obtained for each concentration where the mean and standard deviation (SD) were then calculated. In some cases the three signals were of the same size, *i.e.* SD = 0, the uncertainty due to the reading of the measuring scale will be shown instead (noted by an *). Molecular weight (MW) of 2,4-dichlorophenol is 163 g mol^{-1}.To change the concentration of mM to mg l^{-1}, 163 was multiplied to the mM unit.

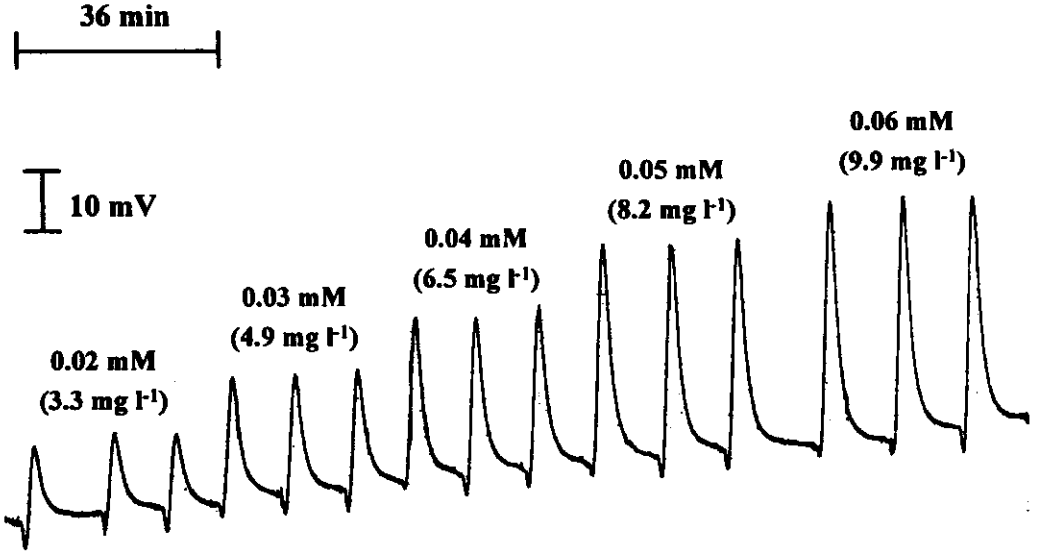


Figure 22 Responses of cell-based biosensor for monitoring 2,4-dichlorophenol using Swedish mixed culture bacteria, 100 µl of 2,4-dichlorophenol, 100 mM potassium phosphate buffer, pH 7.50 and at flow rate of 0.10 ml min^{-1}.

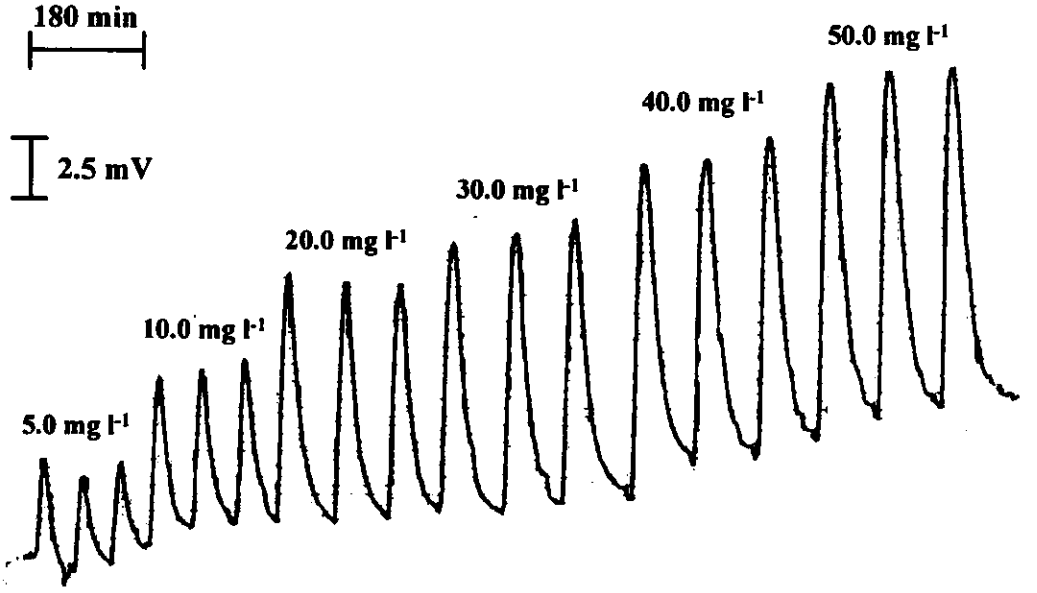


Figure 23 Responses of cell-based biosensor for monitoring 2,4-dichlorophenol using Thai mixed culture microbes, 500 µl of 2,4-dichlorophenol, 100 mM tris-HCl buffer containing 10 mM $CaCl_2$, pH 6.50 at flow rate of 0.10 ml min^{-1}.

3.2 Swedish mixed culture bacteria

3.2.1 Optimization of culture conditions

Using 2,4-dichlorophenol as a carbon source and energy, the mixed culture bacteria utilized oxygen as a terminal electron acceptor and the amount of oxygen decreased with time. Table 3 and Figure 24 show residual amount of oxygen from the culture flask headspace. The results showed a lag phase of 6 days, after which the amount of oxygen decreased rapidly at the concentrations of 50.0 mg l^{-1} and 80.0 mg l^{-1} of 2,4-dichlorophenol (log phase). Meanwhile there was no change at 150.0 mg l^{-1} and only a slight change at 20.0 and 110.0 mg l^{-1}

At high concentration, 2,4-dichlorophenol can have toxic effects, through a number of mechanisms, on target cell constituents such as mitochondria and bacterial cytoplasmic membranes. They can act in several ways as uncouplers,

inhibitors or narcotic agents. If it is possible for a weak acid to pass through the membrane in the anionic form, then it will act as an uncoupler. Uncouplers have been shown to destroy the electrochemical proton gradient by transporting protons back across the cell membrane at a rate faster than the respiratory chain or ATP synthase proton pumps. It has also been proposed that chlorophenols may inhibit ATP synthesis due to an affinity of binding directly to components of the electron transport chain. Substituted phenol can be structurally similar to reduced quinone molecules which are present at reasonably high concentrations in bacteria and carry electrons and proton equivalents. Molecules which resemble quinones can bind to quinone binding site blocking electron flow (Sinclair *et al.*, 1999). Therefore, it can be suggested that the metabolism of the cell is being disrupted by 2,4-dichlorophenol.

At 20.0 mg l^{-1} small reduction of oxygen was also observed. It is possible that at this low concentration 2,4-dichlorophenol had been completely assimilated by the mixed culture bacteria in a short time. So cell growth stopped, *i.e.* no change in respiration activity. Comparing to the amount of oxygen in the controlled experiment (no mixed culture bacteria), that maintained the same level throughout the experiment, this indicated that at 20.0 mg l^{-1} the reduction of oxygen was caused by the used of 2,4-dichlorophenol as a carbon source and energy by the mixed culture bacteria.

The study showed that 50.0 and 80.0 mg l^{-1} of 2,4-dichlorophenol were suitable for the culture of mixed culture bacteria. However, 50.0 mg l^{-1} was chosen to prevent the possible inactivation of bacteria at high concentration of toxic substance.

When 50.0 mg l^{-1} of 2,4-dichlorophenol was used for the cultivation, the active mixed culture bacteria was harvested after 10 days (late exponential phase). It was then immobilized on a Clark type oxygen electrode and used in a microbial biosensor system.

Table 3 Residual amount of oxygen (%) from culture flask headspace at different concentrations of 2,4-dichlorophenol

Days	Residual amount of O_2 (%) in air					
	Control*	20.0 mgl^{-1}	50.0 mgl^{-1}	80.0 mgl^{-1}	110.0 mgl^{-1}	150.0 mgl^{-1}
3	20.5236	21.7665	21.1986	21.0668	20.1827	20.7343
4	20.3405	19.3540	20.0024	21.7082	20.5516	20.7031
5	20.3405	19.7897	20.0259	21.7331	20.9520	20.7010
7	19.0938	19.2101	18.9005	19.9680	20.6772	20.6194
8	22.5040	19.3555	16.2841	18.8263	20.3736	21.6171
9	19.2871	19.0093	15.3504	16.3209	20.7991	22.0155
10	19.5770	19.1707	15.1517	15.1470	20.3101	21.2587
11	19.2494	18.9431	11.8197	13.9465	21.7015	20.2545
12	19.2153	18.8571	11.5347	10.6501	20.2313	20.7009
13	19.2992	17.7930	11.0811	11.7600	19.8339	20.5979
14	19.0234	18.6682	11.2682	9.7247	19.5827	20.4376
15	19.1024	20.4990	11.3747	11.3961	20.0058	21.1152
16	18.9659	19.2298	11.5607	12.1677	19.3676	21.8201
17	19.0099	17.3395	12.0965	11.1353	17.3531	20.6420

(* = Controlled experiment without mixed culture bacteria)

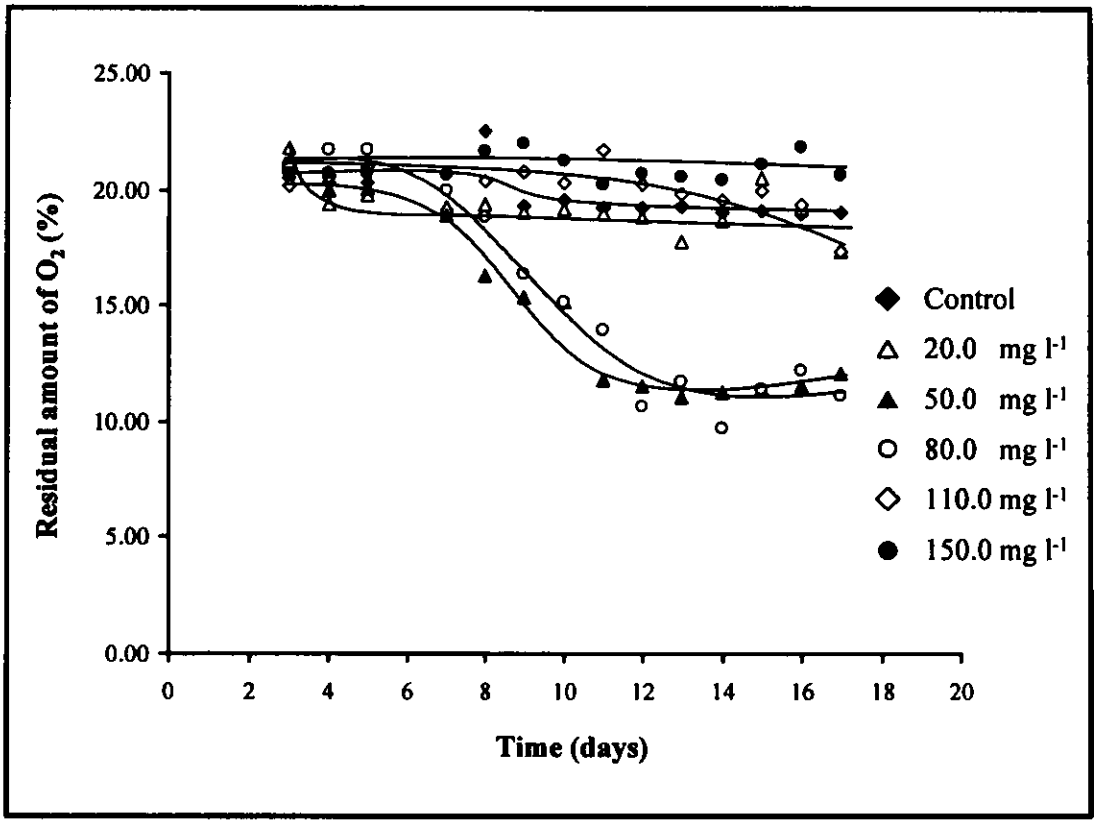


Figure 24 Percentage residual of oxygen from culture flask headspace at different concentration of 2,4-dichlorophenol

3.2.2 Optimization of operational conditions

3.2.2.1 Flow rate

The responses of 0.05 mM to 0.40 mM standard 2,4-dichlorophenol to different flow rates are shown in Table 4 and Figure 25. The peak areas and sensitivities decreased as the flow rate increased. The flow rate would generally affect the dispersion behavior and the retention time of the analyte to contact the microbial electrode (Fang, 1993). A slow flow rate allows more time for a sample to be degraded at the microbial electrode. This would provide a higher peak area, but the analysis time would also be longer. In this work a flow rate of 0.10 ml min^{-1} provided the highest response and sensitivity. Although this flow rate needed an analysis time of up to 18 minutes, the sensitivity was higher than at 0.20 ml min^{-1} by 49%. So it was chosen as an optimum flow rate.

Table 4 Responses of microbial biosensor at different flow rates

Concentration (mM)	Peak area (cm^2) at different flow rates		
	0.10 ml min^{-1} mean ± SD	0.20 ml min^{-1} mean ± SD	0.30 ml min^{-1} mean ± SD
0.05	1.0 ± 0.1	0.48 ± 0.06	0.28 ± 0.03
0.20	4.4 ± 0.3	2.29 ± 0.07	1.30 ± 0.06
0.30	5.6 ± 0.2	3.20 ± 0.06	1.94 ± 0.09
0.40	7.8 ± 0.1	3.83 ± 0.03	2.42 ± 0.02
Analysis time (min)	8-18	5-12	4-9
Sensitivity (cm^2/mM)	19.00	9.66	6.17
r	0.9949	0.9908	0.9974

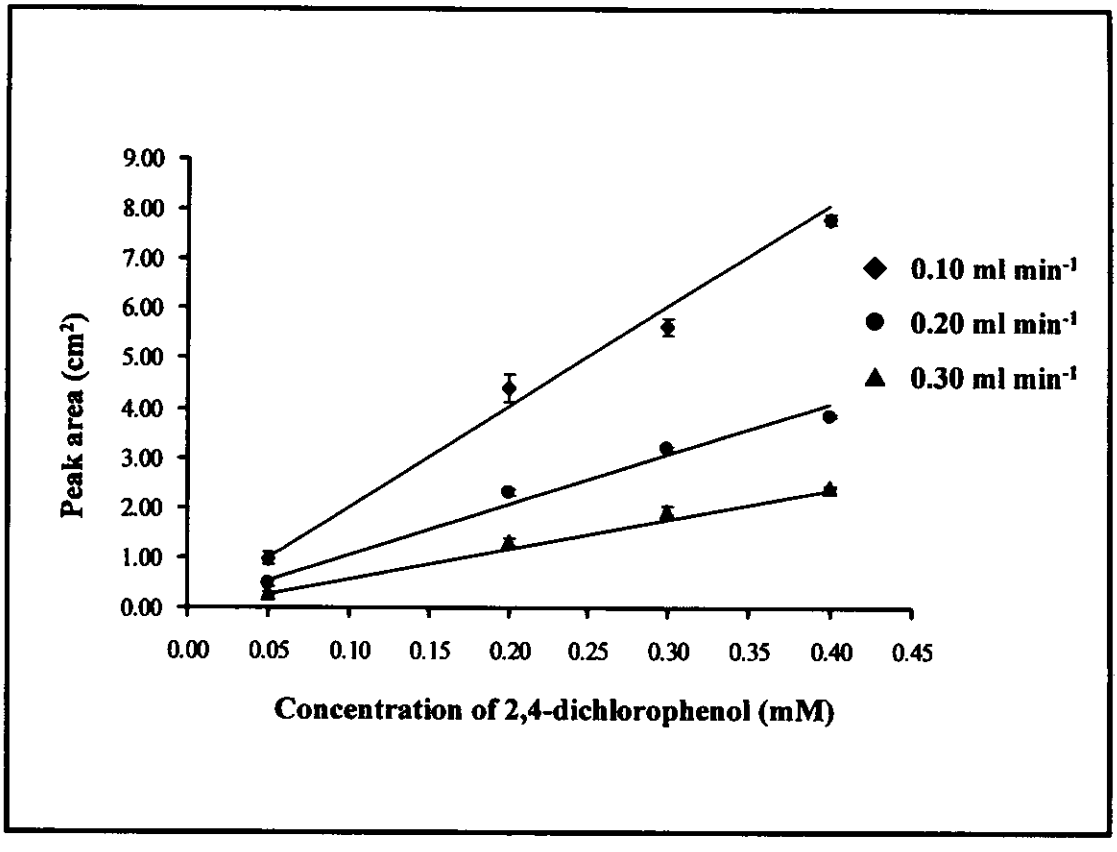


Figure 25 Responses of microbial biosensor at different flow rates

3.2.2.2 Sample volume

The responses of 0.05 mM to 0.40 mM standard 2,4-dichlorophenol at different sample volumes are shown in Table 5 and Figure 26. The response to 10 µl of 0.05 mM standard 2,4-dichlorophenol could not be detected. For the rest, the peak areas and sensitivities increased as the sample volumes increased but the analysis time also increased. The highest sensitivity was obtained at sample volume of 100 µl. Although the analysis time at 100 µl was rather long (22 min), it was chosen as an optimum sample volume to reach the required detection limit of 1 mg l^{-1}. This is the permitted concentration of phenolic compounds in the effluent from industries (Ministry of Industry, Thailand, 1996).

Table 5 Responses of microbial biosensor at different sample volumes

Concentration (mM)	Peak area (cm^2) at different sample volumes			
	10 µl mean ± SD	25 µl mean ± SD	50 µl mean ± SD	100 µl mean ± SD
0.05	ND	1.6 ± 0.2	2.0 ± 0.2	6.0 ± 0.4
0.10	1.1 ± 0.2	2.60 ± 0.08	3.7 ± 0.1	11.5 ± 0.7
0.20	2.0 ± 0.3	4.7 ± 0.4	6.8 ± 0.3	18.6 ± 0.5
0.30	2.78 ± 0.09	6.9 ± 0.4	9.30 ± 0.06	26.1 ± 0.5
0.40	3.6 ± 0.1	7.9 ± 0.9	11.1± 0.2	30.3 ± 0.6
Analysis time (min)	5-8	5-10	7-14	12-22
Sensitivity (cm^2/ mM)	8.36	21.17	29.26	78.21
r	0.9985	0.9999	0.9981	0.9972

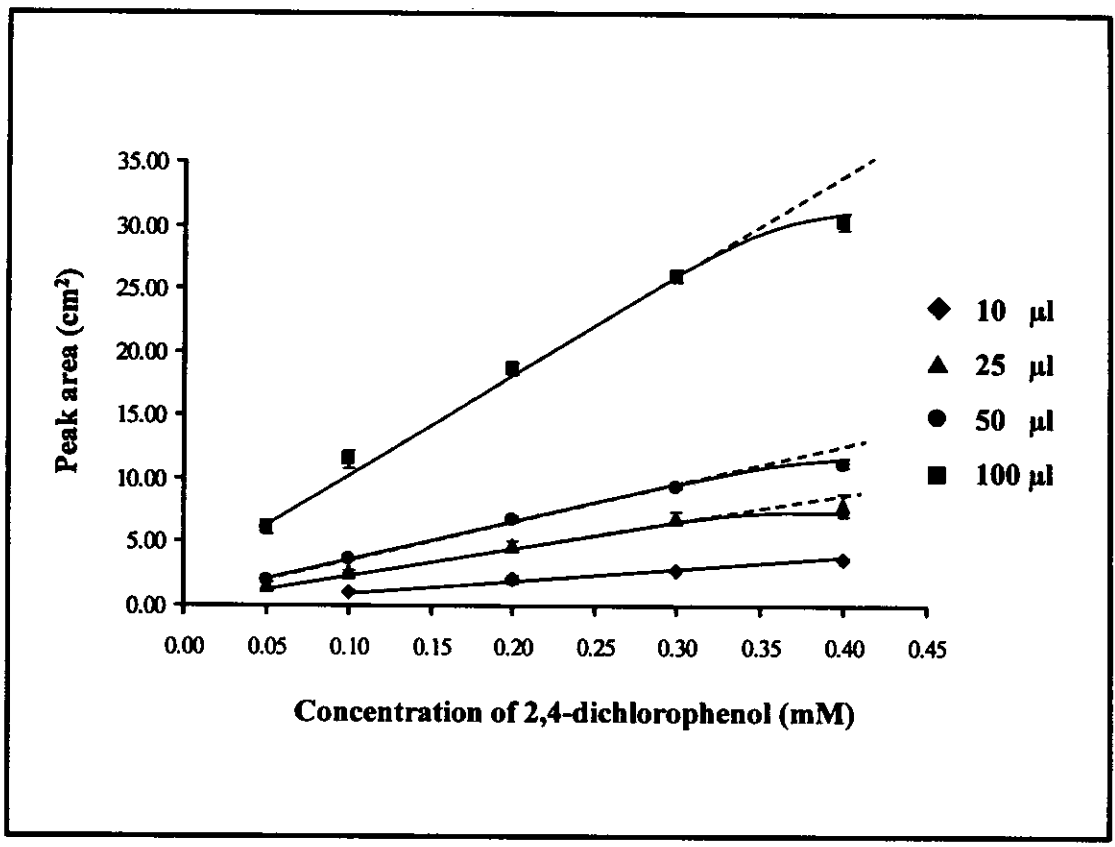


Figure 26 Responses of microbial biosensor at different sample volumes

3.2.2.3 Buffer pH

The influence of buffer pH was optimized by using potassium phosphate buffer. The responses of 0.05 mM to 0.40 mM standard 2,4-dichlorophenol at pH 6.50, 7.00, 7.50 and 8.00 are shown in Table 6 and Figure 27. The peak areas increased as buffer pH increased but decreased at pH 8.00. The highest sensor response was obtained at pH 7.50 and this pH was chosen as an optimum condition. Although the optimum pH for bacterial growth should be around 7.00, this may vary for immobilized cells (Bitton, 1994). The analysis time at different buffer pH was not different.

Table 6 Responses of microbial biosensor at different buffer pH

Concentration (mM)	Peak area (cm^2) at different buffer pH			
	pH 6.50 mean ± SD	pH 7.00 mean ± SD	pH 7.50 mean ± SD	pH 8.00 mean ± SD
0.05	3.2 ± 0.3	4.4 ± 0.1	4.9 ± 0.5	3.4 ± 0.3
0.10	4.7 ± 0.3	6.7 ± 0.6	7.0 ± 0.4	5.4 ± 0.4
0.20	7.40 ± 0.02	8.6 ± 0.3	10.3 ± 0.3	8.1 ± 0.2
0.30	8.9 ± 0.3	10.8 ± 0.2	12.3 ± 0.3	10.3 ± 0.2
0.40	10.6 ± 0.1	12.6 ± 0.5	13.6 ± 0.7	11.6 ± 0.3
Analysis time (min)	8-17	9-16	9-16	9-15

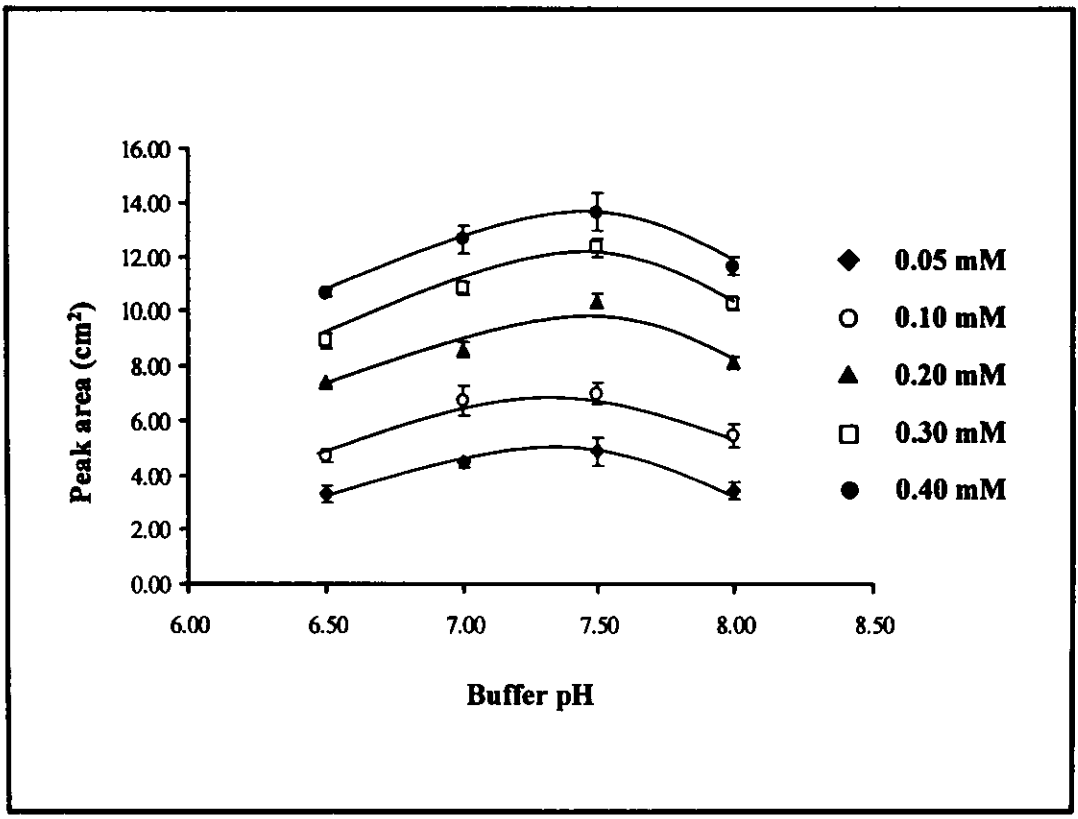


Figure 27 Responses of microbial biosensor at different buffer pH in potassium phosphate buffer

3.2.2.4 Buffer concentration

Potassium phosphate buffer was used as a buffer solution. The responses to 0.05-0.40 mM standard 2,4-dichlorophenol at different buffer concentration, 10, 30, 50 and 100 mM, are shown in Table 7 and Figure 28. The peak areas between 10 and 50 mM buffer solution were not much different. At 100 mM the sensor responses were higher than at 50 mM by 29%. At high concentration of potassium phosphate buffer, the solution present high amount of K^+. This ion is important for transportation of nutrient into cells (Perrin and Dempsey, 1974). Therefore, 100 mM potassium phosphate buffer was chosen as an optimum buffer concentration.

Table 7 Responses of microbial biosensor at different buffer concentrations

Concentration (mM)	Peak area (cm^2) at different buffer concentration			
	10 mM mean ± SD	30 mM mean ± SD	50 mM mean ± SD	100 mM mean ± SD
0.05	3.1 ± 0.2	3.8 ± 0.1	3.0 ± 0.1	4.9 ± 0.5
0.10	4.7 ± 0.3	5.5 ± 0.4	5.0 ± 0.1	7.0 ± 0.4
0.20	6.0 ± 0.2	7.5 ± 0.5	7.1 ± 0.1	10.3 ± 0.3
0.30	7.2 ± 0.4	8.7 ± 0.5	8.4 ± 0.3	12.3 ± 0.3
0.40	7.8 ± 0.3	8.9 ± 0.2	9.8 ± 0.2	13.6 ± 0.7
Analysis time (min)	6-17	6-13	7-14	8-16

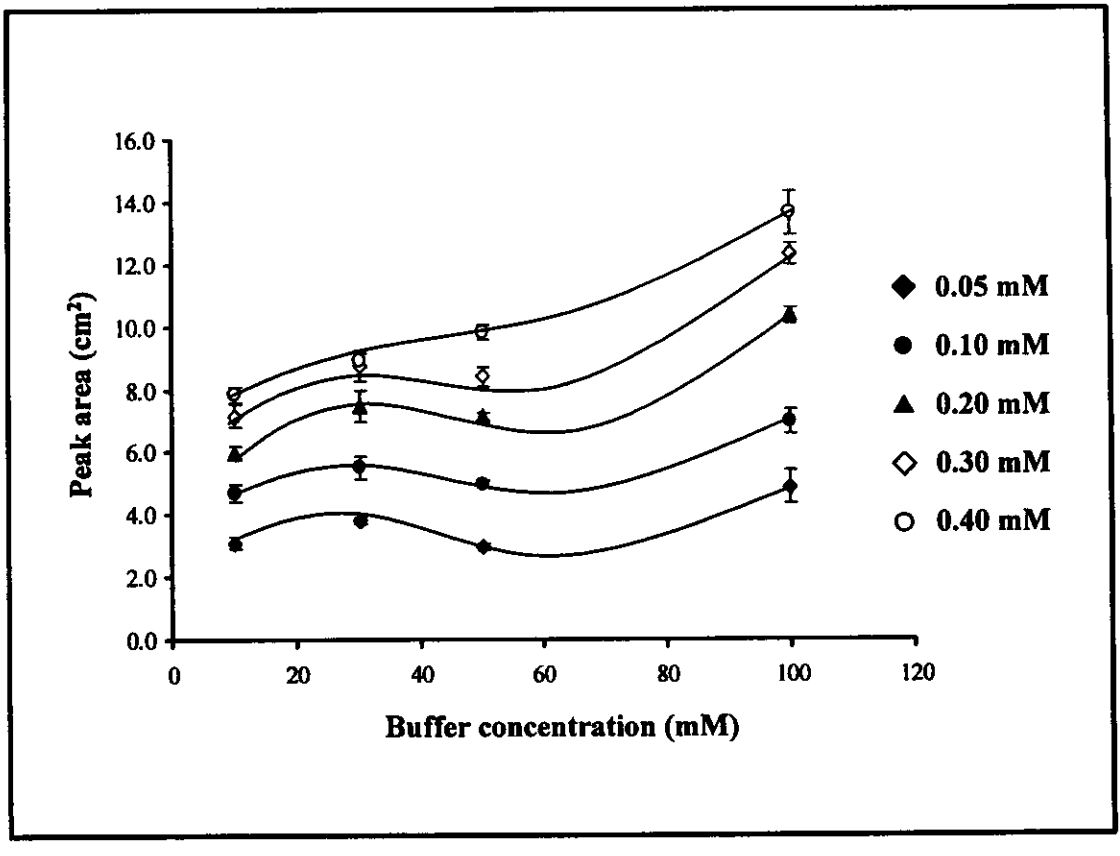


Figure 28 Responses of microbial biosensor at different buffer concentrations

3.2.3 Linearity

Using the optimum conditions, *i.e.* flow rate 0.10 ml min^{-1}, sample volume 100 µl, buffer pH 7.50 and buffer concentration 100 mM, the linearity of the microbial biosensor system to different concentrations of standard 2,4-dichlorophenol is shown in Table 8 and Figure 29. The linear range of immobilized mixed culture bacteria was 0.01 to 0.30 mM. The lower limit of detection was calculated using statistical method (Miller and Miller, 2000) and was found to be 0.02 mM.

Table 8 Responses of the microbial biosensor system to different concentrations of standard 2,4-dichlorophenol at optimum conditions, flow rate 0.10 ml mim^{-1}, sample volume 100 µl, buffer pH 7.50 and buffer concentration 100 mM

Concentration (mM)	Peak area (cm^2)
	Mean ± SD
0.01	0.18 ± 0.01
0.02	0.66 ± 0.04
0.03	1.02 ± 0.06
0.04	1.4 ± 0.1
0.05	1.74 ± 0.07
0.06	1.90 ± 0.06
0.08	2.9 ± 0.1
0.10	3.8 ± 0.1
0.15	6.1 ± 0.1
0.20	7.2 ± 0.1
0.30	10.64 ± 0.07
0.45	14.5 ± 0.4
0.60	18 ± 2
Analysis time (min)	7-21
Sensitivity (cm^2/ mM) of 0.01-0.30 mM standard 2,4-dichlorophenol solution	36.54
r	0.9968

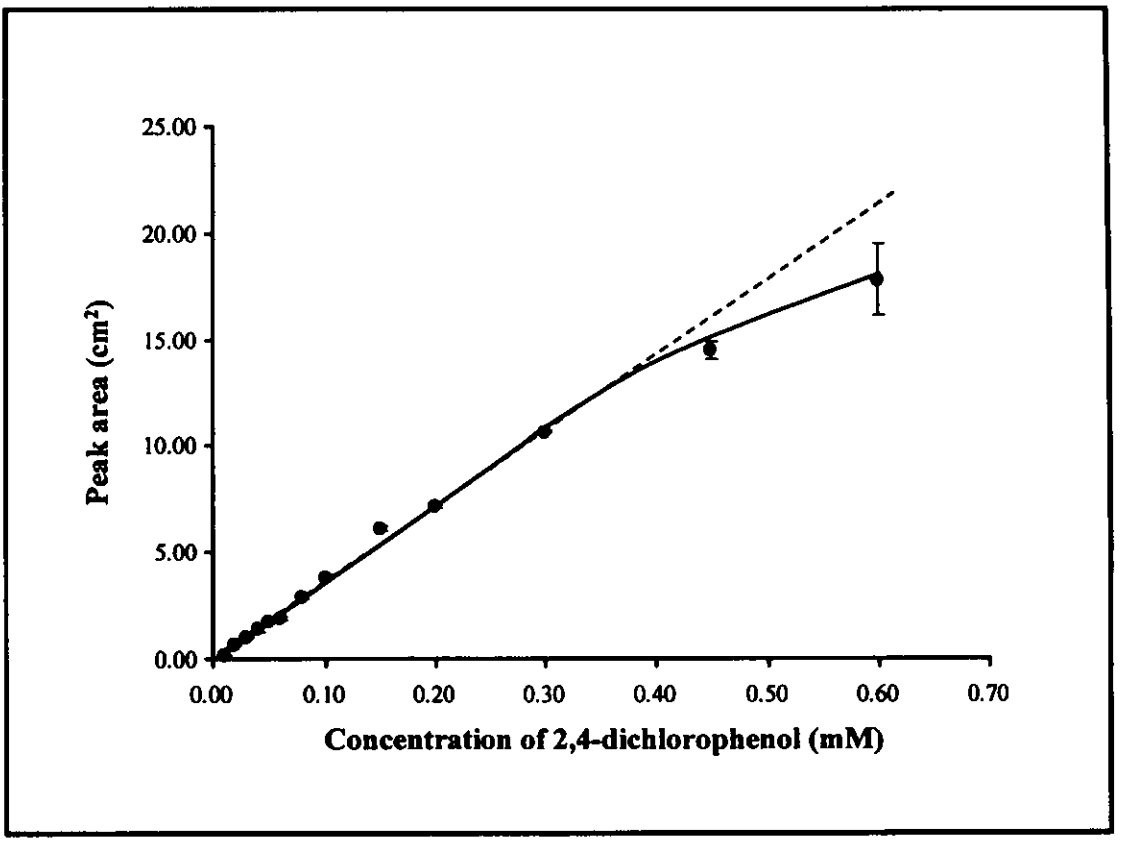


Figure 29 Responses of microbial biosensor system to different concentration of standard 2,4-dichlorophenol at optimum conditions, flow rate 0.10 ml min^{-1}, sample volume 100 µl, buffer pH 7.50 and buffer concentration 100 mM

3.2.4 Stability

During sensor storage in buffer solution without any medium, the variation of microbial strain might occur resulting in a different rate of microbial assimilation. In addition, after the sensor was used for a while, the production and accumulation of toxic metabolites can occur (Bitton, 1994). This would decrease the sensor sensitivity and stability. The death cells might be assimilated by the lysis cells. In such case, the sensor sensitivity can also increase. The response of a Clark type oxygen electrode with immobilized mixed culture bacteria operated at different time after construction are shown in Table 9 and Figure 30. The sensor sensitivity decreased with time. After one week the sensor sensitivity decreased by 39%, but the

responses remained linear. Therefore, if the microbial biosensor is calibrated before use it can be operated for at least one week, or for a total of at least 90 times.

Table 9 Operational stability of microbial biosensor system

Concentration (mM)	Peak area (cm^2) at different day			
	1^{st} mean ± SD	4^{th} mean ± SD	7^{th} mean ± SD	10^{th} mean ± SD
0.02	0.66 ± 0.04	0.32 ± 0.03	0.4 ± 0.1	0.28 ± 0.03
0.05	1.74 ± 0.07	1.46 ± 0.01	1.42 ± 0.07	1.0 ± 0.1
0.10	3.8 ± 0.1	3.6 ± 0.1	2.8 ± 0.2	2.4 ± 0.1
0.15	6.1 ± 0.1	5.4 ± 0.1	3.8 ± 0.3	3.2 ± 0.1
0.20	7.16 ± 0.10	5.7 ± 0.5	4.44 ± 0.02	4.2 ± 0.1
0.30	10.6 ± 0.07	8.4 ± 0.4	6.8 ± 0.3	6.3 ± 0.1
Analysis time (min)	9-21	7-13	6-13	5-13
Sensitivity (cm^2/ mM)	35.73	27.22	21.94	21.25
r	0.9962	0.9819	0.9941	0.9981

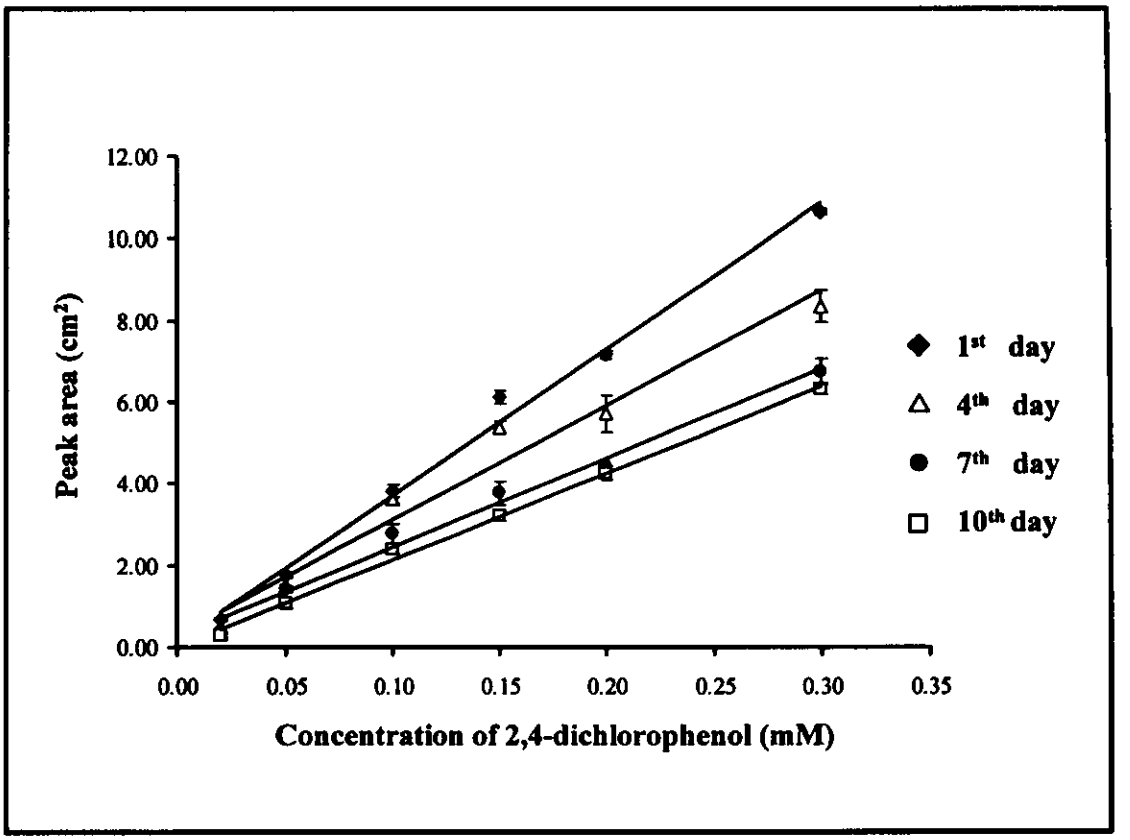


Figure 30 Operational stability of microbial biosensor system

3.2.5 Response characteristic for other compounds

Phenol and three chlorophenols (2-chlorophenol, 4-chlorophenol and 2,4,6-trichlorophenol) were tested for the response from microbial biosensor system. Other generic substances such as glucose, sucrose, sodium acetate and citric acid were also investigated (Table 10 and Figure 31). The sensor was quite selective to phenol and chlorophenols and it exhibited no or very little response to generic substances. Although mixed culture bacteria was acclimated into 2,4-dichlorophenol before using in the biosensor system, the biodegradation of other chlorophenols can occur through the same enzymatic ring fission. In this case the oxygen was consumed by the mixed culture bacteria (Autenrieth *et al.*, 1991) and the amount of oxygen could be measured.

Glucose has been reported as a prefer substrate, when it was in a medium mixture (Autenrieth *et al.*, 1991). Therefore, mixed culture bacteria could utilize glucose and gave the sensor response. For sodium acetate, it is an intermediate

product during biodegradation of 2,4-dichlorophenol in aerobic condition (Autenrieth *et al.*, 1991). Thereby, the microbial biosensor also gave a small response to this compound.

Table 10 Responses of microbial biosensor to other compounds at 0.10 mM of target substances and 0.10 mM 2,4-dichlorophenol

Compounds	Sensor response (%)
	mean ± SD
2,4-dichlorophenol	28.4 ± 0.5
2,4-dichlorophenol + Phenol	57.2 ± 1.4
2,4-dichlorophenol + 2-chlorophenol	51.4 ± 2.4
2,4-dichlorophenol + 4-chlorophenol	66.7 ± 2.6
2,4-dichlorophenol + 2,4,6-trichlorophenol	67± 29
2,4-dichlorophenol + Glucose	32.1 ± 1.3
2,4-dichlorophenol + Sucrose	29.4 ± 1.8
2,4-dichlorophenol + Sodium acetate	32.1 ± 2.1
2,4-dichlorophenol + Citric acid	28.2 ± 0.8

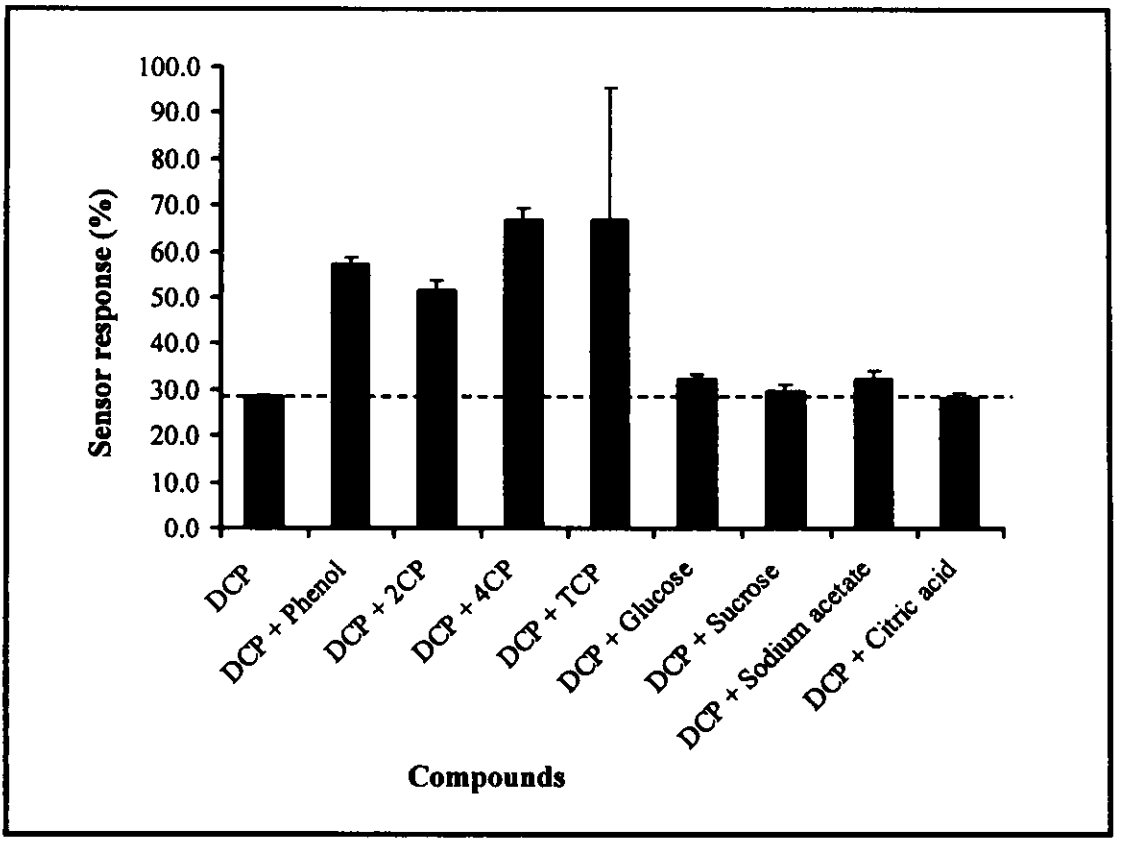
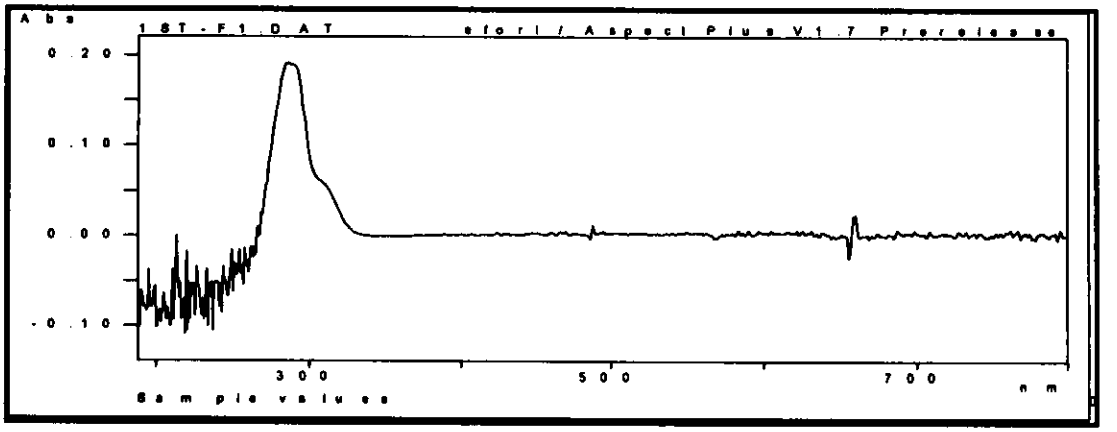


Figure 31 Responses of microbial biosensor to other compounds at 0.10 mM of target substances and 0.10 mM 2,4-dichlorophenol

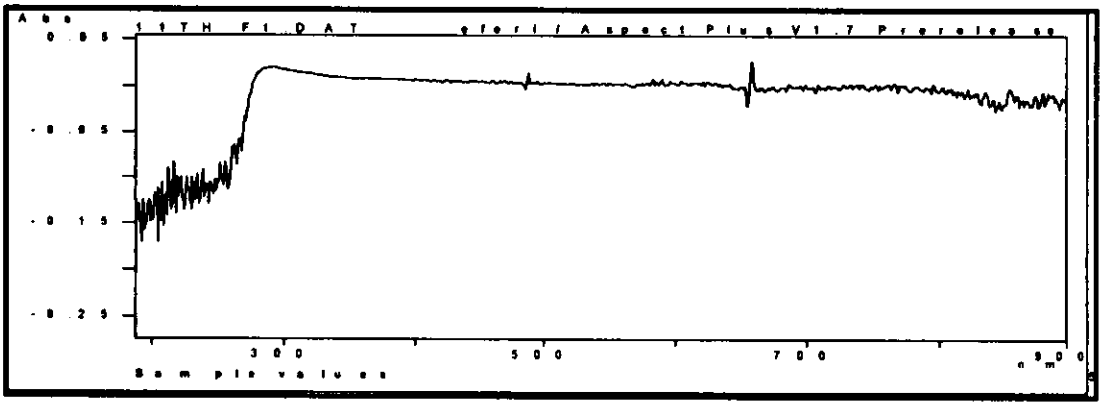
3.3 Thai mixed culture microbes

3.3.1 Optimization of culture conditions

The ability of the mixed culture microbes to assimilate 2,4-dichlorophenol as a carbon source and energy at different concentration was investigated (20.0, 30.0, 40.0, 50.0 and 60.0 mg l^{-1}). The concentration of 2,4-dichlorophenol, that decreased with time, could be detected by a UV spectrophotometer at 285 nm (maximum absorption wavelength) (Figure 32). The concentration of 2,4-dichlorophenol was reported as residual concentration (Table 11 and Figure 33).



(a)



(b)

Figure 32 Absorption spectrum of 2,4-dichlorophenol showing an absorption peak at 285 nm in the presence of 2,4-dichlorophenol (a) and no observed peak in the absence of 2,4-dichlorophenol (b)

Table 11 Residual concentration of 2,4-dichlorophenol detected by UV spectrophotometry (285 nm)

Time (h)	Concentration of 2,4-dichlorophenol (mg l^{-1})				
0	20.0	30.0	40.0	50.0	60.0
1	21.5	29.9	40.2	49.3	56.4
11	21.8	31.4	40.0	49.4	56.7
15	23.3	29.5	39.9	49.3	55.9
21	21.9	29.3	40.3	48.9	55.8
37	14.6	24.3	35.7	45.8	55.5
42	9.2	20.7	34.2	44.1	52.9
61	2.8	2.6	4.7	10.7	27.4
66	2.8	4.7	6.2	9.9	20.4
81	-	-	-	-	9.6

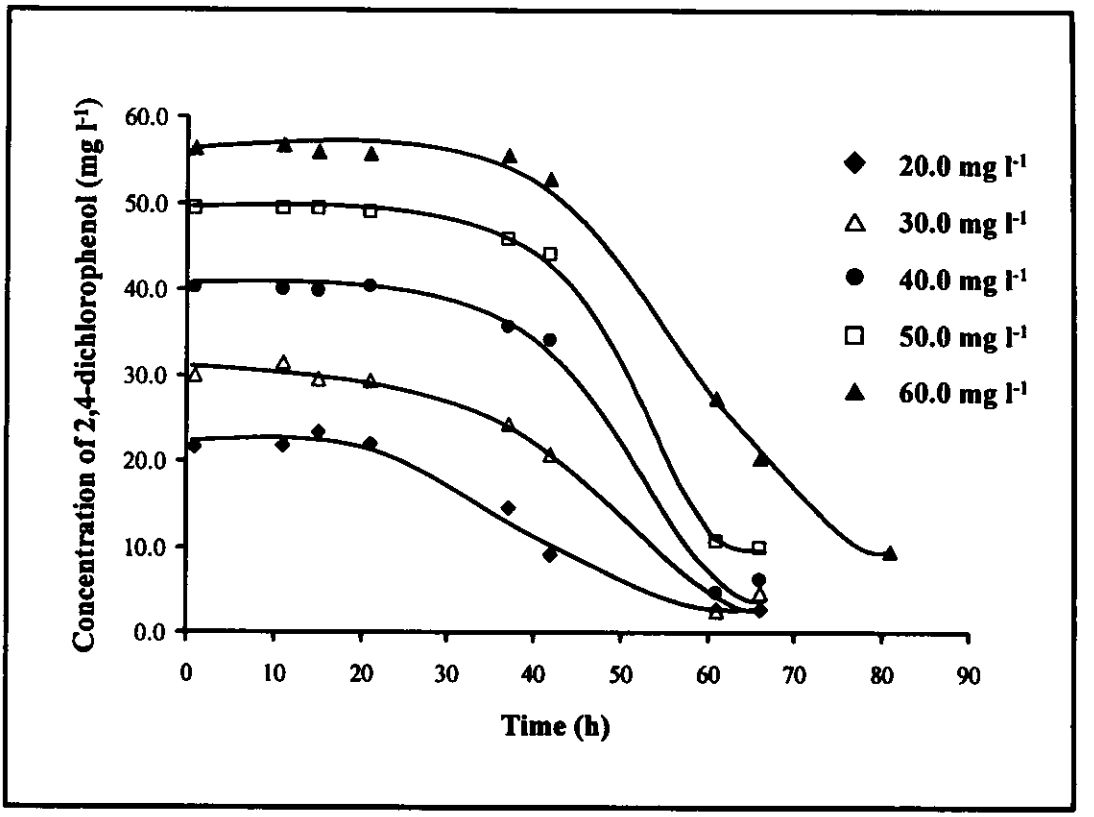


Figure 33 Residual concentration of 2,4-dichlorophenol detected by UV spectrophotometry (285 nm)

The lag phase was 21 hours at concentration of 20.0, 30.0, 40.0 and 50.0 mg l^{-1}, for 60.0 mg l^{-1} it was longer (21-37 hours). At 20.0 and 30.0 mg l^{-1} the mixed culture microbes took 66 hours to degrade 87 and 84 % of 2,4-dichlorophenol, respectively, but only 35 % of 2,4-dichlorophenol could be degraded at 60.0 mg l^{-1}. However, the mixed culture microbes did not show any inhibition by the high concentration. Therefore, 60.0 mg l^{-1} of 2,4-dichlorophenol was chosen as an appropriate concentration for cultivation of mixed culture microbes and to be used in a biosensor system. In this case, the cultivation has a longer lag time.

When compared the activity to a controlled experiment (no mixed culture microbes), it was found that there was a small reduction of 2,4-dichlorophenol (Table 12 and Figure 34). This might be the volatilization of 2,4-dichlorophenol because it is semi-volatile compounds (Henry's law constant between $3 \times 10^{-4} - 10^{-2}$) (Mitra, 2003). Since the reduction of concentration of 2,4-dichlorophenol caused from

microbes growth was much higher, it can be concluded that the mixed culture microbes utilized 2,4-dichlorophenol as a carbon source and energy.

Then 60.0 mg l^{-1} of 2,4-dichlorophenol was used for the cultivation, the active mixed culture microbes was harvested after 80 hours (late exponential phase). They were immobilized in calcium alginate gel and used in a microbial biosensor system.

Table 12 Residual concentration of 2,4-dichlorophenol detected by UV spectrophotometry at 285 nm of controlled experiment

Time (h)	Concentration of 2,4-dichlorophenol (mg l^{-1})	
	Controlled no mixed culture microbes	With mixed culture microbes
0	60.0	60.0
15	57.9	58.3
23	58.4	58.4
39	57.2	54.4
48	53.6	44.6
63	53.3	11.4
73	51.0	7.9

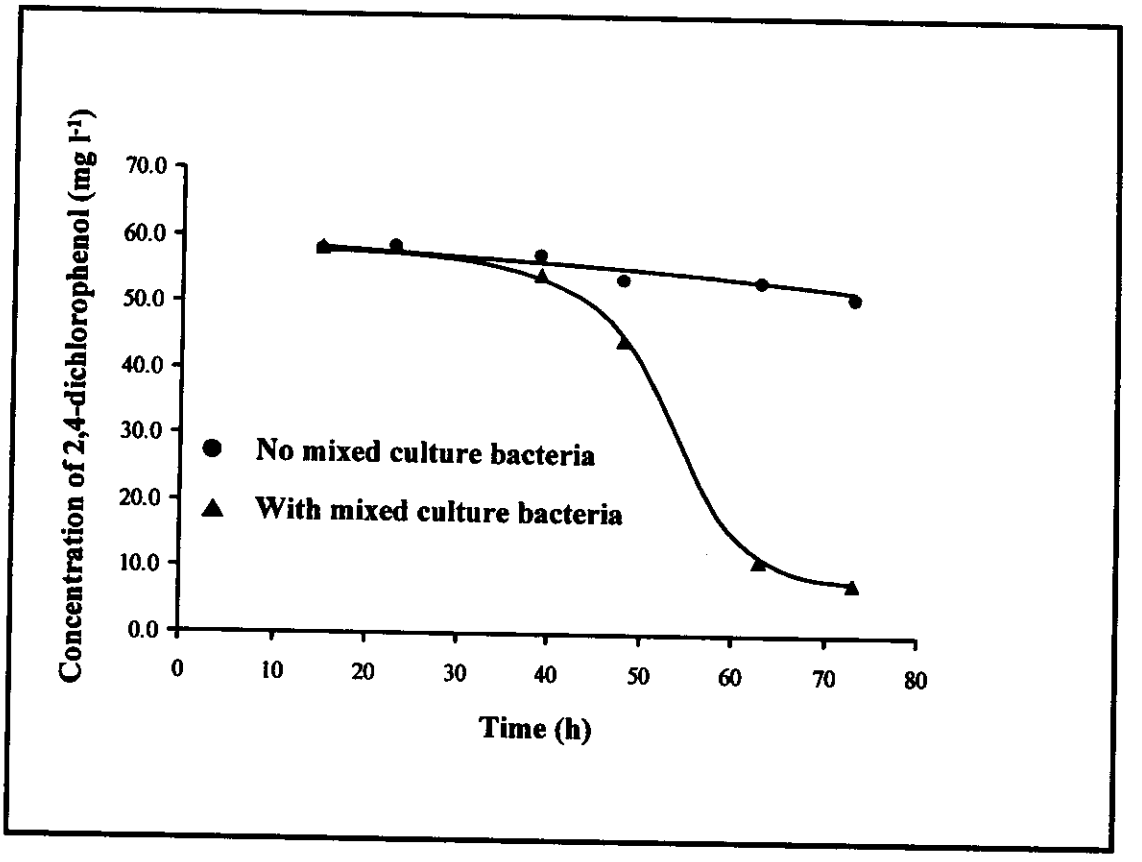


Figure 34 Residual concentration of 2,4-dichlorophenol detected by UV spectrophotometry at 285 nm of controlled experiment

3.3.2 Optimization of operational conditions

3.3.2.1 Flow rate

The responses of 5.0 mg l^{-1} to 40.0 mg l^{-1} standard 2,4-dichlorophenol to different flow rates are shown in Table 13 and Figure 35. The sensor responses and sensitivities decreased as the flow rates increased. The sensitivity at 0.25 ml min^{-1} was less than at 0.10 ml min^{-1} by 75 %. Therefore, 0.10 ml min^{-1} was chosen as an optimum flow rate.

Table 13 Responses of microbial biosensor system at different flow rates

Concentration ($mg\ l^{-1}$)	Sensor response (mV) at different flow rates		
	0.10 ml min^{-1} mean ± SD	0.25 ml min^{-1} mean ± SD	0.50 ml min^{-1} mean ± SD
5.0	3.8 ± 0.2	3.0 ± 0.3	3.7 ± 0.3
10.0	5.9 ± 0.3	5.5 ± 0.3	4.4 ± 0.3
20.0	10.7 ± 0.4	6.8 ± 0.1	4.96 ± 0.07
30.0	15.0 ± 0.4	7.8 ± 0.1	5.83 ± 0.07
40.0	19.1 ± 0.4	8.8 ± 0.4	5.8 ± 0.1
Analysis time (min)	36-60	24-36	12-18
Sensitivity ($mV/\ mg\ l^{-1}$)	0.44	0.11	0.08
r	0.9997	0.9987	0.9886

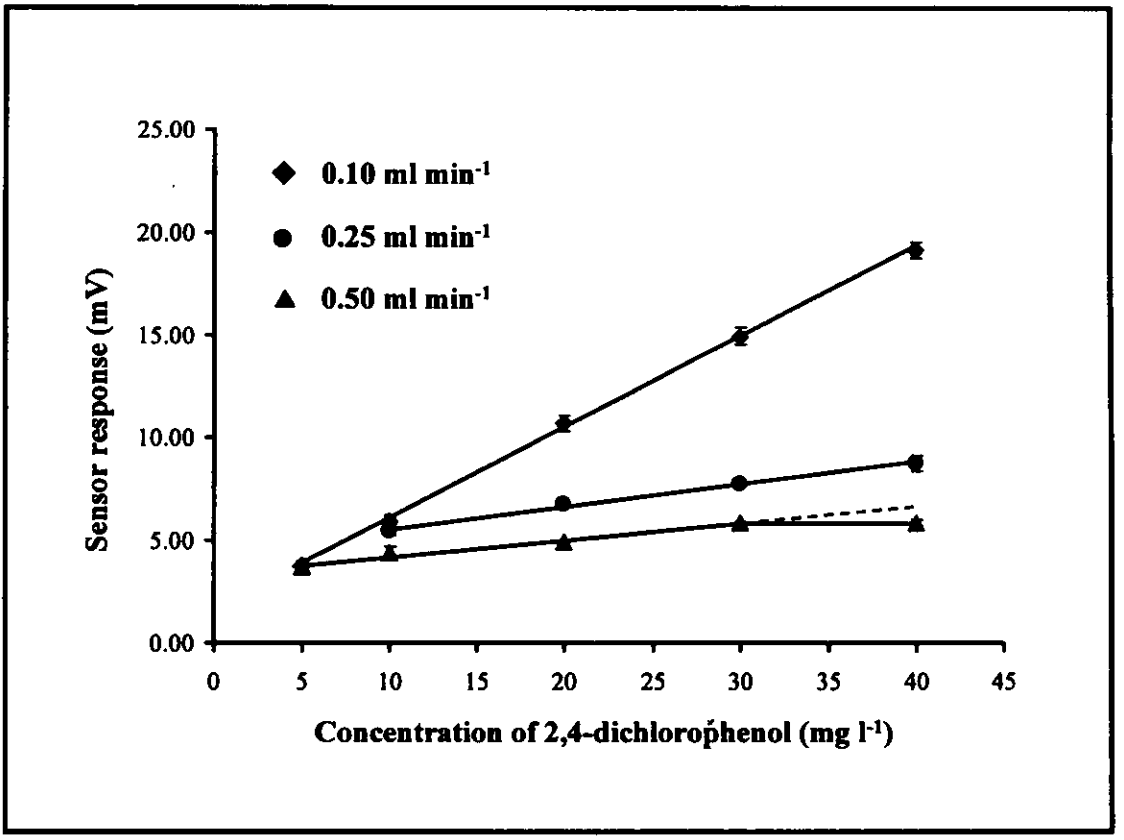


Figure 35 Responses of microbial biosensor system at different flow rates

3.3.2.2 Sample volume

Responses of 0.5 mg l^{-1} to 30.0 mg l^{-1} standard 2,4-dichlorophenol at different sample volumes are shown in Table 14 and Figure 36. The sensor response and sensitivities increased as the sample volumes increased, but the analysis time was also prolonged. At sample volume of 300 µl, the sensor could detect 2,4-dichlorophenol at 5.0 mg l^{-1}, meanwhile at 500 µl the sensor could detect the sample down to 0.5 mg l^{-1}. The sample volume of 500 µl was chosen as an optimum value despite the large volume and the fact that the analysis time was almost an hour. This is to meet the required lower detection limit at 1 mg l^{-1}, the permitted concentration of phenolic compounds of industrial effluent (Ministry of Industry, Thailand, 1996). The large volume should not be a problem since the real samples would be environmental sample, which can easily be collected at large amount.

Table 14 Responses of microbial biosensor system at different sample volumes

Concentration ($mg\ l^{-1}$)	Sensor response (mV) at different sample volumes		
	300 µl mean ± SD	400 µl mean ± SD	500 µl mean ± SD
0.5	ND	ND	1.67 ± 0.06
1.0	ND	1.02 ± 0.03	2.6 ± 0.3
5.0	4.5 ± 0.1*	6.3 ± 0.5	8.0 ± 0.5
10.0	9.3 ± 0.8	10.5 ± 0.6	11.0 ± 0.3
20.0	15.2 ± 1.0	13.3 ± 0.9	13.8 ± 0.9
30.0	16.0 ± 0.7	13.7 ± 0.5	16.5 ± 2.0
Analysis time (min)	24-48	30-48	30-66
Sensitivity ($mV/\ mg\ l^{-1}$)	0.69	1.04	1.38
r	0.9908	0.9918	0.9996

(* = the uncertainty of the measuring scale)

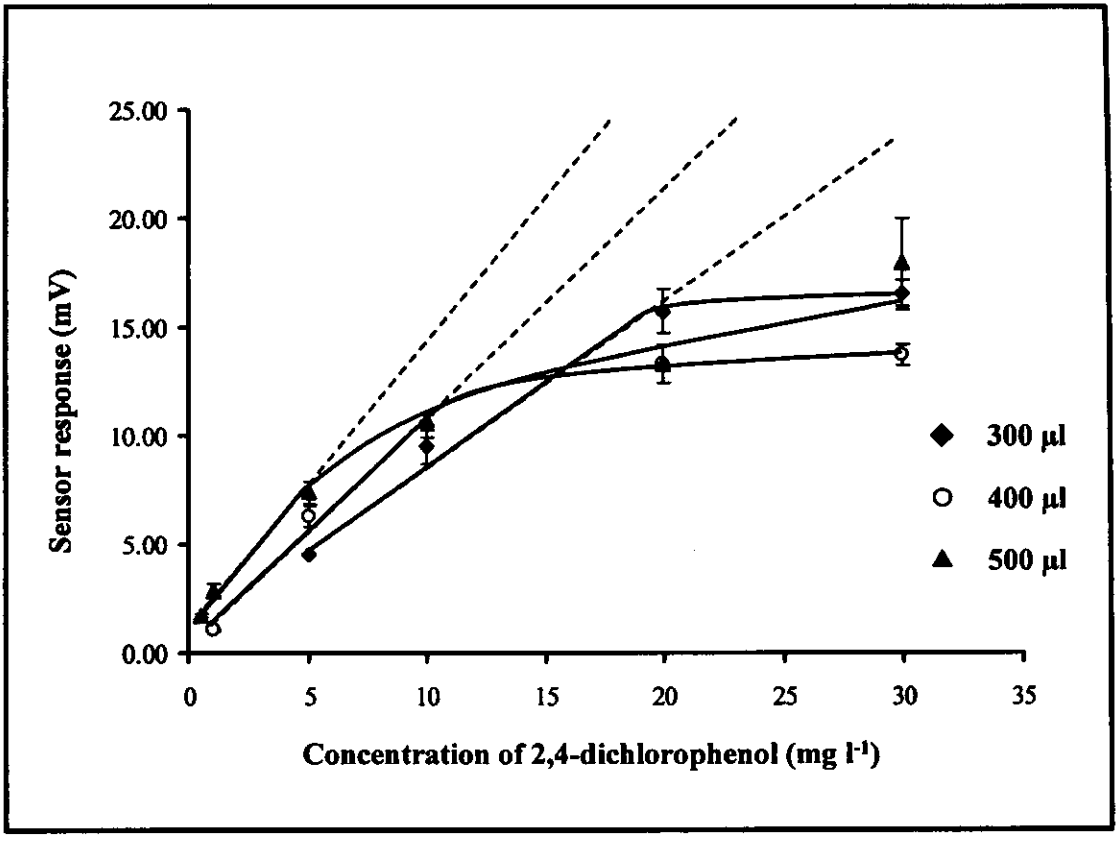


Figure 36 Responses of microbial biosensor system at different sample volumes

3.3.2.3 Buffer pH

The effect of buffer pH was studied by using tris-HCl buffer containing 10 mM $CaCl_2$. $CaCl_2$ was added into buffer solution to stabilize alginate beads (Adinarayana *et al.*, 2004). The responses of 0.3 mg l^{-1} to 5.0 mg l^{-1} standard 2,4-dichlorophenol at pH 6.50, 7.00 and 7.50 are shown in Table 15 and Figure 37. The sensor sensitivities decreased as buffer pH increased. The highest sensitivity was obtained at pH 6.50 and was chosen as an optimum pH.

Table 15 Responses of microbial biosensor system at different buffer pH

Concentration ($mg\ l^{-1}$)	Sensor response (mV) at different buffer pH		
	pH 6.50 mean ± SD	pH 7.00 mean ± SD	pH 7.50 mean ± SD
0.3	5.1 ± 0.1	7.8 ± 0.6	1.4 ± 0.1
0.5	6.8 ± 0.8	8.8 ± 0.3	2.0 ± 0.1
1.0	8.5 ± 0.3	10.0 ± 0.5	2.3 ± 0.1
3.0	20.1 ± 1.3	17.1 ± 0.3	6.8 ± 0.5
5.0	28.3 ± 2.4	22.6 ± 0.7	10.8 ± 0.4
Analysis time (min)	36-72	36-42	36-54
Sensitivity ($mV/ mg\ l^{-1}$)	4.97	3.17	2.02
r	0.9972	0.9985	0.9982

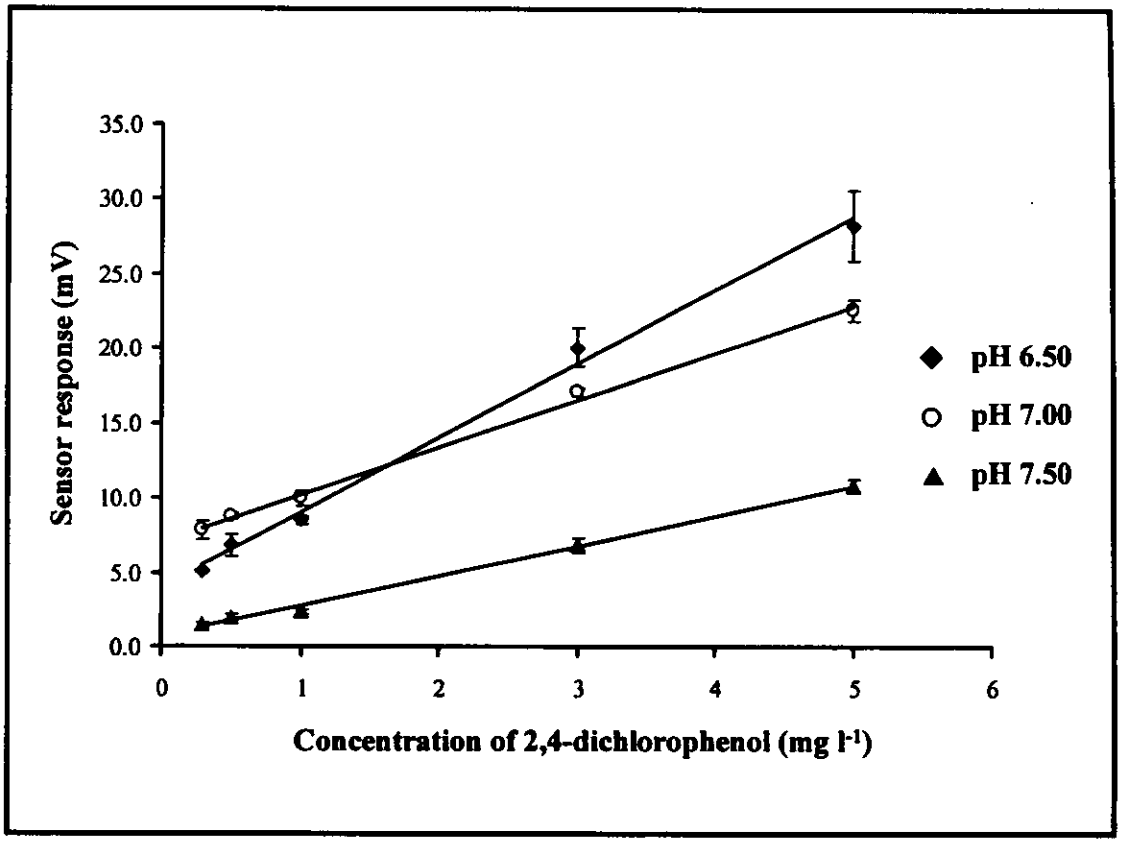


Figure 37 Responses of microbial biosensor system at different buffer pH

3.3.2.4 Buffer concentration

The responses of 5.0 mg l^{-1} standard 2,4-dichlorophenol at different buffer concentration, 50, 100, 150, 200 and 250 mM are shown in Table 16 and Figure 38. Tris-HCl buffer containing 10 mM $CaCl_2$ was used as a buffer solution. The highest sensor response was obtained at buffer concentration of 100 mM, meanwhile there were not much differences in sensor responses at 50, 150, 200 and 250 mM. The decreasing of sensor response at 150-250 mM caused from high concentration of tris-HCl buffer. These include its reactivity as a primary amine and its appreciable solubility in organic solvents which leads to its accumulation in the biological phase of reaction system. Thus, tris-HCl buffer displace the electron transportation (Perrin and Dempsey, 1974). In this study, 100 mM tris-HCl with 10 mM $CaCl_2$ was chosen as an optimum buffer concentration.

Table 16 Responses of microbial biosensor system at different buffer concentrations

Buffer concentration (mM)	Sensor response (mV) mean ± SD	Analysis time (min)
50	4.3 ± 0.1	36
100	6.0 ± 0.5	36
150	3.8 ± 0.3	36
200	4.3 ± 0.3	42
250	4.3 ± 0.5	42

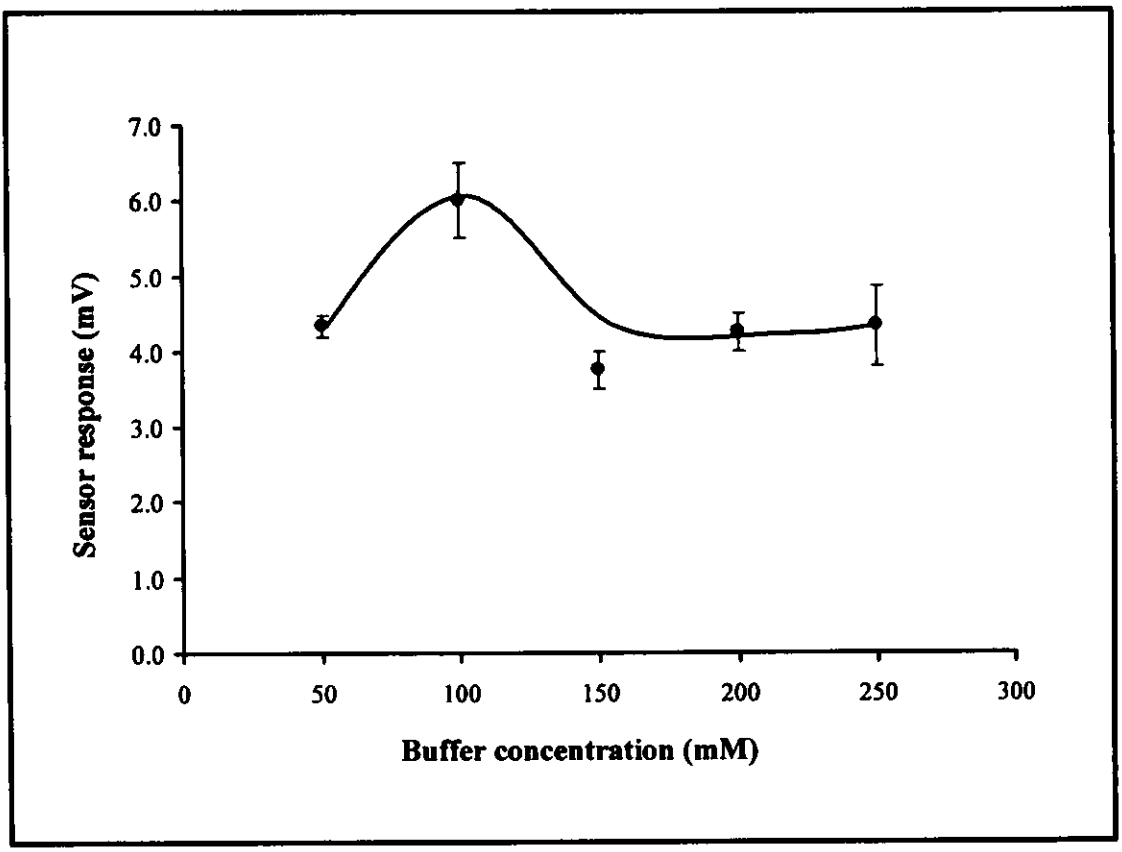


Figure 38 Responses of microbial biosensor system at different buffer concentration

3.3.3 Linearity

The linearity of microbial biosensor system to different concentrations of 2,4-dichlorophenol is shown in Table 17 and Figure 39.

Table 17 Responses of microbial biosensor system to different concentrations of standard 2,4-dichlorophenol

Concentration (mg l^{-1})	Sensor response (mV)
	Mean ± SD
0.001	4.2 ± 0.1
0.01	4.3 ± 0.1*
1.0	4.3 ± 0.1
5.0	7.3 ± 0.5
9.0	10.0 ± 0.3
13.0	11.9 ± 0.3
17.0	14.5 ± 0.3
21.0	18.9 ± 0.1
25.0	22.1 ± 0.2
29.0	27.0 ± 1.0
33.0	28.2 ± 1.4
37.0	33.2 ± 0.8
41.0	38.3 ± 1.3
45.0	43.2 ± 0.3
49.0	45.5 ± 1.8
53.0	51.7 ± 1.6
57.0	55.7 ± 0.8
61.0	60.7 ± 0.3
65.0	49.7 ± 0.6
Analysis time (min)	30-60
Sensitivity (mV/ mg l^{-1}) of 17.0-61.0 mg l^{-1} standard 2,4-dichlorophenol solution	1.04
r	0.9973

(* = the uncertainty of the measuring scale)

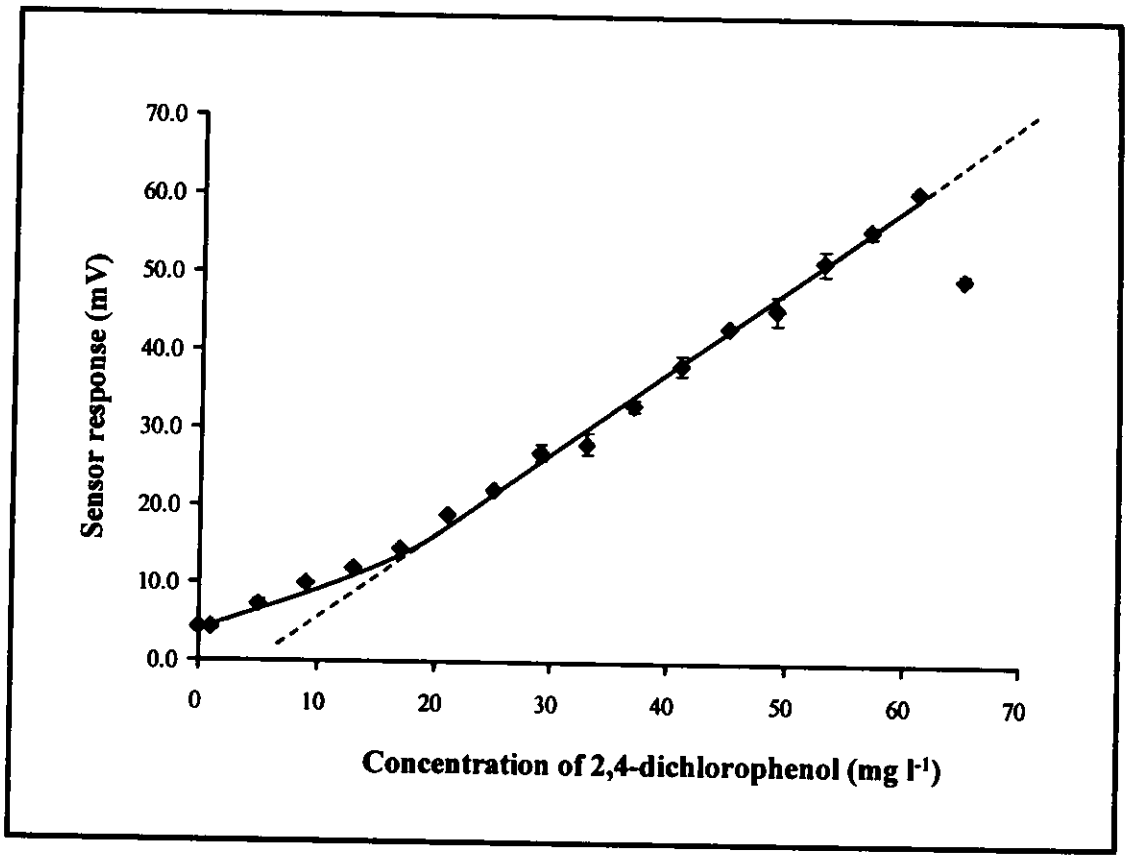


Figure 39 Responses of microbial biosensor system to different concentrations of standard 2,4-dichlorophenol

The linear range of cell-based biosensor was from 17.0 mg l^{-1} to 61.0 mg l^{-1}. At optimum conditions, to determine the lower limit of detection, 10 blanks were injected into the sensor system. It provided the average signal of 3.3 ± 0.3 mV. The lowest detectable signal is determined by using the average blank signal plus 3SD (blank), which was 4.2 mV. When compared this value to responses in Table 17, the limit of detection of this system is 0.01 mg l^{-1} of 2,4-dichlorophenol (Taverniers *et al.*, 2004). Comparing to microbial biosensor used *Rhodococcus* (0.65 mg l^{-1}), the detection limit was in the same range (Riedel *et al.*, 1993). When the limit of detection between Swedish mixed culture bacteria and Thai mixed culture microbes were compared, it was found that Thai mixed culture microbes provided better limit of detection than Swedish mixed culture bacteria (3.26 mg l^{-1}). This could be the use of column with immobilized cells, where the sample had longer time to pass through and contact to the immobilized microbes.

3.3.4 Stability

Besides the variation of microbial strain during storage, the loss of microbial cells from alginate gel during the operation can also occur. This can cause the decrease of sensor sensitivity and stability. The responses of microbial biosensor using immobilized cells in alginate gel with respect to time are shown in Table 18 and Figure 40. The sensor sensitivities of the second and third day are higher than the first day. These may cause by the way the biosensor system was set up. During each experiment consecutive measurements were carried out at room temperature (25 ± 2°C). Between experiments, when the sensor was not used, buffer solution was passed through the reactor column at room temperature. Therefore microbes growth may occur and, thus, increase the biomass in the reactor column. However, on the fourth and fifth day the sensor responses decreased and were not linear at high concentration. It might be because the microbes reached the stationary and death phase.

Table 18 Operational stability of microbial biosensor system

Concentration ($mg\ l^{-1}$)	Sensor response (mV) at different day				
	1^{st} mean ± SD	2^{nd} mean ± SD	3^{rd} mean ± SD	4^{th} mean ± SD	5^{th} mean ± SD
1	4.8 ± 0.3	9.8 ± 1.0	4.0 ± 0.5	4.5 ± 0.5	4.8 ± 0.3
10	8.3 ± 0.4	13.8 ± 1.0	8.5 ± 0.5	11.3 ± 0.8	11.9 ± 1.1
20	10.9 ± 0.6	16.7 ± 2.1	12.3 ± 1.1	13.8 ± 0.3	15.5 ± 1.3
30	13.0 ±1.2	22.8 ± 1.3	14.8 ± 0.3	14.3 ± 1.3	18.2 ± 1.0
Analysis time (min)	24-72	30-60	18-42	30-48	30-48
Sensitivity ($mV/\ mg\ l^{-1}$)	0.3	0.4	0.4	-	-
r	0.9910	0.9890	0.9900	-	-

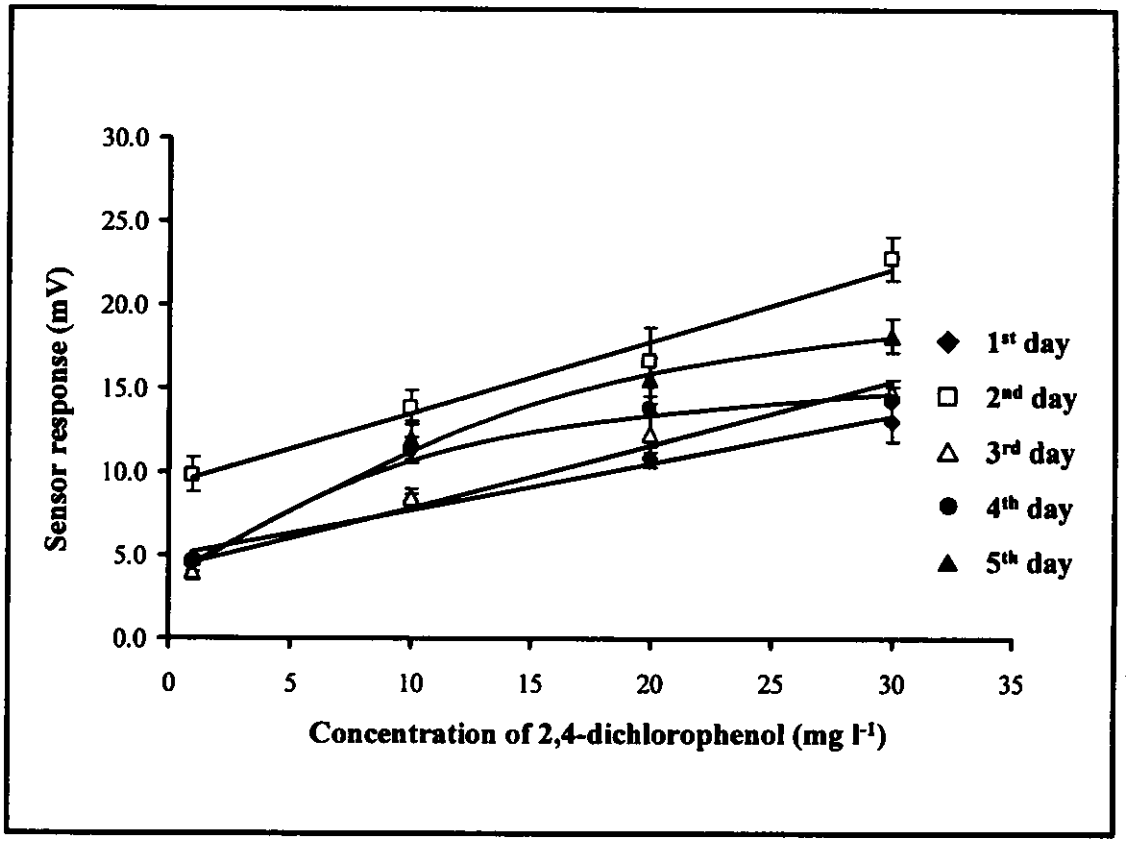


Figure 40 Operational stability of microbial biosensor system

3.3.5 Response characteristic for other compounds

Phenolic, aromatic compounds and generic substances were investigated for their responses (Table 19 and Figure 41). The microbial biosensor response to 9.0 mg l^{-1} standard 2,4-dichlorophenol was calculated as 100%. The immobilized mixed culture microbes could provide response to all phenolic compounds, except PCP. Particularly, catechol showed nearly the same response as 2,4-dichlorophenol. Aromatic compounds, *i.e.* benzene and benzoic acid, also provided the sensor response but was $\leq$ 50 %. However, when benzene was mixed with 2,4-dichlorophenol, the sensor response was the same as when 2,4-dichlorophenol was applied alone (99 and 100 %, respectively). This indicated that benzene would not interfere with the determination of chlorophenols by this microbial biosensor system. Similar behavior was also obtained from glucose.

Table 19 Responses characteristic of microbial biosensor to other compounds (9.0 mg l^{-1} of target substance and 9.0 mg l^{-1} of 2,4-dichlorophenol)

Target substances	Concentration (mg l^{-1})	Sensor response (%)		
		Single	Mixture 2,4-DCP + target substance	Signal 2,4-DCP + target substance
2,4-DCP	9.0	100	-	-
2,4,6-TCP	9.0	76	156	176
PCP	9.0	0	79	100
Phenol	9.0	34	75	134
Catechol	9.0	104	206	204
Benzene	9.0	34	99	134
Benzoic acid	9.0	50	163	150
Glucose	9.0	33	101	133
Sucrose	9.0	61	125	161
Sodium acetate	9.0	175	249	275

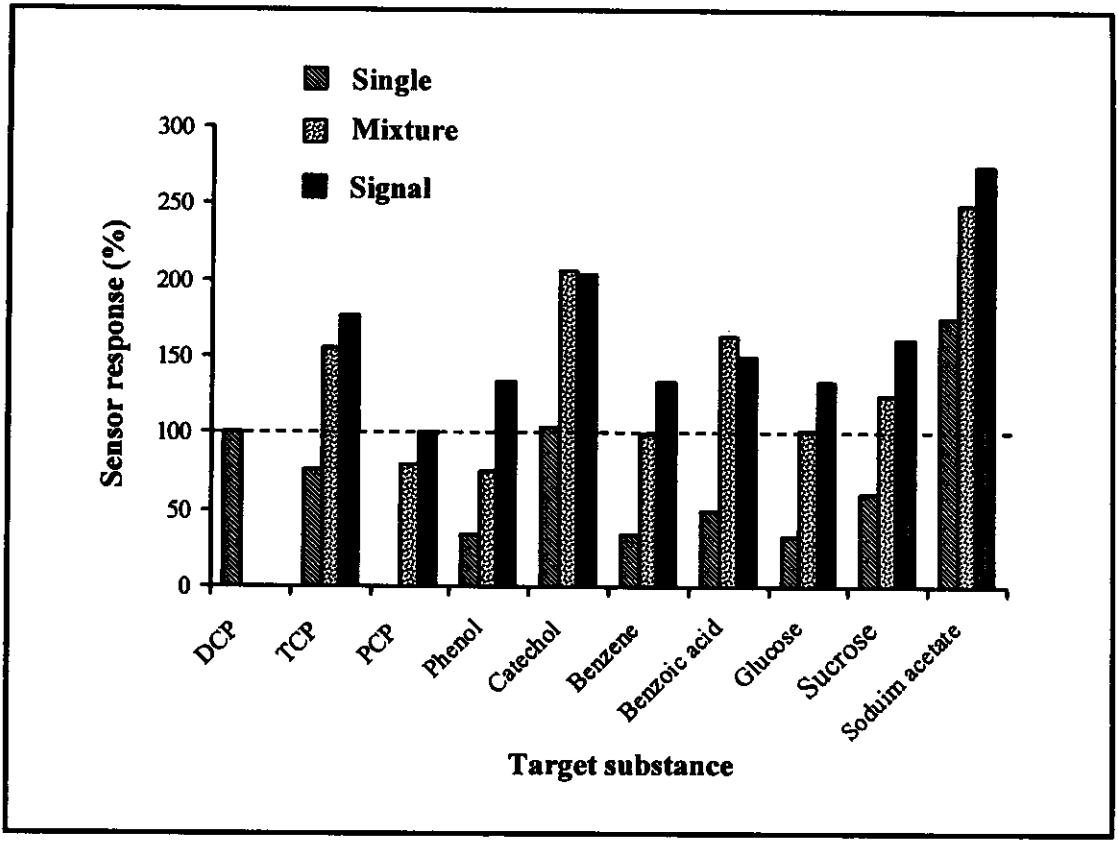


Figure 41 Responses characteristics of microbial biosensor to other compounds

3.3.6 Repeatability

The immobilized cells in calcium alginate beads were packed in the reactor column (5.20 g) to study the repeatability (Table 20 and Figure 42). The sensor responses of eight injections of 7.0 mg l^{-1} standard 2,4-dichlorophenol and the standard deviation were calculated. The percent relative standard deviation (% RSD) is within 10 %.

Table 20 Responses of microbial biosensor system at different injections 7.0 mg l^{-1} 2,4-dichlorophenol

Peak number	Sensor response (mV)
1	6.8
2	7.5
3	7.8
4	6.9
5	8.5
6	7.5
7	8.3
8	8.6
Mean	7.7
SD	0.7
% RSD	9.1 %

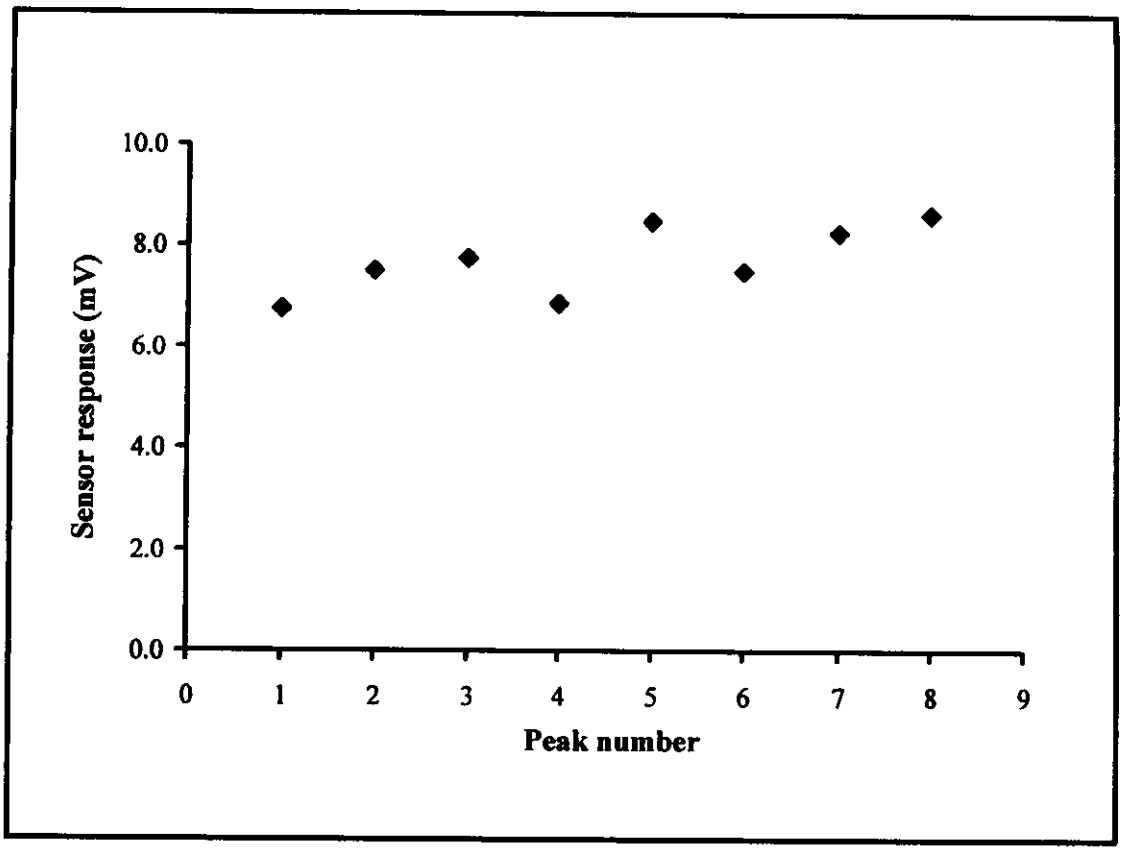


Figure 42 Responses of microbial biosensor system at different injections of 7.0 mg l^{-1} 2,4-dichlorophenol

3.3.7 Reproducibility

The reproducibility was studied by investigating the responses from 4 reactor columns. They were packed with equal amount of immobilized cells in calcium alginate gel (5.20 g). The sensor responses are shown in Table 21 and Figure 43. The significant difference was tested by using the statistical for several means (Miller and Miller, 2000). The sensor responses of column III were not significantly differ from column II and IV ($P < 0.05$), whereas the sensor responses of column I were differ from others. Although each column was packed with equal amount of alginate gel beads (5.20 g), the amount of entrapped cell might be different. Therefore, recalibration when changing the column may be necessary.

Table 21 Responses of microbial biosensor system by using different packed reactor column injected 7.0 mg l^{-1} 2,4-dichlorophenol

Column	Sensor response (mV) Mean ± SD	% RSD
I	6.4 ± 0.4	5.9
II	7.4 ± 0.4	5.1
III	7.6 ± 0.4	5.0
IV	8.2 ± 0.1	1.7

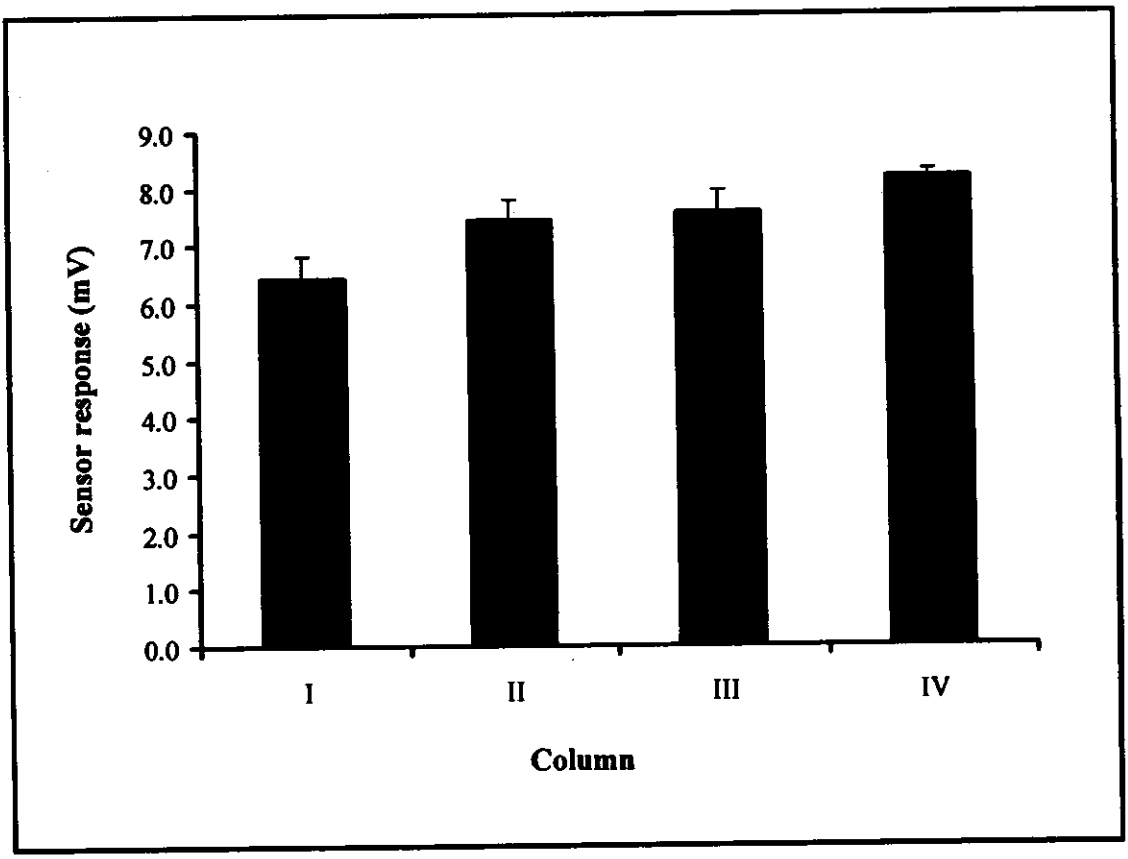


Figure 43 Responses of microbial biosensor system from different reactor columns injected 7.0 mg l^{-1} 2,4-dichlorophenol

3.3.8 Determination of chlorophenols in wastewater

Before the analysis of real sample, standard 2,4-dichlorophenol was injected into GC/MS where the peak appear at a retention time of 9.46 min. The mass fragments of 2,4-dichlorophenol (from the most abundance to the least) were at *m/z* 63 and 162. Real water sample from wastewater treatment pond of Songklanagarind Hospital, Prince of Songkla University was analyzed for 2,4-dichlorophenol by GC/MS monitored at this retention time and mass fragments in SIM mode (Selective Ion Monitoring). 2,4-Dichlorophenol was not found by GC/MS analysis. Then, the scan mode was also studied and showed *m/z* at 149. From the GC/MS library it showed that the compound might be phthalic acid. This compound consists of a benzene ring and two carboxylic substituted groups at *ortho* position.

When cell-based biosensor was used to analyze the real sample, very high sensor responses were obtained. This implied that the biosensor also responded to phthalic acid.

In addition, the study of response characteristics to other compounds showed sensor response to various aromatic compounds. Therefore, it would be possible to use this type of microbial biosensor to be a screening method for aromatic compounds.