CHAPTER 4

RESULTS

4.1 Sediment samples

The sediment samples were collected using the stainless steel grab in May 2003. The sampling sites were chosen with the criteria of the river or canal locatations. All of them must be near the heavy traffic area or/and presents the aquatic transportation in respective river or canal. Due to the water resource, which locate near high level of traffic area may be contaminated by PAH atmospheric deposition (Patarasiriwong and Boonyoy, 2002). Furthermore, the petroleum spillage and exhausted gas from engine oil play as other factors of PAH contamination in sediments (Zakaria *et al.*, 2002). Temperature of sediment samples was in the range of 28-33°C.

4.2 Concentration of PAHs in sediments

The PAH concentrations were determined by dichloromethane extraction and HPLC analysis using HPLC equipped with UV detector at 275 nm; 80% methanol as mobile phase. From the results, fluoranthene in the sediments of Saen-Saeb Canal, Phrachulachomklao Royal Navy Dockyard, the Chao-Phraya River and the Padungkrungkasem Canal was detected at the concentration of 5.4, 3.2 and 0.81 μg/g dry weight, respectively. Phenanthrene could found at the concentration of 0.13 and 0.2 μg/g dry weight for 2 sampling sites (S_1 and S_2) at the Saen-Saeb Canal. Other PAHs could not detect by HPLC. The PAH concentrations were obtained via calculation with comparing the standard curve. The concentration of PAHs, in the collected sediment samples, is presented in Table 4.1. The PAH frequently detected in these sediments was fluoranthene.

Table 4.1 Concentration of PAHs in sediment samples

Sampling sites	PAHs	
	Compounds	**Concentrations (µg/g dry weight of sediment)**
S_1, Ratchathewi pier (The Saen-Saeb Canal)	Fluoranthene Phenanthrene	5.4 0.2
S_2, Pratunam pier (The Saen-Saeb Canal)	Phenanthrene	0.13
S_3, Panfa-leelard pier (The Saen-Saeb Canal)	nd*	nd
S_4, Wat Sri-boon-reung pier (The Saen-Saeb Canal)	nd	nd
S_5, Phrachulachomklao Royal Navy Dockyard	Fluoranthene	3.2
S_6, See-phraya pier (The Chao-Phraya River)	nd	nd
S_7, Sa-thon pier (The Chao-Phraya River)	nd	nd
S_8, Padungkrungkasem Canal	Fluoranthene	0.81

* nd : not detected by HPLC

4.3 Isolation of the PAHs degrading bacteria

4.3.1 Enrichment culture

The PAHs degrading bacteria were enriched and isolated from sediment samples based on their degradability of fluorene, fluoranthene and pyrene at 100 mg/l of the concentration containing in liquid CFMM. The subsequent transfer was conducted when the growth of culture was visualized. The bacterial growth was indicated by colour changing and/or increasing of turbidity when compared with the control (Bouchez *et al.*, 1995). After five successive transfer, the culture from samples of the Saen-Saeb Canal (S_1) and Phrachulachomklao Royal Navy Dockyard, the Chao-Phraya River (S_5) increased turbidity and the colour of the culture was changed from colourless to yellow or pale pink, whereas pyrene and fluoranthene crystals were depleted.

4.3.2 Isolation the PAHs degrading bacterial strains

The enrichment cultures from samples of the Saen-Saeb Canal (S_1) and Phrachulachomklao Royal Navy Dockyard (S_5) were spreaded on CFMM plates and sprayed with either pyrene or fluoranthene solubilized in diethyl ether at 2 % (w/v), then incubated at 30°C. The colonies with clear zone showed the capability of PAHs degradation (Figure 4.1). These colonies were inoculated in liquid CFMM supplemented with 100 mg/l of respective enrichment substrate. After the colour of medium was changed, the culture were diluted and spreaded on CFMM agar with respective PAH vapor phase. The enriched culture from the Saen-Saeb Canal (S_1) consisted of 3 strains, namely, strain PY1, PY2 and PY3. For the enriched culture from Phrachulachomklao Royal Navy Dockyard, the Chao-Phraya River (S_5) contained 2 strains, strain FT1 and FT2. The colonies growing on CFMM plate were characterized and the results were shown in Table 4.2.

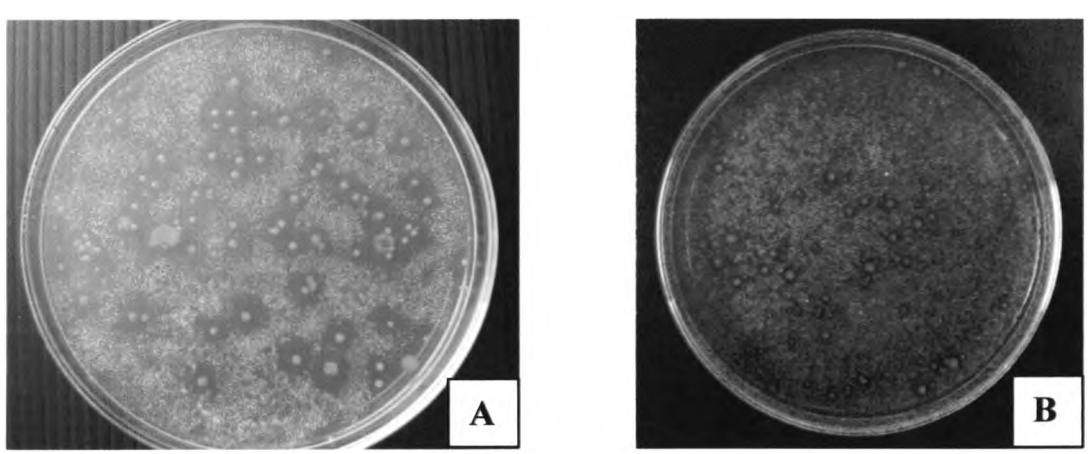

Figure 4.1 The clear zone around the bacterial colony of enriched culture from S_1 (A) and S_5 (B).

Table 4.2 Colony characteristics of bacteria from S_1 and S_5

Sites	Substrate	Strain	Colony characteristics
S_1	Pyrene	PY1	- Small and round shape, smooth and sticky surface, yellow pigment
		PY2	- Irregular shape, pale yellow colour
		PY3	- Round shape, smooth surface, orange pigment
S_5	Fluoranthene	FT1	- Large and round shape, smooth surface, yellow pigment
		FT2	- Small and round shape, cream colour

Each strain was individually innoculated in liquid CFMM supplemented with 100 mg/l of respective enrichment PAH. After incubation, only two strains namely PY1 and FT1 could grow in CFMM, which represented by decreasing of PAH crystals and the medium colour change. The colony characterization of the isolated strain PY1 and strain FT1 were demonstated in Figure 4.2. Moreover, the colour of culture media had change therefore, the strain PY1 and FT1 were chosen for further identification and evaluation of PAH degrading ability.

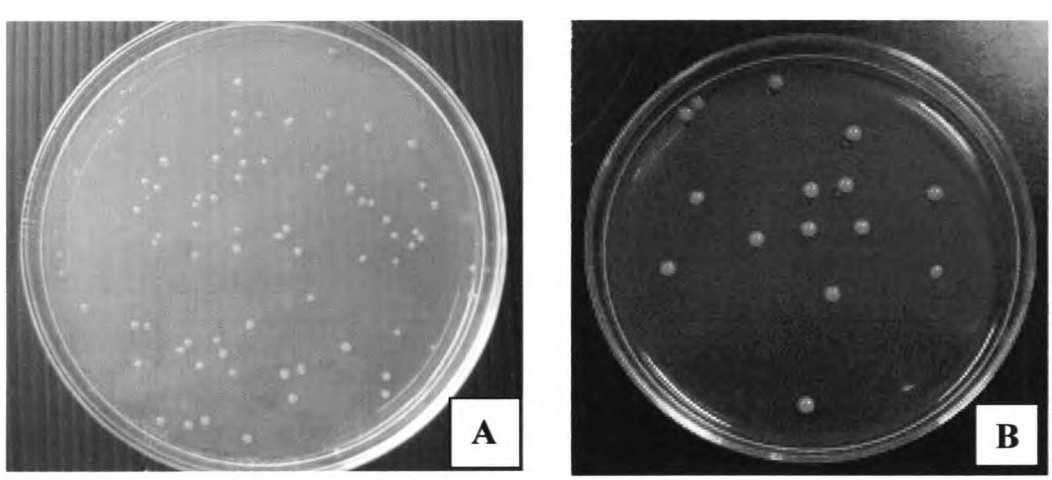

Figure 4.2 Colony characterization of isolated strain PY1 (A) and strain FT1 (B).

4.4 Identification and characterization the isolated strains

4.4.1 Morphological and biochemical characterizations

The pyrene degrading bacteria strain PY1 was obtained from sediment sample from The Saen-Saeb Canal. This strain was Gram positive, acid-fast positive, dimorphism cell form and the colony was smooth with yellow pigmented. Detailed characteristics of strain PY1 were described in Table 4.3. Strain FT1 or fluoranthene degrader, which was isolated from sediments of Phrachulachomklao Royal Navy Dockyard, was rod shape, Gram negative, large and smoothed-yellow colony. Table 4.4 showed the bacteriological and biochemical characterizations of the strain FT1. The microscopic morphologies of the strains PY1 and FT1 were stained by acid-fast technique and Gram staining, respectively, which are shown in Figure 4.3.

Table 4.3 Morphological and biochemical characterizations of the isolated strain PY1

Characteristics	Results*
Microbiological characteristics	
Colonial morphology	Yellow colour colony
Gram reaction	Gram positive
	Rod shape
Motility	-
Acid-fast stain	+
Growth tests	
Glucose	-
Mannitol	+
Fructose	-
Sorbitol	-
Simmon citrate	-
Succinate	-
Biochemical characteristics	
Oxidase	-
Nitrate reduction	-
Oxidation-fermentation test	-/-
Hydrolysis	
Gelatin	-
Starch	-
Tween 80	+
Acid from lactose	-
Hydrogen sulfide production	-
Urease	-

* + : positive, - : negative

Table 4.4 Morphological and biochemical characterizations of the isolated strain FT1

Characteristics	Results*
Microbiological characteristics	
Colonial morphology	Yellow colour colony
Gram reaction	Gram negative
	Rod shape
	No endospore forming
Motility	-
Growth tests	
Glucose	-
Galactose	-
Mannitol	-
Fructose	-
Sorbitol	-
Simmon citrate	-
Biochemical characteristics	
Oxidase	+
Nitrate reduction	-
Oxidation-fermentation test	Oxidation
Esculin hydrolysis	+
Gelatinase	-
Hydrogen sulfide production	-
Urease	+

* + : positive, - : negative

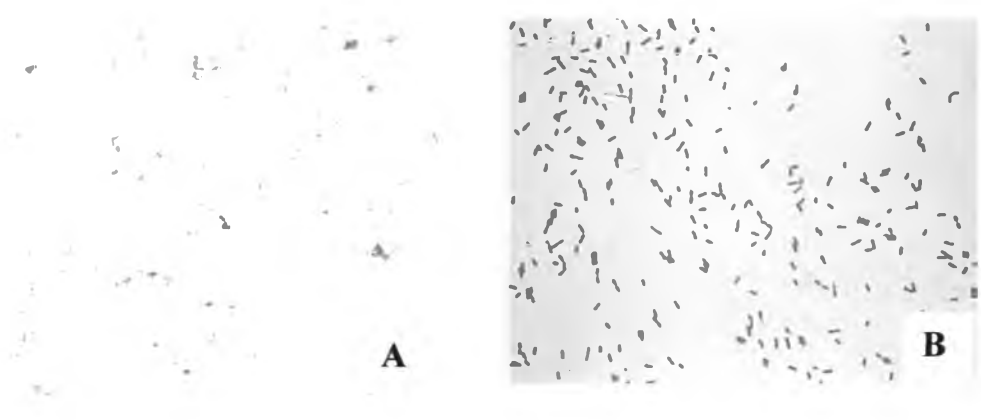

Figure 4.3 Microscopic characteristics of acid-fast stain of strain PY1 (A) and the Gram stain of FT1 (B).

4.4.2 16S rDNA sequence analysis

16S rDNA of these 2 isolated strains were amplified by PCR. The PCR products were approximately 1.5 Kb. Nucleotide sequence analysis of these two PCR products were carry out by the BioService Unit, National Centre for genetic Engineering and Biotechnology, National Science and Technology Development Agency. Nucleotide sequences were compared with those available sequences in GenBank using BlastN program (Altschul *et al.*, 1997). The nucleotide sequence of isolated strain PY1 and FT1 (Appendix C) are similar to *Mycobacterium gilvum* for 99% and *Sphingomonas* sp. strain B1 for 99%, respectively. Therefore, on the basis of 16S rDNA sequences along with morphological and biochemical characterizations, it could be concluded that the strain PY1 belongs to the Genus *Mycobacterium* sp., designated as *Mycobacterium* sp. strain PY1 and the strain FT1 belongs to *Sphingomonas* sp., designated as *Sphingomonas* sp. strain FT1.

4.5 PAHs degradation

4.5.1 Utilization of pyrene by *Mycobacterium* sp. strain PY1

Utilization of pyrene as the sole carbon source and energy source was indicated by the increase of bacterial cell number with concurrent disappearance of pyrene. *Mycobacterium* sp. strain PY1 in CFMM containing pyrene 100 mg/l of pyrene, after 14 days of incubation, pyrene was remained approximately 9.6 % and the cell number has increased from 6.7 to 7.2 log CFU/ml (Figure 4.4) whereas the remaining of pyrene in substrate control was 89.9%. Moreover, the colourless of medium was turned to pale yellow and the depletion of pyrene crystal was observed as demonstrated in Figure 4.5.

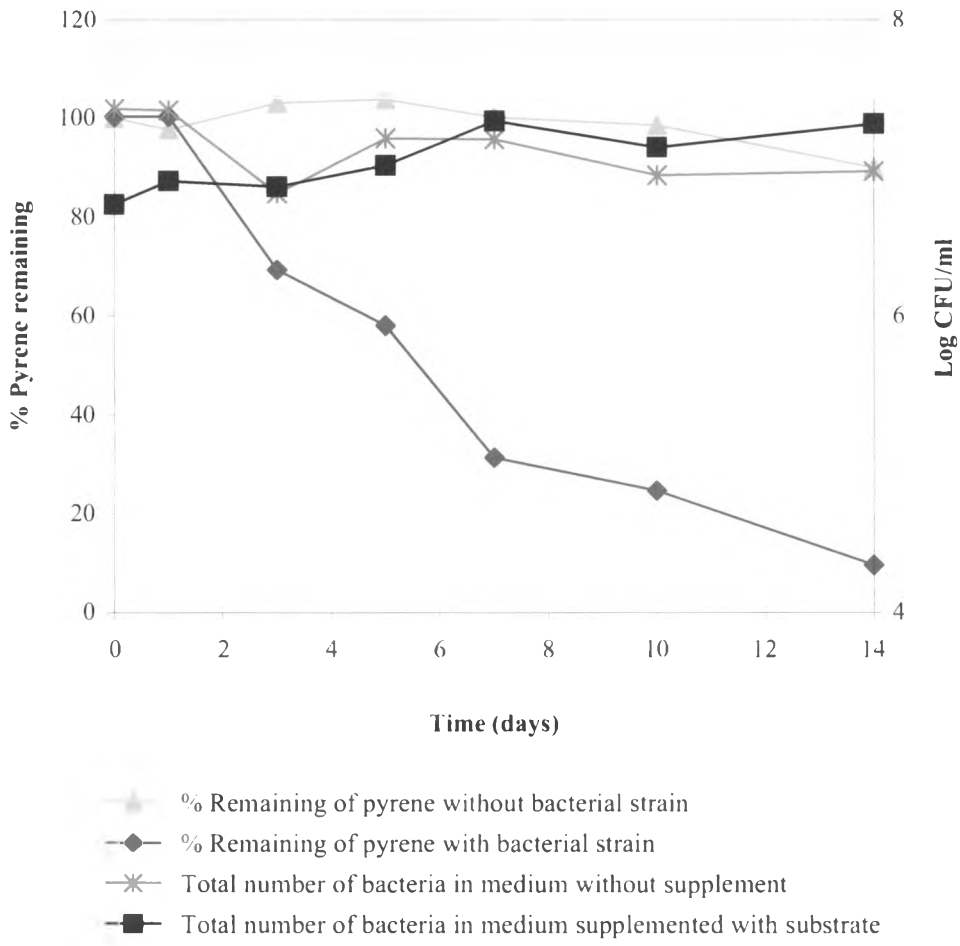

Figure 4.4 Time course of pyrene degradation, with initial concentration of 100 mg/l, by *Mycobacterium* sp. strain PY1.

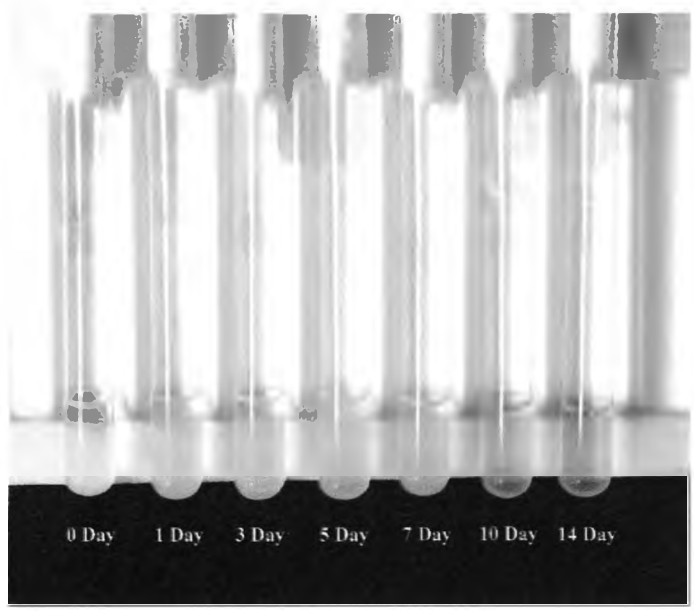

Figure 4.5 The change of liquid CFMM in pyrene degradation experiment.

4.5.2 Utilization of fluoranthene by *Sphingomonas* sp. strain FT1

Sphingomonas sp. strain PY1 in CFMM supplemented with fluoranthene 100 mg/l, after 14 days of incubation, fluoranthene was remained approximately 61.6% and the cell number were increased from 6.9 to 8.1 log CFU/ml (Figure 4.6), meanwhile remaining of fluoranthene in substrate control was 94.4%. Moreover, the unknown pale pink metabolite had appeared after the third day of incubation and the colour of medium was darker as shown in Figure 4.7.

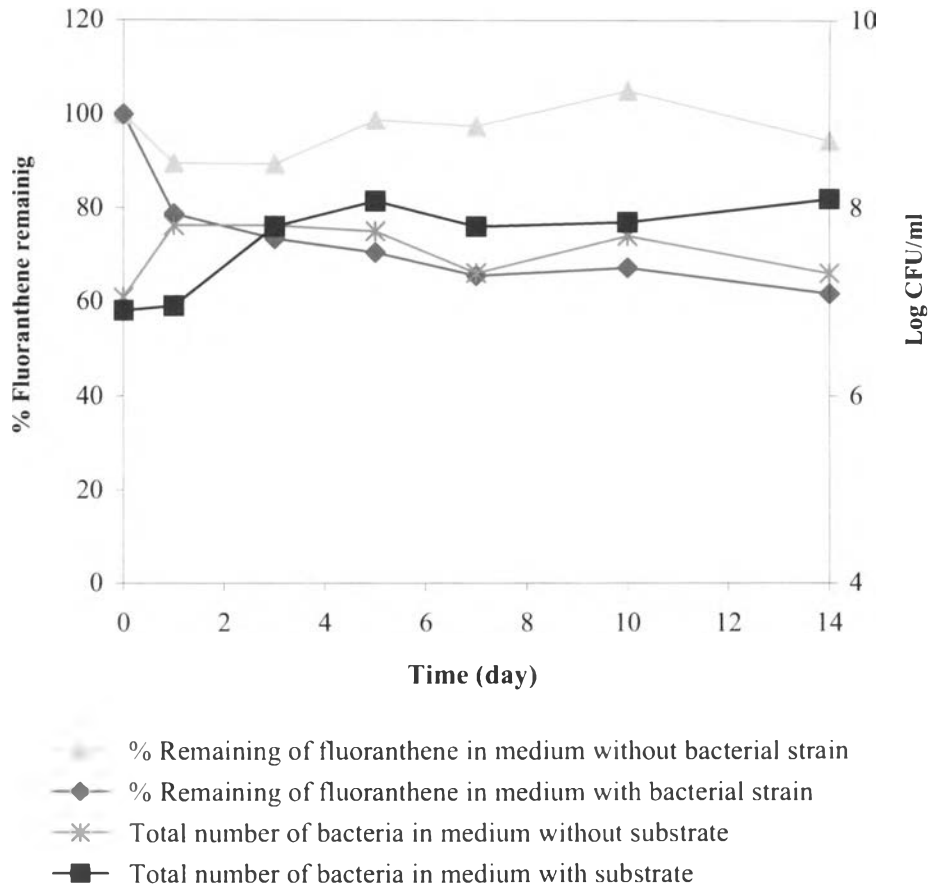

Figure 4.6 Time course of fluoranthene degradation, with initial concentration of 100 mg/l, by *Sphingomonas* sp. strain FT1.

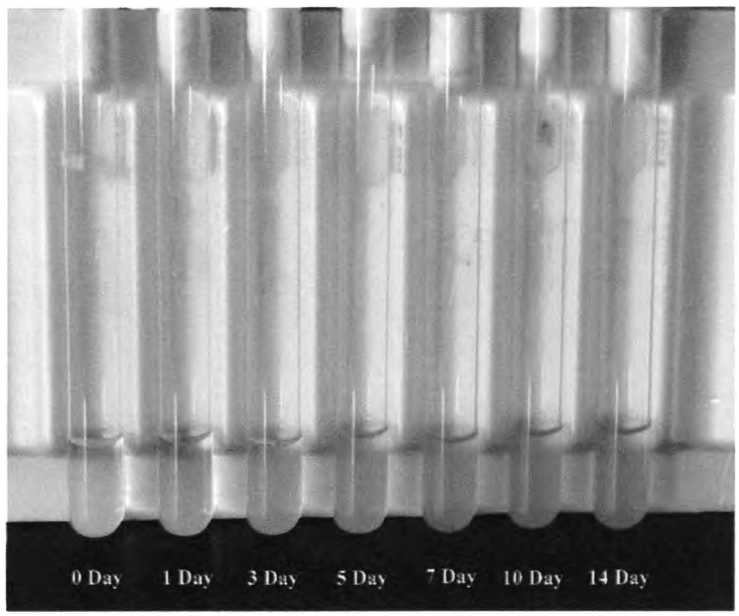

Figure 4.7 The change of liquid CFMM in fluoranthene degradation experiment.

4.6 Other PAHs utilization

The substrate specificity experiment was performed to evaluate the degrading ability of other PAH compounds namely acenaphthylene, acenaphthene, dibenzofuran, and phenanthrene as carbon and energy source. These PAH compound were selected to perform in this experiment because they are usually found in the contaminated sediments (Stout *et al.*, 2001). During the incubation stage on CFMM with growth substrate and other PAH compounds of both isolates, the yellow or orange metabolites causing changed of medium colour were formed. Since the transformation of PAH compound led to accumulate of metabolites such as catechol-like compounds and their meta-ring cleavage products may be ascribed to this colour change (Mueller *et. al.*, 1990b). Therefore, this is an evidence of PAH degradation by both of isolated strains.

The disappearance of PAH compound occurred corresponding to the increasing of bacterial cell number and changing of medium colour as shown in Figure 4.8. It was found that *Mycobacterium* sp. strain PY1 could degrade all substrates within 7 days. Acenaphthylene, acenaphthene, dibenzofuran, and phenanthrene were degraded up to 98.59, 99.37, 99.64 and > 99.95%, respectively (Figure 4.9).

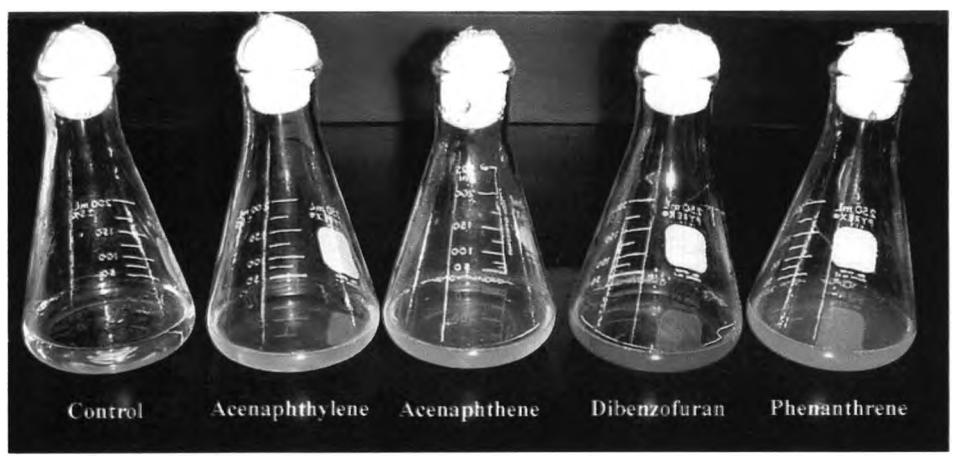

Figure 4.8 Medium colour of *Mycobacterium* sp. strain PY1 in liquid CFMM supplemented with individual substrate compared with control.

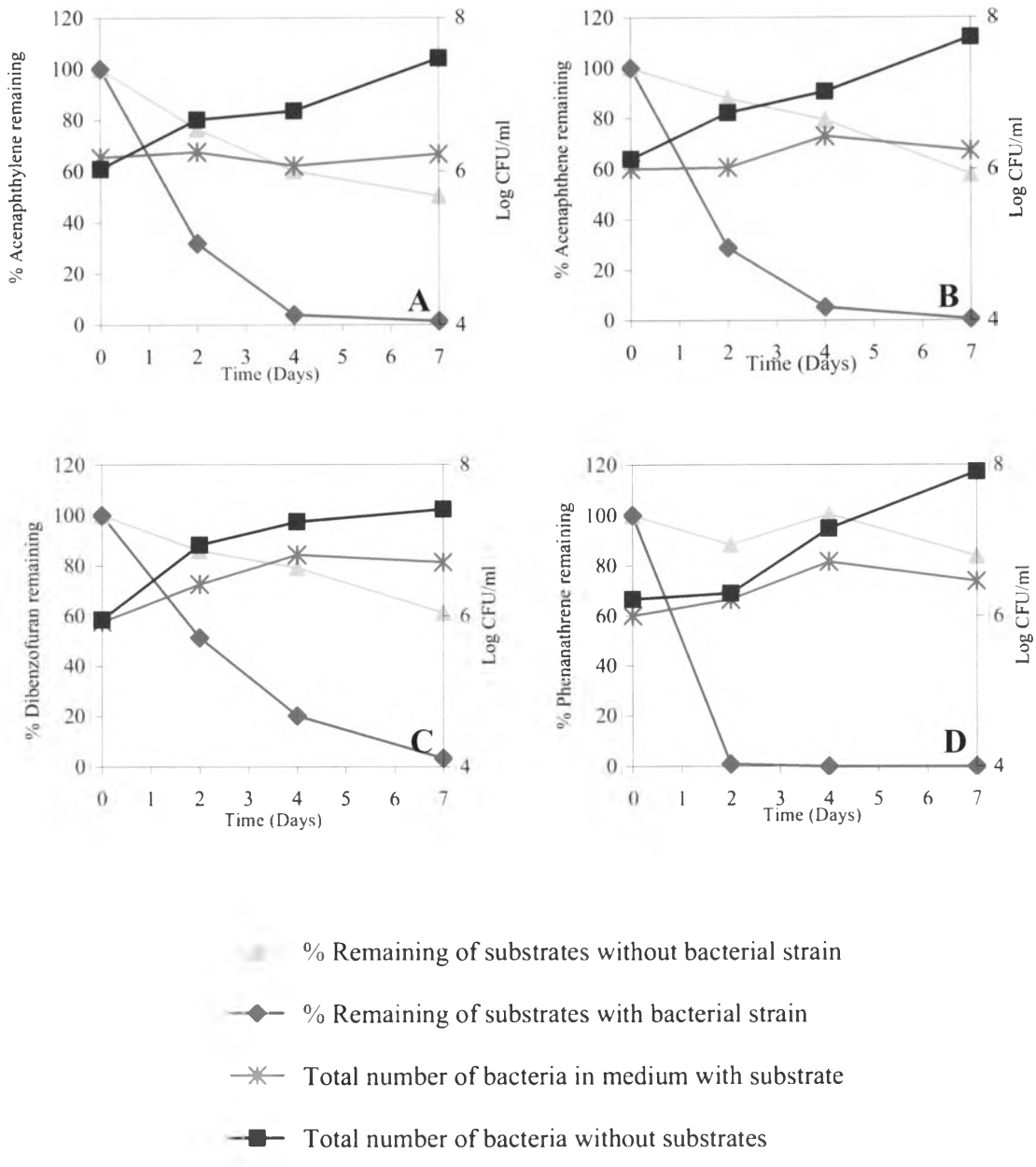

Figure 4.9 Time course of other PAHs degradation at concentration of 100 mg/l by Mycobacterium sp. strain PY1 as following; acenaphthylene (A), acenaphthene (B), dibenzofuran (C) and phenanthrene (D).

Sphingomonas sp. strain FT1 showed the similar results as *Mycobacterium* sp. This strain could change medium color from colorless to pale yellow or orange as shown in Figure 4.10. Acenaphthylene, acenaphthene, dibenzofuran, and phenanthrene were degraded up to 98.90, 89.85, 67.48 and 99.90%, respectively (Figure 4.11).

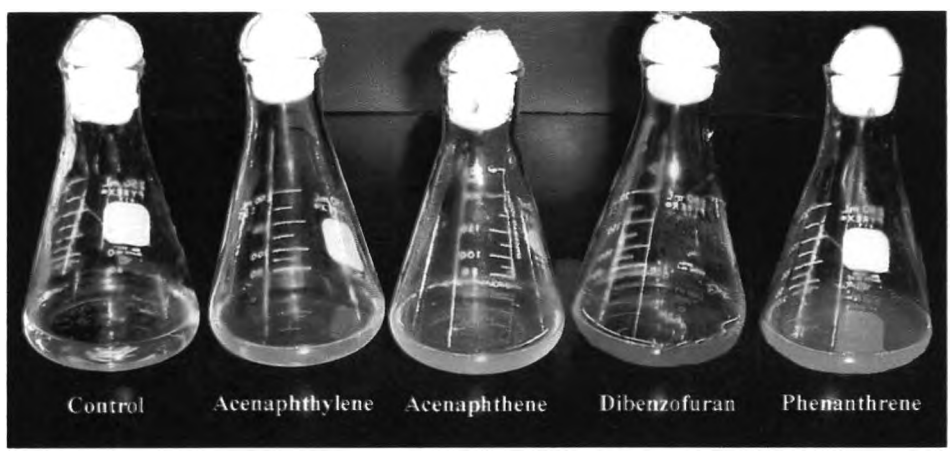

Figure 4.10 Medium colour of *Sphingomonas* sp. strain FT1 in liquid CFMM supplemented with individual substrate compared with control.

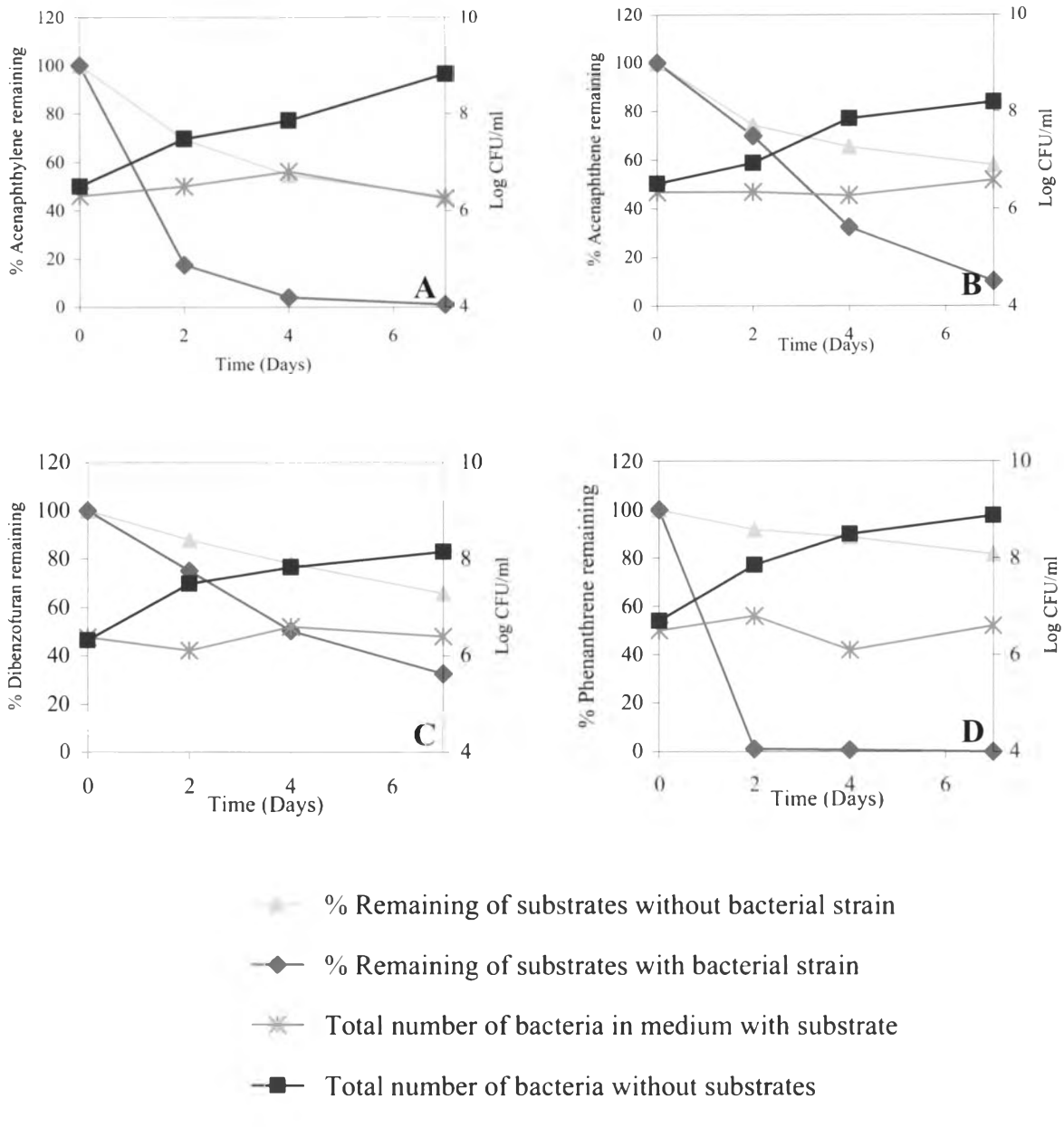

Figure 4.11 Time course of other PAHs degradation at concentration of 100 mg/l by *Sphingomonas* sp. strain FT1 as following; acenaphthylene (A), acenaphthene (B dibenzofuran (C) and phenanthrene (D).