ABSTRACT

COMPARISON OF OPTICAL DENSITY, TOTAL CELL PROTEIN, AND NUMBER OF VIABLE CELLS (VIA FLUORESCENT STAINING) AS MEASURES OF MICROBIAL GROWTH KINETICS IN SUSPENDED AND BIOFILM CULTURES DURING BIODEGRADATION OF NAPHTHALENE

by
Jeong Seop Shim

Most studies on bacterial growth kinetics have been dependent on theoretical modeling with general biomass measurements using either dry weight or optical density (OD), without distinguishing live from dead bacteria or debris. As a result, there remains considerable uncertainty in reliably predicting rates of biodegradation for design of treatment processes for environmental pollutants.

This research focused on measurement of bacterial growth rates and activities in suspended cultures and biofilms using *Pseudomonas putida* (ATCC 17484) for biodegradation of naphthalene. As expected, the rates of biodegradation differed between suspended and immobilized cultures. A comparison was made of the impact of three biomass measures: optical density, total cell protein, and living cell number on the calculated rate of naphthalene disappearance. Living cell number was determined by a fluorescent staining technique and use of epifluorescence microscopy. More than 90% of total cells remained viable over the course of each experiment (35 to 54 hours).

All three techniques experienced difficulties reconciling calculated values of biomass growth and naphthalene disappearance. This was considered to be a consequence of the production of intermediate products detected in the chromatograms, and possibly adsorption and subsequent release of naphthalene, which resulted in a lag time between the disappearance of naphthalene and the appearance of biomass. Inclusion of a lag time in

the integrated Monod expression improved the agreement between experimental and calculated values of biomass and naphthalene concentrations. However, further improvements will require more detailed kinetics of the actual biochemical pathway.

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COMPARISON OF OPTICAL DENSITY, TOTAL CELL PROTEIN, AND NUMBER OF VIABLE CELLS (VIA FLUORESCENT STAINING) AS MEASURES OF MICROBIAL GROWTH KINETICS IN SUSPENDED AND BIOFILM CULTURES DURING BIODEGRADATION OF NAPHTHALENE

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This dissertation is dedicated to
my parents and wife

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CHAPTER 1

INTRODUCTION

1.1 Viable Bacteria in Suspended and Biofilm Cultures

Most researchers have focused on estimating cell concentration in suspended culture using general parameters such as OD, dry weight of biomass, and cell protein concentration [Best (1997), Karel and Robertson (1989), Götz and Reuss (1997), Kennedy et al. (1992)]. However, cell quantitation by these methods measures not only viable cells, but also dead cells and debris. Pour plate techniques with nutrient agar have also been used to count the number of viable cells. However, researches have shown that the plate count (PC) method only reveals those bacteria that are capable of forming colonies on agar plates [Roszak and Colwell (1987), Stewart et al. (1994), Yu and McFeters (1994), Jacques and Morris (1995)], and this is often only a small fraction of the total bacteria present in the system.

Microbial cells attached to a surface produce extracellular polymers (called slime), which provide a supporting structure for the biofilm [Sandford et al. (1995)]. Biofilms are composed of living cells, dead cells, and cell debris in a polysaccharide matrix. Also, a thick biofilm may contain both aerobic and anaerobic environments due to oxygen diffusion limitations within the biofilm. [Characklis and Marshall (1990), Bishop (1996)]. Bio-oxidation in a biofilm is directly related to an active thickness corresponding to the depth of substrate penetration into the biofilm [Trulear and Characklis (1982), Liu and Capdeville (1996), Swope and Flickinger (1996)]. Active cells in aerobic systems usually grow in a thin film near the source of substrate and oxygen.

1.2 Use of Fluorescent Staining Techniques

Fluorescent probes have been used to assess bacterial viability and function as well as antibiotic susceptibility [Comas and Vives-Rego (1998), Jacobsen et al. (1997), Llobet-Brosa et al. (1998), Lebaron et al, (1998)]. Molecular Probes Inc. has developed LIVE/DEAD® BacLight™ Bacterial Viability Kit (LDBBVK) to distinguish live bacteria (with intact plasma membranes) from dead bacteria with compromised membranes [Haugland (1996)]. The LDBBVK provides a novel two-color fluorescence assay of bacterial viability by staining with bacterial nucleic acid, using a mixture of SYTO 9 for green fluorescence and propidium iodide (PI) for red fluorescence. LDBBVK has also been used to observe viable and non-viable cells using epifluorescence microscopy [Terzieva et al. (1996), Taghi-Kilani et al. (1996), Braux et al. (1997), Duffy and Sheridan (1998), Strathmann and Flemming (2000)], confocal laser scanning microscopy [Neu and Lawrence (1997)], or flow cytometry [Virta et al. (1998)].

The SYTO 9, when used alone, labels all bacteria in a population which have either intact membranes or damaged membranes. In the presence of both dyes, PI (which is a membrane-impermeable DNA stain) penetrates only those cells with damaged membranes, competing with the SYTO 9 for nucleic acid binding sites. The excitation/emission maxima of these dyes are about 480 nm to 500 nm for the SYTO 9 and 490 nm to 635 nm for the PI. Therefore, in the presence of both dyes, bright green fluorescence indicates live bacteria with intact membranes, while bright red fluorescence indicates dead bacteria with damaged membranes.

1.3 Determination of Viability in Surface Biofilms

By far, most information about biofilms has been extrapolated from those studies with bacterial suspensions. Several researchers [Massol-deyá et al. (1995), Sanford et al., (1996), Bauer-Kreisel et al. (1996)] typically extracted cells from attached biofilms into suspension (using phosphate-buffered saline and ultrasonication) and subsequently stained and counted them. As a result, there is a need for *in-situ* measurement of biofilm characteristics, and the properties of the surface microenvironments by direct microscopic observation.

To directly assess viability of bacteria in biofilms without disturbing the integrity of the interfacial community, a non-destructive *in-situ* direct viable count (DVC) method was developed by Yu et al. (1993). *Klebsiella pneumoniae* Kp1 was applied for the biofilm cultures on stainless steel coupons in a stirred batch reactor under aerobic conditions. The biofilm cultures were limited to monolayers in order to be countable by epifluorescence microscopy. They fixed immobilized cells with formaldehyde followed by immersion in 0.02% acridine orange solution for 2 minutes. Although the *in-situ* DVC method has some constraints imposed by optical microscopy, it can still provide rapid and accurate determination of viable cells. They reported that the in situ DVC method showed higher viable cell counts than plate counts and conventional-DVC methods without the bias caused by aggregated bacteria.

A great deal of research on the structure of biofilms has been conducted at the Center for Biofilm Engineering at Montana State University (Characklis, de Beer, Stoodley, Lewandowski, etc.). Their research has generally involved the use of

microprobes to determine e.g. oxygen and pH profiles in biofilms. However, individual cell viability has not been addressed.

CHAPTER 2

LITERATURE REVIEW

2.1 Enumeration of Viable Cells by Fluorescence Microscopy

5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has been used as an indicator of electron transport activity for direct microscopic visualization of actively respiring cells in drinking water or other aquatic environmental samples under aerobic conditions [Rodriguez et al. (1992), Schaule et al. (1993), Winding et al. (1994), Yu et al. (1995), An and Friedman (1997)]. As a redox fluorescent probe, it is readily reduced via the electron transport chain of an active bacterial population from a colorless, nonfluorescent, and oxidized form to fluorescent and insoluble CTC-formazan (CTF) crystals accumulating intracelluarly. To allow concurrent determination of total cells (i.e. viable and nonviable) and respiring cells (i.e. cells forming CTF) by an epifluorescence microscope, the CTC-treated samples (*Pseudomonas putida* 54g) were counterstained with the DNA-binding fluorochrome 4′,6-diamidino-2-phenylindole (DAPI). Using the ratio of CTC and DAPI counts in conjunction with biofilm cryogenic sectioning techniques, Villaverade et al. (1997) and Villaverade and Fernández-Polanco (1999) were able to evaluate fractions of total respiring cells in a biofilm, which they compared to respiration rates at various distances from the surface using oxygen microsensors. The biofilm was cultured in an aerobic system with a flat-plate vapor phase biological reactor.

Another indicator of bacterial respiration, a redox dye 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), has been applied for direct observation of active cells by the reduction of INT to dark-red intracellular INT-formazan (INF) crystals. Zimmermann et al. (1978) and Tarbor and Neihof (1982) tested bacterial

samples taken from natural aquatic environments. Okabe et al. (1996) investigated spatial distributions of nitrifiers and heterotrophs in mixed-population biofilms under aerobic conditions using a partially submerged rotating biological contactor (RBC). Smith and McFeters (1997) tested both tetrazolium salts of INT and CTC under aerobic and anaerobic conditions with *Escherichia coli* K-12. They reported that both INT and CTC were significantly reduced under most anaerobic conditions, particularly glucose fermentation. However, CTF makes it relatively easier (compared to INT) to directly visualize respiring cells in biofilms on an opaque background (e.g. wood, metal, and plastic, etc.) by epifluorescence microscopy [Rodriguez et al. (1992), Smith, and McFeters (1997)].

For counting viable cells in thin biofilms (monolayers) on stainless steel coupons with *Klebsiella pneumoniae*, Yu and McFeters (1994) compared four different enumerating methods. These methods were: (1) CTC reduction technique, (2) use of rhodamine 123 (Rh-123), (3) *in-situ* direct viable count (DVC), and (4) a modified drop plate count. Rh-123 is a proton motive force-driven dye, which is only taken up by viable cells. The DVC method is related to the counting of elongated cells stained with acridine orange (AO) as viable cells, using nalidixic acid and nutrients to halt cell division [Kogure et al. (1979), Peele and Colwell (1981), Liebert and Barkay (1988), Singh et al. (1990)]. Yu and McFeters (1994) reported that the percent cell viability varied from 95% obtained by the CTC reduction method, to 43% obtained by the PC method.

For the direct enumeration of injured *Escherichia coli* cells, Braux et al. (1997) also tested four different methods such as the DVC for testing biosynthesis capacity, the reduction assay of the CTC or sodium 3′-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-

bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) for respiratory activity, and the LDBBVK assay with SYTO 9 and PI for membrane integrity. They concluded that the use of dual staining with LDBBVK appears to be the most sensitive test among those assays to assess *E. coli* viability.

Using two strains of *Escherichia coli*, Boulos et al. (1999) reported that the LDBBVK assay resulted in the highest viable (green) and total (green and red) counts among several staining techniques (CTC, CTC with DAPI counterstaining, and a modified CTC method with SYTO 9 counterstaining). They found that storage after treatment with glutaraldehyde decreased total and viable counts of some coliform strains when using LDBBVK assay. Therefore, they suggested that immediate processing of samples is preferable. In this research with LDBBVK assay, formalin was not used to preserve bacterial samples because all live cells treated with formaldehyde (2% in the final concentration) showed red fluorescence, indicating that the use of preservative chemicals damaged the integrity of bacterial membranes.

2.2 Biofilm Research

As general parameters for biofilm characterization, many researchers [Stewart et al. (1993), Lauvvik and Bakke (1994), Tanyolaç and Beyenal(1996, 1997), Pavasant et al. (1996), Peyton (1996), Freitas dos Santos and. Livingston (1995a, 1995b)] have used biofilm thickness and total dry weight. However, these parameters are not sufficient to describe biofilm characterization and biomass activity. Lazarova et al. (1994) proposed an integrated approach for biofilm analysis: 1) direct binocular microscopic observation and scanning electron microscopy for external biofilm structure and thickness; 2)

chemical oxygen demand, total organic carbon, and dry weight for total biofilm amount; 3) exopolysaccharides, total protein, total cell count, and viable heterotrophic count for specific biofilm components; 4) electron transport system activity, active cell count, and specific activity for estimating fixed biomass activity; and 5) nucleic acid probes for study of population dynamics (in particular of the autotroph/heterotroph competition in biofilms).

For the surface biofilm studies, a few researchers have performed experiments using direct microscopic observations of microorganisms under *in-situ* aerobic conditions using small continuous-flow slide culture chambers [Caldwell and Lawrence (1988), Lawrence et al. (1989), Korber et al. (1990), Lawrence et al. (1991), Caldwell et al. (1992), Lawrence et al. (1994)]. They have developed *in-situ* techniques for observing cells attached on glass surfaces, and have performed computer image analyses of the microscopic views. They studied bacterial growth kinetics by looking at the cell doubling time, and by counting cells on the upper and lower surfaces. However, they did not distinguish between live and dead cells.

Microelectrodes for oxygen (O_2), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), or pH have been used to study distribution of bacterial activity in biofilms by measuring the microprofiles of the constituents with biofilm depth. Thus, active or inactive zones, in terms of bacterial activity, can be distinguished with biofilm depth. De Beer et al. (1993) used the microelectrodes to study nitrifying bacterial aggregates grown in an aerobic fluidized-bed reactor. Lewandowski et al. (1993) measured oxygen concentration profiles across a biofilm grown in an aerobic continuous flow reactor.

Schramm et al. (1997) measured microprofiles of O_2 consumption and NO_3^-/NO_2^- production in a trickling filter biofilm with nitrifying bacteria.

Neu and Lawrence (1997) have studied the development of biofilm structures in raw river water, using removable polycarbonate slides for biofilm sampling on a rotating annular bioreactor, and confocal laser scanning microscopy (CLSM) with the LIVE/DEAD fluorescent probe (Molecular Probes, Inc.). The dual channel images obtained by the CLSM were able to show the distribution of live (green) and dead (red) cells in sagittal sections (or xz axis) of biofilms. The vertical distribution of cells (live and dead) showed the same general trend of increasing with distance away from the attachment surface toward the nutrient source. They reported that in mature biofilms, red fluorescence was obscured by the autofluorescence of organic matter incorporated into the biofilm, but both living and dead cells could be resolved within individual microcolonies at high magnification. They indicated that a high proportion of the biofilm showed relatively non-viable state (red fluorescence). This was consistent with the findings of this dissertation which observed a problem with direct staining of biofilms, as mentioned in Section 4.5.2.

Using CLSM, de Beer et al. (1994) and Stoodely et al. (1994) developed a noninvasive technique for microscale measurement of flow velocity profiles near and within biofilms cultured in an aerobic reactor. They used fluorescein microinjection technique to determine the presence of flow in cell clusters and voids. They found that liquid can flow through the voids and is stagnant in the cell clusters. De Beer et al. concluded that in the voids mass transport might take place by both convection and diffusion, whereas in the cell clusters only diffusion can occur.

Characklis and Marshall (1990) described several ways in which bacteria attach to a range of surfaces. Some bacteria respond gradually to the presence of a surface, attaching more firmly with time. Differences in attachment depend on the type of bacteria, type of surface (and its conditioning), and nutrient conditions.

By using rectangular glass tubing to simulate flow in industrial pipes, Stoodley et al. (1999) studied biofilm structures under laminar and turbulent flows. Biofilms grown under laminar flow were patchy and consisted of cell clusters separated by interstitial voids. Biofilms grown under turbulent flow formed filaments, which had a more complex structure and were formed by the colonization of filamentous sheathed bacteria with microcolonies of non-filamentous bacteria.

By cryosectioning biofilms covered with hydrophilic polymers as an embedding agent that maintains a degree of plasticity when frozen, Murga et al. (1995) reported that biofilms with two species of bacteria (*Pseudomonas aeruginosa* and *Klebsiella pneumoniane*) grown in an aerobic system were structurally distinct from monopopulations, due to differences in such characteristics as capsule structure and motility.

2.3 Biodegradation of Polycylic Aromatic Hydrocarbons

Guerin and Boyd (1992, 1997) reported that *Pseudomonas putida* (ATCC 17484) had superior characteristics in both the rate and extent of naphthalene mineralization.

Many researchers [Stucki and Alexander (1987), Rockne and Strand (1998), Erickson et al. (1993)] have focused on biodegradation of polycyclic aromatic hydrocarbons (PAHs), which are principal components of coal tar. Naphthalene is the

most abundant and simplest PAH compound in coal tar. Rockne and Strand reported that bicylics and PAHs could be degraded in anaerobic enrichments such as nitrate- and sulfate-reducing conditions. Erickson et al. investigated loss of PAHs at a manufactured gas plant (MGP) site. Ghoshal and Luthy (1996, 1998) and Ghoshal et al. (1996) presented kinetic data for naphthalene degradation, including the following Monod parameters for using a mixed culture isolated from PAH contaminated soil and grown on naphthalene over a long period of time in aerobic slurry systems: Y (yield coefficient) = 0.25±0.08 (mg dry biomass per mg naphthalene degraded); μ_m (maximum specific growth rate) = 0.067 h^{-1} and K_s (half saturation constant) = 3.4 mg/L.

Wilson and Madsen (1996) detected a characteristic metabolite (1,2-dihydorxy-1,2-dihydronaphthalene) during aerobic degradation of naphthalene at a coal tar-contaminated field site.

Biodegradation of PAHs may be limited by their low solubility, coupled with strong binding and sorption onto soils. Liu et al. (1995) evaluated the effects of two nonionic surfactants on bacterial mineralization of naphthalene: an alkylethoxylate (Brij 30) and an alkylphenolethoxylate (Triton X-100). Both enhanced bioavailability without inhibiting the rate of biodegradation by a mixed aerobic culture isolated from PAH-contaminated soils.

Marx and Aitken (2000) suggested that bacterial chemotaxis to a pollutant can overcome the mass transfer limitations that may govern biodegradation rates in contaminated environments. They showed that a motile wild strain of *Pseudomonas putida* exhibited superior naphthalene degradation when compared to a nonmotile mutant or a mutant deficient in naphthalene chemotaxis.

CHAPTER 3

OBJECTIVES

The objectives of this research were to study the activities and microbial growth rates of viable bacteria in suspended culture and immobilized biofilm by comparing optical density (OD), cell number, and total cell protein (TCP) as measurement tools. In order to distinguish living cells from dead cells, a fluorescent staining technique was developed, with *Pseudomonas putida* (ATCC 17484) and naphthalene as model system.

The specific objectives were to:

- develop a fluorescent staining technique to distinguish live from dead cells
- compare biomass measures - dry biomass weight, OD, total cell number (TCN), living cell number (LCN), and total cell protein – for the model system
- examine both suspended culture and fixed biofilm
- relate measures of biomass to naphthalene degradation rate

CHAPTER 4

MATERIALS AND EXPERIMENTAL METHODS

4.1 Materials

4.1.1 Preparation of Growth Medium

Naphthalene was used as sole carbon source. About 2 to 3 g of naphthalene crystals were added to autoclaved deionized (DI) water in 4-L glass containers. The solution with crystal naphthalene was dissolved by a magnetic stirrer for about one week to make it saturated. The saturated solution (approximately 30 ppm) was finally sterilized by passing through an autoclaved membrane filter with 0.22 μm pore size. The filter-sterilized naphthalene solution was stored in 2-L glass containers at room temperature and sealed completely. For experiments with suspended cultures, this stock solution was diluted with autoclaved DI water and inorganic medium to reach the desired concentration.

The inorganic growth medium is shown in Table 4.1. Medium A and B were prepared separately to avoid formation of white precipitates during autoclaving. Potassium phosphate (K_2HPO_4) was used to maintain culture solution at pH 7. Each medium was autoclaved at 121 °C and 16 psi for 20 minutes and stored in a refrigerator.

Nutrient broth was prepared by dissolving 4 g of "BBL Nutrient Broth" with 1-L of DI water, which was then autoclaved. This solution was used for reviving frozen cultures initially.

Table 4.1 Composition of inorganic medium for culture of *Pseudomonas putida*.

	Chemicals	Final concentration	
		(g/L)	(mmol/L)
Medium A	Potassium Phosphate, Monobasic (KH_2PO_4)	0.2	1.5
	Potassium Phosphate, Dibasic (K_2HPO_4)	0.26	1.5
Medium B	Ammonium Chloride (NH_4Cl)	0.3	4.6
	Sodium Chloride (NaCl)	1.0	17.1
	Magnesium Chloride, Heptahydrate ($MgCl_2$ $6H_2O$)	0.4	2.0
	Calcium Chloride, Dihydrate ($CaCl_2$ $2H_2O$)	0.2	1.0
	Potassium Chloride (KCl)	0.5	6.7
	Sodium Sulfate (Na_2SO_4)	0.2	1.4
Trace elements		(mg/L)	(mmol/L)
	Ferrous Chloride, Tetrahydrate ($FeCl_2$ $4H_2O$)	1,500	7.5
	Zinc Chloride ($ZnCl_2$)	70	0.5
	Manganous Chloride, Tetrahydrate ($MnCl_2$ $4H_2O$)	100	0.5
	Boric Acid (H_3BO_3)	6	0.1
	Cobalt Chloride, Heptahydrate ($CoCl_2$ $6H_2O$)	190	0.8
	Cuprous Chloride, Dihydrate ($CuCl_2$ $2H_2O$)	2	0.02
	Nickel Chloride, Heptahydrate ($NiCl_2$ $6H_2O$)	24	0.1
	Sodium Molybdate, Dihydrate (Na_2MoO_4 $2H_2O$)	36	0.15
	Hydrochloric Acid (HCl, 37%)	5.795 mL	70

4.1.2 Fluorescent Probes for Staining Bacteria

LIVE/DEAD® BacLight™ Bacterial Viability Kit (LDBBVK, L-7012) was used to provide the fluorescent probes for fluorescence microscopy (Molecular Probes, Inc., Eugene, OR). This kit contains 3.34 mM SYTO 9 solution and 20 mM propidium iodide (PI) solution (each dissolved in anhydrous dimethyl sulfoxide: DMSO), and 10 mL of BacLight mounting oil. The mounting oil provides a high viscosity environment, which minimizes Brownian motion of stained cells, and serves as an anti-fading agent to reduce the bleaching of the dyes. The excitation/emission maxima of these dyes are about 480 nm/500 nm for the SYTO 9 and 490 nm /635 nm for the PI.

Based on results of pre-screening tests, the mixture of SYTO 9 and PI with a 1:1 ratio by volume was used as mentioned in the manufacturer's instructions. Although the manufacturer suggests the use of 0.3% DMSO in the final suspension (i.e. 3 μL of dye mixture per 1 mL of suspension), pre-screening tests indicated that 0.2% DMSO gave equivalent results with a saving of the expensive dye.

4.1.3 BCA Protein Assay Kit

The BCA protein assay, purchased from Pierce (Rockford, IL), is a detergent-compatible formulation based on bicinchoninic acid (BCA), for the colorimetric detection and quantitation of total protein. The BCA protein assay is performed under the following reaction schemes:

1) Protein (peptide bonds) + $Cu^{+2} \xrightarrow{OH-}$ Tetradentate-Cu^{+1} complex

2) Cu^{+1} + 2 Bicinchoninic acid $\longrightarrow$ BCA-Cu^{+1} complex (purple color).

The purple-colored reaction product exhibits a strong absorbance at 562 nm. The BCA protein assay kit includes Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2N sodium hydroxide), Reagent B (4% cupric acid solution), and Albumin standard ampules (containing bovine serum albumin at a concentration of 2.0 mg/mL). The BCA working reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B.

4.1.4 Biofilm Glass Supports

Rectangular glass pieces were used as biofilm supports in column reactors with continuous medium flow. The biofilm supports (BF-supports) were designed to be 25.5

cm in length, 3.4 cm in width and 0.22 cm in thickness, providing about 186 cm^2 in the total surface area and about 19 cm^3 in volume (Shin Glass Inc., Palisades Park, NJ). Before starting experiments, each glass piece was scratched using a glass cutter, thereby dividing it into three regions. After staining the biofilm, the scratched glass pieces were snapped off just before microscopic observation in order to obtain replicate counts and average values for each original piece. The glass pieces were cleaned by sonication, then rinsed with detergent, hot water and distilled water, before autoclaving.

4.2 Pure Culture of *Pseudomonas putida* (ATCC 17484)

Pseudomonas putida biotype A, which was obtained from American Type Culture Collection (ATCC 17484) by Jay Best (previous student at NJIT), was used to degrade naphthalene as sole carbon source. The strain of *Pseudomonas putida* grows under aerobic conditions at 20 to 30 °C (Best, 1997). The original culture used for this research had been stored at -20 °C for about one year. The frozen culture was melted at room temperature for reviving cells.

When the fluorescent staining dye was tested with *Pseudomonas putida* at the beginning of this research, only the nutrient broth culture was used for rapid and convenient bacterial growth instead of naphthalene and inorganic medium. However, it was found that the strain of *Pseudomonas putida* lost the ability to degrade naphthalene after being cultured with nutrient broth for a prolonged period. As a result, the following method was adopted.

About 1 mL of the melted seed culture was transferred to a 250 mL flask containing about 30 mL of nutrient broth. It was then cultured in an incubator shaker

(Series 25, New Brunswick Scientific Co.), which was set at 25 °C and 200 rpm, for about 2 days. The culture indicated signs of growth by becoming cloudy. After that, the culture was transferred to a 1-L baffled flask for acclimation with naphthalene (about 5 mg/L in concentration) and inorganic medium. Flasks containing inoculated suspension were also placed in the incubator shaker to maintain the appropriate temperature and air-supply. After about 24 hours, the culture also showed signs of growth. When the carbon source was depleted, a small amount of crystal naphthalene was added periodically to the suspension, sometimes also adding inorganic medium. Seed cultures were prepared by this method about 2 to 3 days prior to each experiment.

4.3 Experimental Set Up

4.3.1 Preparation of Suspended Cultures

Experiments for suspended cultures with *Pseudomonas putida* were simultaneously performed with 13 shake flasks (1-L glass Erlenmeyer flasks), which had 4 different sets (indicated as a, b, c and d) of naphthalene concentration as shown in Table 4.2. Each concentration set consisted of 3 flasks, with flasks A and B as duplicates and flask C for sterile control (no bacteria). One additional flask (D) contained bacteria but no naphthalene. Each flask contained 300 mL of suspension: 20 mL of seed culture, 13.3 mL of inorganic media (3 mL of medium A, 7 mL of medium B and 0.3 mL of trace elements), and 269.7 mL of naphthalene solution. The naphthalene concentrations were measured by HPLC, as described in subsequent sections. The suspended cultures were put in an incubator shaker at 25 °C and 200 rpm. Each flask was plugged with a cap.

There was sufficient air in the headspace of a shake flask because fresh air was introduced into the flask by opening the cap at every sampling time.

Table 4.2 Arrangement of shake flasks for suspended cultures.

Duplicate flasks	a-A	b-A	c-A	d-A
	a-B	b-B	c-B	d-B
Sterile control	a-C	b-C	c-C	d-C
Culture control	D			

4.3.2 Preparation of Fixed Biofilms

At least 13 BF-supports were used to perform one set of biofilm experiment. 10 supports were installed on two column reactors, and 3 supports as initial samples were prepared to obtain more reliable data for bacterial enumeration. An autoclavable glass container (8.5 inches in diameter, 17.5 inches in height, and 15-L in volume) was prepared to culture seed biofilms on glass supports.

At the beginning of the biofilm tests, all glass supports were irregularly placed in the container as shown in Figure 1A. However, there were difficulties obtaining a uniform biofilm distribution on all glass supports. To overcome this problem, a round holder made with Teflon material (Figure 1B) was designed to make all BF-supports stand vertically and evenly spaced in the container. Aeration (about 40 mL/minute) was provided by a laboratory-based air line through 4 in-line air filters (Bacterial Air Vent, Gelman Sciences) and a glass tube with a fritted end. The top of the container was covered with aluminum foil while culturing the biofilms. Bacterial suspension in the container was continuously mixed using a magnetic stirrer (Cimarec 3, Thermolyne).

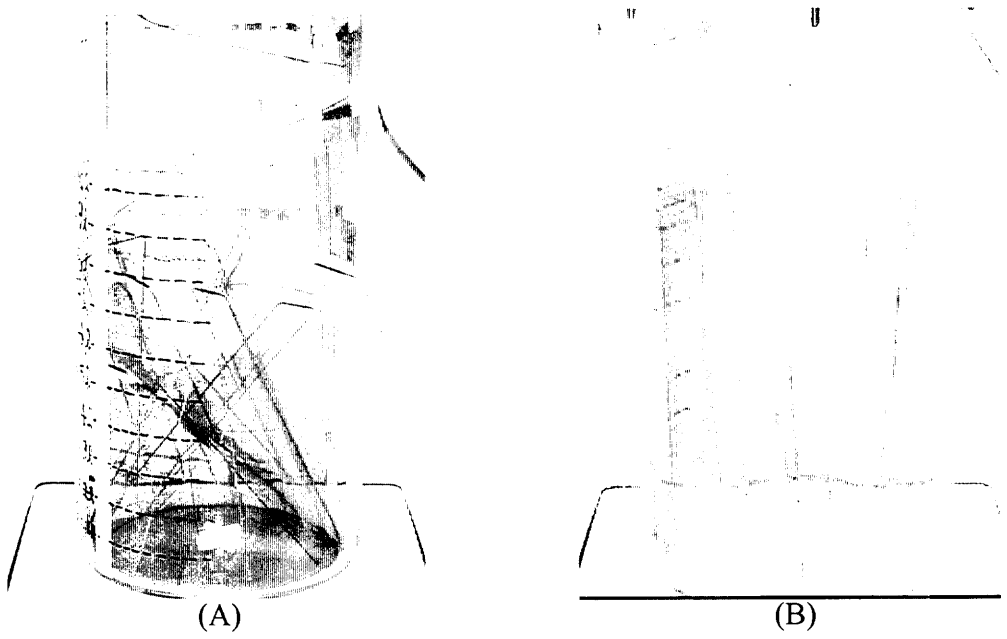


Figure 4.1 Seed biofilm containers: (A) Non-uniform biofilms and (B) Uniform biofilms.

4.3.3 Column Reactors for Biofilm Cultures in Continuous Flow System

A schematic diagram of the experimental set-up for biofilm cultures on glass supports is presented in Figure 4.2 and C (A) in Appendix C. The two columns in the diagram were custom-made (Ace Glass, Vineland, NJ). Each column was a heavy-walled glass process pipe with 5 cm inside diameter and 12 inches (30.5 cm) in length. There were 5 sampling ports along the height of each column. Figure 4.3 and C (B) in Appendix C show the details of the column reactor. There were 5 glass supports for biofilm culture in each column. Two thick polymeric end plates were tightly connected to each end of the column using a rubber o-ring. The bottom end plate (inlet) was specially re-designed by a machine shop (N. & J. Machine Prod. Inc., Newark, NJ) with slots for installing 5 glass BF-supports vertically onto the inlet plate, maintaining almost the same interval (4~5

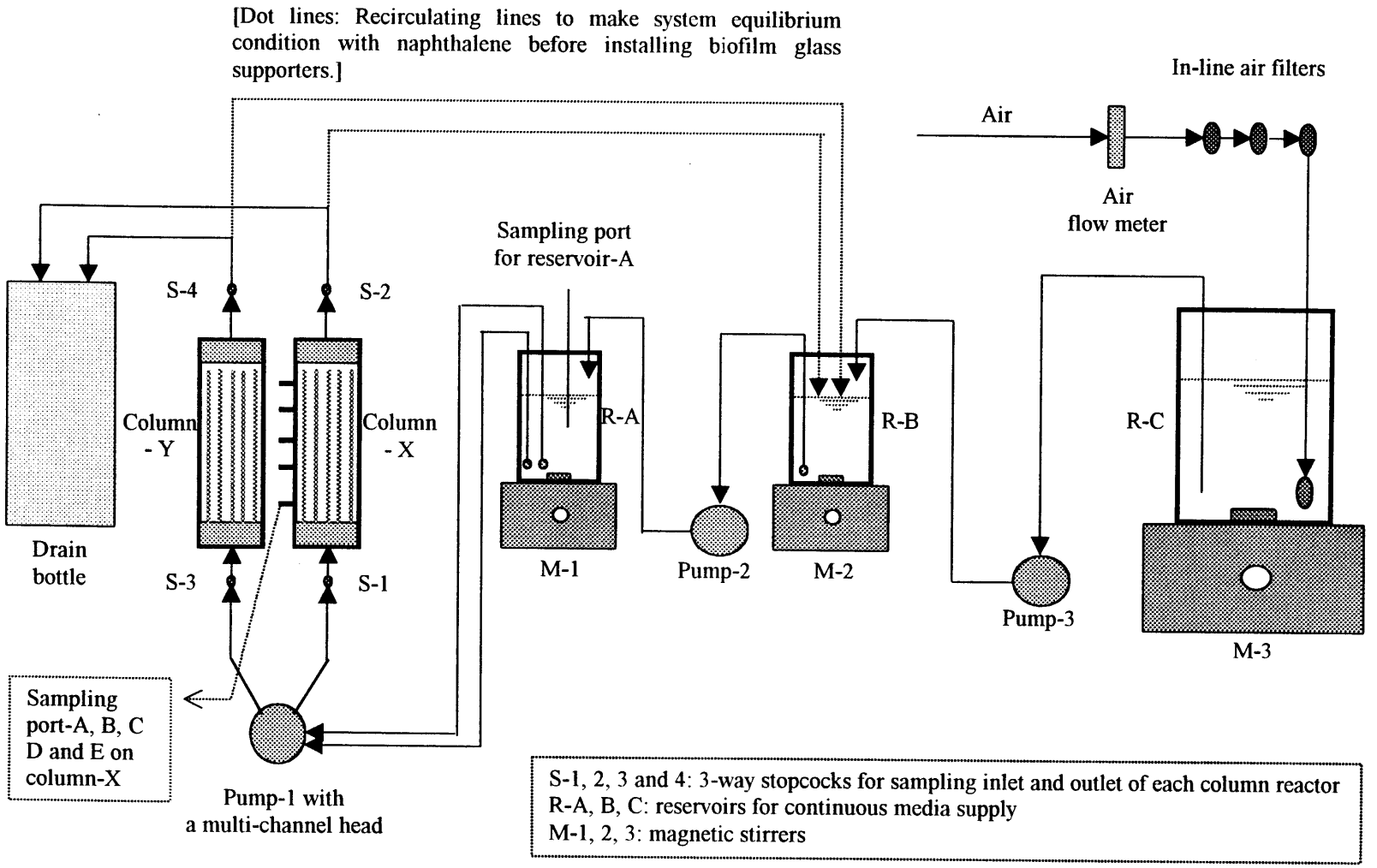


Figure 4.2 Experimental set-up of a continuous flow system with two column reactors and glass supports for biofilm cultures. (R-A and R-B contained excess of crystal naphthalene and inorganic media. R-B was used to damp sudden change of naphthalene concentration in R-A when fresh inorganic medium without naphthalene was continuously supplied from R-C.)

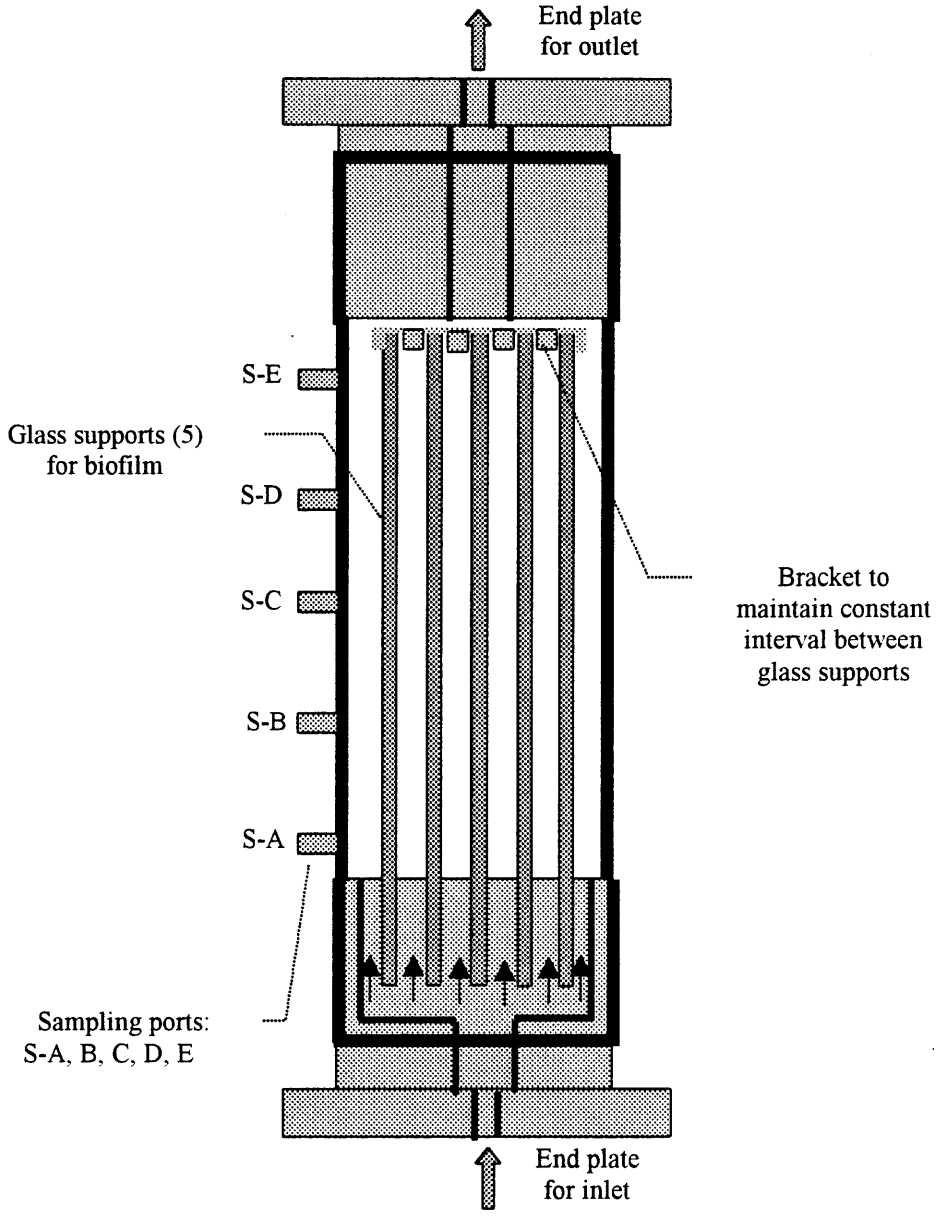


Figure 4.3 Design of a column reactor with 5 glass supports for biofilm cultures in continuous flow system during biodegradation of naphthalene by *Pseudomonas putida*. (Glass support: 25.5 cm in length, 3.4 cm in width, and 2.2 mm in thickness; surface area of each glass support = 186 cm^2; interval between each glass = 4~5 mm)

mm) between each BF-support. Also included was a stainless steel bracket on the top of the glass supports. BF-supports on each column were numbered for sampling order.

The two columns (X and Y) in the continuous flow system were set up in a parallel position as shown in Figure 4.2. Three pumps (Pump-1, 2, 3), three reservoirs (R-A, B, C), and three magnetic stirrers (M-1, 2, 3) were installed in this system, connected with polypropylene tubing (ID: 3/32", OD: 1/8", Cole-Parmer Instrument Co.), Tygon® tubing (ID: 1/16", OD: 3/16", Cole-Parmer Instrument Co.) or PharMed® tubing (L/S™ 14, Cole-Parmer Instrument Co.). Pump-1 was operated with two cartridges on a multi-channel pump head to maintain the same flow rate for both column reactors.

Because of adsorption of naphthalene on Tygon® and PharMed® tubing, polypropylene tubing was used whenever possible. However, polypropylene is a rigid material that cannot be used in the pump heads. Tygon® and PharMed® are superior in those applications. Tygon® tubing was used only on the pump heads for pumps-1 and -2. The PharMed® tubing was used on the line from R-C to R-B because R-C contained only inorganic media and trace elements. Some straight fittings for 1/16" tubing ID (polypropylene, Cole-Parmer Instrument Co.) and small pieces of PharMed® tubing were used to connect the system lines. Four three-way stopcocks with luer lock (polypropylene, Cole-Parmer Instrument Co.) were installed before and after both column reactors for taking naphthalene samples.

It was observed that there was difficulty in maintaining a constant concentration of naphthalene in R-A with aeration. It was decided to maintain R-A at a saturated condition using an excess of crystal naphthalene. R-A and R-B (2-L PYREX® bottles) contained an excess of crystal naphthalene and were supplied continuously with

inorganic media solution from R-C (5-gallon PYREX® bottle). R-B was used to damp sudden change of naphthalene concentration in R-A when fresh inorganic media without naphthalene was continuously supplied from R-C. The media solution in R-C was continuously aerated at about 125 mL/minute by a laboratory-based air line through 3 in-line air filters (Bacterial Air Vent, Gelman Sciences). A stainless steel filter was installed at the inlet of each pumping line in R-A and R-B, as well as at the sampling port for R-A, in order to prevent crystalline naphthalene from affecting the HPLC system.

Experimental results using suspended cultures with shake flasks indicated that the exponential phase of bacterial growth usually lasted for approximately 10 hours. Considering that the water capacity of the column reactor with 5 BF-supports was 441 mL, a flow rate at 1 mL/minute was desirable to maintain a proper residence time (7.3 hours) for the culture medium. Water capacity without BF-supports was 536 mL (8.9 hours residence time).

The flow rate of pump-1 was fixed at 1 mL/minute, while flow rates of the other pumps were periodically adjusted to maintain a constant liquid volume (about 2-L) in R-A and R-B. It was necessary to maintain the same flow rate (1 mL/minute) at the inlets of both columns. The inlet concentration of naphthalene was increased when the flow rate of pump-1 was increased. As the flow rate increased, less adsorption of naphthalene was observed in the system. Above a certain flow rate, the inlet concentration of naphthalene became steady. When this system was recirculated for equilibrium condition with naphthalene, it was necessary to keep the same flow rate of the medium having a constant concentration on the inlets of the column reactors during one run of biofilm culture.

With the fixed flow rate (1 mL/minute) in both column reactors, whenever a BF-support in column-Y was taken out as a sample for microscopic enumeration, the residence time of the liquid medium gradually increased (from 7.3 to 8.9 hours) due to the replacement of the glass supports with liquid volume.

Autoclavable 3-way stopcocks with luer locks (polypropylene, Cole-Parmer Instrument Co.) were used for HPLC and microscopic samples of suspended cells at the inlet and outlet of each column. 3-way plastic stopcocks with luer locks (Cole-Parmer Instrument Co.) were also used to take samples of suspension in column-X. The plastic stopcocks were sterilized by 3% hydrogen peroxide and washed with autoclaved water.

4.4 Analytical Procedures

4.4.1 Optical Density of Bacterial Suspension

The biomass concentration in shake flask tests for suspended cultures was determined by measuring optical density (OD) of the sample suspension. About 5 mL of bacterial suspension from a shake flask was taken into a test tube. OD of each sample was measured at wavelength of 540 nm using a spectrophotometer (Spectronic 20D, Milton Roy Company), and related to biomass using a calibration curve (Figure A-1, Appendix A).

4.4.2 Dry Biomass of Bacterial Suspension

A gravimetric method by centrifugation was applied to separate the biomass from suspended culture (Best, 1997), using a General Purpose Centrifuge (Clay Adams™ DYNAC™ II, BD Sciences).

Four 1-L shake flasks with 300 mL of bacterial suspension were cultured with different concentrations of biomass, using different concentrations of naphthalene. When the bacterial culture reached a proper range of OD, 150 mL of suspension was taken from each flask at four different sampling times. The OD of each sample was measured at 540 nm and then transferred to three 50-mL centrifuge tubes for triplicate tests. They were centrifuged at about 2,400 rpm for 45 minutes and supernatant was decanted. The bacterial pellet on the bottom of each tube was rinsed with 50 mL of DI water and then centrifuged again. After that, the supernatant was decanted a second time. Each biomass pellet was diluted with 25 mL of DI water and then mixed by vortex. The diluted suspensions were transferred into pre-tared aluminum weighing dishes. They were placed in a drying oven at about 110 °C overnight. After drying, the weight of biomass was measured, and the dry biomass concentration was determined by averaging data from three replicates.

Once a calibration curve was developed (Figure A-1), experiments relied on OD to determine biomass concentration, since this was easier to obtain.

4.4.3 Measurement of Total Cell Protein

To measure total cell protein (TCP) of suspended cultures, about 1.5 mL of each sample used for OD measurement were placed in a microcentrifuge tube, centrifuged at 17,000 ×g for 3 minutes (Centra-M centrifuge, International Equipment Company). The supernatant was then discarded, and 0.3 mL of 0.1 N-NaOH were added to the pellet on the bottom of the centrifuge tube and thoroughly mixed by vortex. Also, 0.1 N-NaOH without biomass was prepared for the blank control. This was subtracted from

concentrations of actual bacterial samples. All bacterial samples in NaOH solution were stored in the freezer for the future analysis.

The frozen samples in microcentrifuge tubes were boiled for 30 minutes by using floating bubble racks to hold the tubes. Final volume of the boiled samples was adjusted to 0.5 mL by adding about 0.2 mL of DI water. They were mixed by vigorous vortexing and then centrifuged at 17,000 × g for 3 minutes.

As a sample for the BCA protein assay, 0.15 mL of each supernatant was placed in a test tube. Also, 0.15 mL of each standard with known concentrations of bovine serum albumin (BSA), which was previously diluted with DI water, was pipetted into appropriately labeled test tubes. 3 mL of the BCA working reagent (WR) was added to each test tube and then mixed well (sample to WR ratio = 1:21).

All samples including standard solutions were incubated at 75 °C for 30 minutes in a drying oven. The incubating temperature was increased from 60 °C to 75 °C to detect lower concentrations of samples. On the enhanced protocol of the BCA protein assay, the working range at 60 °C for 30 minutes was 5 to 250 μg/mL in total protein.

After incubation, all sample tubes were kept on ice to prevent further color development. The absorbance of each sample was measured at 562 nm by a spectrophotometer (Varian, DMS 300). A typical calibration curve for all standard solutions is shown in Figure A-2 in Appendix A. Using the standard curve, the TCP concentration for each sample was determined.

4.4.4 Analysis of Naphthalene

Concentrations of naphthalene were analyzed using a Waters HPLC with: (1) Tunable Absorbance Detector (Model 484); (2) System Controller (Model 600E); (3) Multisolvent Delivery System (Model# 600); and (4) Autosampler (Ultra WISP Sample Processor, Model# 715); (5) a chromatographic column (MercK 50822; Lichrospher® 100 RP-8, 5 μm).

Isocratic elution of naphthalene was obtained by a mobile phase consisting of 70% methanol and 30% water with 1% acetic acid. The flow rate of the mobile phase was maintained at 1.2 mL/minute. By adjusting the ratio and flow rate of the mobile phase, the production of three intermediates due to biodegradation of naphthalene could be detected. The mobile phase with 80% methanol and 20% water could also separate the naphthalene peak at 1 mL/minute, but it was not useful for separating the intermediates. The retention time of naphthalene was approximately 2.7 minutes in an older HPLC column (3.5 minutes with a new column), but it was observed to fluctuate with varying room temperature or column condition.

Both methanol and water were vacuum filtered through a 0.45 μm-membrane filter and preserved by adding 1% acetic acid. The solutions were then degassed by a continuous helium purge (Ultra High Purity, Matheson gas Products). The UV-detector was set at a wavelength of 254 nm. The data were processed and integrated by Nelson Chromatography Software (PE Nelson Model 2600, rev. 5.10) using a Nelson 900 Series interface.

About 1 mL sample for biofilm experiments was transferred to a 1.2 mL-vial for HPLC autosampler. In case of suspended cultures with shake flasks, about 1.2 mL of a

sample was filled up to the top of each vial. Two different calibration curves were applied for both cases, plotting 5 known standard naphthalene solutions for each curve, and checked periodically. As an example, a plot is shown in Figure A-3 in Appendix A. The standard solutions were prepared with pure methanol. Each sample was preserved with approximately 20 μL of 1:1 hydrochloric acid (HCl) to stop any further biological activity. The volume injected by the autosampler was set at 8 μL, which was reduced from 20 μL to avoid pressure built up due to use of an on-line pre-filter kit on the HPLC column (instead of using a separate filtering step to remove bacteria from the sample suspension).

Initially, for pre-treatment of HPLC samples, about 1.2 mL of bacterial sample was filtered through a 0.2 μm-membrane using a Swinney filter holder, which was attached to a syringe. However, loss of naphthalene due to adsorption on the membrane filter averaged about 36.5 % as shown in Table B-1 and Figure B-1 in Appendix B. Therefore, an on-line prefilter kit with 0.5 μm pore size (Alltech Associates, Inc.) was used instead to protect the HPLC column and avoid a separate filtration step. The on-line filter was continuously exposed to the mobile phase (70% methanol and 30% water with 1% acetic acid), which resulted in a much lower loss of naphthalene.

4.4.5 Observation of Live and Dead Cells by Fluorescence Microscopy

A Nikon epifluorescence microscope (Eclipse TE200) equipped with a 100W-mercury lamp (Chiu Technical Corporation) was used with a Nikon B-2A filter set for fluorescein isothiocyanate (FITC); 470±20 nm for exciter filter, 505 nm for dichroic mirror and 520 nm for barrier filter. The excitation/emission maxima of the dyes are about 480 nm/500

nm for the SYTO 9, and 490 nm/635 nm for the PI. Therefore, the fluorescence from both live and dead bacteria can be viewed simultaneously with the FITC filter set. Two objective lenses were used: 60× for suspended cultures and 100× for biofilm cultures.

Photomicrographs of bacterial images were collected with an image controller (Optronics, DEI-750D CE Digital Output, Model S60675) and Image Pro 4.0 (software). However, actual enumeration of bacterial samples was performed by visual counting with 10× eyepiece lens, using 3 cell counters (Hand Tally Counter, Fisher Scientific) for live cells, dead cells, and number of focusing fields. Because the size of photographic image is only about 20% of countable area (a square) in a focusing field, visual counting with the eyepiece lens was a better choice, in which a square with known area (0.1×0.1 mm^2 on 100× objective, and 0.162×0.162 mm^2 on 60× objective) is marked with 100 small grids.

4.5 Fluorescent Staining Techniques for Microscopic Counting of Cells

4.5.1 Screening Tests of LIVE/DEAD® BacLight™ Bacterial Viability Kit

To examine the capability of LIVE/DEAD® BacLight™ Bacterial Viability Kit (LDBBVK) for distinguishing live and dead cells on a fluorescence microscope, the dual-staining mixture of SYTO 9 and propidium iodide (PI) was pre-screened by adjusting the ratio of both components volumes and concentration of dimethylsulfoxide (DMSO) in a bacterial sample. Also, the incubation time in staining *Pseudomonas putida* was observed.

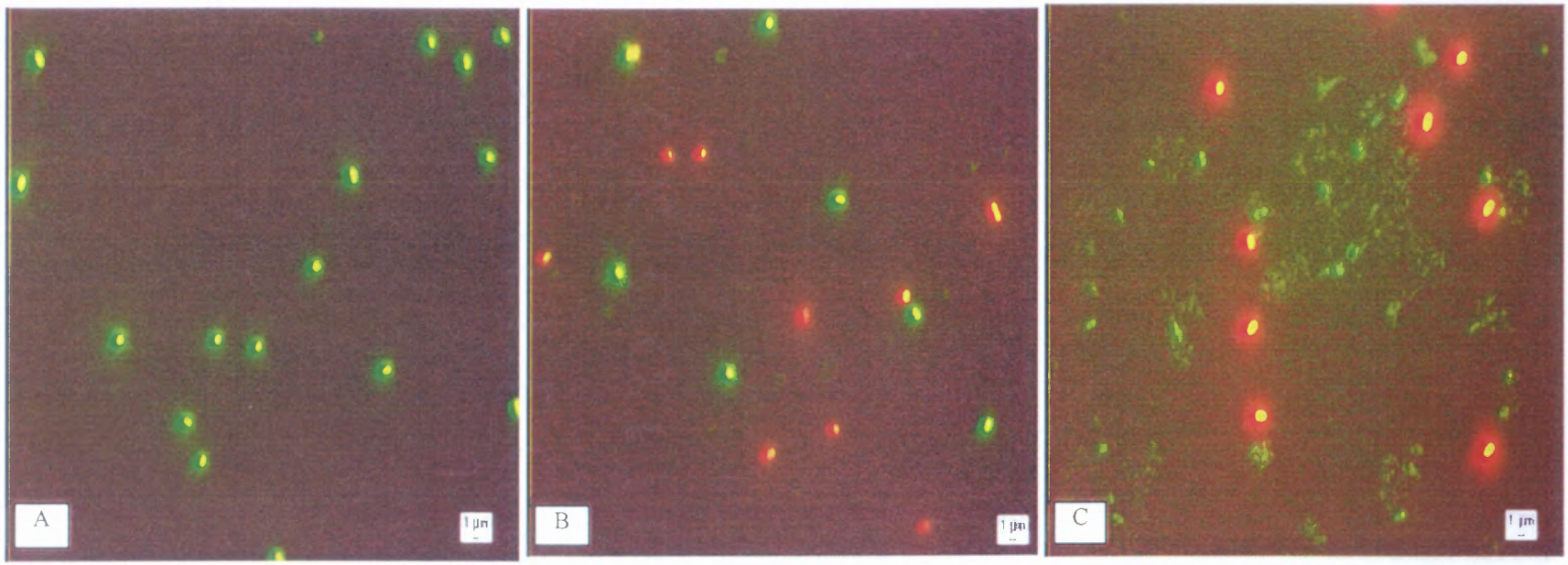
When bacterial suspensions were tested with LDBBVK, three different methods as shown in Table 4.3 were evaluated for enumerating cell density as well as

distinguishing both live and dead cells. The general method with a regular slide glass and a coverslip showed the best result, making the staining technique more rapid, simpler and more reliable than the others.

Table 4.3 Evaluation of staining methods for enumerating live and dead cells in bacterial suspension. (Dual staining of SYTO 9 and propidium iodide)

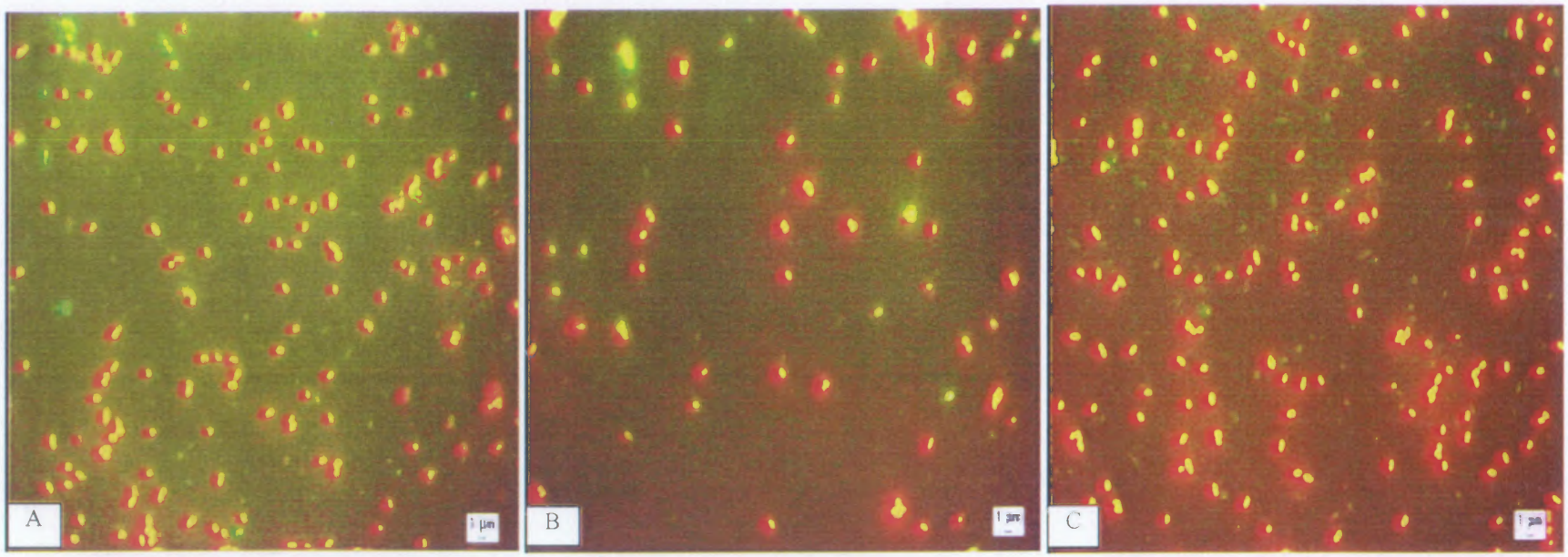
	Filtration with black membrane (13 mm in diameter)	Multi-well slide glass (8 mm in diameter)	Regular slide glass + cover slip (22 x 22 mm^2)
Sample condition after staining	Dry or wet	Dry	Wet
Available sample volume	Large (depending on cell density)	Small (2 – 6 μL)	Small (4 μL)
Reliability in distinguishing live and dead cells	Uncertain	Uncertain	Very good
Precision in measuring surface area of stained sample	Good	Good	Sufficient
Bacterial motion for visualization	Little	No	Brownian motion (No problem on visual counting)
Accumulation of red probe on living cell wall	Possible	Possible	No (within about 5 hours)
Uniform distribution of cells	Good	Bad	Sufficient

As an example of the staining method with a regular slide glass and a coverslip, Figure 4.4 shows fluorescence photomirographs observed under the wet condition of bacterial samples stained with the mixture of SYTO 9 and PI (0.2% DMSO); a) 100% live cells, b) 50/50 of live and dead cells, c) 100% dead cells. This staining method was highly reliable in distinguishing live and dead cells. On the other hand, the two methods



(100 % live cells) (1:1 of live: dead cells) (100 % dead cells)

Figure 4.4 Epifluorescence photomicrographs of *Pseudomonas putida* stained (in wet condition) with the mixture of SYTO 9 and propidium iodide under FITC optic filter. Wet condition of stained bacterial samples under microscopic observation resulted in more reliable images for enumerating live and dead cells (60× objective lens, 2.8× relay lens).



(100 % live cells) (1:1 of live: dead cells) (100 % dead cells)

Figure 4.5 Epifluorescence photomicrographs of *Pseudomonas putida* stained (in dry condition) with the mixture of SYTO 9 and propidium iodide under FITC optic filter. Mounting oil was placed right after drying out stained samples on a multi-well slide glass at room temperature. When stained samples were dried out, microscopic images were not reliable for distinguishing live and dead cells (60× objective lens, 2.8× relay lens).

using dry stained samples were unreliable because of the accumulation of PI on live cell wall showing partially green or orange (or red) fluorescence as illustrated in Figure 4.5.

The most important clue for optimizing the staining technique with LDBBVK was to maintain wet (non-dried) condition in a stained sample. It was necessary to avoid keeping a stained sample sealed with a slide glass and a coverslip for more than about 1 hour. Also, it was preferable to keep the stained sample in a microcentrifuge tube. Right before microscopic observation, the stained sample in the tube needs to be mixed by vortex to make uniform cell distribution, and subsequently placed on a slide glass and covered with a coverslip sealed with nail polish. The vortexing step may have an effect on the removal of the PI accumulated on the surface of viable cells.

As mentioned by Lawerence et al. (1997), it was found that the red probe (PI) was partially coated on a sample specimen with live cells showing orange or red fluorescence on the FITC filter set. Based on experimental results and the reference, the reason was inferred as follows: When the stained sample was kept for a long time (more than about one hour) under a coverslip sealed with nail polish, the aqueous portion of the red probe, which is presumably hydrophilic, was partially dried out. Consequently, it could be easily coated on the surface of living cells due to the characteristic of the PI.

4.5.1.1 Staining Method by Vacuum Filtration with PCTE Black Membrane

A polycarbonate track-etched (PCTE) black membrane (13 mm in diameter and 0.22 um in pore size, Osmonics Inc.) for epifluorescence microscopy was used in conjunction with a drain disk supporter (PreSep Mesh Spacer, 13 mm, Osmonics Inc.) for promoting uniform distribution of bacteria on the filter surface. A Swinney filter holder (Millipore),

with the black membrane and the drain disk-supporter, was assembled with a disposable 3 mL syringe, which had no plunger.

After 1 mL of bacterial suspension was stained with 3 μL of the dye mixture (0.3% DMSO) and then incubated at room temperature in the dark for about 15 minutes, 1 mL of phosphate buffered saline (PBS) was added into a syringe assembled with the filter set. A proper volume (about 2 to 50 μL) of stained bacterial suspension was then added to the PBS in the syringe, accompanied with vortexing for enhanced uniform distribution of the stained bacteria on the black membrane. The reason that a small amount of stained sample was diluted with the PBS solution was to wash out the coated red probe (PI). The stained cells were filtered through the black membrane under low vacuum. 2 μL of filter-sterilized water was placed on a slide glass, and then, only the membrane without the drain disk-supporter was removed from the filter set and placed on top of the water droplet. About 3 to 4 μL of BacLight mounting oil (provided with LDBBVK) was added to the top of the filter. An oversized 22-mm square coverslip (No.1, Fisher Scientific) was placed on the top of the mounting oil, and gentle pressure was applied to spread the fluid over the filter. The coverslip was sealed with nail polish. Finally, the sample was observed under a fluorescence microscope equipped with proper filter sets.

Bacterial staining using vacuum filtration caused irregular results under a fluorescence microscope: sometimes showing clear bacterial images with wet condition of stained samples, and sometimes showing false images from dried specimen due to excess vacuum suction of stained liquid.

4.5.1.2 Staining Method Using Multi-Well Slide Glass

Instead of using membrane filters, multi-well slide glasses (25 × 75 mm), with known surface area of stained liquid sample, were applied as an alternative method. Multi-well slide glasses with 8 mm diameter and 8 wells (HTC®, Black; Cel-Line/Erie-Scientific Co.) were coated with a solution of 0.1% gelatin and 0.01% $KCr(SO_4)_2$, dipping each glass into the solution for a second and drying out at room temperature. The coated glasses were stored in the refrigerator.

100 μL of original suspended culture was stained with 0.2 μL of the 1:1 dye mixture (0.2% DMSO). 2 to 6 μL of the stained sample were placed on a well of a multi-well slide glass and then spread uniformly on the well using a Pipetman tip. The stained sample was incubated and dried out simultaneously at room temperature for about 5 to 10 minutes. After the drying step, BacLight mounting oil was placed on the dried sample and then covered with a coverslip (25 × 50 mm, No.1). This sample was observed under a fluorescence microscope.

4.5.1.3 Staining Method Using Regular Slide Glass

100 μL of original suspended culture were mixed with 0.2 μL of the 1:1 dye mixture (0.2% DMSO) without rinsing steps, and incubated at room temperature in the dark for about 15 minutes. Right before microscopic observation, the stained sample in a microcentrifuge tube was mixed again by vortex. Exactly 4 μL of the sample was subsequently put on a regular slide glass (3 × 1 inches, 1 mm in thickness, Fisher Scientific) and covered with a 22 × 22 mm-coverslip (Fisher Scientific, No.1) sealed with nail polish. The 4 μL-sample was uniformly spread out in the size of the coverslip.

If the stained sample is not immediately used for microscopic observation, it is necessary to avoid keeping the stained sample in a slide glass sealed with a coverslip for more than about one hour. This causes drying out of the sample and accumulation of the red probe (PI) on live cell wall. Regardless of the incubation time instructed by the manufacturer, the stained bacterial suspension kept in a microcentrifuge tube was still useful within about 4~5 hours, showing intensive green or red fluorescence on either live or dead cells under the microscope equipped with FITC filter set.

4.5.1.4 Optimization of Staining Method with Live and Dead Cells

The dual-staining mixture was tested with two types of samples: (1) bacterial suspension with live cells only (cultured in nutrient broth); and (2) dead bacterial suspension (killed by 70% isopropyl alcohol, according to the instructions with the LDBBVK). These two samples were prepared from the same original suspension (*Pseudomonas putida*) grown in a shake flask at room temperature for one or two days.

The two components of the dye mixture were tested with different volumetric ratios of SYTO 9 and PI (4:1, 3:1, 2:1, 3:2, 1:1, 2:3, 1:2, 1:3, 1:4 and 1:10). The optimal result was obtained with a 1:1 mixture of SYTO 9 and PI, as instructed in LDBBVK.

Also, the concentration of DMSO was tested by staining living cells with different amount of the 1:1 dye mixture (1%, 0.1%, 0.2 % and 0.3%). The result with 1% DMSO showed red fluorescence which gave a false indication of dead cells. The results with 0.1%, 0.2% and 0.3% showed clear green fluorescence images in staining live bacteria. As a result, the 0.2% DMSO was applied for all subsequent testing of bacterial suspensions.

4.5.2 Staining Methods for Biofilms on Glass Supports

Both direct and indirect staining methods were pre-tested for enumeration of biofilms on glass supports, distinguishing live and dead bacteria. Based on the results of the test, it was recognized that each case had its drawbacks in performing bacterial enumeration with the fluorescent staining technique.

The direct staining method (see below) was not effective in distinguishing live from dead cells, since red fluorescence was observed irregularly on active (live) biofilm due to accumulation of the red probe on live cell membrane. In order to overcome this problem, a longer incubation time (1 to 3 hours) was used with 0.3% DMSO. However, the longer incubation time under a coverslip caused the stained active biofilms to dry out and show the red fluorescence irregularly. Hence, the direct staining method usually underestimated the number of viable cells. However, this method was useful to enumerate total (live and dead) cells on biofilm monolayers (Microscopic observation indicated that the biofilm were generally laid down as a monolayer for growth periods of 2 to 3 days).

For the indirect staining method (see below), it was expected that there would be loss or damage of cells when they were detached from a BF-support by a scraper. The indirect staining method was not useful for biofilm monolayers because of the technical problem in detaching cells from a glass support into water. There was difficulty establishing a consistent technique for lifting immobilized cells.

Due to the difficulties of both staining methods, a combined method was used with direct staining for total cell count on the BF-supports, and general staining of proximate suspended cells for the ratio of live to dead cells.

4.5.2.1 Direct Staining

For direct staining of biofilms, 3 μL of the stock dual-staining dye mixture was diluted to 1 mL of autoclaved-DI water (0.3% DMSO) using a 1.5 mL-microcentrifuge tube. Before starting biofilm cultures, about 10 tubes were prepared and stored in the freezer. It was mixed by vortex prior to use.

After taking a glass support with tripartite biofilms (as mentioned in Section 4.1.4), it was first rinsed by immersing it into autoclaved DI water for about 15 seconds. Then, about 50 μL of the staining solution (0.3% DMSO with autoclaved DI water) was gently added to the center of each part of the BF-support, repeated 3 to 4 times, and incubated at room temperature in the dark for about 15 to 30 minutes. A cover glass (22 x 22 or 25 x 25 mm) was placed on the stained biofilm. To reduce Brownian motion of stained cells under the fluorescence microscope, the excess staining solution under the coverslip was removed by touching (or gently pressing) cotton swabs around (or over) the edge of the coverslip. The coverslip was sealed with nail polish. Finally, non-stained areas of the BF-support were cleaned with 70% isopropyl alcohol using a small piece of paper towel and a tong.

4.5.2.2 Indirect Staining

A BF-support taken from a column reactor was gently rinsed with DI water to remove any unattached cells. Biofilm attached to the surface was then harvested using a cell scraper (Fisher Scientific), and mixed with about 100 mL of autoclaved DI water in a 250 mL beaker. Once samples were prepared, the procedure followed that for cell enumeration with bacterial suspension as described in Section 4.5.1.3.

4.6 Enumeration of Bacterial Samples by Microscopic Observation

4.6.1 Suspended Cultures in Shake Flasks

Using 4 μL of stained sample prepared as instructed in Section 4.5.1.3, the number of bacteria under a 22 × 22 mm-coverslip (484 mm^2) was estimated by counting cell number visually from 30 to 60 objective fields (counting about 100 to 700 cells) under the microscope. The enumeration step was performed by counting live and dead cells only in a square outlined on the objective field. The area of the square was equal to 0.162 × 0.162 mm^2 (0.026244 mm^2) on 60× objective. The objective field was moved in a zigzag pattern, beginning in the upper left hand corner of the cover slip and proceeding across back and down. 30 to 60 objective fields covered about 0.16% to 0.32% of the cover slip area.

The average cell number of live or dead cells on the square of the objective field was then converted to cell density (cells/mL) in bacterial suspension using the following equation.

$$Cell\ density\ (cells/mL) = \frac{Sum\ of\ counted\ cells}{Number\ of\ observed\ fields} \frac{Field}{Area\ of\ square\ on\ objective} \frac{1000\ \mu L}{4\ \mu L} \frac{Area\ of\ coverslip}{1\ mL}$$

4.6.2 Biofilm Cultures in a Continuous Flow System with Column Reactors

In culturing biofilms by the continuous flow reactor, an alternative method was applied for enumerating cells immobilized on glass support, as well as distinguishing live and dead cells. The direct staining method with diluted mixture of SYTO 9 and PI was not effective in distinguishing live from dead cells (although it could be used to count total cell number).

To count total cells on BF-supports, direct staining was applied as described in Section 4.5.3.1 using an objective lens with 100× magnification. It was desirable to use a high magnification lens because sometimes it was difficult to distinguish cells from debris or salts on stained samples. The number of total cells was counted from at least 30 randomly chosen objective fields. However, the ratio of live to dead cells was presumed to be the same as that of the suspended cells that were in proximity to the glass supports in the continuous flow system.

When cell density in the square with 100 small grids on an objective field was increased, the number of small grids used for counting cells was gradually reduced from 100 to 20, counting about 250 to 1800 in total cell number. For cell density of biofilms, the number of total cells per unit surface area of BF-support was calculated using following equation. The area of the square was equal to 0.1 × 0.1 mm^2 (0.0001 cm^2) on 100× objective.

$$Cell\ density\ (cells/cm^2) = \frac{Sum\ of\ counted\ cells}{Number\ of\ observed\ fields} \frac{Field}{Area\ of\ square\ on\ objective} \frac{100\ grids\ on\ a\ square}{Number\ of\ grids\ used}$$

4.7 Experimental Procedure

4.7.1 Suspended Cultures in Shake Flasks

Suspended cultures with 4 different sets of naphthalene concentration were initially prepared as described in Section 4.3.1. At every sampling time, 5 mL of suspension from each flask were transferred to a test tube for measuring OD. 100 μL of the OD sample were immediately stained with 0.2 μL of the dye mixture, followed by the enumerating procedure of suspended cells as described in Section 4.5.1.3. At the same time, about 1.2 mL of the OD sample were transferred to a vial and analyzed quantitatively by HPLC as

mentioned in Section 4.4.4. Measurement of total cell proteins was conducted by the BCA protein assay using about 1.5 mL of the OD sample, as described in Section 4.4.3. All materials and apparatus were already sterilized by autoclaving or filtering with 0.22 μm pore size prior to use.

4.7.2 Biofilm Cultures on Glass Supports in Continuous Flow System

4.7.2.1 Culture of Seed Biofilms

13 glass supports for biofilm, prepared as described in Section 4.1.4, were uniformly placed into the seed biofilm container with the round glass-holder as shown in Figure 1B. The glass holder was useful to have uniform distribution of biofilms on all seed BF-supports. Also, a magnetic bar and a glass tube with a fritted end for aeration were placed into the container. This container was covered with aluminum foil and then sterilized by autoclaving.

About 6.4-L of autoclaved DI water was poured into the autoclaved container, followed by adding 90 mL of medium A, 210 mL of the medium B, and 9 mL of the trace element solution. Finally, about 300 mL of bacterial suspension, pre-acclimated with naphthalene, were added to the container, mixed by the magnetic stirrer, and aerated at about 40 mL/minute. This bacterial suspension was cultured for about 3~4 days to develop initial seed biofilms for the column reactors. To increase the reliability for enumeration of the initial biofilms, 3 of the seed BF-supports were stained with the diluted dye mixture (0.3% DMSO). Before staining with the dye, they were carefully immersed into autoclaved DI water for about 15 seconds to remove any unattached cells.

Using a tong and a pair of forceps sterilized by a flame burner, all glass supports with seed biofilms were transferred to 2 column reactors in the continuous flow system.

4.7.2.2 Culture of Biofilms in Continuous Flow System

Before transferring the seed biofilms from the original container, the medium in the reactor system was recirculated at 1 mL/minute for 2~3 days in order to reach equilibrium in naphthalene concentration, as shown in Figure 4.2. After confirming equilibrium in the system by measuring naphthalene concentration periodically, the recirculating lines were disconnected and connected to a drain bottle for continuous flow of the medium.

Because of refraction of the liquid medium, and close spacing of the BF-supports, it was difficult to install them properly into slots on the inlet end plate of each column. Thus, it was necessary to take out all medium in each column right before transferring the BF-supports into the column. The medium in each column reactor was separately transferred into 2 autoclaved 500 mL beakers. After that, each seed BF-support was rinsed by immersing it carefully into autoclaved DI water for about 15 seconds and then immediately transferred into both column reactors. After installing 5 BF-supports, the medium kept in the 500 mL beaker was immediately re-filled into each column using a glass funnel with long neck. A stainless steel bracket was fitted onto the top of the 5 glass supports, and then each outlet end plate was tightly re-connected to the top of each column.

To observe the adsorption of naphthalene on the BF-supports at the beginning of a run, it was necessary to measure naphthalene concentration as soon as possible at the inlet and outlet of both column reactors.

During this experiment, column-Y was specially used to take a BF-support at every sampling time, measuring concentration of naphthalene at both inlet and outlet, while column-X was used to observe viability of suspended cells in the column as well as naphthalene concentration of suspension. After taking out all biofilm supports from column-Y, all 5 biofilm supports in column-X were taken out at the same time as the last samples for biofilm cultures in the continuous flow system.

Due to the adsorption of naphthalene on a 3 mL-syringe, HPLC sample was taken by dropping liquid medium into a vial through a 3-way stopcock on both inlet and outlet of each column. Secondly, bacterial suspension (100 μL) from the sampling ports-A, C, E on column-X, and the outlet of column-Y, were immediately stained with 0.2 μL of the 1:1 dye mixture. As the third step, after the BF-support taken from column-Y was rinsed with water as mentioned above, it was immediately stained with diluted dye mixture (0.3% DMSO). After finishing sampling, the stainless bracket was carefully re-placed on the top of the remaining BF-supports using a tong and a pair of forceps, and then the top end plate was re-connected to the column. BF-supports pre-numbered on column-Y were taken out for analysis after a prescribed time.

All apparatus and materials used in this experiment were sterilized prior to use by autoclaving, or using 70% isopropyl alcohol or 3% hydrogen peroxide for plastic or rubber materials such as sampling ports and o-rings on the column end plates.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Suspended Cultures of *Pseudomonas putida* in Shake Flasks

Two sets of shake flask experiments were conducted at a constant temperature of 25 °C (±1 °C) and 200rpm, each using *Pseudomonas putida* (ATCC 17484). In each set, there were four different values of the initial naphthalene concentration, ranging from about 3 to 16 mg/L (the solubility of naphthalene in water at room temperature is about 30 mg/L). For each initial naphthalene concentration, four parameters were measured over time (experimental set #1 lasted 54 hours, and set #2 lasted a total of 35 hours): naphthalene concentration, optical density (OD), total cell protein (TCP), total cell number (TCN), and living cell number (LCN). The last four of these measurements are all related to biomass. All data (Tables and Figures) are presented in Appendix D, and will be discussed below in detail. TCP data for set#2 were discarded because for unknown reasons measured values of most samples were below those of the distilled water blank. Decreases in naphthalene concentration for the sterile controls are primarily due to volatilization.

5.1.1 Kinetic Rate Constants for Biodegradation of Naphthalene

The rate of biomass growth is given by the following equation [Sundstrom and Klei, (1979)]:

$$\frac{dB}{dt} = \mu B \qquad \mu = \frac{dB/dt}{B} = \frac{d(LnB)}{dt} \tag{5-1}$$

where, B = concentration of biomass at time t
t = time

dB/dt = biomass growth rate [concentration/time]
μ = specific growth rate [time^{-1}]

Since there was no evidence of naphthalene toxicity up to a concentration of 16 mg/L, the Monod equation was used to describe the specific growth rate in this range:

$$\mu = \frac{\mu_m S}{K_s + S} \tag{5-2}$$

where, μ_m = maximum specific growth rate [time^{-1}]
S = concentration of limiting substrate [mg/L]
K_s = half-velocity constant [mg/L]

Substituting equation 5-2 into equation 5-1, bacterial growth rate is:

$$\frac{dB}{dt} = \frac{\mu_m \cdot S \cdot B}{Ks + S} \tag{5-3}$$

However, only a fraction of the substrate is converted to cell mass. Defining the yield coefficient (Y) as mg/L of biomass produced per mg/L of substrate degraded:

$$Y = -\frac{dB}{dS} = -\frac{dB/dt}{dS/dt} \tag{5-4}$$

And the decrease in naphthalene concentration is given as:

$$\frac{dS}{dt} = -\frac{1}{Y}\frac{dB}{dt} = -\frac{\mu B}{Y} \tag{5-5}$$

Substituting equation 5-2 for the specific growth rate (μ):

$$\frac{dS}{dt} = r_s = -\frac{1}{Y}\left(\frac{\mu_m \cdot S \cdot B}{K_s + S}\right) \tag{5-6}$$

where, r_s = rate of naphthalene degradation

The key issue that this dissertation focused on is the determination of biomass concentration (B).

During the exponential growth phase of *Pseudomonas putida* in shake flasks, the specific growth rate was determined by preparing plots of Ln(biomass) versus time and taking the initial slope. Original data are given in Appendix E, and the results are summarized in Table 5.1, and Figures 5.1 to 5.4.

Kinetic rate constants (μ_m and K_s) for the Monod equation were obtained using SigmaPlot® 2.0 (Jandal Scientific Software), which employs a Marquardt-Levenberg algorithm to perform a non-linear regression of the specific growth rate vs. initial naphthalene concentration data. The curves shown in Figures 5.1 to 5.4 are the regressed curves.

Table 5.1 Regression of specific growth rates to obtain kinetic rate constants for Monod equation. (experimental data sets 1 and 2 combined)

Initial concentration (mg/L)	OD Experiments (h^{-1})	OD Regression (h^{-1})	LCN Experiments (h^{-1})	LCN Regression (h^{-1})	TCP Experiments (h^{-1})	TCP Regression (h^{-1})
3.07	0.034	0.046	0.071	0.045	0.093	0.090
6.23	0.097	0.084	0.043	0.078		
7.21	0.068	0.094	0.111	0.087	0.150	0.145
9.54	0.130	0.116	0.089	0.104		
11.49	0.115	0.132	0.140	0.115	0.155	0.173
12.60	0.179	0.141	0.095	0.121		
15.16	0.169	0.158	0.120	0.134		
16.19	0.137	0.165	0.162	0.138	0.203	0.192
	μ_m = 0.412		μ_m = 0.264		μ_m = 0.261	
	K_s = 24.33		K_s = 14.83		K_s = 5.83	
All data combined			μ_m = 0.291 h^{-1}			
			K_s = 13.55 mg/L			

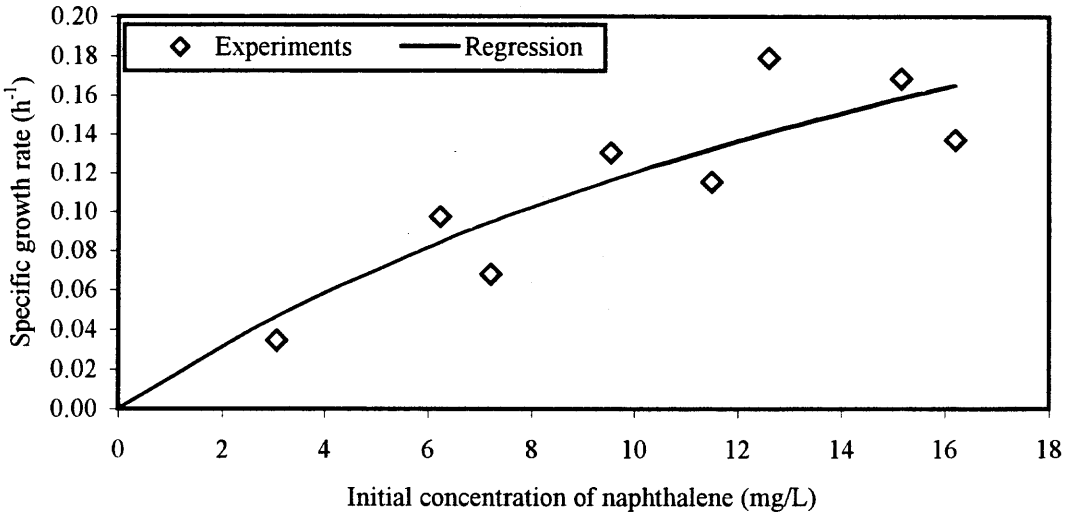


Figure 5.1 The best fit of specific growth rates in the original measurements of OD for determination of μ_m and K_s (Experiments-1&2 combined).: $\mu_m = 0.412\ h^{-1}$, $K_s = 24.33$ mg/L

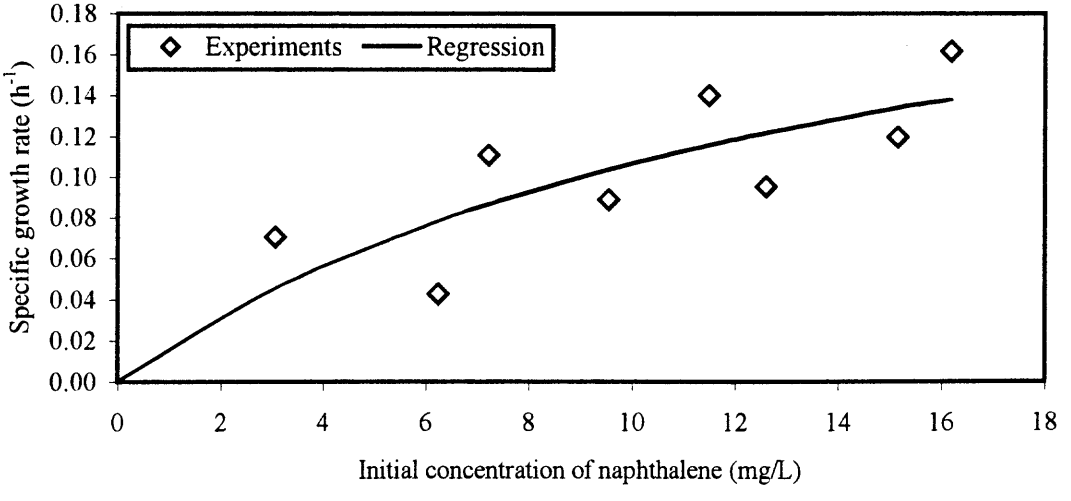


Figure 5.2 The best fit of specific growth rates in the original measurements of LCN for determination of μ_m and K_s (Experiments-1&2 combined).: $\mu_m = 0.264\ h^{-1}$, $K_s = 14.83$ mg/L

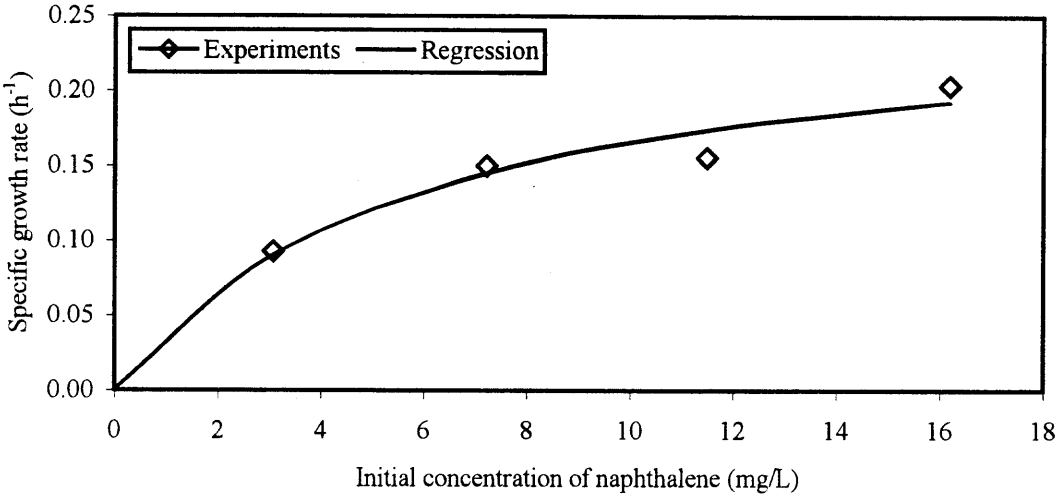


Figure 5.3 The best fit of specific growth rates in the original measurements of TCP for determination of μ_m and K_s (Experiment-1 combined).: $\mu_m = 0.261\ h^{-1}$, $K_s = 5.83$ mg/L

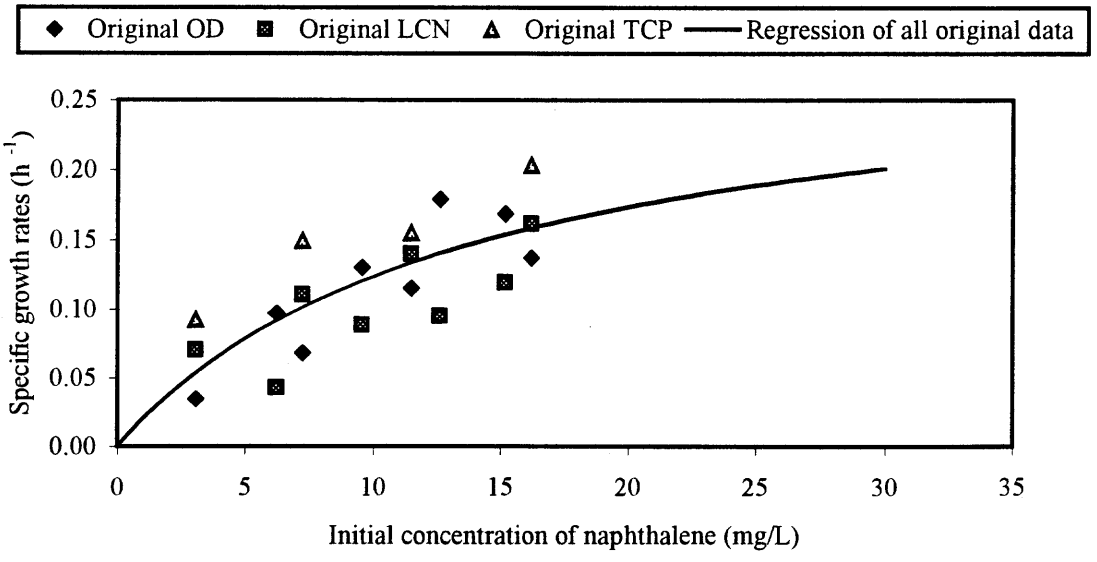


Figure 5.4 The best fit of specific growth rates in all original biomass measurements for determination of μ_m and K_s (Experiments 1&2 combined).: $\mu_m = 0.291 h^{-1}$, $K_s = 13.55$ mg/L

5.2 Yield Coefficient (Y)

Pseudomonas putida (ATCC 17484) is a very effective naphthalene degrader. As a result, there were at most two measurements of naphthalene concentration in each run prior to its complete disappearance after 4 to 8 hours. Consequently, the following procedure was adopted.

First, all biomass data were converted to mg/L dry biomass using appropriate conversion factors. A conversion factor (F_{OD} = 373 mg/L of dry biomass per unit OD) was obtained by preparing a plot of dry biomass versus OD as shown in Figure A-1 in Appendix A. Conversion factors (f) for total cell number (TCN), living cell number (LCN) and total cell protein (TCP) to optical density were determined by plotting OD versus TCN, LCN and TCP as shown in Figures F-1 through F-7 in Appendix F. All measurements were converted to dry biomass using the factors in Table 5.2.

Table 5.2 Conversion of OD, TCN, LCN and TCP to dry biomass (mg/L) for *Pseudomonas putida* with naphthalene in shake flasks.

Experimental set	Expression
Experiment-1	$F_{OD} = 373 \times OD$
	$F_{TCN} = 373 \times (8.81\times10^{-10} \times TCN + 0.0061)$
	$F_{LCN} = 373 \times (9.20\times10^{-10} \times LCN + 0.0059)$
	F_{TCP}: (1-a) = $373 \times (0.005 \times TCP + 0.0115)$ (1-b) = $373 \times (0.0055 \times TCP + 0.0076)$ (1-c) = $373 \times (0.0068 \times TCP - 0.0034)$ (1-d) = $373 \times (0.0068 \times TCP - 0.0239)$
Experiment-2	$F_{OD} = 373 \times OD$
	$F_{TCN} = 373 \times (8.06\times10^{-10} \times TCN + 0.001)$
	$F_{LCN} = 373 \times (8.32\times10^{-10} \times LCN + 0.007)$
Experiment-1&2 combined	$F_{TCN} = 373 \times (8.06\times10^{-10} \times TCN + 0.0049)$
	$F_{LCN} = 373 \times (8.30\times10^{-10} \times LCN + 0.0049)$

Then, the yield coefficient for each experiment was determined by approximating equation 5-4 as $-\Delta B/\Delta S$, as shown in Table 5.3. This resulted in an average Y of 0.20 mg/L dry biomass produced per mg/L naphthalene degraded.

Table 5.3 Determination of yield coefficient using initial slopes.

Experiment	Concentration of naphthalene (mg/L)	Naphthalene degraded (-ΔS, mg/L)	Dry biomass produced (ΔB, mg/L)	Y (-ΔB/ΔS)
1-a	NG	-	-	-
1-b	NG	-	-	-
1-c	11.49 at 0 hours 1.05 at 4 hours	10.44	ΔB_{OD}=2.99 ΔB_{LCN}=0.50 ΔB_{TCP}=5.21	0.29 0.05 0.50
1-d	16.19 at 0 hours 7.53 at 4 hours	8.66	ΔB_{OD}=1.31 ΔB_{LCN}= - 0.67 ΔB_{TCP}=0.98	0.15 - 0.11
2-a	NG	-	-	-
2-b	9.54 at 0 hours 2.43 at 4 hours	7.11	ΔB_{OD}=1.87 ΔB_{LCN}=0.65	0.26 0.09
2-c	12.60 at 0 hours 5.25 at 4 hours	7.35	ΔB_{OD}=2.23 ΔB_{LCN}=0.84	0.30 0.11
2-d	15.16 at 0 hours 8.25 at 4 hours	6.91	ΔB_{OD}=1.49 ΔB_{LCN}=0.88	0.22 0.13
Average Y				Y_{OD} = 0.24 Y_{LCN} = 0.10 Y_{TCP} = 0.30
Overall average Y (Standard deviation)				0.20 (± 0.13)

Another method for determining Y was used which involved averaging the four to five biomass measurements at or after 13 hours of run time. This was assumed to be the final biomass concentration. Since the final naphthalene concentration was zero in all cases: Y = (final biomass - initial biomass)/(initial naphthalene), as shown in Table 5.4. This resulted in an average yield coefficient of 0.96.

Table 5.4. Yield coefficient determined by averaging the final biomass concentration. (mg/L biomass produced per mg/L naphthalene degraded)

Experiment	TCP	LCN	TCN	OD	Averge
1-a	0.82	1.05	1.06	1.03	0.99
1-b	0.99	1.18	1.18	1.05	1.10
1-c	1.04	1.06	1.06	1.14	1.08
1-d	0.96	0.76	0.76	0.98	0.86
2-a	-	0.58	0.60	1.03	0.73
2-b	-	0.91	0.91	1.01	0.94
2-c	-	0.92	0.90	1.11	0.98
2-d	-	0.95	0.95	1.04	0.98
Average	0.95	0.93	0.93	1.05	0.96
Standard deviation	0.09	0.19	0.19	0.05	0.15

5.3 Prediction of Naphthalene Loss and Biomass Growth in Suspended Cultures

Once the Monod kinetic parameters (μ_m and K_s) have been obtained, a comparison can be made between experimental data and mathematical predictions of the naphthalene and biomass profiles. The decrease in naphthalene concentration (S) and the growth of biomass (B) are predicted by the following algorithm:

For constant substrate, equation 5-3 can be integrated, resulting in:

$$\ln(\frac{B}{B_0}) = (\frac{\mu_m \cdot S}{K_s + S}) \cdot \Delta t \qquad (5\text{-}7)$$

Equation 5-4 can be approximated as:

$$\Delta S = -\frac{\Delta B}{Y} \qquad (5\text{-}8)$$

$$S = S_0 - \frac{B - B_0}{Y} \qquad (5\text{-}9)$$

Equations 5-7 and 5-9 were then used in a stepwise algorithm as presented in Table 5.5.

Table 5.5 Prediction method for naphthalene and biomass concentration in suspended cultures.

Time (hours)	Concentration of dry biomass (mg/L)	Concentration of naphthalene (mg/L)	Specific growth rate (h^{-1})
t_0	B_0	S_0	$\mu_0 = \frac{\mu_m \cdot S_0}{K_s + S_0}$
t_1	$B_1 = B_0 \cdot e^{\mu_0 (t_1 - t_0)}$	$S_1 = S_0 - \frac{B_1 - B_0}{Y}$	$\mu_1 = \frac{\mu_m \cdot S_1}{K_s + S_1}$
t_2	$B_2 = B_1 \cdot e^{\mu_1 (t_2 - t_1)}$	$S_2 = S_1 - \frac{B_2 - B_1}{Y}$	$\mu_2 = \frac{\mu_m \cdot S_2}{K_s + S_2}$

Footnote: $\mu_m = 0.291\ h^{-1}$; $K_s = 13.55$ mg/L

Two different yield coefficients were applied (Y=0.20 and Y=0.96), as discussed in Section 5.2. Typical results are shown in Figures 5.5 to 5.9. These results were checked using a fourth order Runge-Kutta method, which solved the simultaneous differential equations 5-3 and 5-6 (MATLAB® Version 5.3.1). Predicted values of naphthalene and biomass concentrations were indistinguishable within the experimental error using either method.

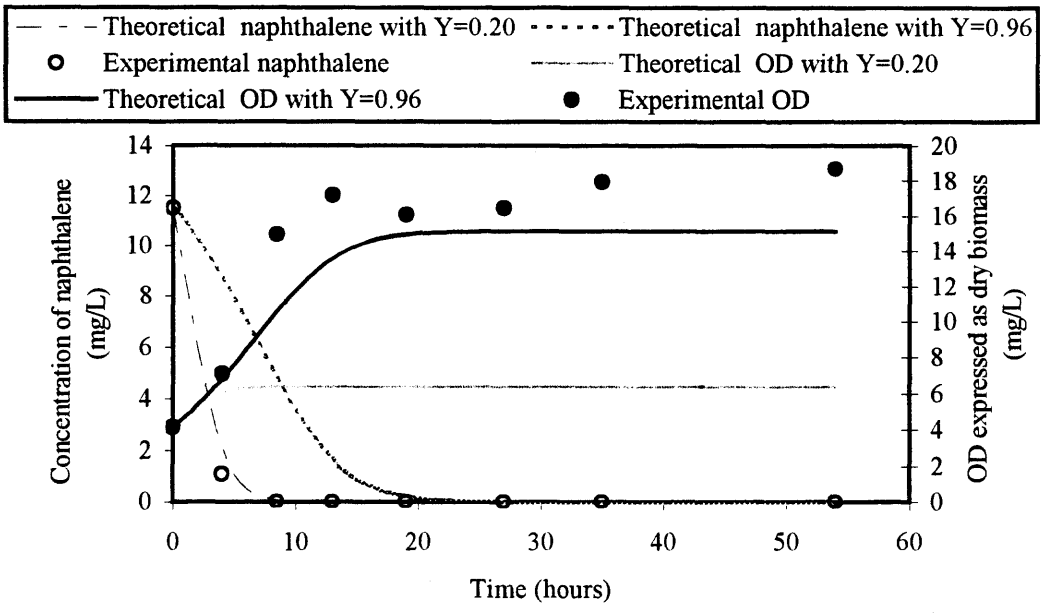


Figure 5.5 Model predicted profiles for naphthalene and OD (Exp-1-c).

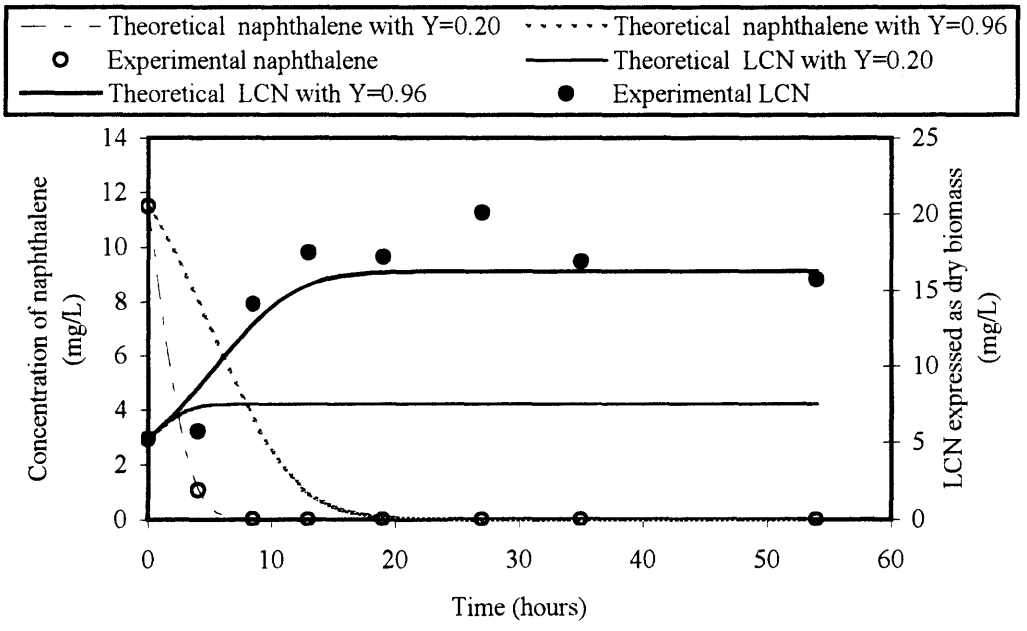


Figure 5.6 Model predicted profiles for naphthalene and LCN (Exp-1-c).

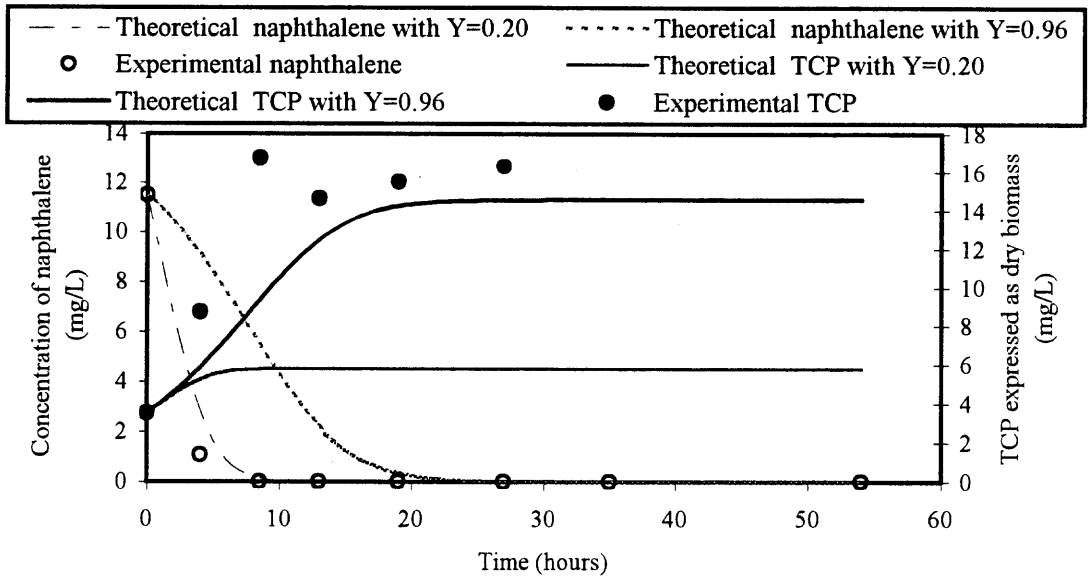


Figure 5.7 Model predicted profiles for naphthalene and TCP (Exp-1-c).

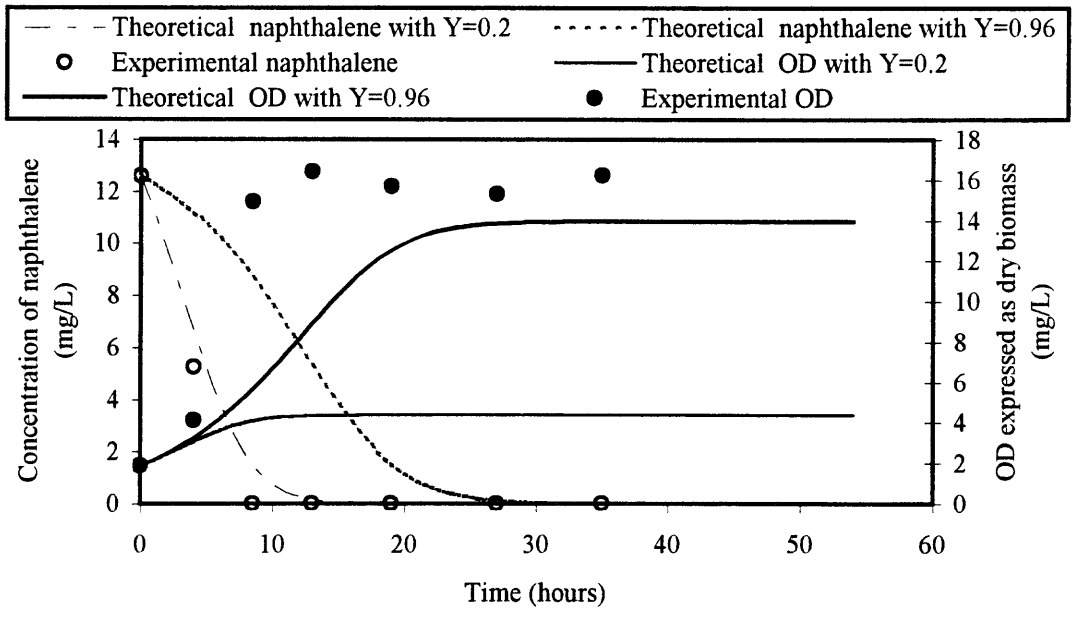


Figure 5.8 Model predicted profiles for naphthalene and OD (Exp-2-c).

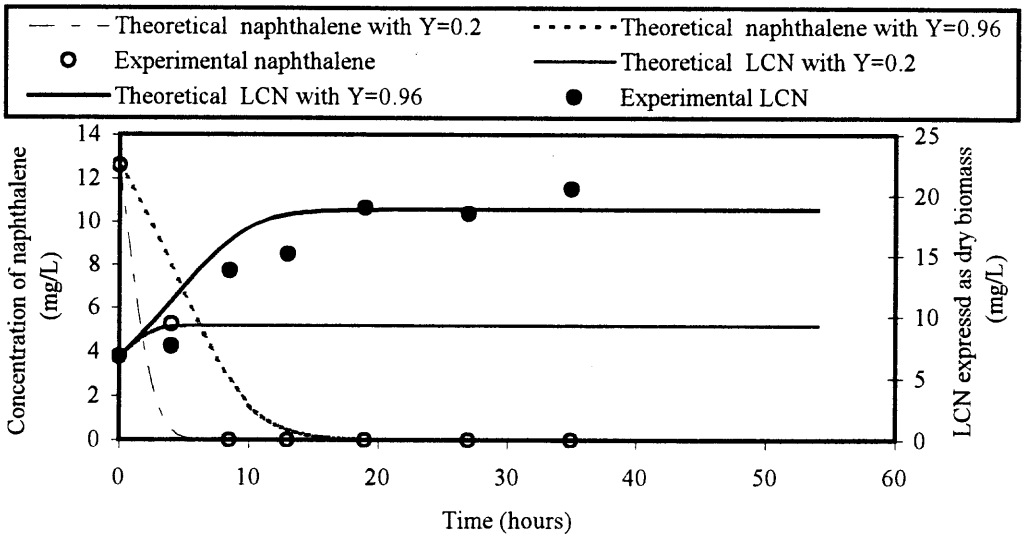


Figure 5.9 Model predicted profiles for naphthalene and LCN (Exp-2-c).

Results were also predicted using the specific values of μ_m, K_s, and Y for each type of biomass measurement (OD, LCN, TCP) rather than average values. Again, they were indistinguishable from those given above.

The problem which these figures illustrate is a disjuncture between the disappearance of naphthalene and biomass growth. After naphthalene disappears, biomass continues to grow, most likely as a result of the formation and disappearance of intermediate products (see Section 5.5 below). Adsorption and subsequent release of naphthalene from surfaces in the flasks may also play an important role. Such a lag cannot be handled by the conventional Monod model. One possible remedy is to add a lag time (T) to the biomass equation (5-7):

$$\ln(\frac{B}{B_0}) = (\frac{\mu_m \cdot S}{K_s + S})(\Delta t + T) \qquad (5\text{-}10)$$

If a lag time of about 1 hour is assumed, the corresponding predictions are shown in

Figures 5.10 to 5.14. Agreement is much improved when Y reflects the total change in biomass divided by the total change in naphthalene concentration (i.e. Y=0.96).

Note that the specific methodology used to characterize biomass (OD, LCN, or TCP) is less important than the need to define a lag time, which in turn depends on the specific biochemical pathways and their detailed kinetics.

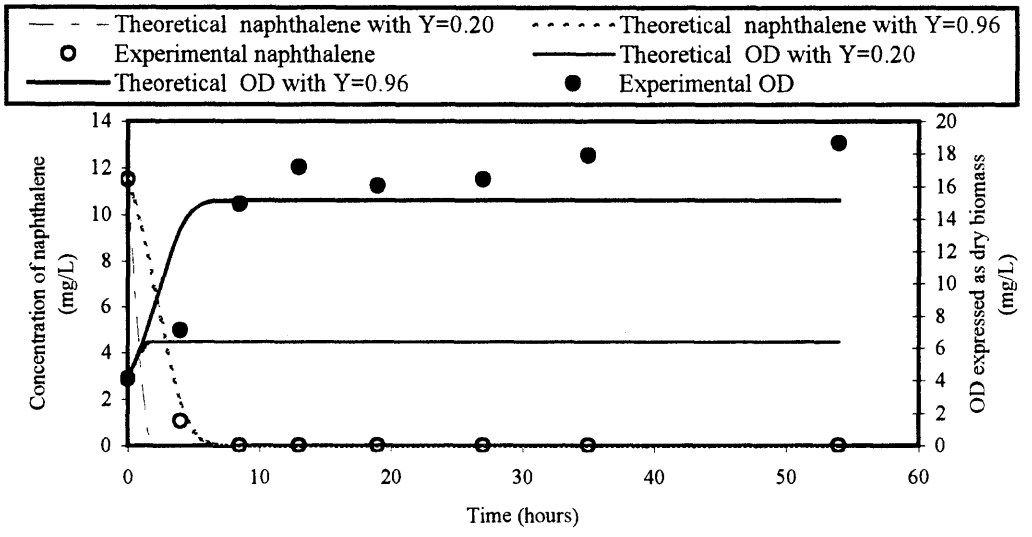


Figure 5.10 Model predicted profiles for naphthalene and OD, with 1 hour lag time (Exp-1-c).

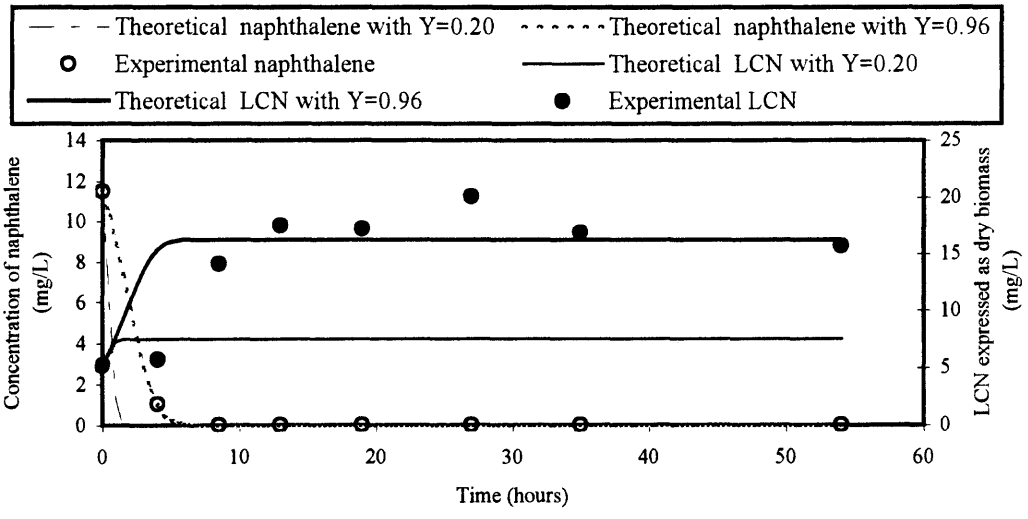


Figure 5.11 Model predicted profiles for naphthalene and LCN, with 1 hour lag time (Exp-1-c).

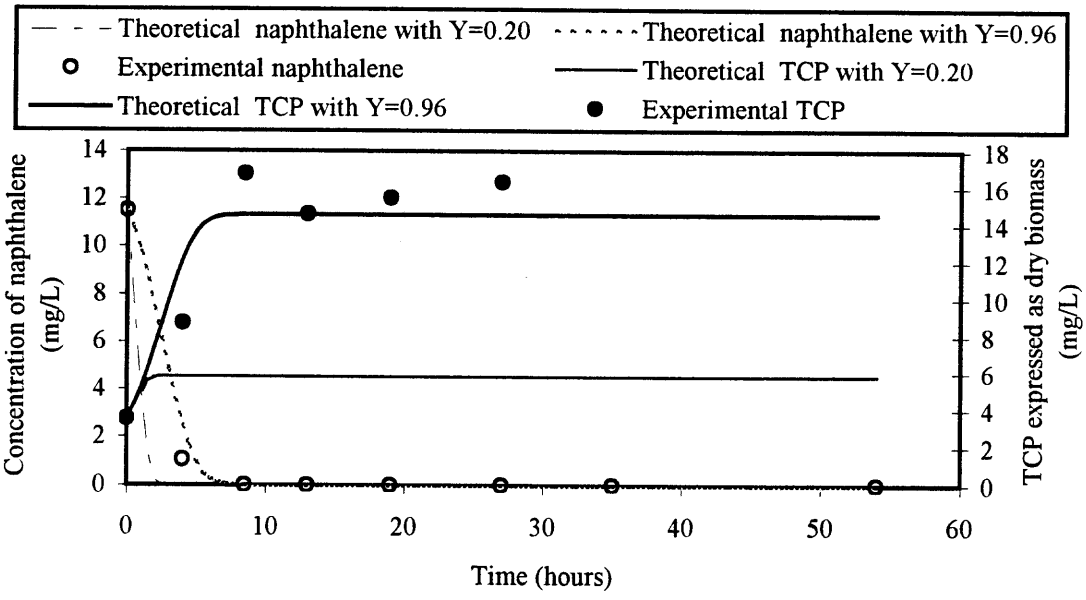


Figure 5.12 Model predicted profiles for naphthalene and TCP, with 1 hour lag time (Exp-1-c).

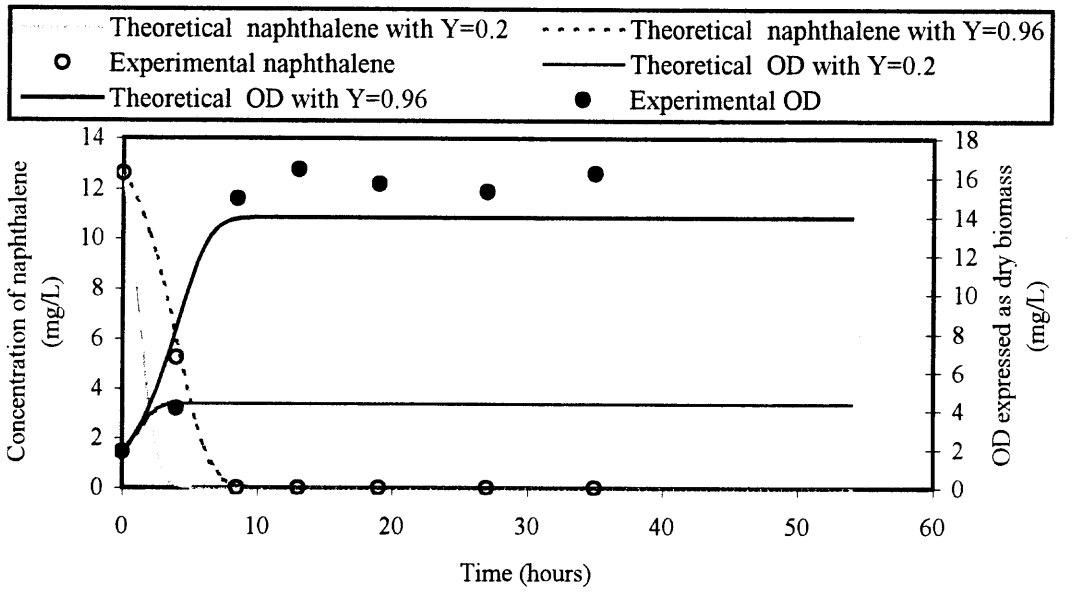


Figure 5.13 Model predicted profiles for naphthalene and OD, with 1 hour lag time (Exp-2-c).

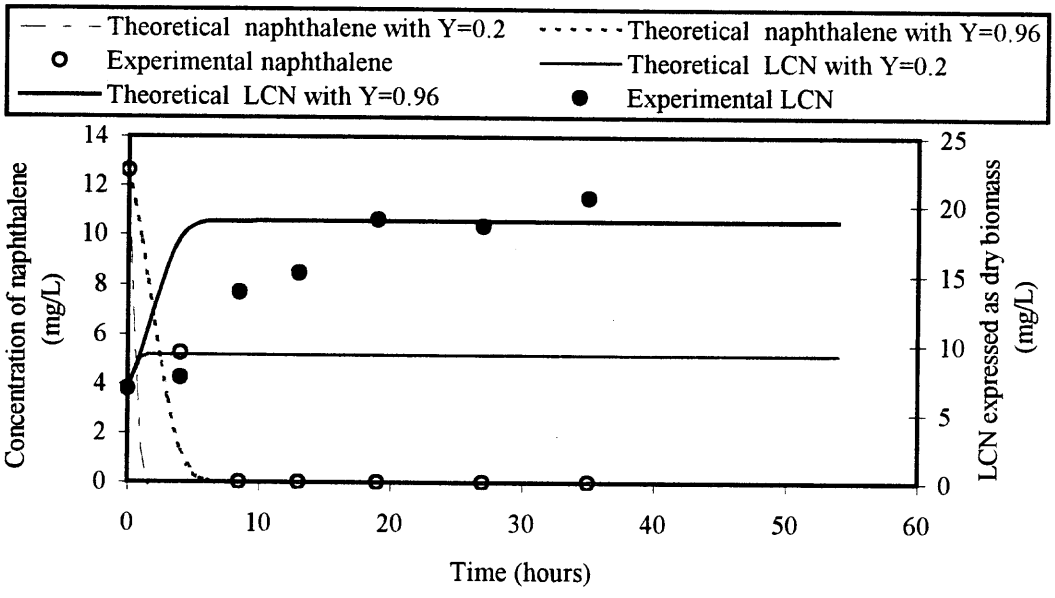


Figure 5.14 Model predicted profiles for naphthalene and LCN, with 1 hour lag time (Exp-2-c).

5.4 Determination of Doubling Time during Exponential Growth Phase

To determine the time for biomass concentration to double, equation 5-1 was used as an approximation at the initial substrate concentration:

$$\ln(\frac{B}{B_0}) = \ln 2 = (\frac{\mu_m \cdot S}{K_s + S})\Delta t_D \qquad (5\text{-}11)$$

where, Δt_D = doubling time

Using equation 5-11 and the specific growth rates in Table 5.1, the doubling times were calculated as shown in Table 5.6. Figure 5.15 indicates that (as expected) the doubling times decrease with increasing initial concentration of naphthalene. The trends are similar for all three biomass measures (OD, LCN, TCP).

Table 5.6 Doubling times.

Initial concentration of naphthalene (mg/L)	Doubling time (hours)		
	OD	LCN	TCP
(Using specific growth rates by original biomass measurements)			
3.07	-	9.8	7.5
7.21	10.2	6.3	4.6
11.49	6.0	5.0	4.5
16.19	5.1	4.3	3.4
6.23	7.1	-	NA
9.54	5.3	7.8	NA
12.60	3.9	7.3	NA
15.16	4.1	5.8	NA

(NA: not available)

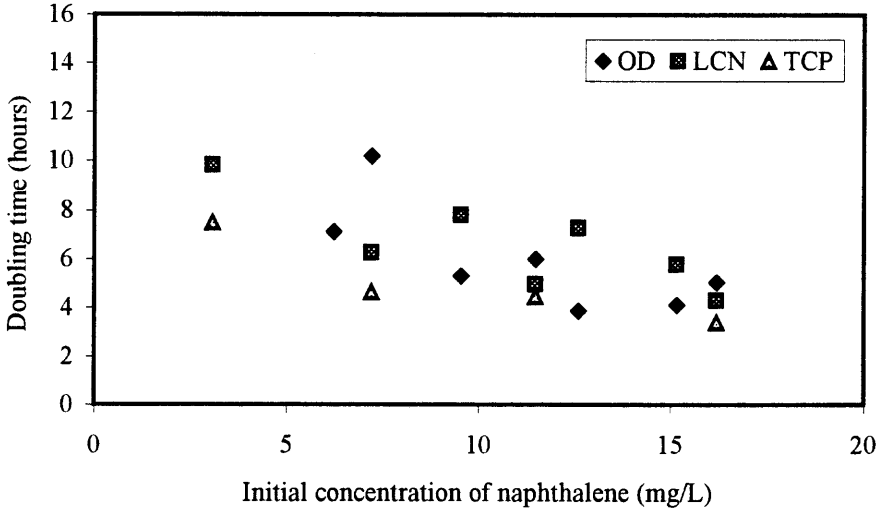


Figure 5.15 Doubling time of suspended cultures of *Pseudomonas putida* in shake flasks with naphthalene.

5.5 Intermediate Products

As shown in Figures G-1 through G-8 in Appendix G, three intermediate peaks were observed during HPLC analysis. In general, the peak corresponding to the shortest retention time remained in the samples even after 50 hours, while the other two peaks disappeared presumably due to further biodegradation.

5.6 Viability of *Pseudomonas putida* Suspension in Shake Flask Test

Numbers of living and dead cells determined by fluorescence microscopy are given in Appendix D. The percentage of live cells ranged from about 93% to 99% as shown in Figures 5.16 and 5.17. Viability in culture controls without naphthalene decreased after one day to about 90 %.

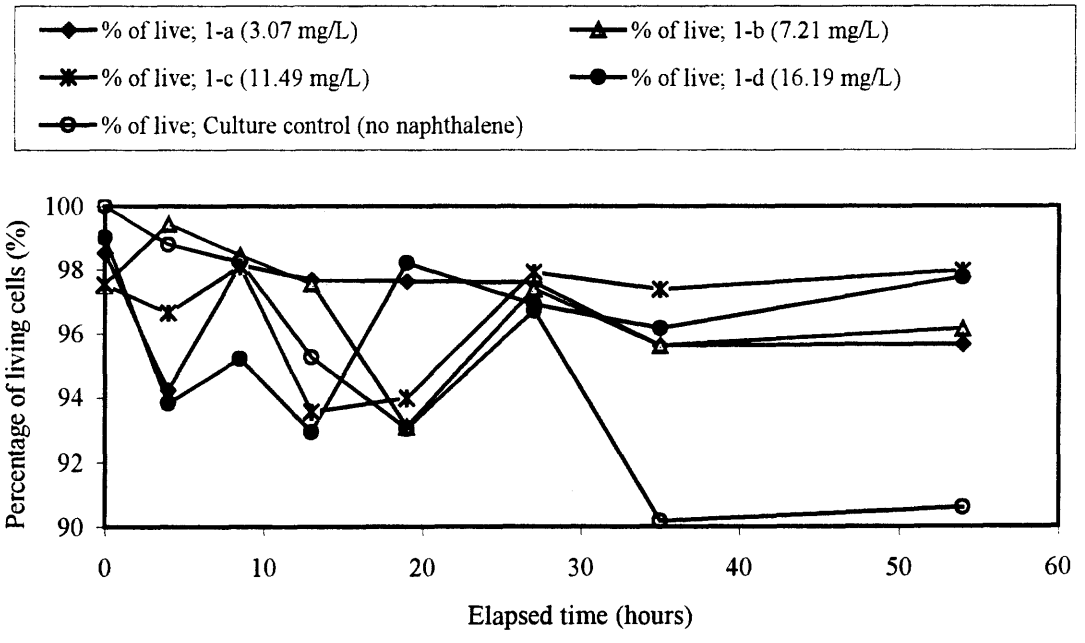


Figure 5.16 Viability of *Pseudomonas putida* (Experiment-1).

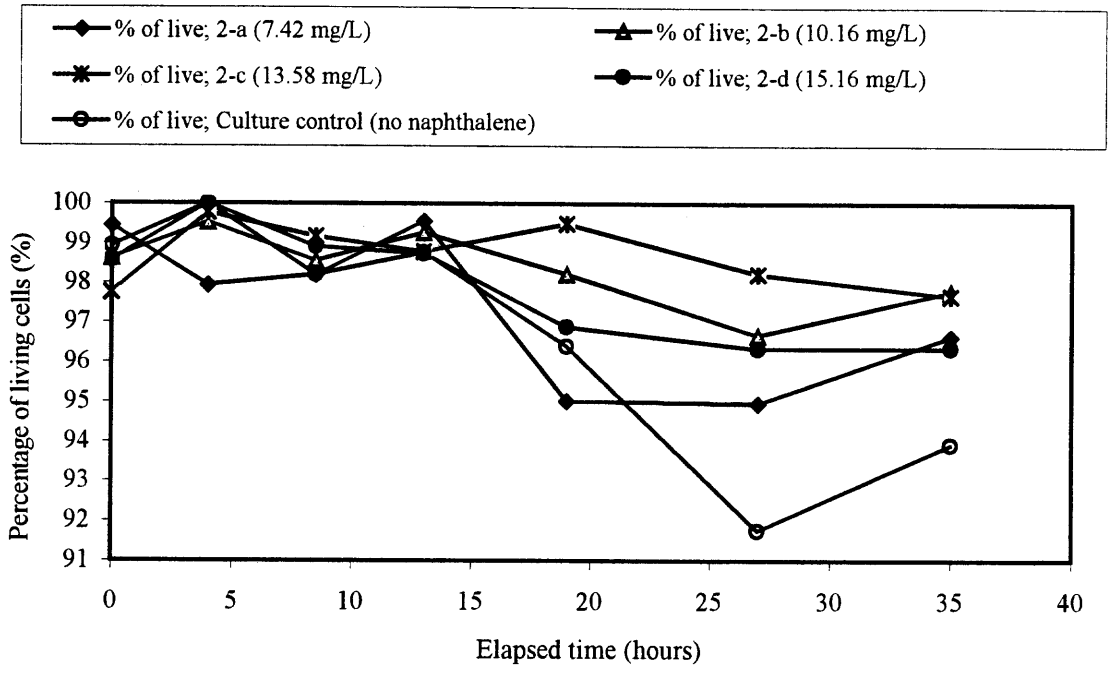


Figure 5.17 Viability of *Pseudomonas putida* (Experiment-2).

5.7 Biofilm Cultures in Continuous Flow Reactors

5.7.1 Cell Distribution in Seed Biofilm Container

A study was made of the cell distribution on the glass biofilm support in the seed container prior to running continuous flow experiments. 12 supports were exposed to a naphthalene concentration of 5.1 mg/L, and total cell numbers were determined for the bottom third, middle third, and upper third of 3 supports removed from the holder at periodic time intervals. Results are shown in Table 5.7 and Figure 5.18.

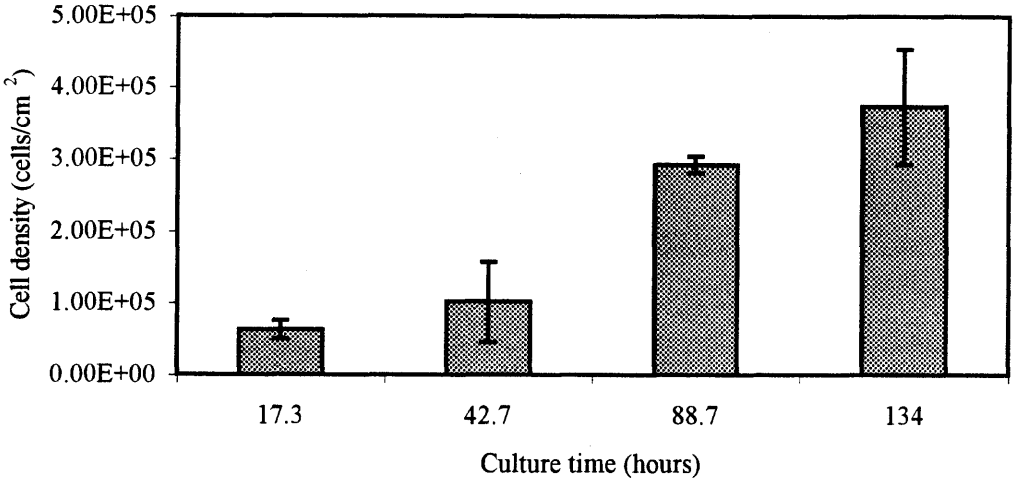


Figure 5.18 Cell distribution of initial seed biofilm on glass supports with 5.1 mg/L of naphthalene (each value is an average of 3 glass supports)

Table 5.7 Cell distribution (total cells/cm^2) on glass supports ($\times 10^4$).

Time (hours)		A glass support				Overall average	Standard deviation
		Upper	Middle	Bottom	Average		
17.3	Support-1	7.57	4.77	5.23	5.86	6.20	1.25
	Support-2	6.63	10.0	6.10	7.59		
	Support-3	8.20	3.83	3.47	5.17		
42.7	Support-1	9.40	6.47	11.4	9.08	10.1	5.55
	Support-2	12.1	19.7	16.5	16.1		
	Support-3	8.20	3.83	3.47	5.17		
88.7	Support-1	29.7	29.0	32.6	30.4	29.2	1.15
	Support-2	25.5	28.0	33.7	29.1		
	Support-3	36.4	21.3	26.7	28.1		
134	Support-1	28.3	38.9	44.9	37.4	37.4	8.03
	Support-2	23.9	23.7	40.3	29.3		
	Support-3	38.9	36.7	60.6	45.4		

5.7.2 Suspended Cells in Seed Biofilm Container

Suspended cells were evident by visual observation in the seed biofilm container. For the cell distribution experiments described above in Section 5.7.1, the suspended cell concentrations are shown in Table 5.8 and Figure 5.19.

Table 5.8 Suspended cells in seed biofilm container.

Time (hours)	Suspended cells/mL ($\times 10^7$)
17.3	1.17
42.7	1.13
88.7	1.90
134	2.45

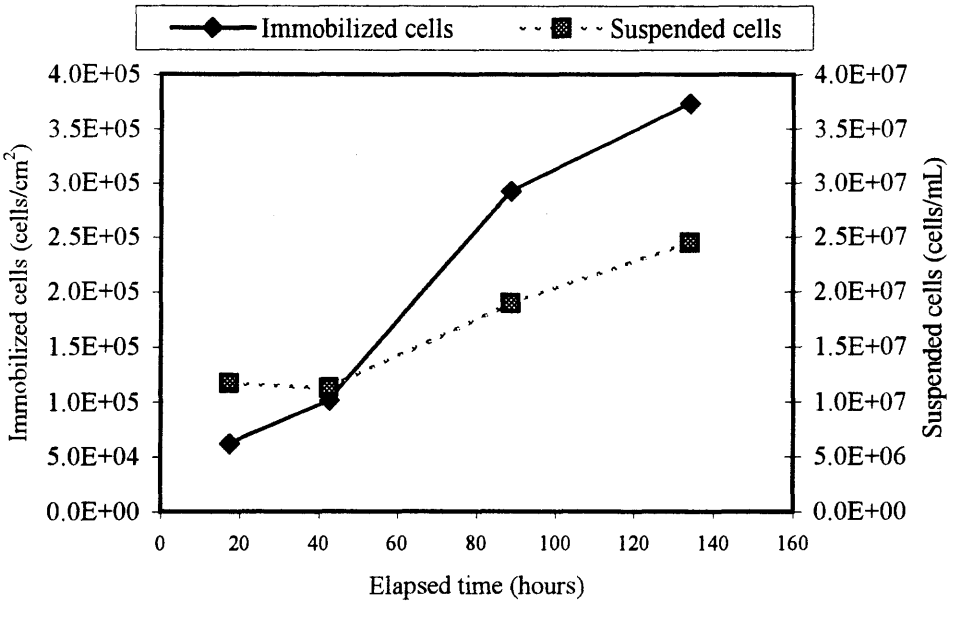


Figure 5.19 Growth comparison of immobilized cells/cm^2 and suspended cells/mL in a seed biofilm container with 5.1 mg/L of naphthalene initially.

From microscopic observation, it was found that the cell size became gradually smaller in both suspended and biofilm cultures due to the lack of carbon source after about 24 hours. Bacterial images during cell division were rarely viewed under the microscope. As shown in Table 5.9, the viability of suspended cells persisted even after more than 4 days in the absence of a carbon source (naphthalene).

Table 5.9 Viability of suspended cells of *Pseudomonas putida* in the seed biofilm container.

Time (hours)	cells/mL			Viability (%)
	Live cells	Dead cells	Total cells	
17.3	1.13E+07	4.03E+05	1.17E+07	97
42.7	1.13E+07	0.00E+00	1.13E+07	100
88.7	1.90E+07	0.00E+00	1.90E+07	100
134	2.45E+07	0.00E+00	2.45E+07	100

(5.1 mg/L of naphthalene initially in 9 L media; aerated at about 40 mL/min)

5.7.3 Biofilm Cultures on Glass Supports in Continuous Flow System

After initial seed biofilms were transferred into two column reactors in the continuous flow system as mentioned in Section 4.7.2, they were cultured on glass supports for 51.3 hours. Figure 5.20, Tables H-1 and H-2 in Appendix H show experimental data for growth of biofilms on glass supports, and difference of naphthalene concentration between the inlet and outlet for each column reactor (X and Y).

Initial biomass data were obtained from the seed biofilm container by averaging 3 supports as described in Section 5.7.1. Intermediate biomass measurements were

obtained by removing glass supports sequentially from column Y. Final biomass data were confirmed by averaging all supports (5) remaining in column-X.

After a glass support was taken out of column Y for a biofilm sample, about 19 mL of media took its place, which increased the residence time from 7.4 hours at the beginning of the run to 8.9 hours at the end. As shown in Figure 5.20, the growth pattern of total live cells in column-Y was similar to that in column-X. However, the naphthalene concentration difference between inlet and outlet in column-Y was greater by about 1 to 2 mg/L than in column-X, probably a result of the increased retention time in column-Y.

To distinguish live and dead cells, both direct and indirect staining methods were applied, as described in Section 4.5.2. Results for suspended and fixed cells are shown in Figure 5.21 and Appendix H. Approximately 90 to 100% of suspended cells were viable, and these data were applied to predict % live cells on the biofilm.

Both fixed and suspended cells are present in the continuous biofilm reactor. As a result, the observed decrease in naphthalene concentration is a consequence of the presence of both types of cells.

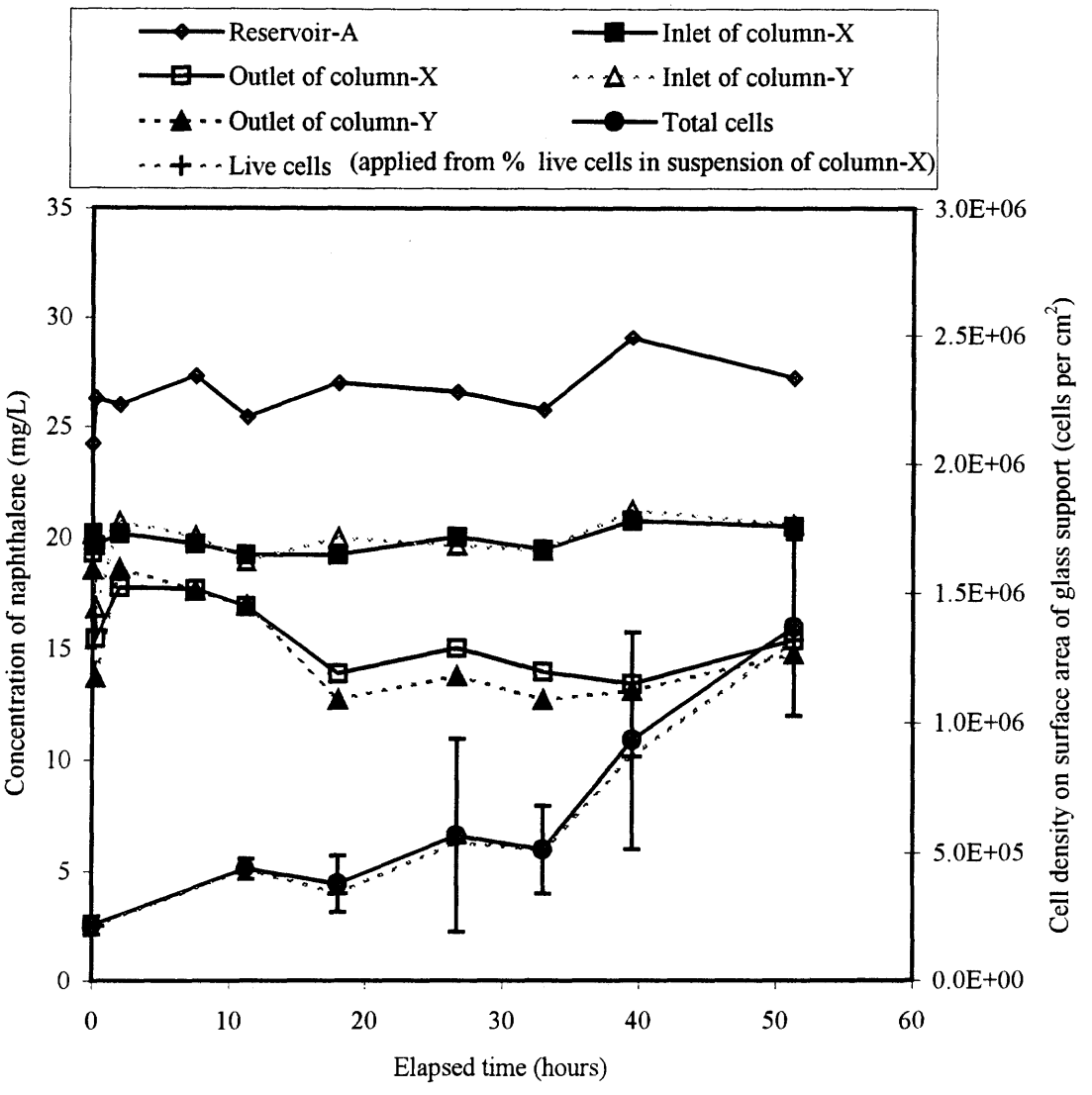


Figure 5.20 Biodegradation of naphthalene and growth of biofilm attached on glass supports in a continuous flow system.
(media flow rate = 1 mL/min., residence time with 5 glass supports = 7.3 hours)

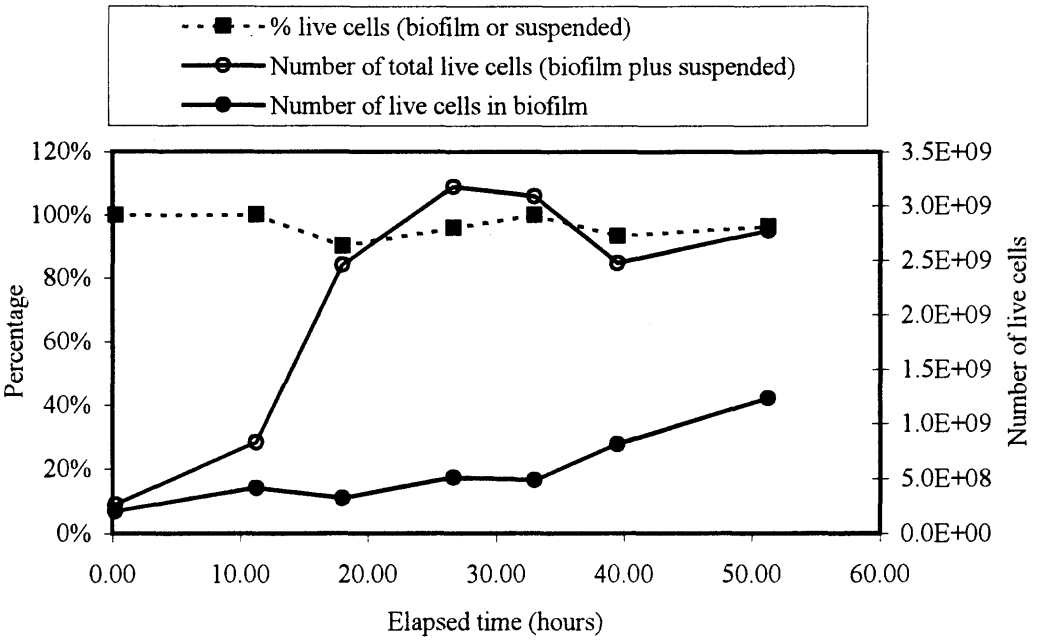


Figure 5.21 Viability of cells in column-X.

5.7.4 Determination of Specific Growth Rates in Continuous Flow System

In the continuous flow biofilm reactors, the steady-state material balance equation is as follows:

$$0 = QS_0 - QS + V \cdot r_s \qquad (5\text{-}12)$$

where, V = liquid media volume in column-X (441 mL)
Q = flow rate (60mL/hour)
S_0 = inlet concentration of naphthalene (mg/L)
S = outlet concentration of naphthalene (mg/L)
r_s = rate of naphthalene degradation $(= -\frac{dS}{dt})$

assuming a well-mixed reactor:

$$-\frac{dS}{dt} = \left(\frac{\mu_m \cdot S}{K_s + S}\right)\frac{B}{Y} = \frac{\mu \cdot B}{Y} \qquad (5\text{-}13)$$

Substituting for $-dS/dt$ and rearranging equation 5-12;

$$(S_0 - S) = (\frac{\mu_m \cdot S \cdot B}{K_s + S})\frac{\theta}{Y} = \mu_B \cdot \frac{B \cdot \theta}{Y} \qquad (5\text{-}14)$$

where, θ = feed residence time ($\frac{Q}{V}$ = 7.35 hours)

μ_B = specific growth rate in the continuous flow reactor (h^{-1})

B = total dry biomass (live cells in both biofilm and suspended growth on column-X; mg/L)

Total live cells (LCN_T, live cells/mL) in column-X are obtained by:

$$LCN_T = \frac{B_f \cdot A_f + B_s \cdot V}{V} \qquad (5\text{-}15)$$

where, B_f = live cells in biofilm (cells/cm^2)

A_f = total surface area of 5 glass supports in column-X (930.5 cm^2)

B_s = live cells in suspension of column-X (cells/mL)

The total live cells are then converted to dry biomass as presented in Table 5.2, using the overall factor (F_{LCN}) for experiments 1 and 2 combined. The average yield coefficient (Y = 0.96) obtained from Table 5.4 was used in equation 5-14, and the specific growth rates for the continuous flow reactor were determined as shown in Table 5.10.

An approximate calculation can be made of the biofilm specific growth rate as follows:

$$\frac{(S_0 - S_s) \cdot Y}{B_s \cdot \theta} = \frac{\mu_m \cdot S_s}{K_s + S_s} = \mu_s \qquad (5\text{-}16)$$

where, μ_s = specific growth rate for the suspended cells only

B_s = biomass for suspended cells only

S_s = what the outlet concentration of naphthalene would have been without the biofilm

$\mu_m = 0.291 h^{-1}$

K_s = 13.55 mg/L

Table 5.10 Biomass growth and naphthalene degradation in column-X of the continuous flow system.

Time (hours)	Concentration of naphthalene (mg/L)			Live cells/cm^2 in biofilm (B_f)	Live cells/mL in suspended growth (B_s)	Total dry biomass (B) (mg/L)
	Inlet (So)	Outlet (S)	Difference (So-S)			
0.3	19.63	15.45	4.17	2.16E+05	1.34E+05	2.01
11.3	19.23	16.90	2.34	4.39E+05	9.41E+05	2.41
18.0	19.22	13.87	5.36	3.42E+05	4.84E+06	3.55
26.7	20.04	15.02	5.02	5.43E+05	6.05E+06	4.06
33.0	19.45	13.96	5.50	5.13E+05	5.92E+06	4.00
39.5	20.77	13.39	7.38	8.70E+05	3.76E+06	3.56
51.3	20.52	15.37	5.15	1.32E+06	3.50E+06	3.77

For Y = 0.96, S_s = 16.5 mg/L, and μ_s = 0.16 h^{-1}

Then, $$\mu_f = \left[\frac{(S_0 - S) - (S_0 - S_s)}{B_f \cdot \theta}\right] \cdot Y \qquad (5\text{-}17)$$

where, μ_f = specific growth rate for the biofilm only
B_f = biomass in the biofilm only

For Y=0.96, and using average steady-state values for S_0 and S (after 18 hours), μ_f=0.25 h^{-1}

The overall specific growth rate is:

$$\mu_B = \frac{(S_0 - S) \cdot Y}{B \cdot \theta} = \frac{(5.26mg/L)(0.96)}{(3.79mg/L)(7.35h)} = 0.18h^{-1} \qquad (5\text{-}18)$$

Thus, for Y = 0.96, the biofilm specific growth rate appears to be somewhat greater than that of the suspended culture (for the same organism).

For Y = 0.20, S_s = 8.32 mg/L, μ_s = 0.11 h^{-1}, $\mu_B = \frac{(S_0 - S) \cdot Y}{B \cdot \theta} = 0.038h^{-1}$, but μ_f is negative. Therefore, as with prediction of the naphthalene and biomass profiles (incorporating a lag time), the overall yield coefficient (Y = 0.96) produces more consistent results.

5.7.5 Formation of Biofilms on Glass Supports in Continuous Flow System

Over a two-day period, biofilms developed from separate cells attached to the glass support, to large groups of cells approximating a continuous biofilm of monolayer thickness. Fluorescence photomicrographs in Figures 5.22 and 5.23 show general patterns of biofilm development according to elapsed time.

After 51 hours, it became difficult to count cells because of their high density. Thus, the last samples were usually roughly counted by observing 30 fields using only 20% (0.002 mm^2) of the square on the objective field (100×).

Figure 5.24 shows various patterns of cell growth on the glass supports. In photomicrographs (A) and (B), cells were dividing in curved and ring patterns at the beginning of biofilm growth. In (C) and (D), cells began growing like chains, while (E) and (F) show more common modes of cell accumulation on the glass supports.

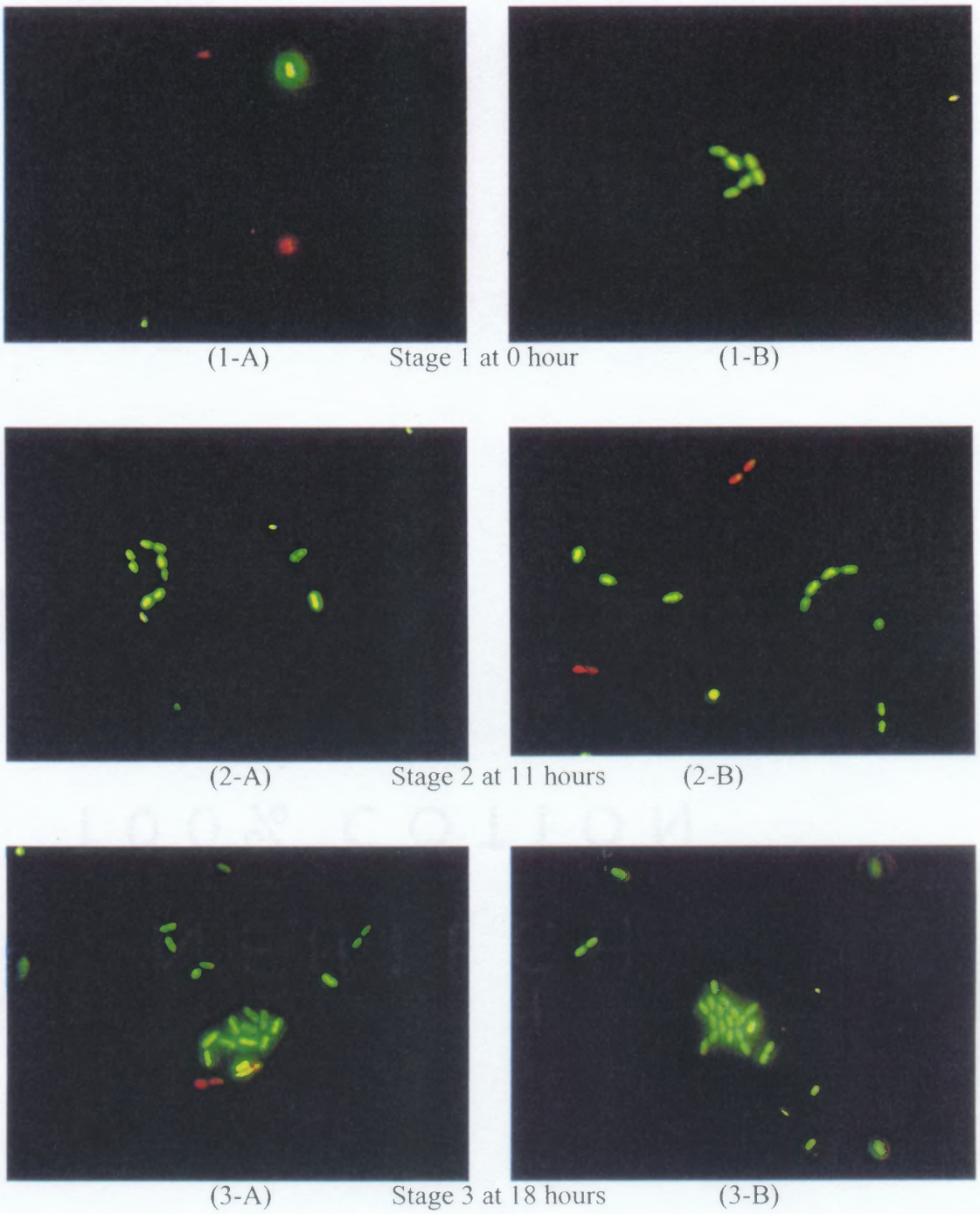


Figure 5.22 General patterns of biofilm development by culturing *Pseudomonas putida* on glass supports in a continuous flow reactor (low cell density). (All photomicrographs were obtained from different samples; observed by 100x objective.)

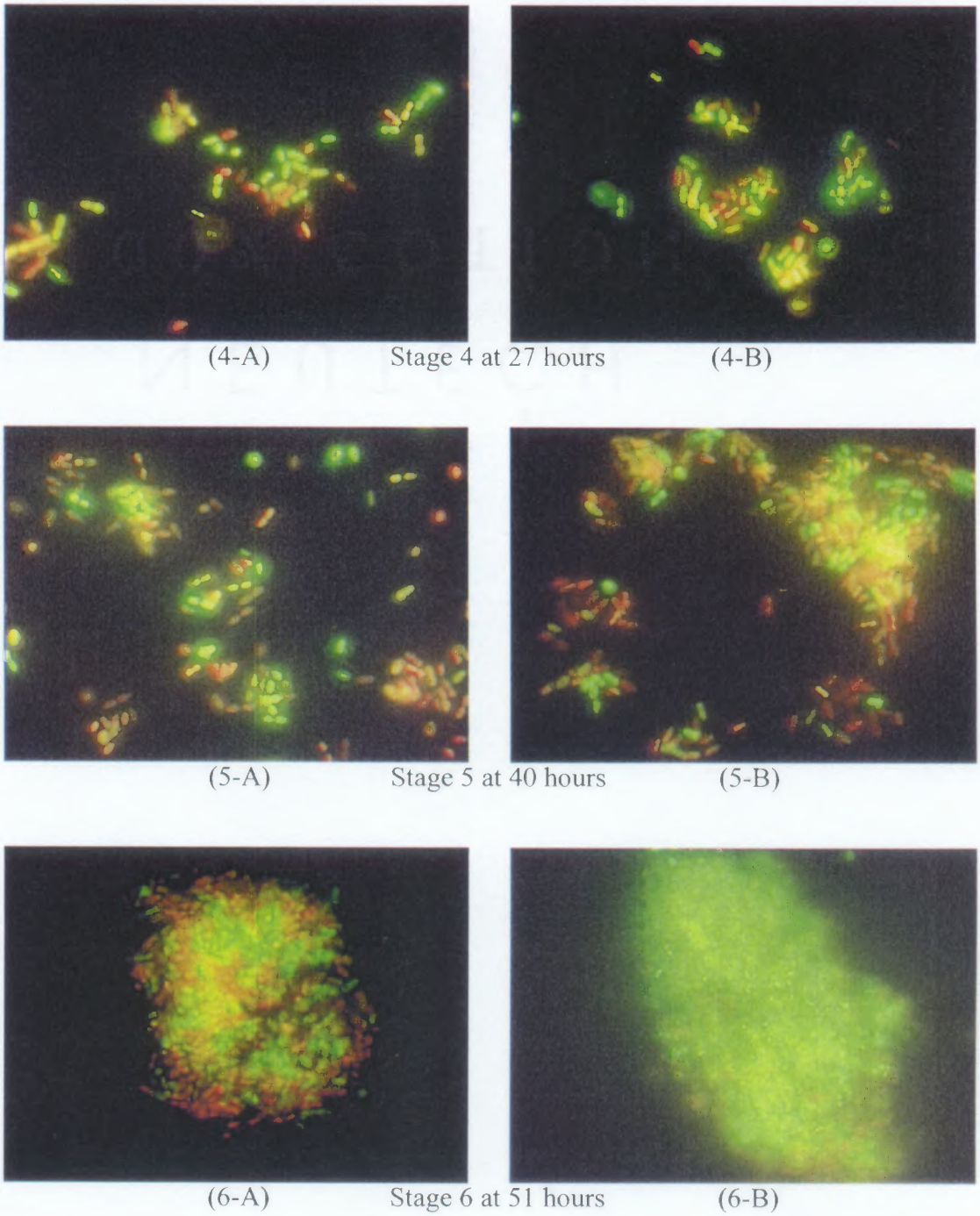
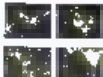
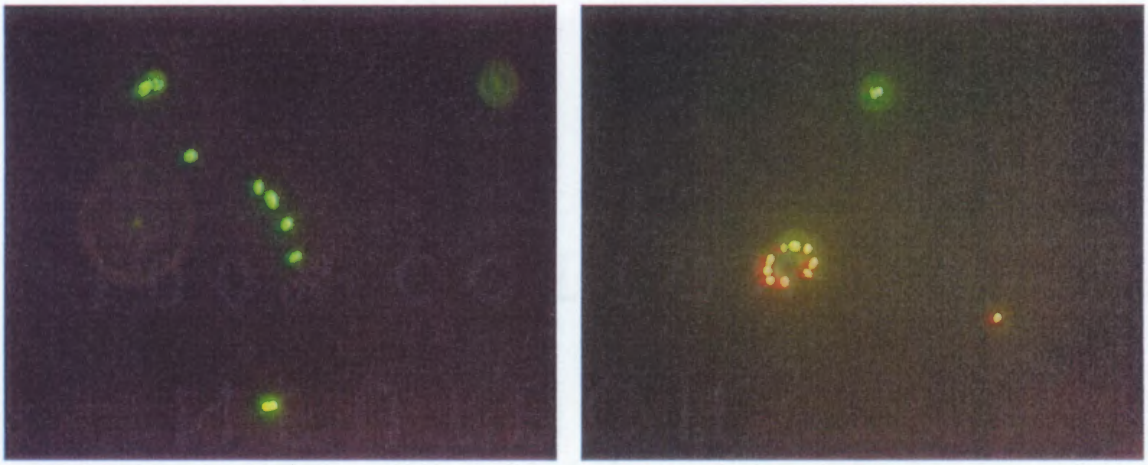
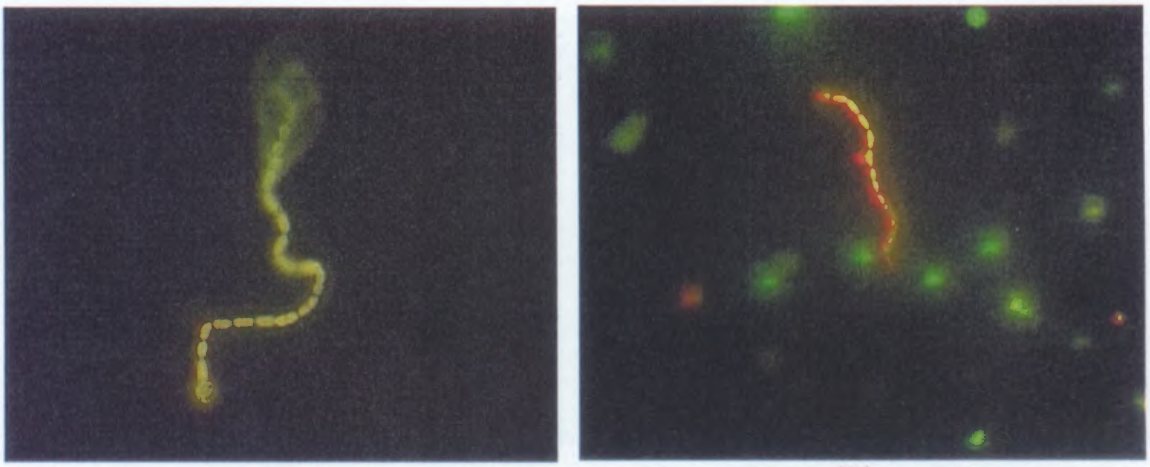


Figure 5.23 General patterns of biofilm development by culturing *Pseudomonas putida* on glass supports in a continuous flow reactor (higher cell density). (All photomicrographs were obtained from different samples; observed by 100x objective; Many live cells show orange fluorescence due to the difficulty of direct staining method.)

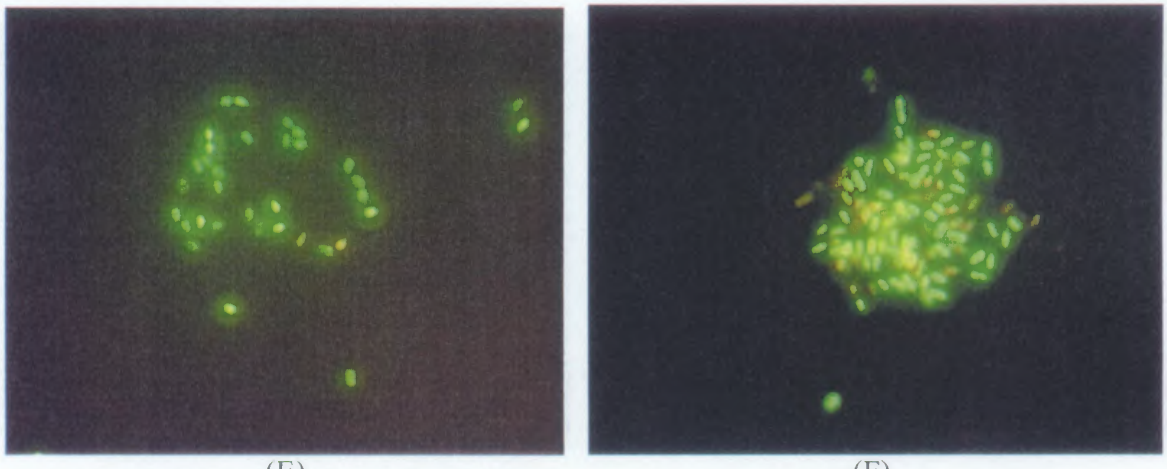




(A) (B)



(C) (D)



(E) (F)

Figure 5.24 Various patterns in biofilm growth on glass supports in a continuous flow system. (observed by 100x objective; Some live cells show orange fluorescence due to the difficulty of direct staining method.)

CHAPTER 6

CONCLUSIONS

6.1 Fluorescent Staining Technique

A satisfactory methodology was developed to distinguish live cells (with intact membranes) from dead cells using *Pseudomonas putida* (ATCC 17484). The methodology was a modification of the techniques originally developed by Molecular Probes, Inc. Use of a slide glass and cover slip under wet conditions using 4 μl of sample volume considerably improved the ability of the method to enumerate cells and distinguish between green fluorescence (living cells) and red fluorescence (dead or damaged cells). By avoiding dry conditions, precipitated salts from the nutrient medium could be avoided, as well as pre-washing steps. Thus, it was possible to protect fresh bacterial samples from damage by the pre-treatment steps, and the staining method was simpler and more rapid

6.2 Comparison of Biomass Measures

Results for biomass measures (OD, LCN, and TCP), and their impact on calculated values of the Monod expression, were similar. Biomass, by whatever the measure, continued to increase significantly after the complete disappearance of naphthalene. This resulted in uncertainty regarding determination of the yield coefficient, and its subsequent impact on calculated values of substrate and biomass concentrations. This problem was reconciled by inclusion of a lag time into the Monod expression. The lag time between biomass growth and naphthalene disappearance could be a result of two factors: (1) adsorption and subsequent release of naphthalene from reactor surfaces, and (2)

formation of detected (but as yet unidentified) intermediate products during biodegradation. Consequently, future work should focus on the exposition of a more detailed biochemical pathway and its attendant kinetics.

6.3 Suspended vs. Immobilized Cells

Not surprisingly, suspended cells were easier to study than immobilized cells. With biofilms, a problem remains of cell layering, with the resulting inability to count or otherwise determine the number of cells below the upper surface in a non-destructive manner. In this study, total cells could be counted in a surface monolayer using fluorescence staining, but it was difficult to distinguish live from dead cells in the biofilms because it was not possible to mix the fluorescent dye with the cells, and this resulted in an inconsistent response.

The apparent specific growth rate for the biofilm appears to be greater than for the suspended cells in the continuous flow reactor.

6.4 Monod Parameters for Biodegradation of Naphthalene Using *Pseudomonas putida* (ATCC 17484) in Suspended Culture

Table 6.1 Summary of Monod parameters.

	OD	LCN	TCP	Overall	Previous literature[1]
μ_m (h^{-1})	0.412	0.264	0.261	0.291	0.067
K_s (mg/L)	24.33	14.83	5.83	13.55	3.4
Y (mg/L per mg/L)	0.24[a] 1.05[b]	0.10a 0.93[b]	0.30[a] 0.95[b]	0.20[a] 0.96[b]	0.25

a: $(\Delta B/\Delta S)$ using initial slope, b: average of $\Delta B_{final}/S_0$

(1) Ghoshal and Luthy(1998), using a mixed culture from a contaminated site

APPENDIX A

CALIBRATION CURVES

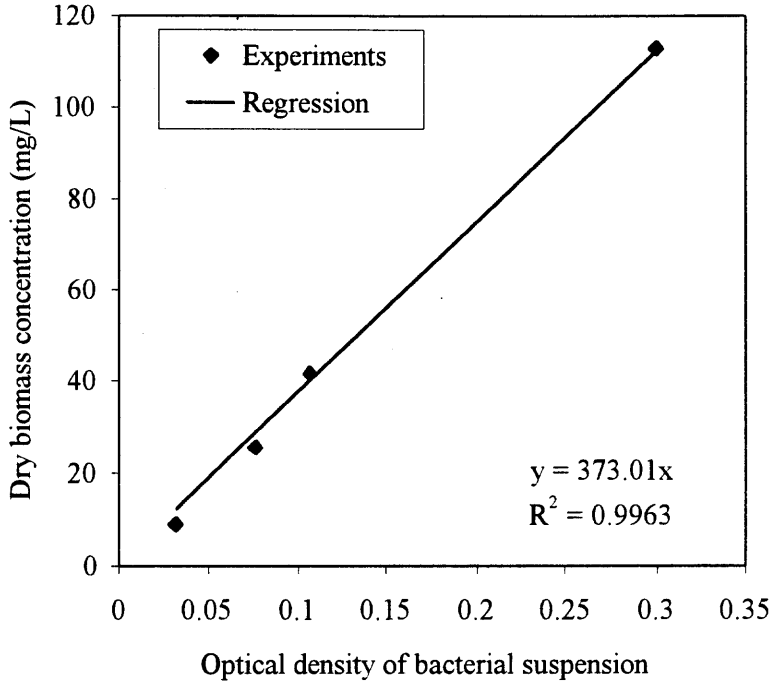


Figure A-1 Calibration curve for determining biomass concentration from optical density by culturing *Pseudomonas putida* with naphthalene.

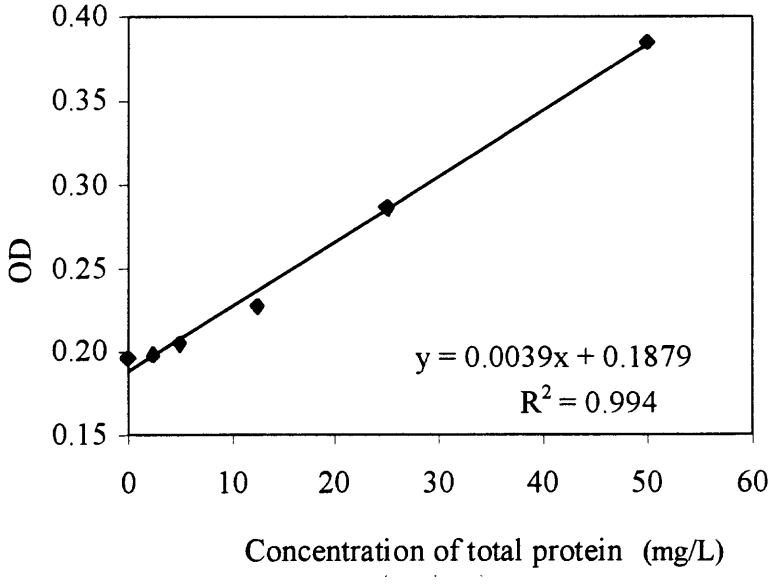


Figure A-2 Standard calibration curve of total protein with bovine serum albumin. (Enhanced protocol of BCA protein assay; incubation at about 75 ° C for 30 min)

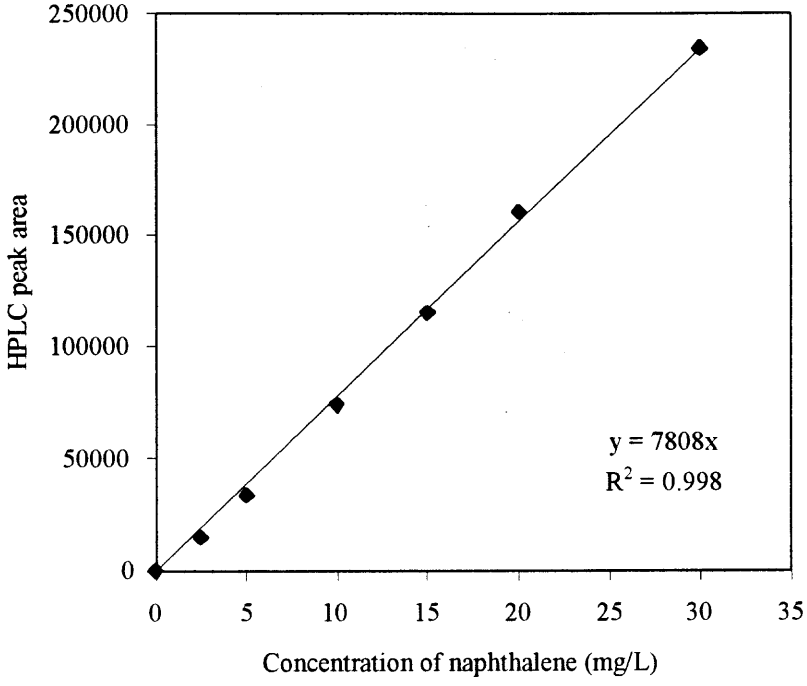


Figure A-3 Calibration curve for naphthalene concentration measurements.

APPENDIX B

ADSORPTION OF NAPHTHALENE ON FILTRATION FOR PRE-TREATMENT OF HPLC SAMPLES

Table B-1 Adsorption of naphthalene on a membrane with a Swinney filter holder for pre-treatment of HPLC samples. (13 mm in diameter and 0.22 μm in pore size; sample volume: about 1.2 mL)

Concentration of naphthalene (mg/L)		Loss of naphthalene (%)
Original solution	Filtered solution	
2.6	1.6	37.14
8.2	4.7	43.20
10.0	6.1	39.11
11.7	7.7	34.16
14.0	9.4	32.80
13.7	9.8	28.78
16.8	10.2	39.32
18.7	14.7	21.62
21.7	10.9	49.79

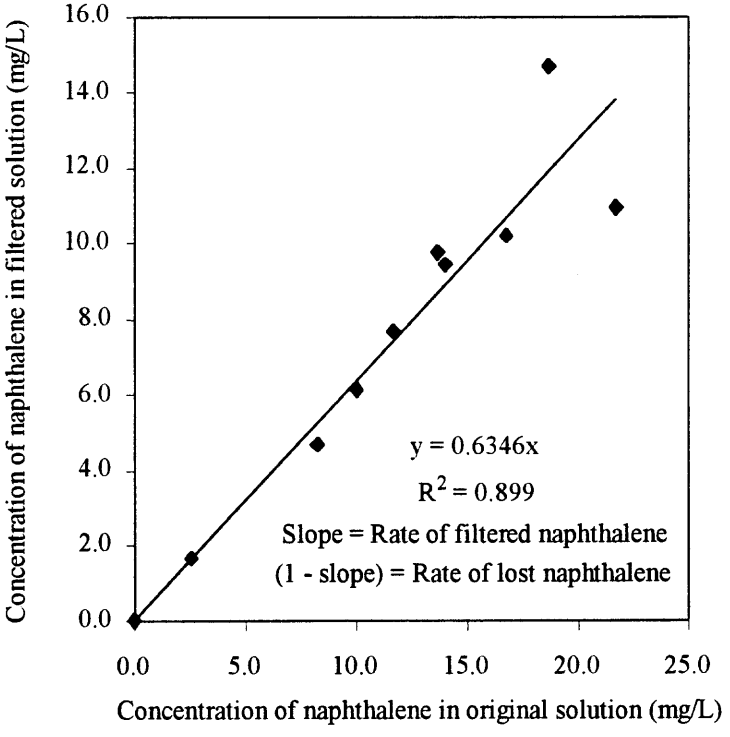
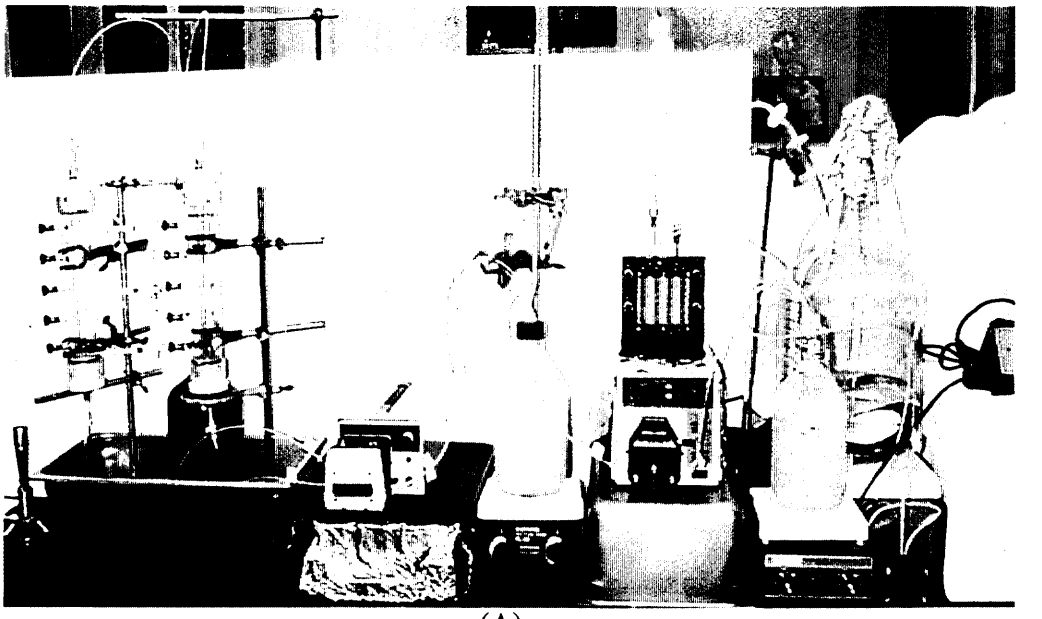


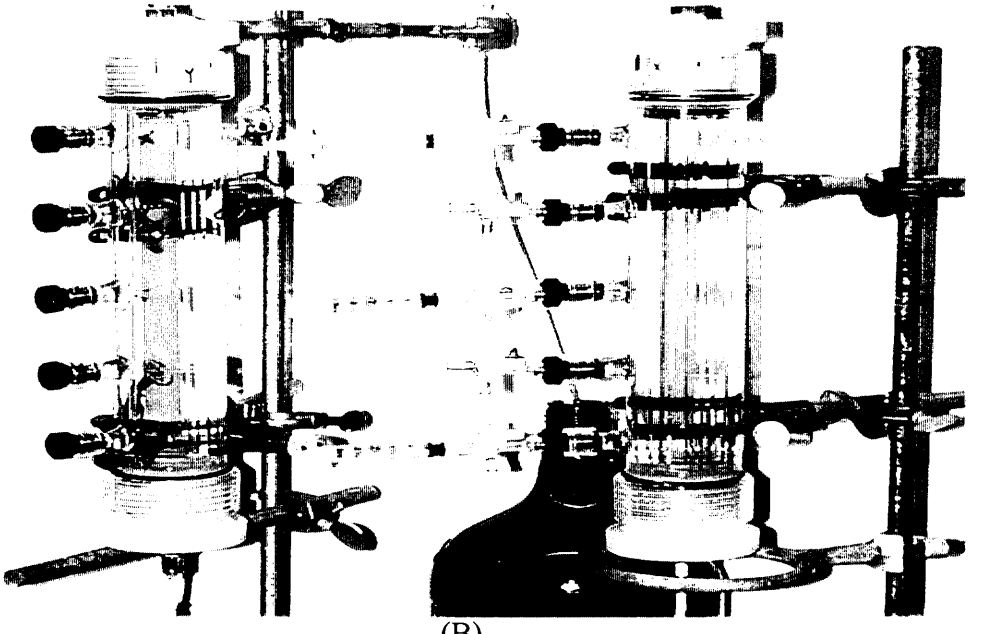
Figure B-1 Adsorption of naphthalene on a membrane with a Swinney filter holder for pre-treatment of HPLC samples.

APPENDIX C

EXPERIMENTAL SET-UP OF CONTINUOUS FLOW REACTORS FOR BIOFILM CULTURES



(A)



(B)

Figure C Experimental set-up of continuous flow reactors for biofilm cultures: (A) experimental set-up and (B) column details.

APPENDIX D

TABLES AND FIGURES OF EXPERIMENTAL RESULTS OBTAINED FROM SUSPENDED CULTURES OF *Pseudomonas putida*

Table D-1 Experimental data obtained from shake flask experiment (1-a).

Time (hours)	Naphthalene concentration (mg/L)	Total cell protein (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
			Live	Dead	Total		
0	3.07	0.48	1.06E+07	1.59E+05	1.08E+07	0.013	4.66
4	0	0.69	1.10E+07	6.75E+05	1.16E+07	0.016	5.78
8.5	0	1.16	2.23E+07	4.21E+05	2.27E+07	0.017	6.34
13	0	1.55	2.32E+07	5.56E+05	2.37E+07	0.020	7.46
19	0	2.15	1.63E+07	3.97E+05	1.67E+07	0.020	7.46
27	0	2.02	2.10E+07	5.21E+05	2.16E+07	0.021	7.65
35	0	1.80	2.17E+07	9.99E+05	2.27E+07	0.023	8.39
54	0	1.63	1.77E+07	8.09E+05	1.85E+07	0.022	8.21

Table D-2 Experimental data obtained from shake flask experiment (1-b).

Time (hours)	Naphthalene concentration (mg/L)	Total cell protein (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
			Live	Dead	Total		
0	7.21	1.03	9.33E+06	2.38E+05	9.57E+06	0.014	5.04
4	0	3.26	1.39E+07	7.94E+04	1.39E+07	0.021	7.65
8.5	0	3.77	3.54E+07	5.56E+05	3.59E+07	0.028	10.26
13	0	3.68	3.41E+07	8.60E+05	3.50E+07	0.033	12.31
19	0	4.71	2.73E+07	2.03E+06	2.93E+07	0.032	11.94
27	0	4.41	2.71E+07	7.25E+05	2.78E+07	0.034	12.68
35	0	4.37	4.61E+07	2.12E+06	4.82E+07	0.035	13.06
54	0	5.39	3.61E+07	1.46E+06	3.75E+07	0.036	13.24

Table D-3 Experimental data obtained from shake flask experiment (1-c).

Time (hours)	Naphthalene concentration (mg/L)	Total cell protein (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
			Live	Dead	Total		
0	11.49	1.889	8.83E+06	2.24E+05	9.05E+06	0.011	4.10
4	1.05	3.940	1.03E+07	3.57E+05	1.06E+07	0.019	7.09
8.5	0	7.103	3.47E+07	6.75E+05	3.53E+07	0.040	14.92
13	0	6.248	4.45E+07	3.08E+06	4.76E+07	0.046	17.16
19	0	6.590	4.37E+07	2.80E+06	4.65E+07	0.043	16.04
27	0	6.932	5.21E+07	1.11E+06	5.32E+07	0.044	16.41
35	0		4.27E+07	1.15E+06	4.39E+07	0.048	17.90
54	0		3.93E+07	8.23E+05	4.01E+07	0.050	18.65

Table D-4 Experimental data obtained from shake flask experiment (1-d).

Time (hours)	Naphthalene concentration (mg/L)	Total cell protein (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
			Live	Dead	Total		
0	16.19	5.82	7.98E+06	7.94E+04	8.06E+06	0.011	4.10
4	7.53	6.25	6.03E+06	3.97E+05	6.43E+06	0.015	5.41
8.5	0	15.61	2.67E+07	1.35E+06	2.81E+07	0.048	17.72
13	0	12.96	4.94E+07	3.77E+06	5.32E+07	0.054	20.14
19	0	12.57	3.28E+07	5.97E+05	3.34E+07	0.052	19.21
27	0	11.85	3.61E+07	1.15E+06	3.73E+07	0.053	19.77
35	0	12.10	4.77E+07	1.92E+06	4.96E+07	0.056	20.70
54	0	13.21	5.22E+07	1.20E+06	5.34E+07	0.055	20.33

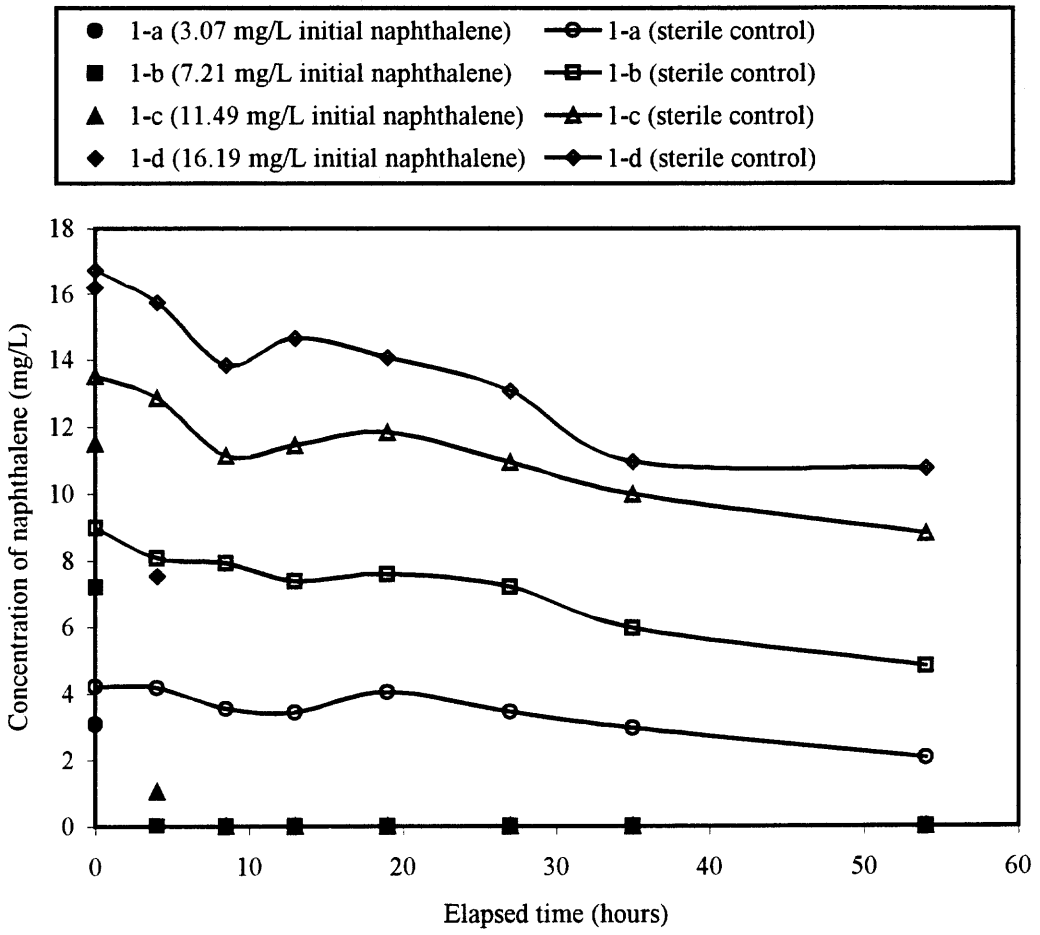


Figure D-1 Biodegradation of naphthalene by *Pseudomonas putida* in shake flasks (Experiment-1).

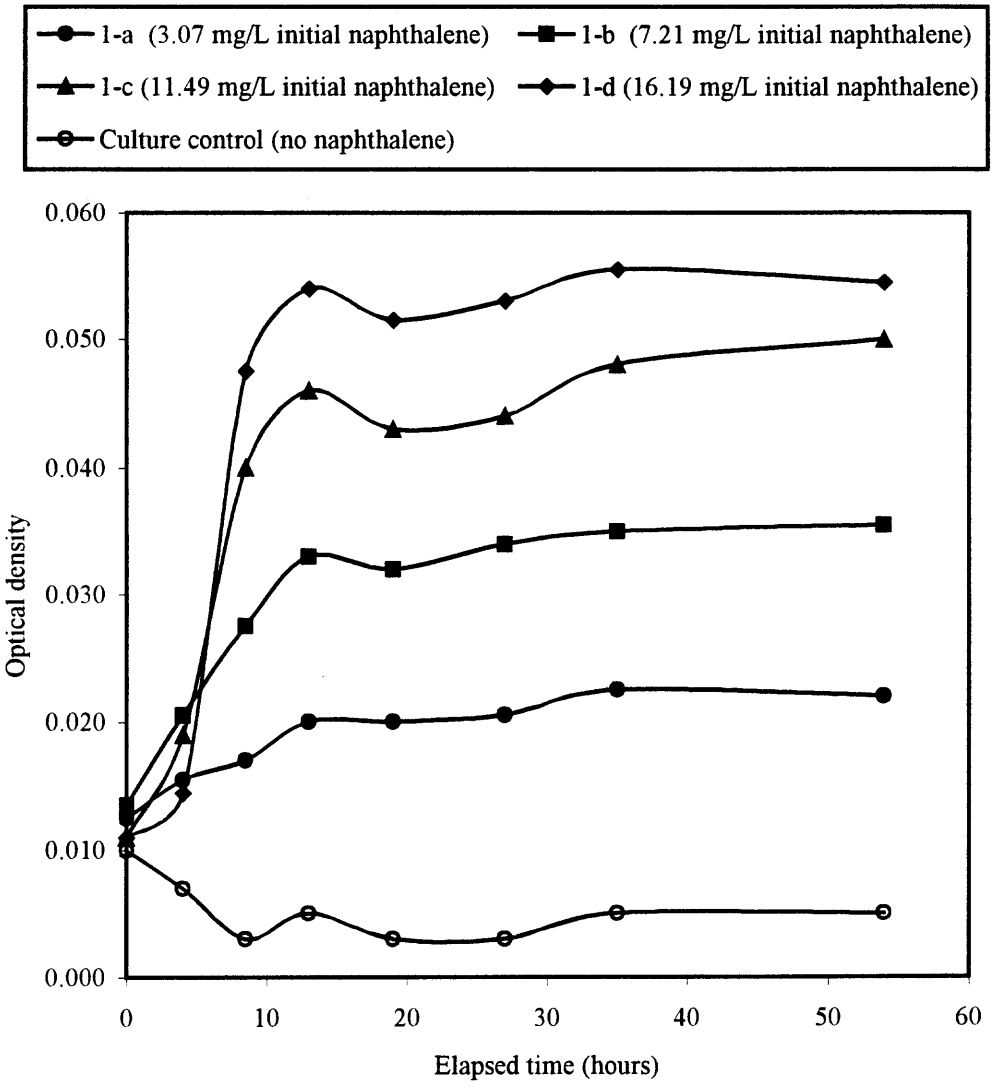


Figure D-2 Optical density during naphthalene degradation in shake flasks using *Pseudomonas putida* (Experiment-1).

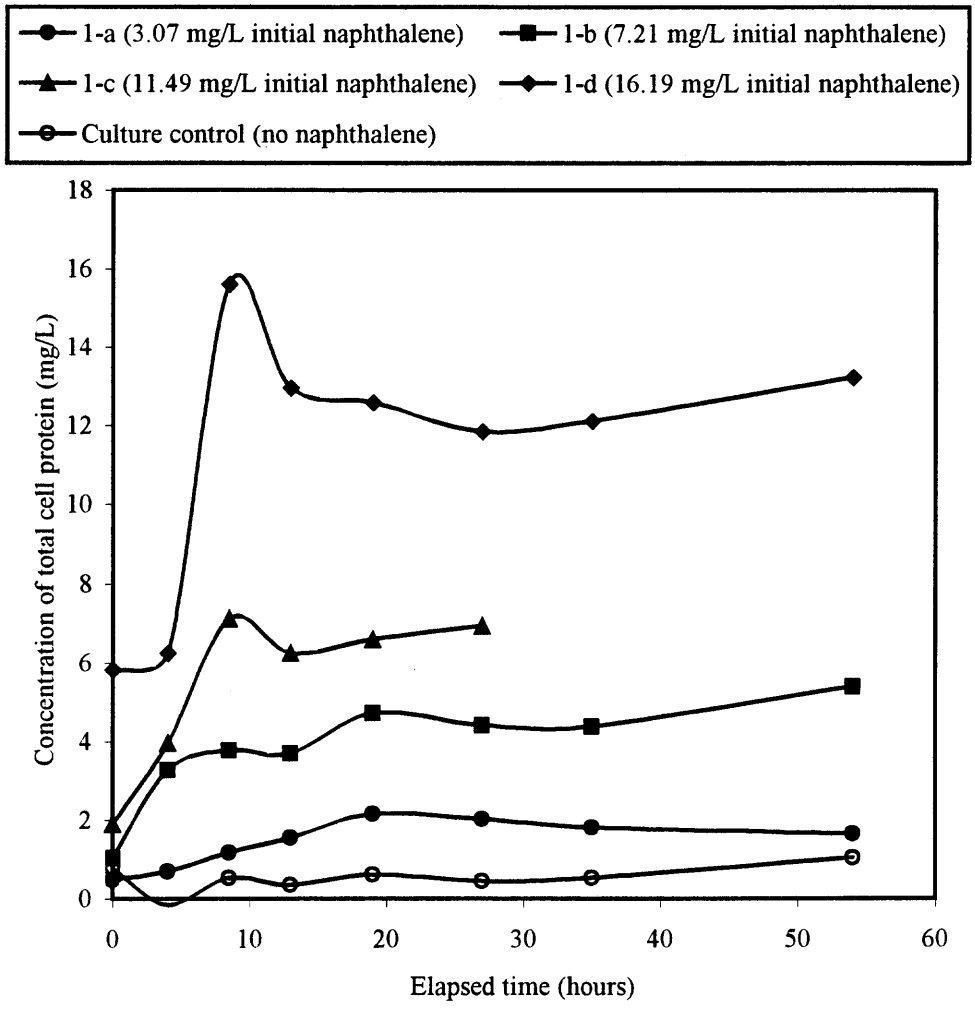


Figure D-3 Total cell protein during naphthalene degradation in shake flasks using *Pseudomonas putida* (Experiment-1).

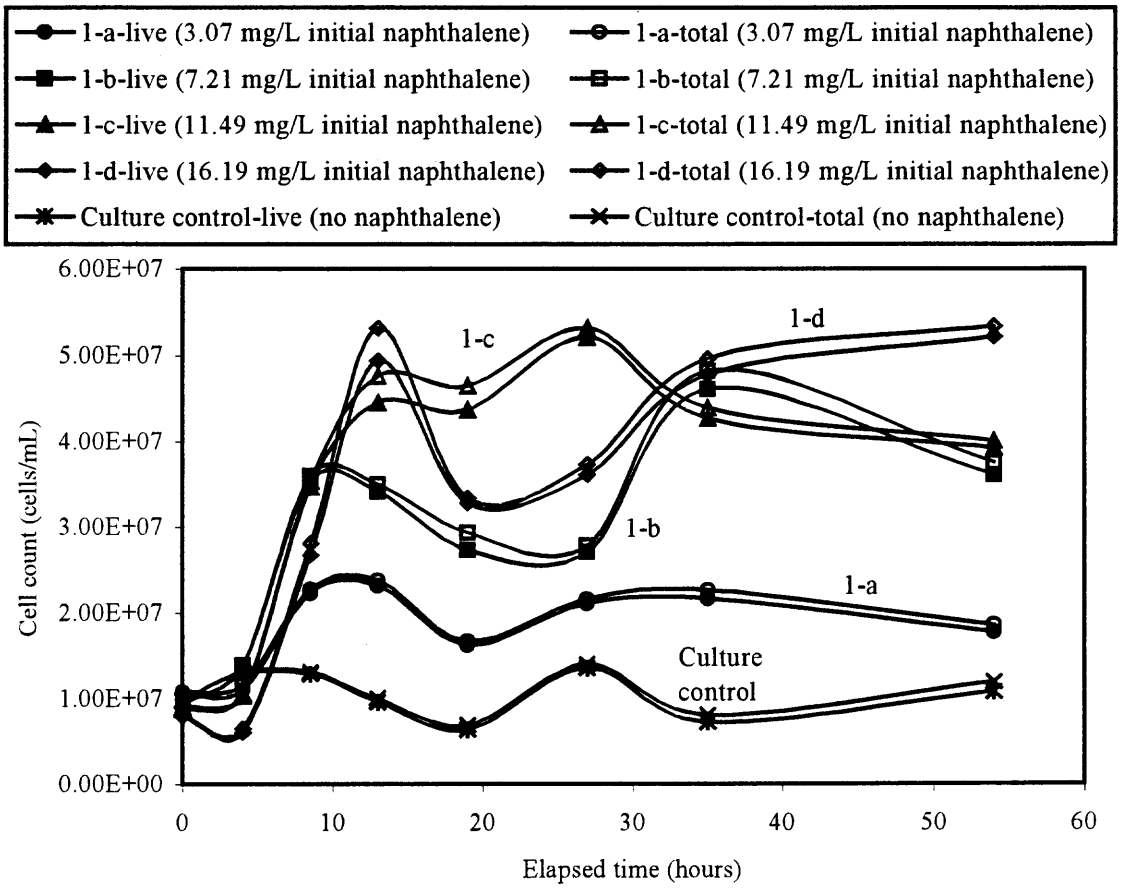


Figure D-4 Enumeration of total and live cells during naphthalene degradation in shake flasks using *Pseudomonas putida* (Experiment-1).

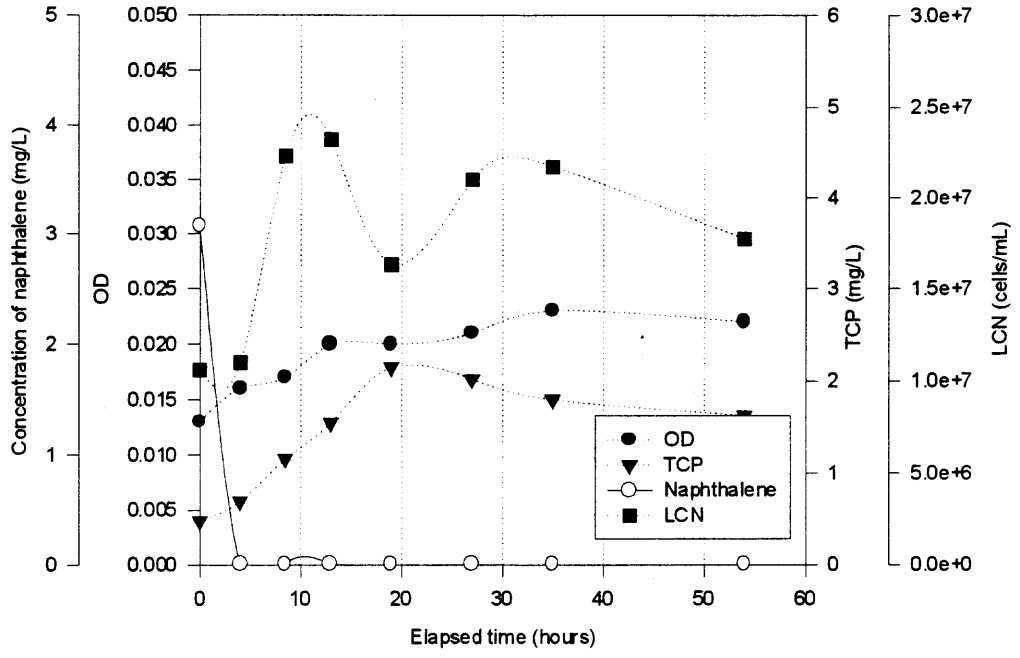


Figure D-5 Growth patterns of *Pseudomonas putida* in OD, TCP and LCN due to biodegradation of naphthalene (initial=3.07 mg/L); Experiment 1-a.

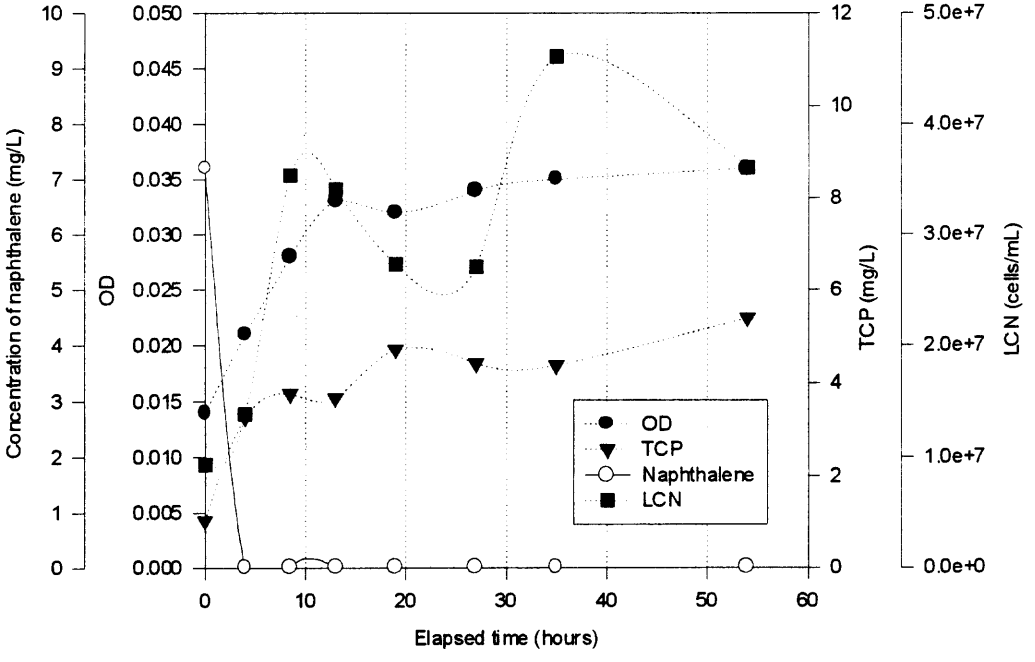


Figure D-6 Growth patterns of *Pseudomonas putida* in OD, TCP and LCN due to biodegradation of naphthalene (initial=7.21 mg/L); Experiment 1-b.

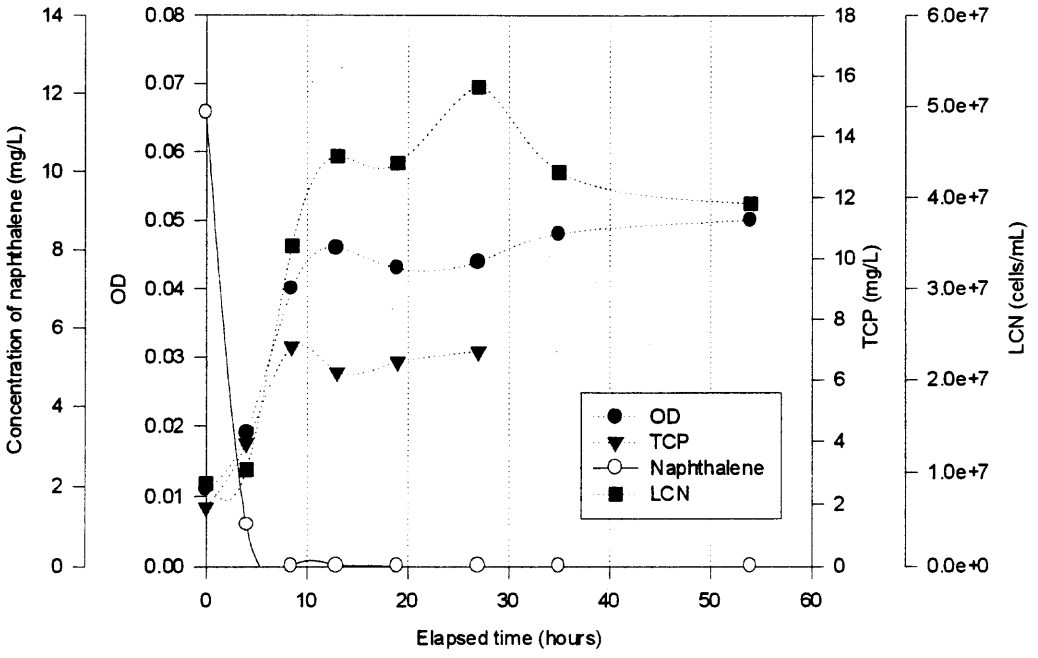


Figure D-7 Growth patterns of *Pseudomonas putida* in OD, TCP and LCN due to biodegradation of naphthalene (initial=11.49 mg/L); Experiment 1-c.

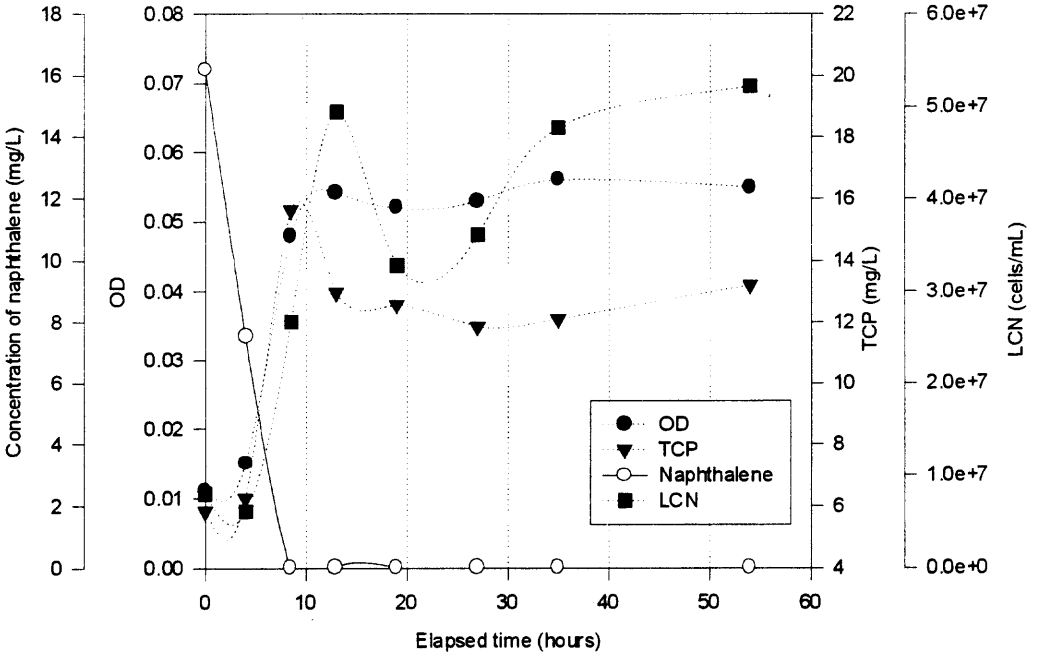


Figure D-8 Growth patterns of *Pseudomonas putida* in OD, TCP and LCN due to biodegradation of naphthalene (initial=16.19 mg/L); Experiment 1-d.

Table D-3 Experimental data obtained from shake flask experiment (1-c).

Time (hours)	Naphthalene concentration (mg/L)	Total cell protein (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
			Live	Dead	Total		
0	11.49	1.889	8.83E+06	2.24E+05	9.05E+06	0.011	4.10
4	1.05	3.940	1.03E+07	3.57E+05	1.06E+07	0.019	7.09
8.5	0	7.103	3.47E+07	6.75E+05	3.53E+07	0.040	14.92
13	0	6.248	4.45E+07	3.08E+06	4.76E+07	0.046	17.16
19	0	6.590	4.37E+07	2.80E+06	4.65E+07	0.043	16.04
27	0	6.932	5.21E+07	1.11E+06	5.32E+07	0.044	16.41
35	0		4.27E+07	1.15E+06	4.39E+07	0.048	17.90
54	0		3.93E+07	8.23E+05	4.01E+07	0.050	18.65

Table D-4 Experimental data obtained from shake flask experiment (1-d).

Time (hours)	Naphthalene concentration (mg/L)	Total cell protein (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
			Live	Dead	Total		
0	16.19	5.82	7.98E+06	7.94E+04	8.06E+06	0.011	4.10
4	7.53	6.25	6.03E+06	3.97E+05	6.43E+06	0.015	5.41
8.5	0	15.61	2.67E+07	1.35E+06	2.81E+07	0.048	17.72
13	0	12.96	4.94E+07	3.77E+06	5.32E+07	0.054	20.14
19	0	12.57	3.28E+07	5.97E+05	3.34E+07	0.052	19.21
27	0	11.85	3.61E+07	1.15E+06	3.73E+07	0.053	19.77
35	0	12.10	4.77E+07	1.92E+06	4.96E+07	0.056	20.70
54	0	13.21	5.22E+07	1.20E+06	5.34E+07	0.055	20.33

Table D-5 Experimental data obtained from shake flask experiment (2-a).

Time (hours)	Naphthalene concentration (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
		Live	Dead	Total		
0	6.23	1.36E+07	7.68E+04	1.36E+07	0.007	2.42
4	0	1.82E+07	3.84E+05	1.86E+07	0.016	5.97
8.5	0	2.13E+07	3.94E+05	2.17E+07	0.021	7.65
13	0	2.40E+07	1.08E+05	2.41E+07	0.025	9.33
19	0	1.96E+07	1.03E+06	2.06E+07	0.023	8.58
27	0	2.38E+07	1.27E+06	2.51E+07	0.023	8.58
35	0	3.32E+07	1.15E+06	3.43E+07	0.024	8.77

Table D-6 Experimental data obtained from shake flask experiment (2-b).

Time (hours)	Naphthalene concentration (mg/L)	Number of cells/mL			Optical density	Dry biomass (= OD x 373.01) (mg/L)
		Live	Dead	Total		
0	9.54	1.37E+07	1.92E+05	1.39E+07	0.007	2.61
4	2.43	1.58E+07	7.68E+04	1.59E+07	0.012	4.48
8.5	0	3.04E+07	4.50E+05	3.08E+07	0.030	11.00
13	0	3.97E+07	2.96E+05	4.00E+07	0.035	12.87
19	0	3.62E+07	6.62E+05	3.69E+07	0.032	11.94
27	0	4.01E+07	1.38E+06	4.15E+07	0.033	12.12
35	0	5.09E+07	1.16E+06	5.21E+07	0.033	12.12

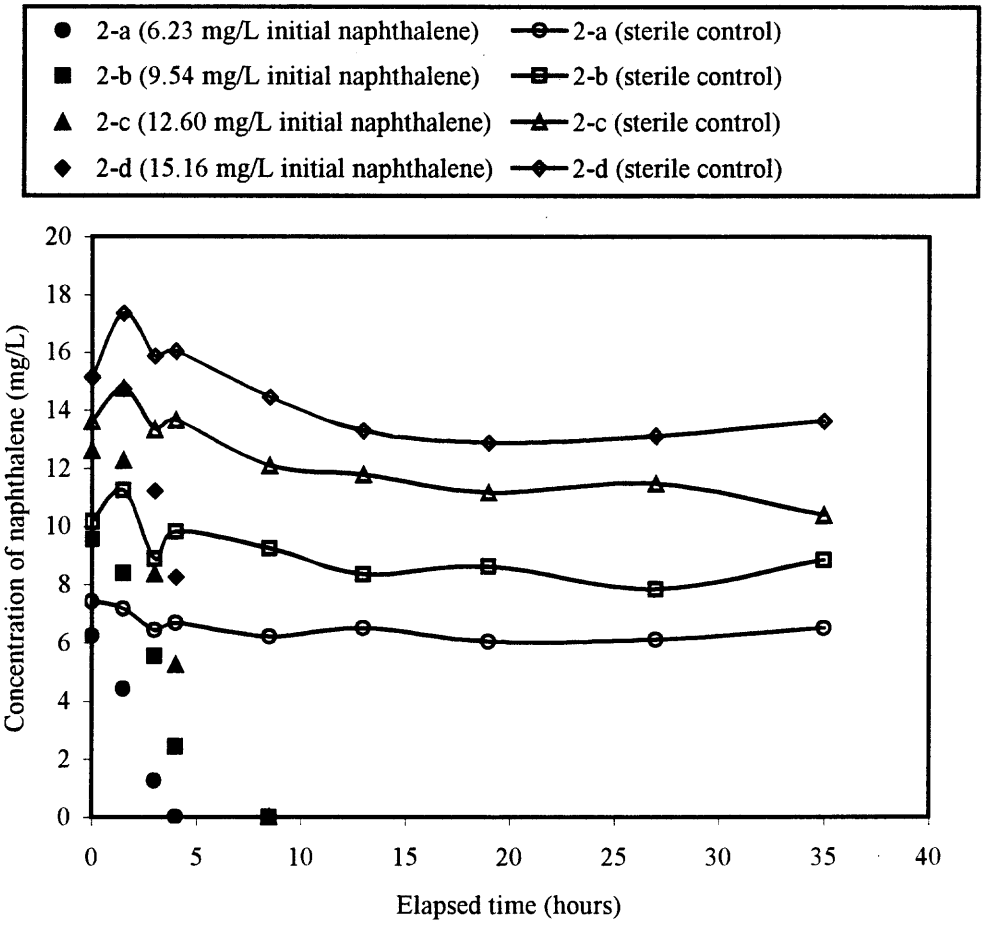


Figure D-9 Biodegradation of naphthalene by *Pseudomonas putida* in shake flasks (Experiment-2).

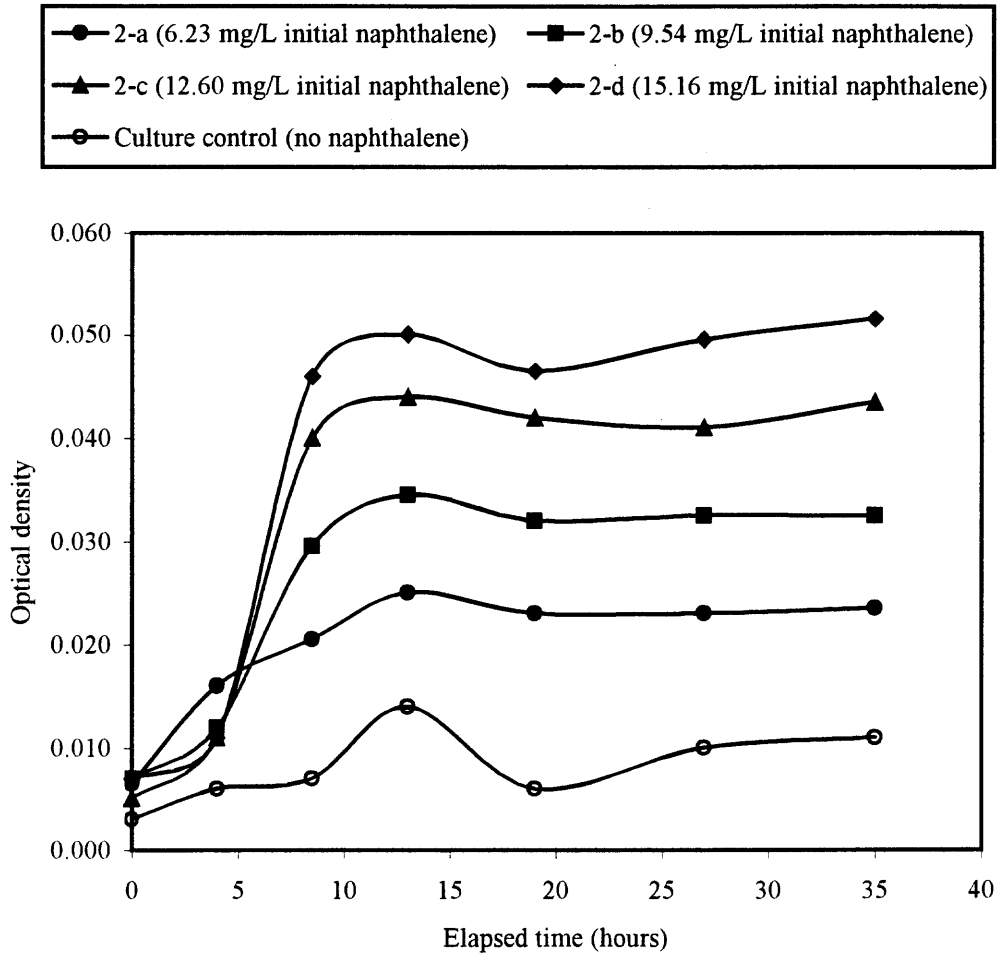


Figure D-10 Optical density during naphthalene in shake flasks using *Pseudomonas putida* (Experiment-2).

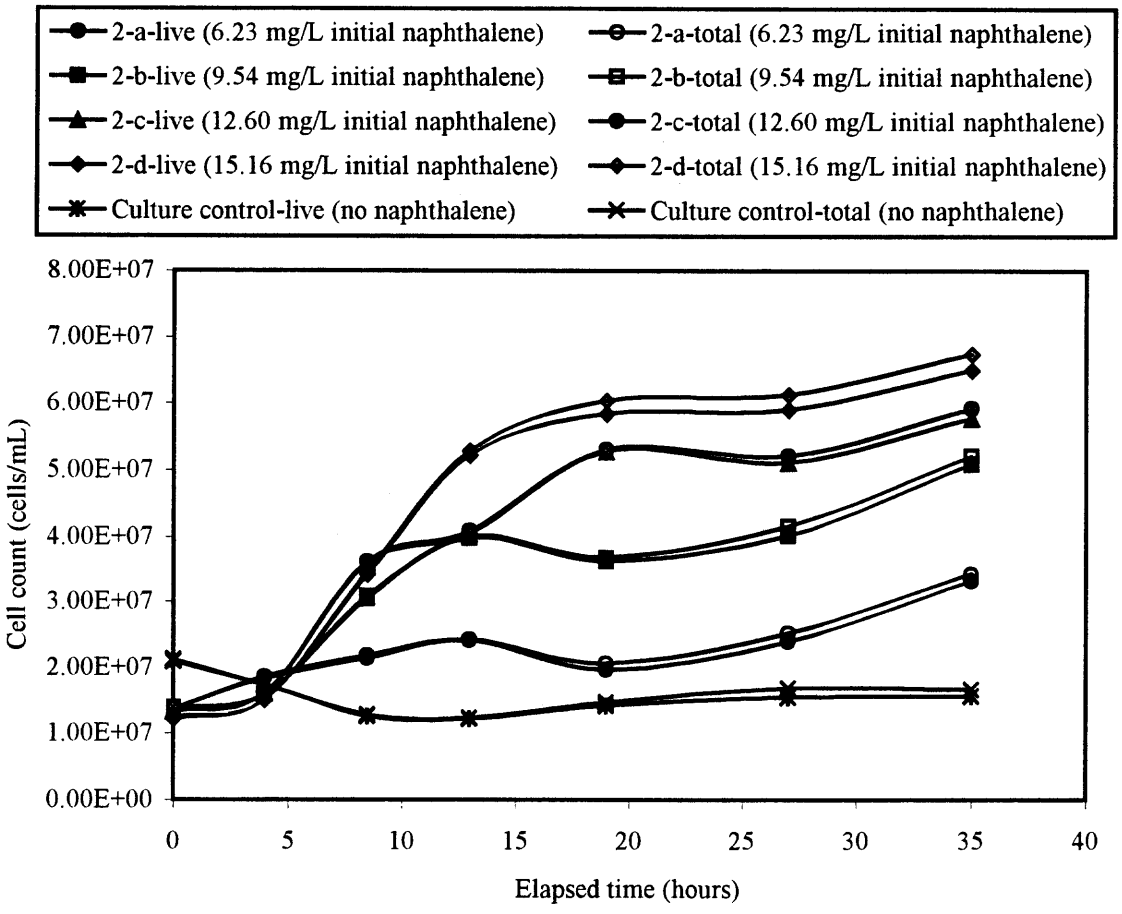


Figure D-11 Enumeration of total and live cells during naphthalene degradation in shake flasks using *Pseudomonas puitda* (Experiment-2).

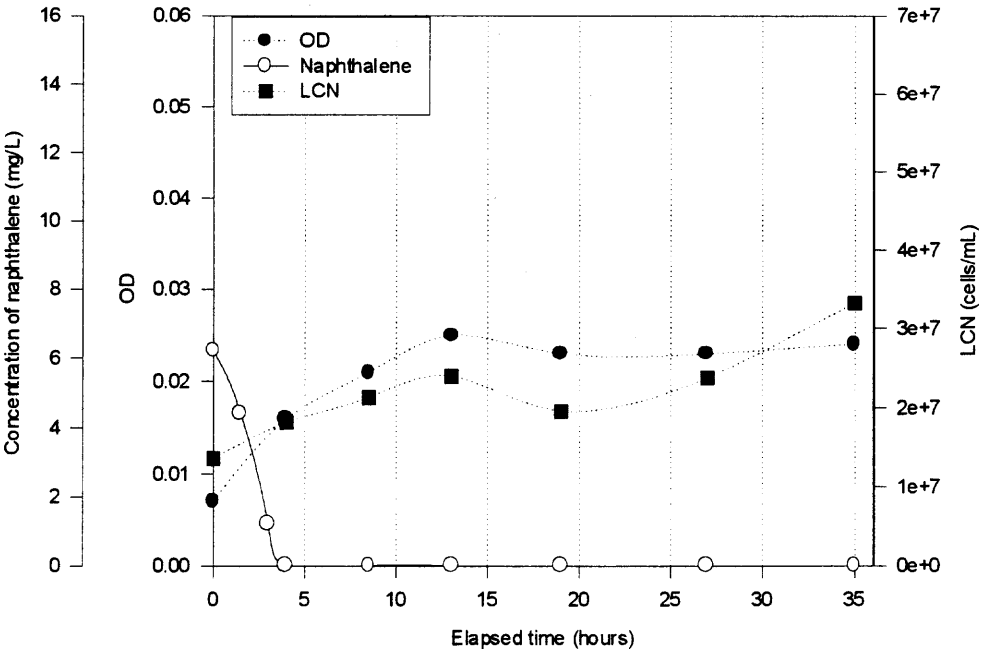


Figure D-12 Growth patterns of *Pseudomonas putida* in OD and LCN due to biodegradation of naphthalene (initial=6.23 mg/L); Experiment 2-a.

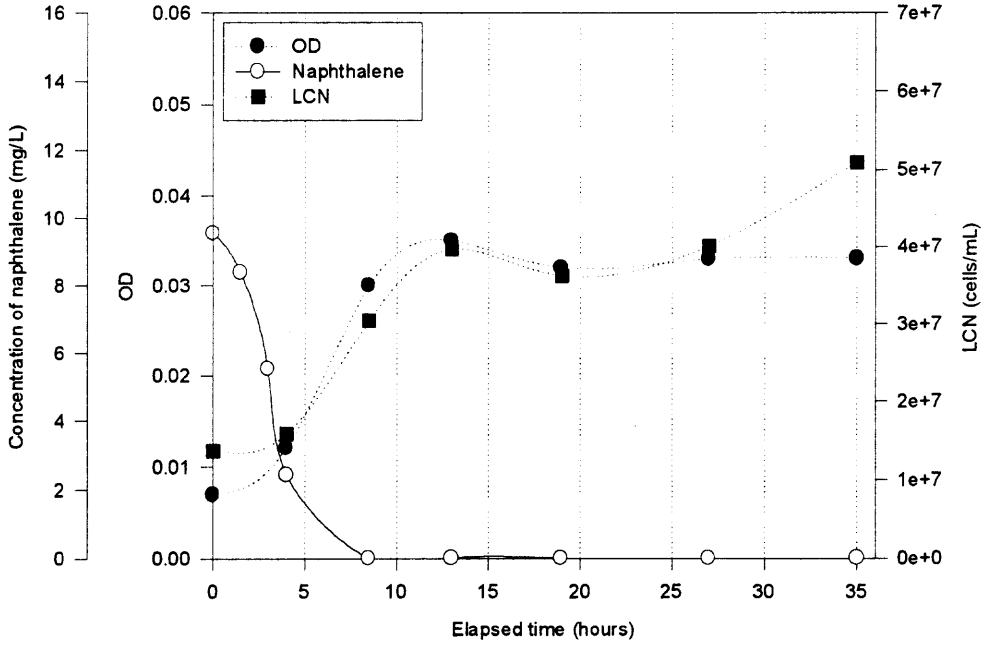


Figure D-13 Growth patterns of *Pseudomonas putida* in OD and LCN due to biodegradation of naphthalene (initial=9.54 mg/L); Experiment 2-b.

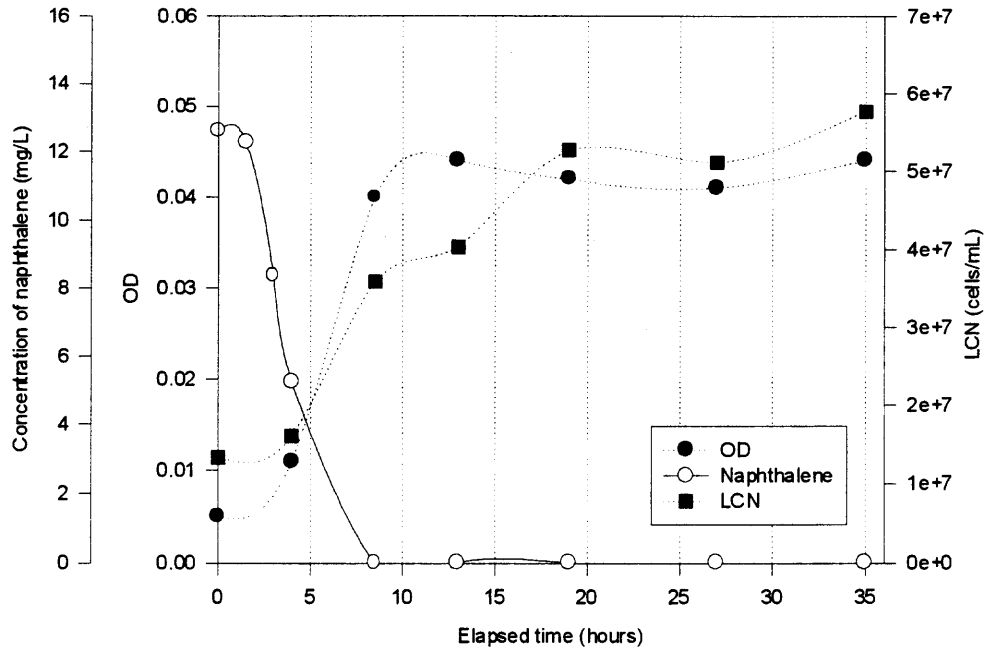


Figure D-14 Growth patterns of *Pseudomonas putida* in OD and LCN due to biodegradation of naphthalene (initial=12.60 mg/L); Experiment 2-c.

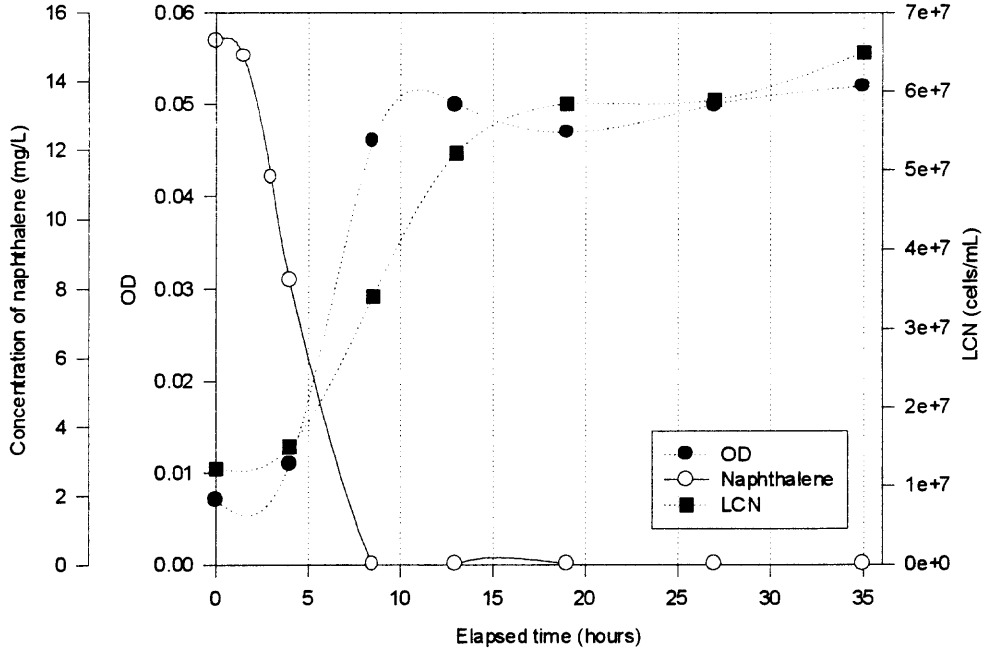


Figure D-15 Growth patterns of *Pseudomonas putida* in OD and LCN due to biodegradation of naphthalene (initial=15.16 mg/L); Experiment 2-d.

APPENDIX E

DETERMINATION OF SPECIFIC GROWTH RATES USING ORIGINAL BIOMASS MEASUREMENTS

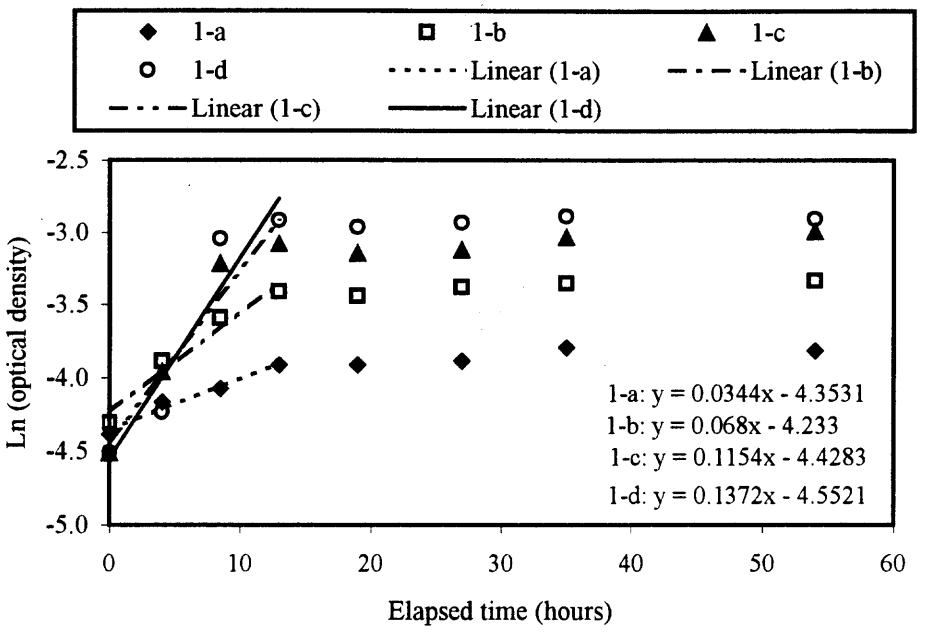


Figure E-1 Determination of specific growth rates of *Pseudomonas putida* suspension using optical density (without conversion factor): Experiment-1.

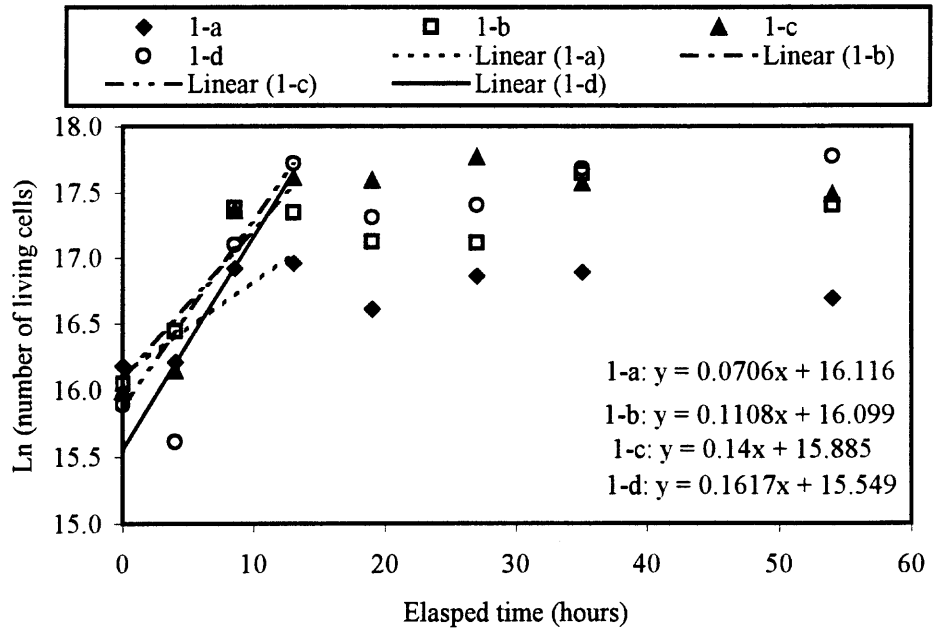


Figure E-2 Determination of specific growth rates of *Pseudomonas putida* suspension using living cell number (without conversion factor): Experiment-1.

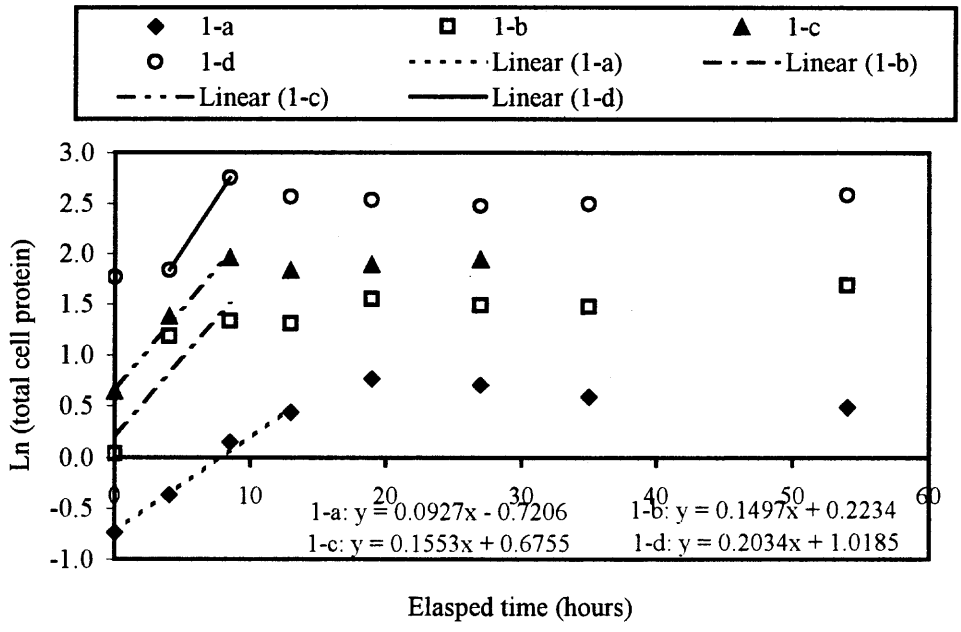


Figure E-3 Determination of specific growth rates of *Pseudomonas putida* suspension using total cell protein (without conversion factor): Experiment-1.

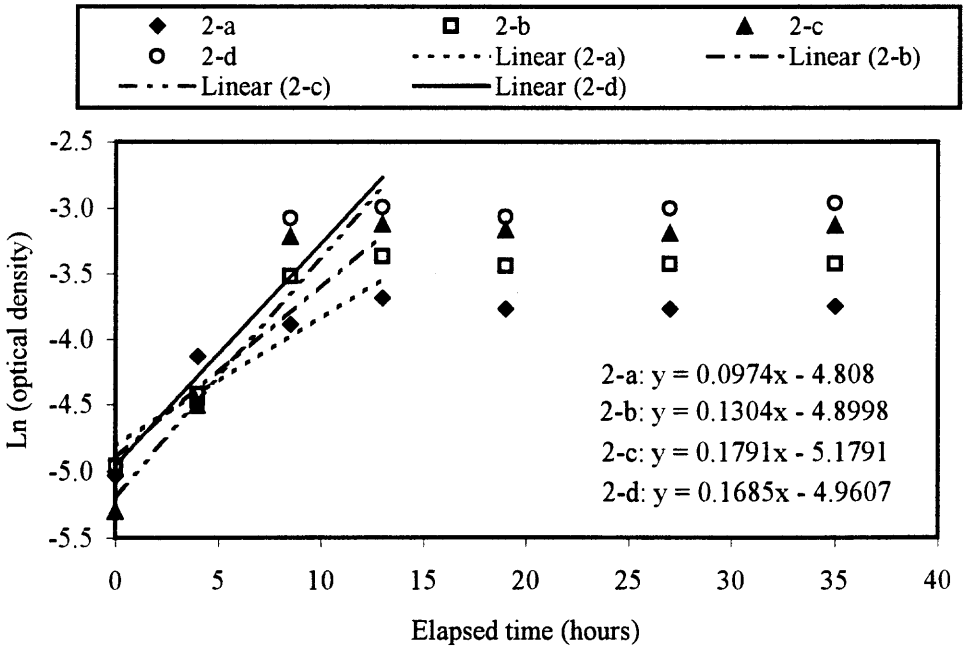


Figure E-4 Determination of specific growth rates of *Pseudomonas putida* suspension using optical density (without conversion factor): Experiment-2.

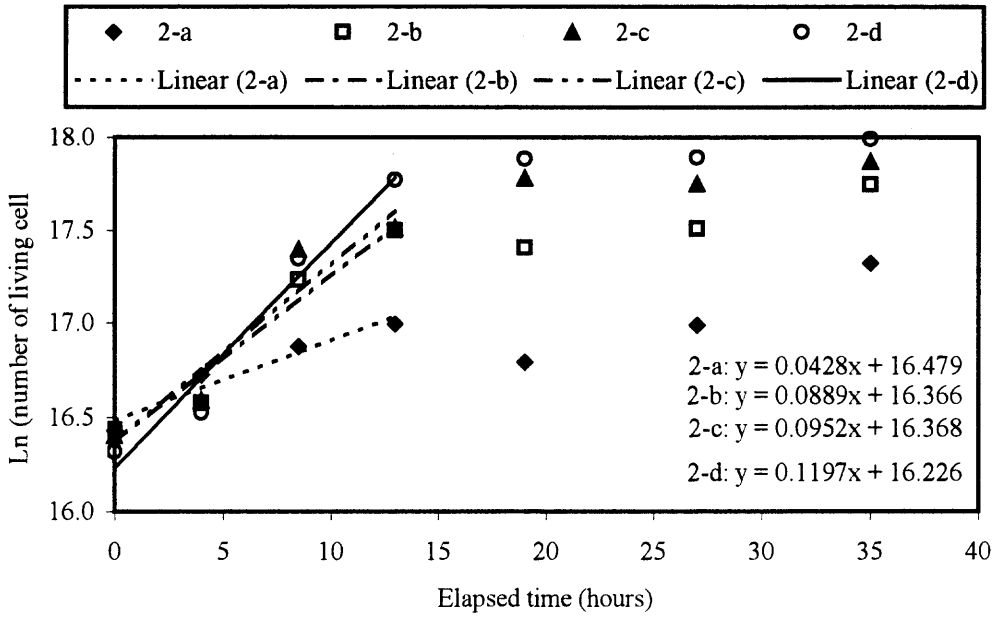


Figure E-5 Determination of specific growth rates of *Pseudomonas putida* suspension using living cell number (without conversion factor): Experiment-2.

APPENDIX F

CONVERSION FACTORS FOR TOTAL CELL NUMBER, LIVING CELL NUMBER, AND TOTAL CELL PROTEIN TO OPTICAL DENSITY

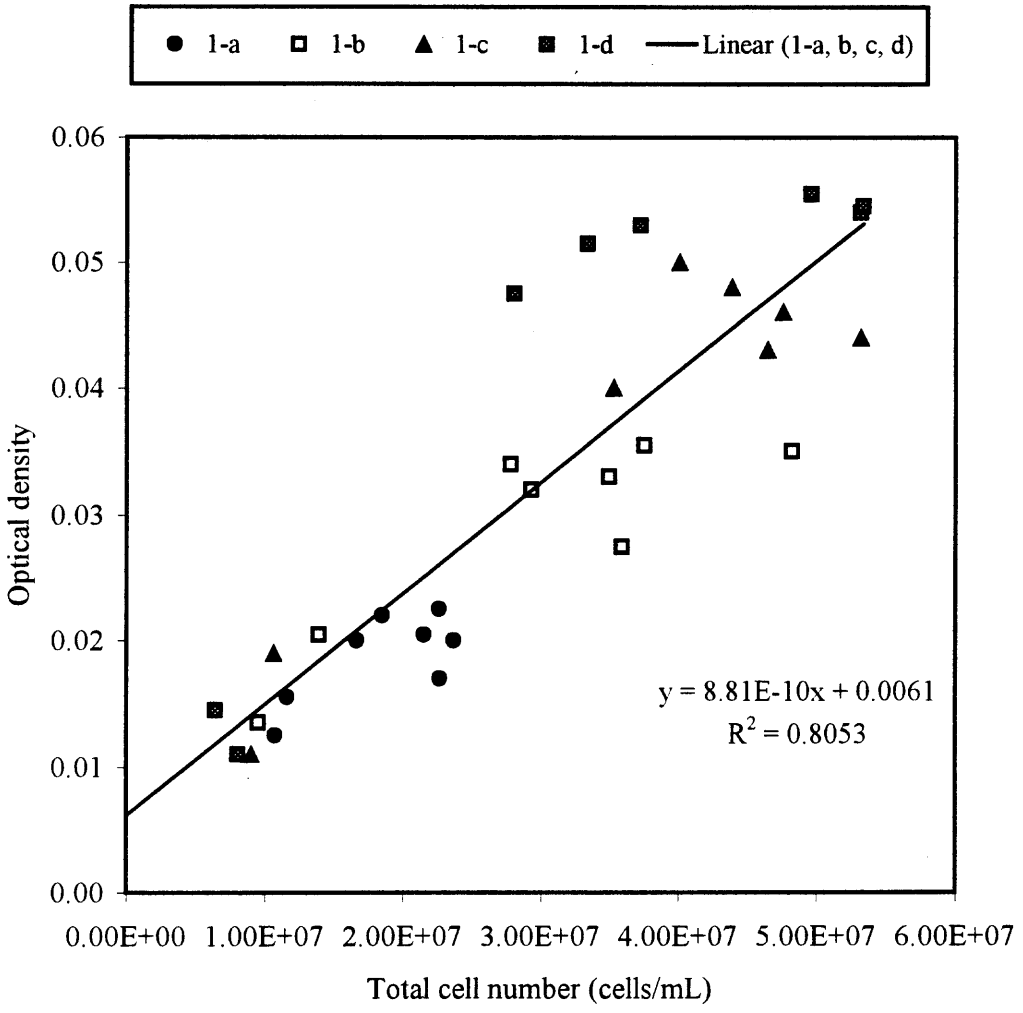


Figure F-1 Conversion factor (f_{TCN}) for total cell number to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks (Experiment-1).

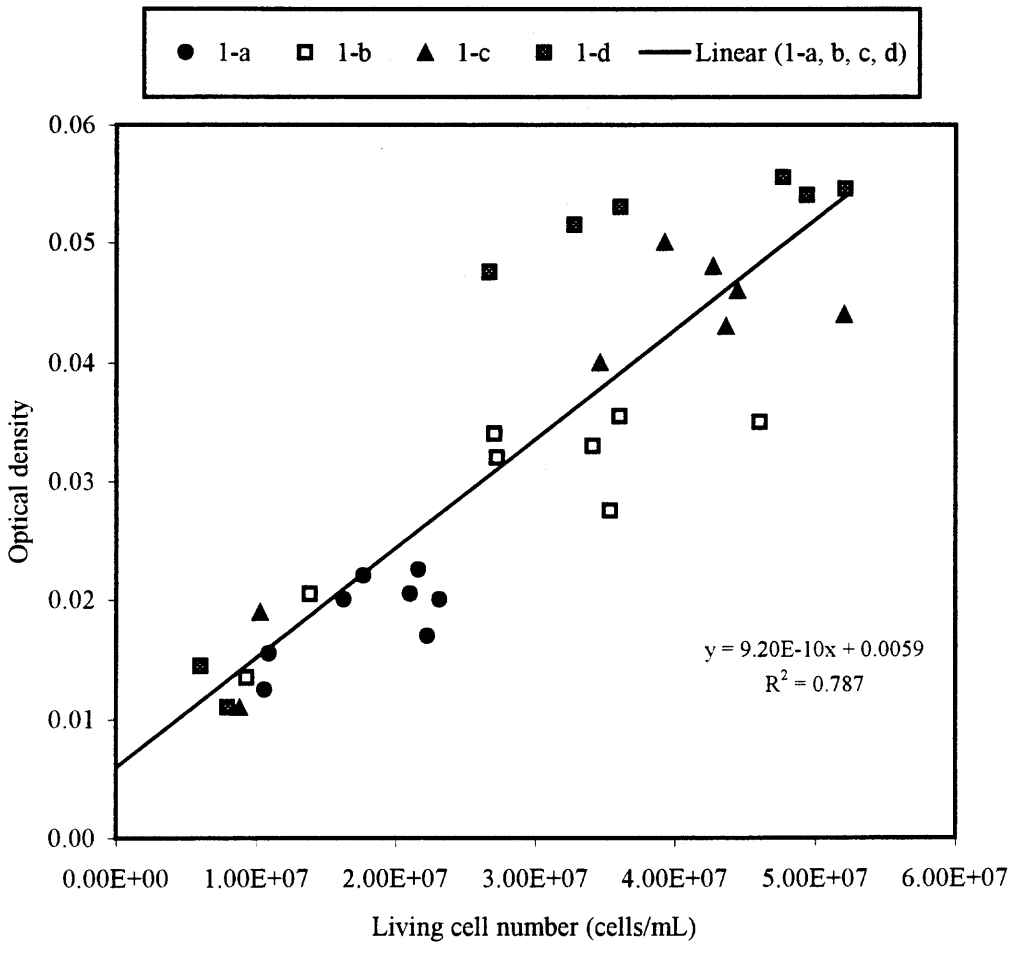


Figure F-2 Conversion factor (f_{LCN}) for living cell number to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks (Experiment-1).

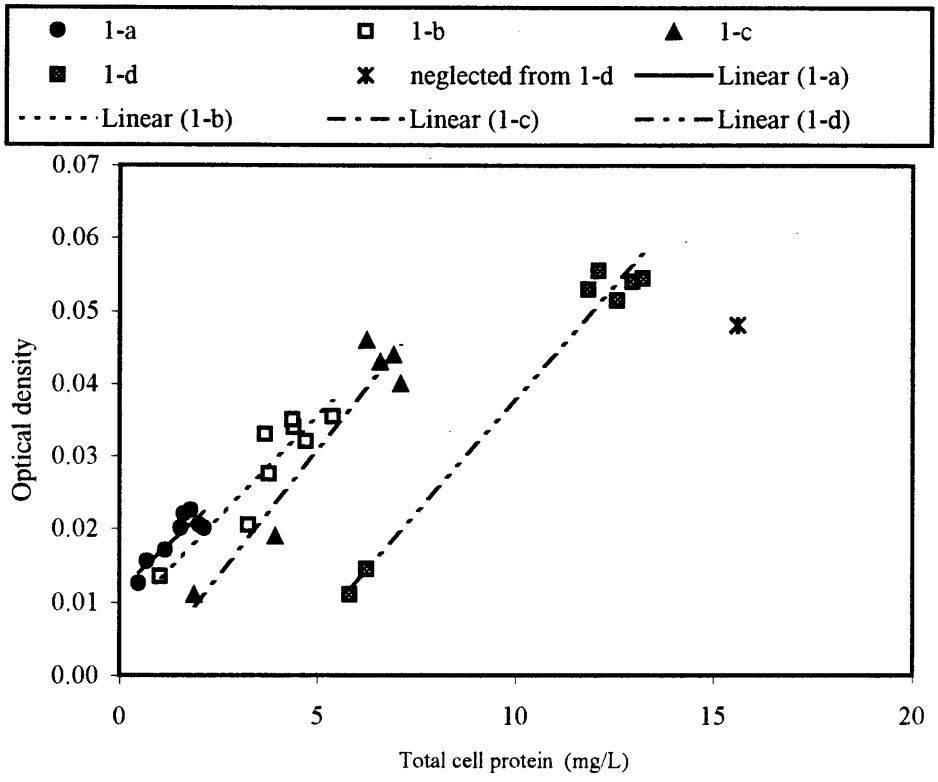


Figure F-3 Conversion factor (f_{TCP}) for total cell protein to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks (Experiment-1).

Experiment	f_{TCP} (converted OD)
1-a	y = 0.005x + 0.0115
1-b	y = 0.0055x + 0.0076
1-c	y = 0.0068x – 0.0034
1-d	y = 0.0062x – 0.0239

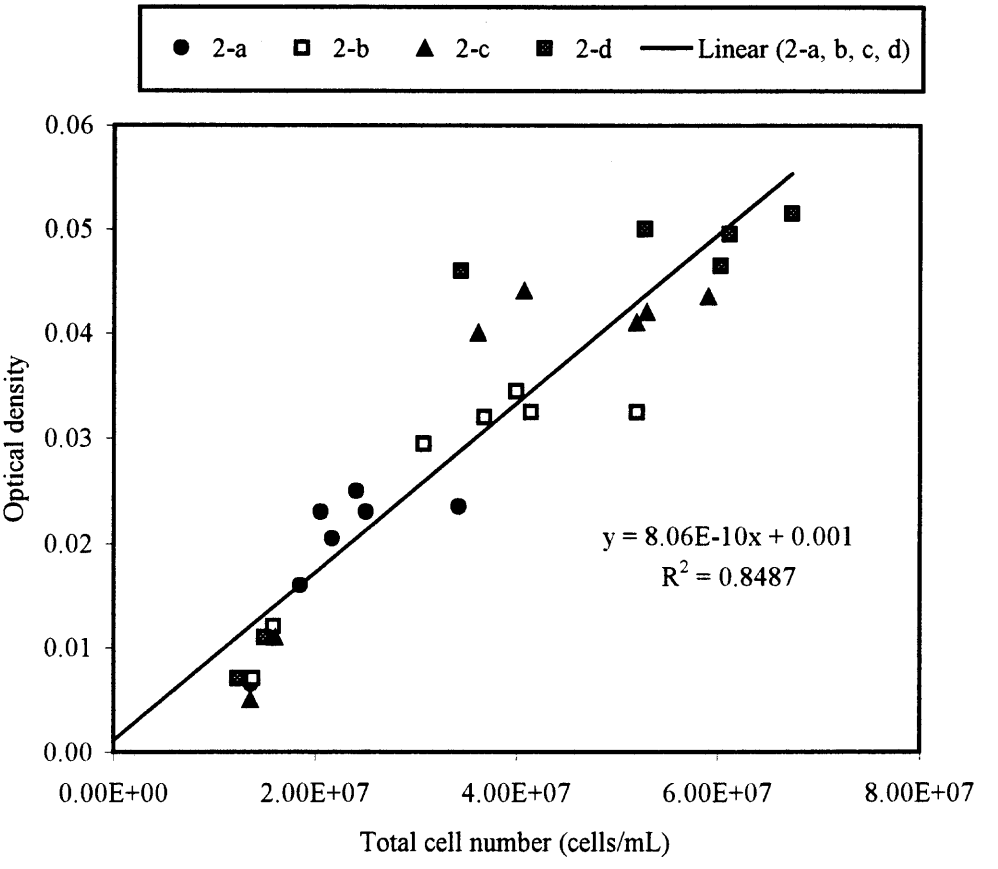


Figure F-4 Conversion factor (f_{TCN}) for total cell number to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks (Experiment-2).

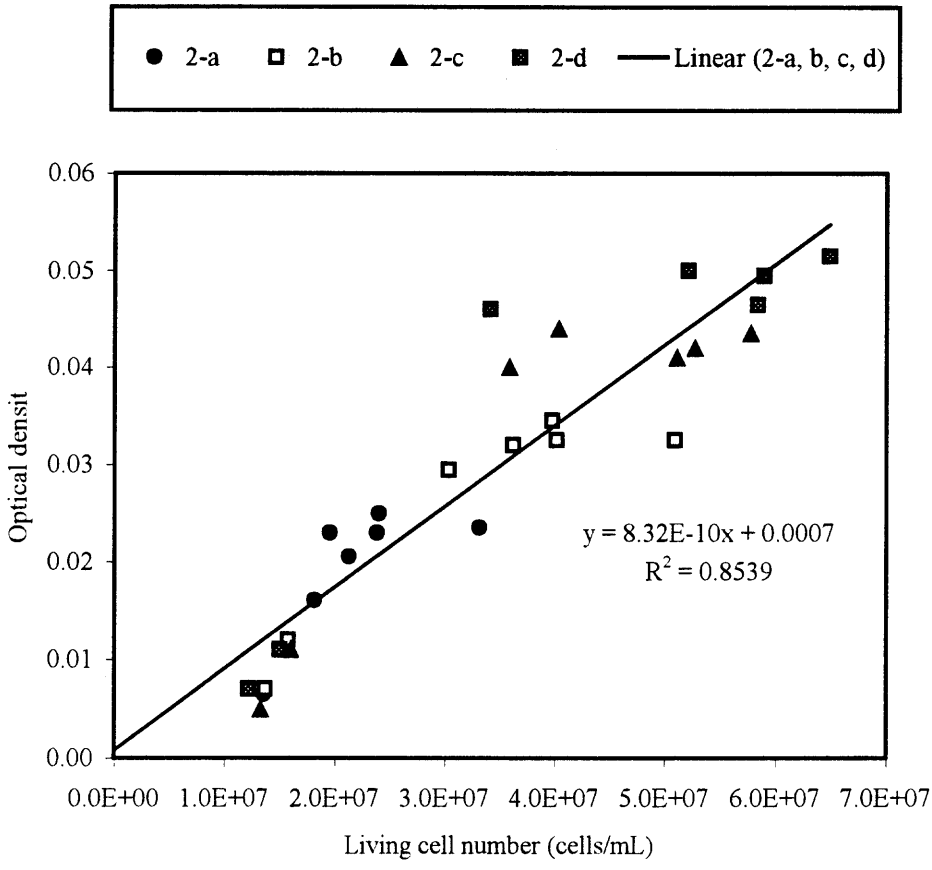


Figure F-5 Conversion factor (f_{LCN}) for living cell number to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks (Experiment-2).

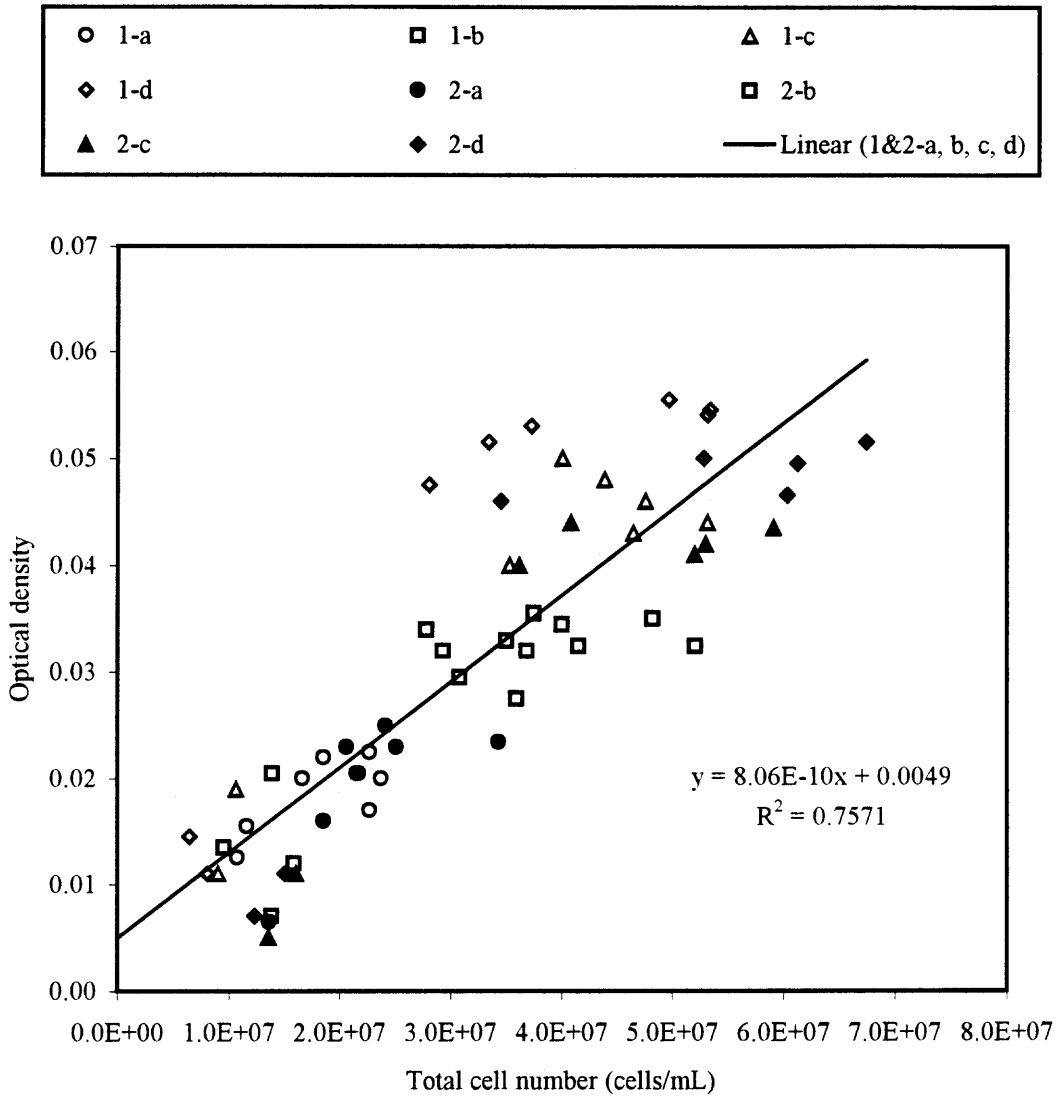


Figure F-6 Combined conversion factor (f_{TCN}) of experiment-1&2 for total cell number to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks.

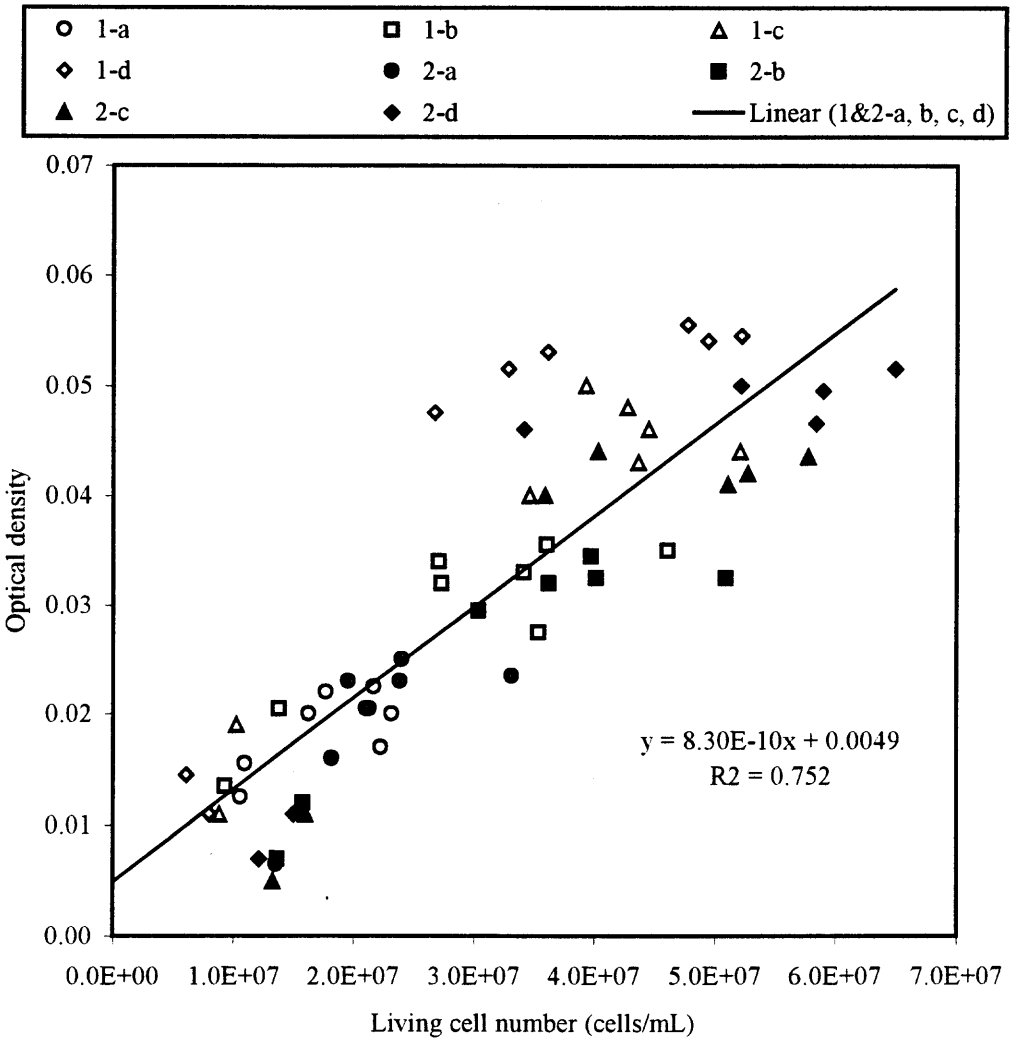


Figure F-7 Combined conversion factor (f_{LCN}) of experiment-1&2 for living cell number to optical density for suspended cultures of *Pseudomonas putida* with naphthalene in shake flasks.

Table F-1 Dry biomass by using conversion factors from OD, TCN, LCN and TCP obtained by suspended culture with *Pseudomonas putida* (Experiment-1).

(1-a)

Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)			
		TCP	LCN	TCN	OD
0	3.07	5.18	5.84	5.81	4.66
4	0	5.58	5.96	6.10	5.78
8.5	0	6.46	9.84	9.73	6.34
13	0	7.17	10.16	10.08	7.46
19	0	8.30	7.79	7.75	7.46
27	0	8.06	9.42	9.36	7.65
35	0	7.65	9.64	9.72	8.39
54	0	7.33	8.29	8.37	8.21

(1-b)

Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)			
		TCP	LCN	TCN	OD
0	7.21	4.96	5.40	5.42	5.04
4	0	9.52	6.95	6.85	7.65
8.5	0	10.57	14.34	14.08	10.26
13	0	10.39	13.91	13.78	12.31
19	0	12.50	11.57	11.91	11.94
27	0	11.88	11.50	11.42	12.68
35	0	11.79	18.02	18.12	13.06
54	0	13.90	14.58	14.60	13.24

(Continued from Table F-1)

(1-c)

Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)			
		TCP	LCN	TCN	OD
0	11.49	3.52	5.23	5.25	4.10
4	1.05	8.73	5.73	5.77	7.09
8.5	0	16.75	14.09	13.88	14.92
13	0	14.58	17.47	17.91	17.16
19	0	15.45	17.18	17.55	16.04
27	0	16.31	20.07	19.75	16.41
35	0		16.86	16.69	17.90
54	0		15.68	15.45	18.65

(1-d)

Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)			
		TCP	LCN	TCN	OD
0	16.19	4.55	4.94	4.92	4.10
4	7.53	5.53	4.27	4.39	5.41
8.5	0	27.18	11.37	11.50	17.72
13	0	21.05	19.16	19.75	20.14
19	0	20.16	13.46	13.26	19.21
27	0	18.48	14.59	14.52	19.77
35	0	19.07	18.58	18.59	20.70
54	0	21.64	20.11	19.82	20.33

Table F-2 Dry biomass by using conversion factors from OD, TCN and LCN obtained by suspended culture with *Pseudomonas putida* (Experiment-2).

(2-a)

Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)		
		LCN	TCN	OD
0	6.23	6.82	4.48	2.42
4	0	8.25	5.95	5.97
8.5	0	9.23	6.90	7.65
13	0	10.07	7.63	9.33
19	0	8.68	6.56	8.58
27	0	10.01	7.92	8.58
35	0	12.90	10.69	8.77

(2-b)

Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)		
		LCN	TCN	OD
0	9.54	6.86	4.54	2.61
4	2.43	7.51	5.14	4.48
8.5	0	12.04	9.64	11.00
13	0	14.94	12.41	12.87
19	0	13.85	11.46	11.94
27	0	15.07	12.86	12.12
35	0	18.41	16.02	12.12

(Continued from Table F-2)

(2-c)

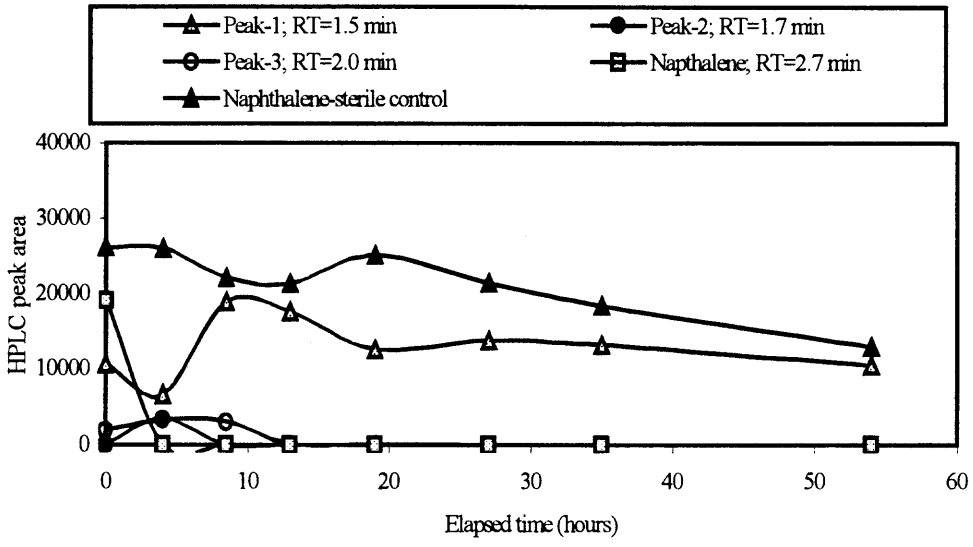
Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)		
		LCN	TCN	OD
0	12.60	6.74	4.46	1.87
4	5.25	7.58	5.20	4.10
8.5	0	13.75	11.25	14.92
13	0	15.12	12.64	16.41
19	0	18.97	16.30	15.67
27	0	18.46	16.01	15.29
35	0	20.53	18.15	16.23

(2-d)

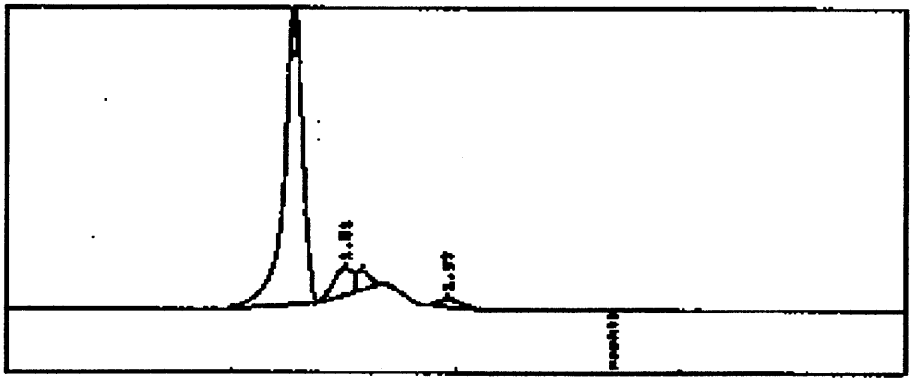
Time (hours)	Naphthalene concentration (mg/L)	Dry biomass (mg/L)		
		LCN	TCN	OD
0	15.16	6.39	4.08	2.61
4	8.25	7.27	4.89	4.10
8.5	0	13.20	10.75	17.16
13	0	18.79	16.25	18.65
19	0	20.73	18.50	17.34
27	0	20.91	18.77	18.46
35	0	22.75	20.63	19.21

APPENDIX G

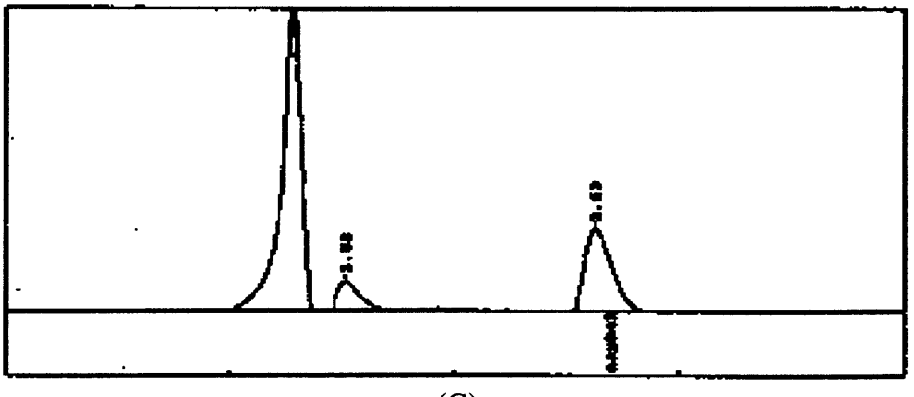
PRODUCTION OF INTERMEDIATES DURING BIODEGRADATION OF NAPHTHALENE BY *Pseduomonas putida*



(A)

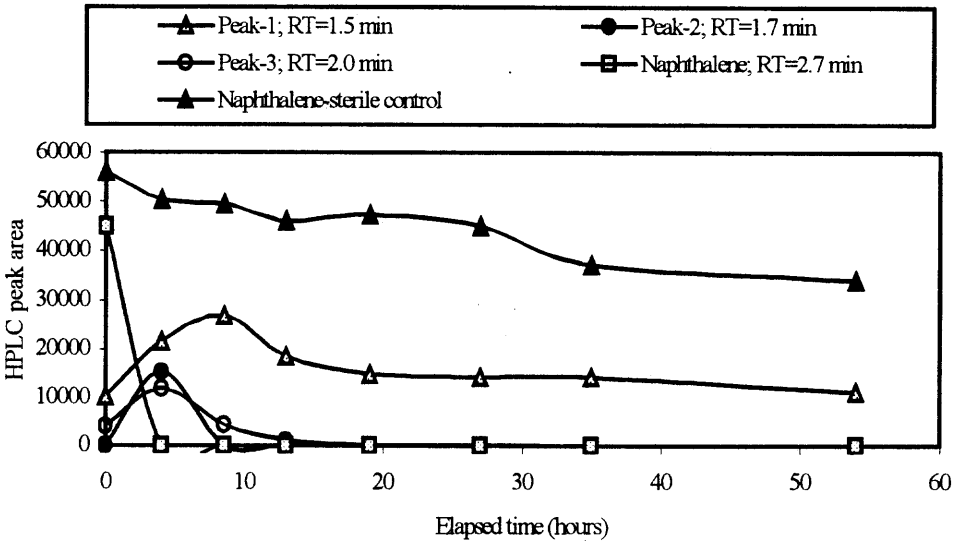


(B)

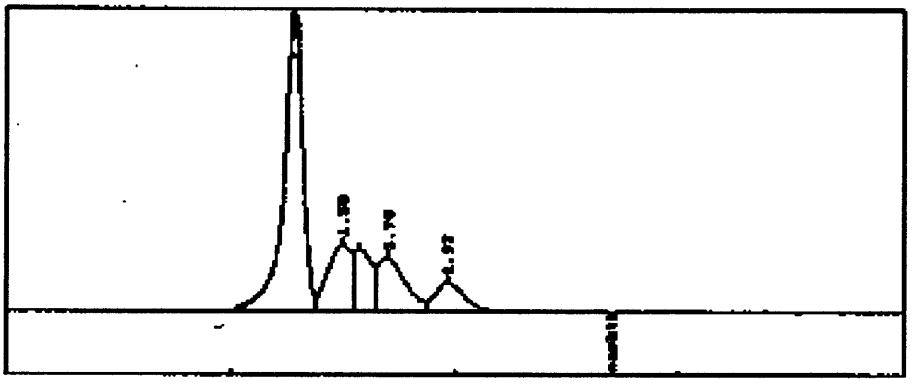


(C)

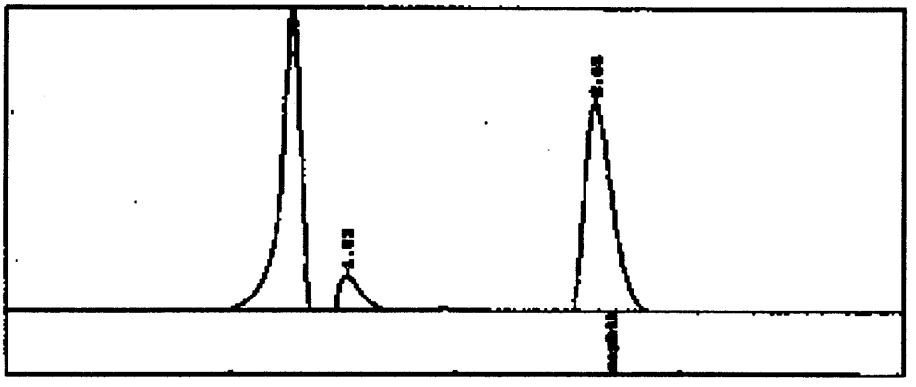
Figure G-1 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-1-a.



(A)

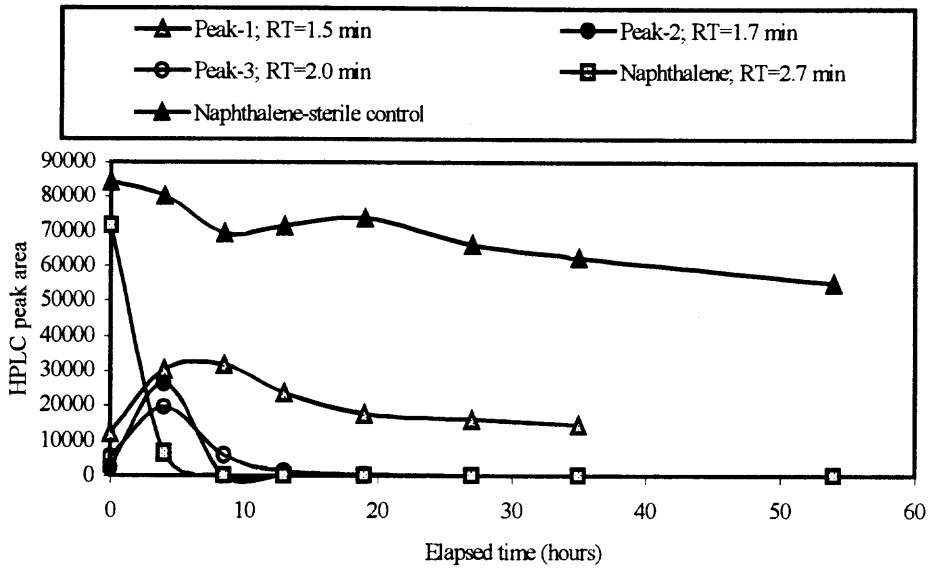


(B)

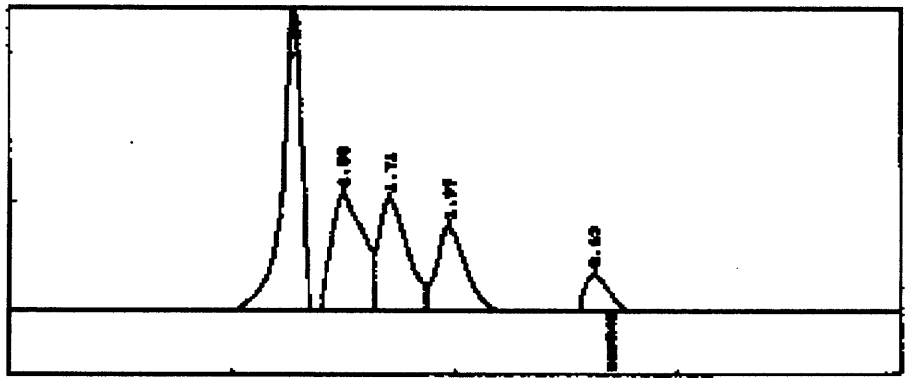


(C)

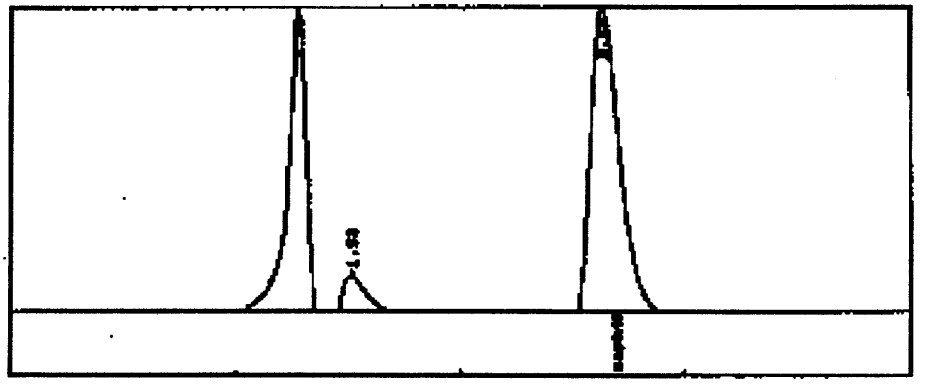
Figure G-2 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-1-b.



(A)

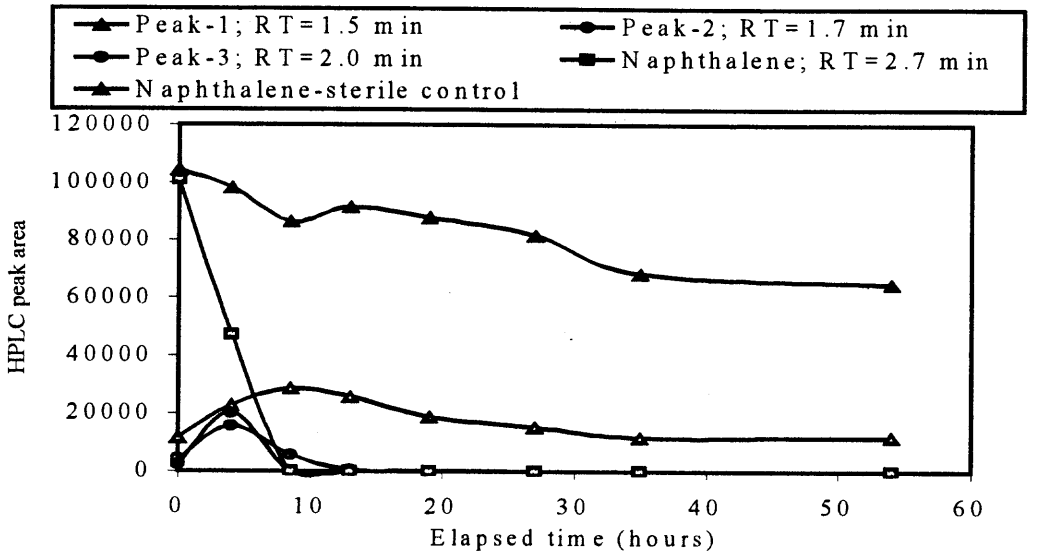


(B)

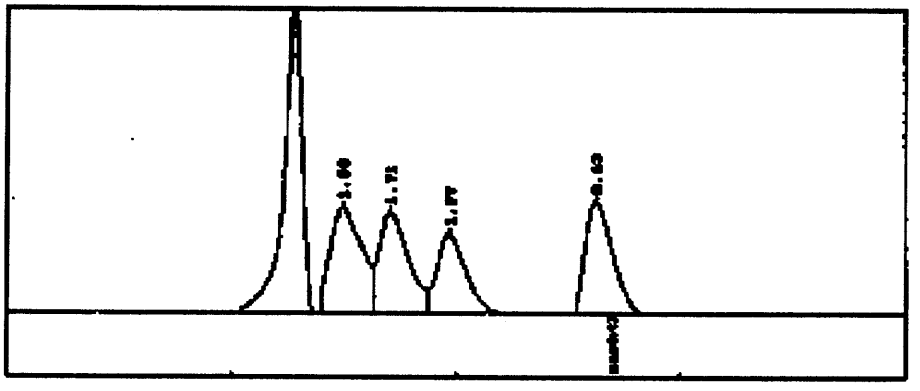


(C)

Figure G-3 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-1-c.



(A)



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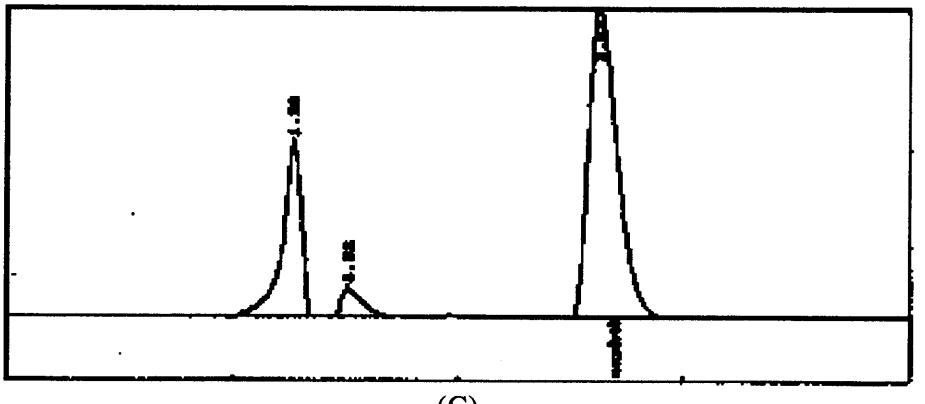
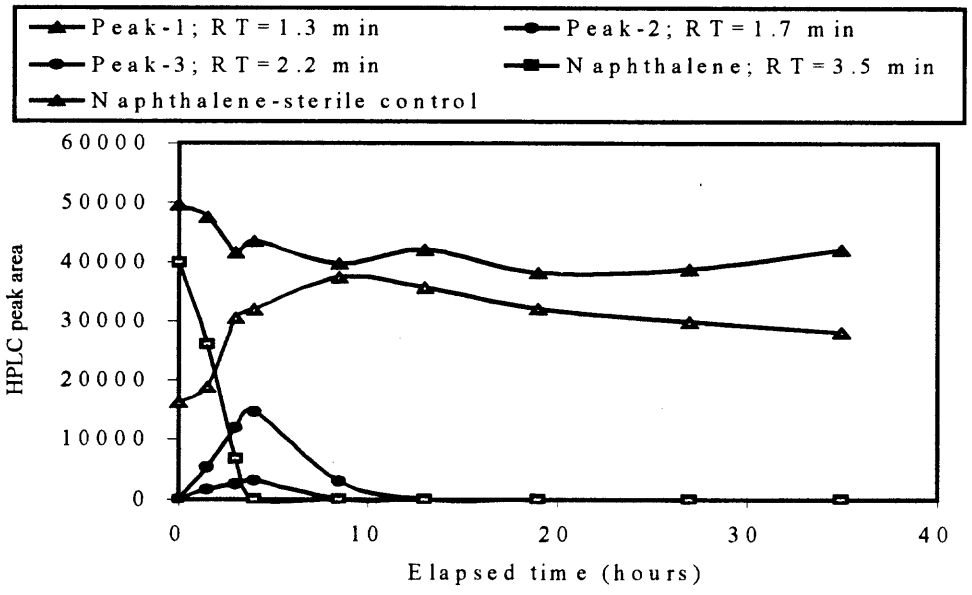
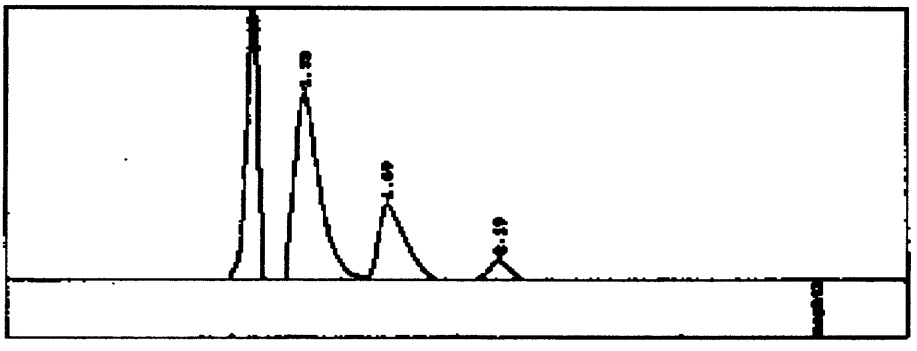


(C)

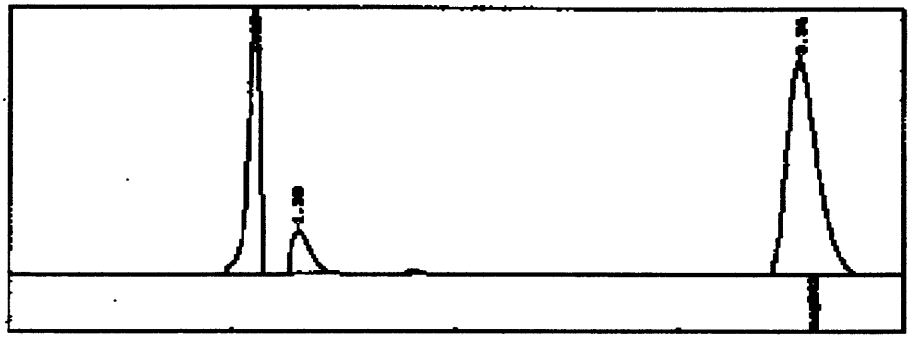
Figure G-4 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-1-d.



(A)

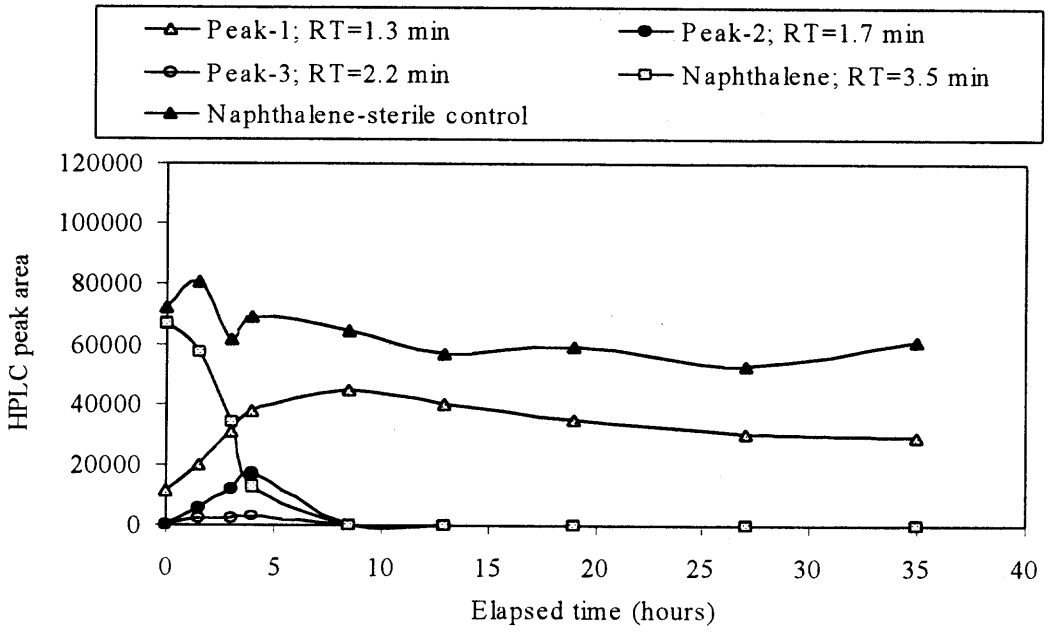


(B)

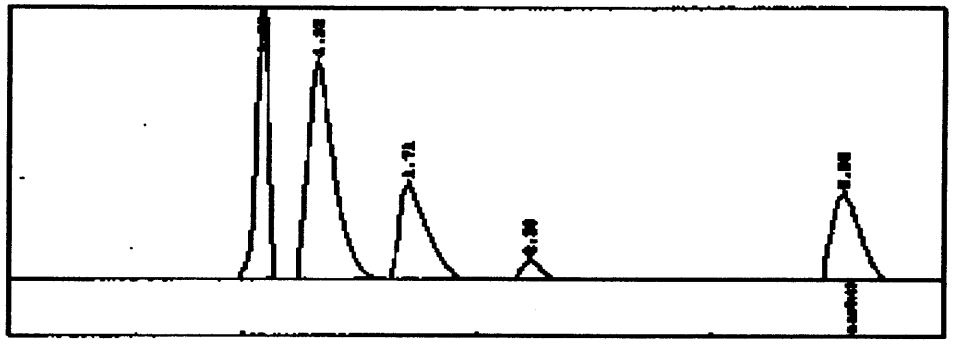


(C)

Figure G-5 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-2-a.



(A)



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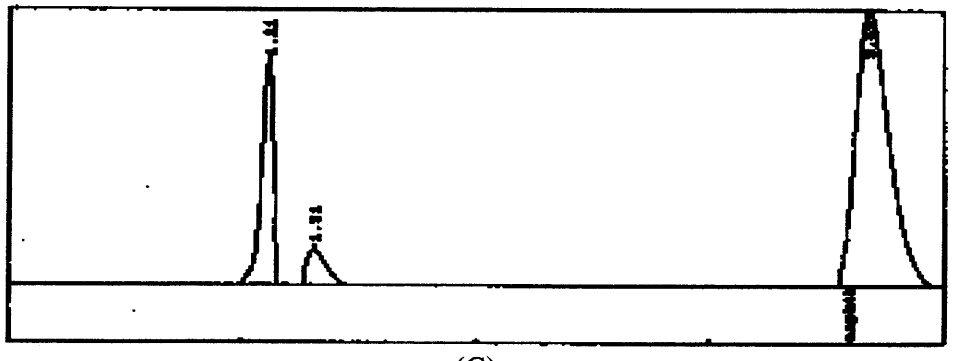


(C)

Figure G-6 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-2-b.

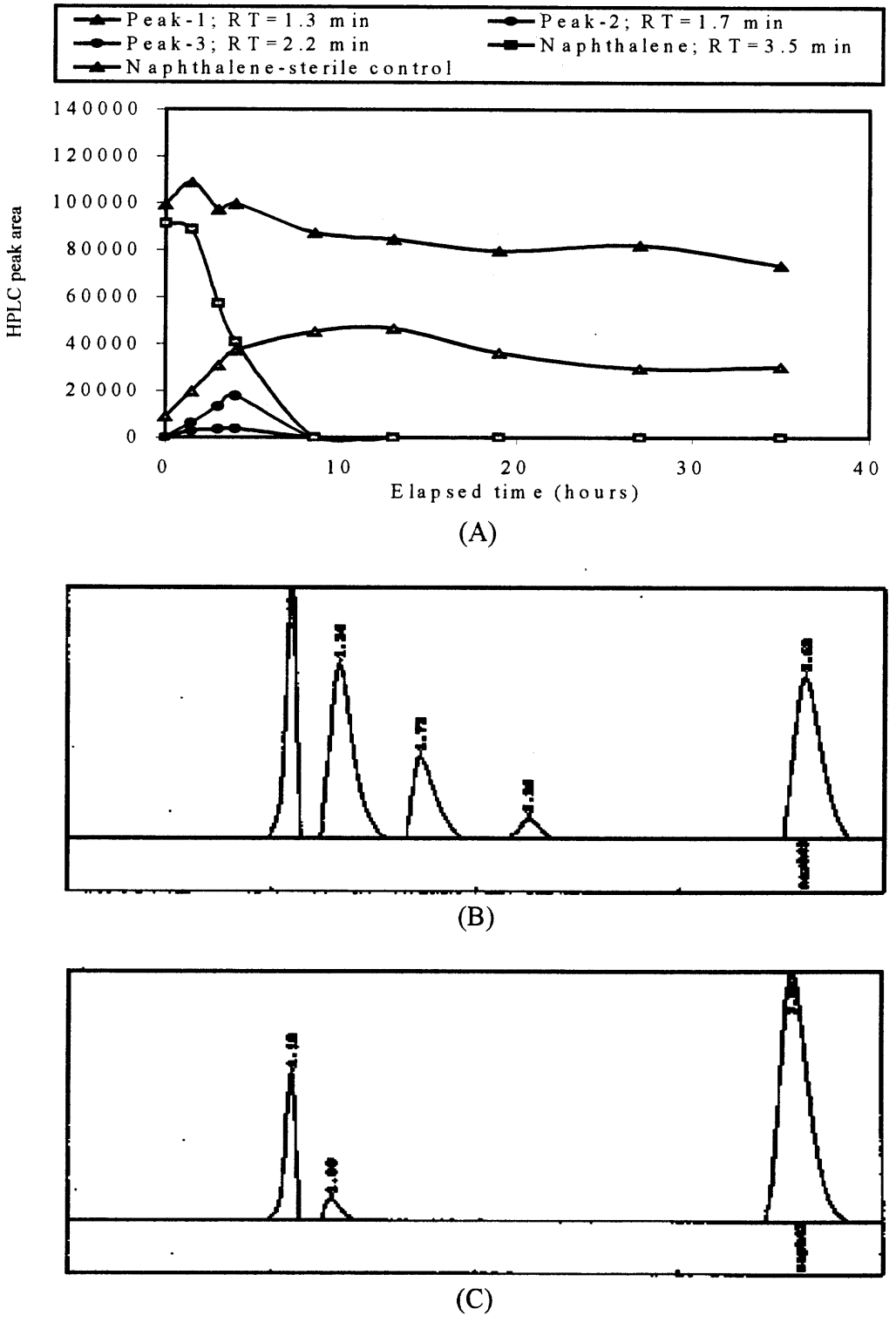
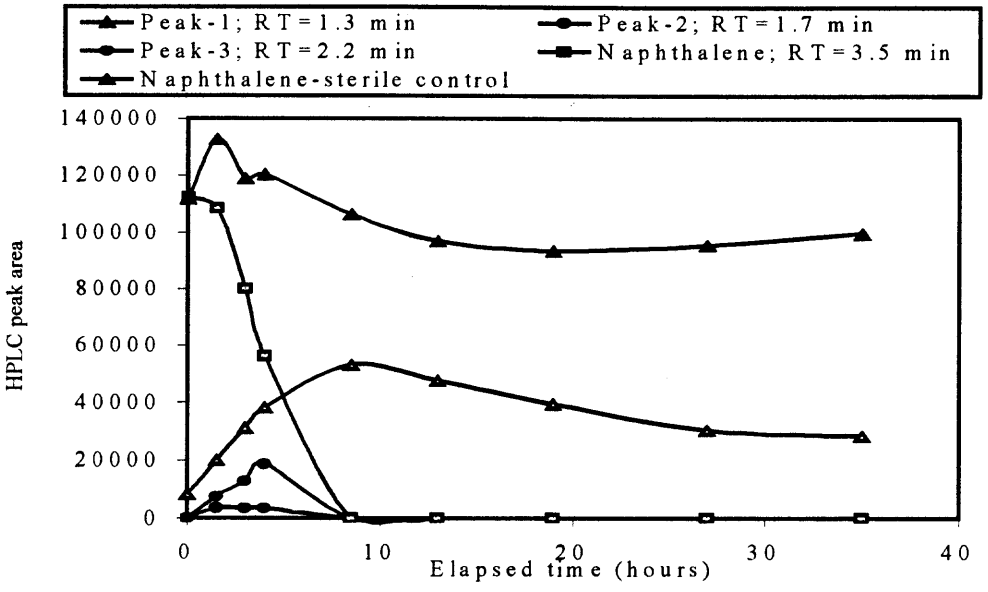
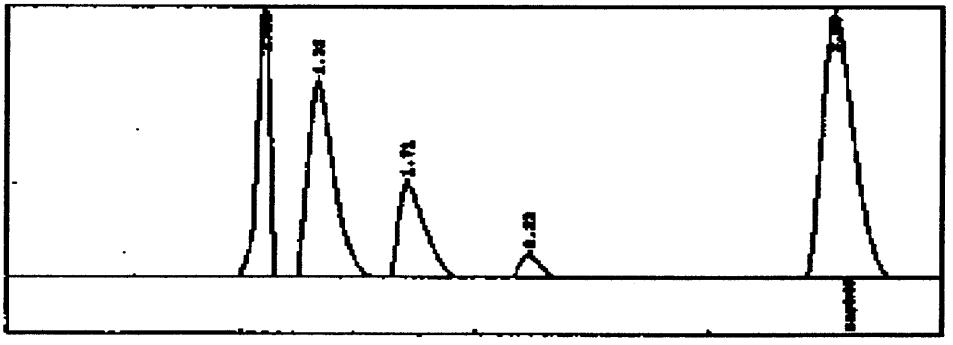


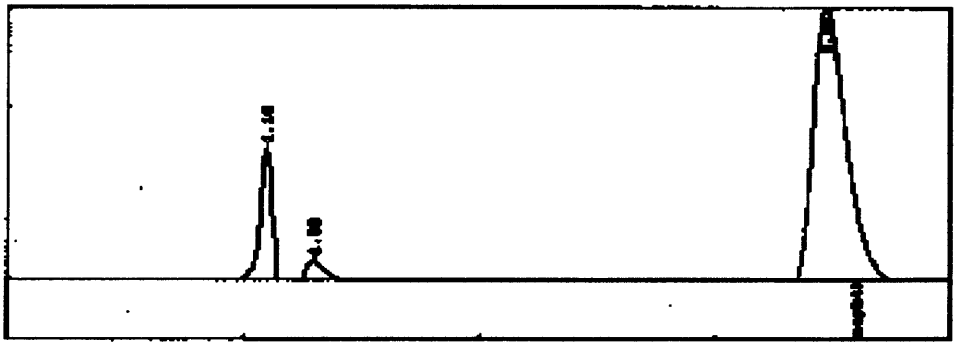
Figure G-7 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-2-c.



(A)



(B)



(C)

Figure G-8 Concentration profiles during biodegradation of naphthalene (A); sample HPLC chromatogram (B) and sterile control (C) at time 4 hours: Experiment-2-d.

APPENDIX H

EXPERIMENTAL DATA FOR BIOFILIM CULTURES ON GLASS SUPPORTS IN CONTINUOUS FLOW SYSTEM

Table H-1 Naphthalene concentration.

Elapsed time (hour)	Room temperature (°C)	Reservoir-A (mg/L)	Column-X (mg/L)			Column-Y (mg/L)		
			Inlet	outlet	difference	Inlet	outlet	difference
0.0	22.0	24.2	20.2	19.3	0.9	20.2	18.6	1.6
0.3	22.0	26.3	19.6	15.5	4.2	16.8	13.7	3.2
2.0	21.7	26.0	20.2	17.7	2.4	20.7	18.6	2.1
7.5	20.7	27.3	19.7	17.7	2.0	20.1	17.6	2.5
11.3	20.7	25.4	19.2	16.9	2.3	18.9	16.9	2.0
18.0	23.8	27.0	19.2	13.9	5.4	20.0	12.7	7.3
26.7	21.7	26.6	20.0	15.0	5.0	19.7	13.8	5.9
33.0	21.0	25.8	19.5	14.0	5.5	19.4	12.7	6.7
39.5	23.0	29.1	20.8	13.4	7.4	21.2	13.1	8.1
51.3	21.8	27.2	20.5	15.4	5.1	20.6	14.8	5.8

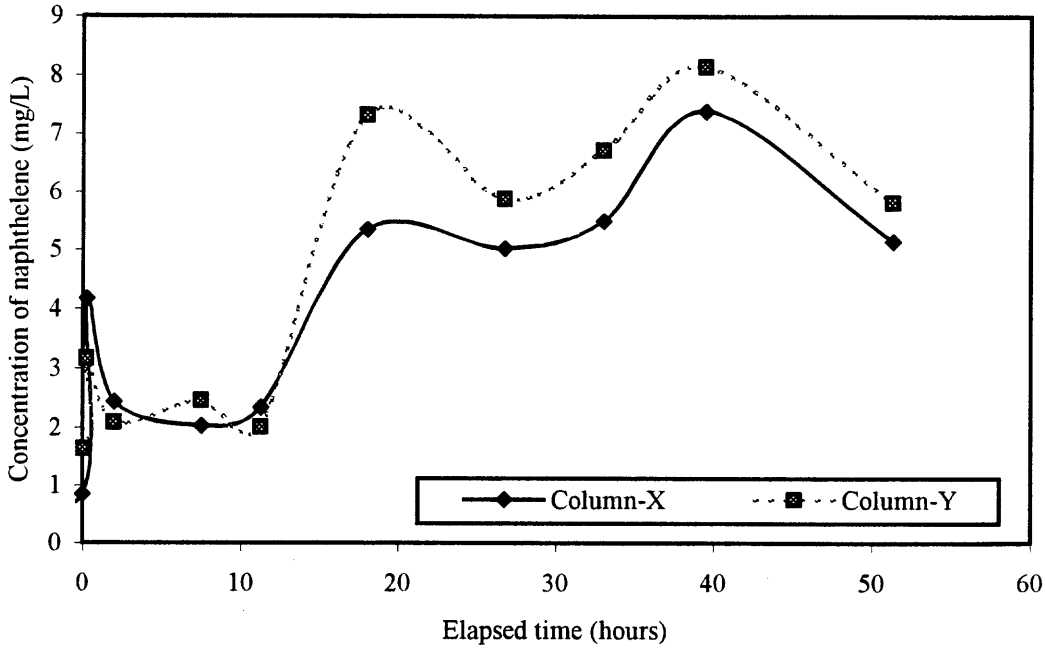


Figure H-1 Comparison of naphthalene concentration in column-X and Y, difference between inlet and outlet for biofilm cultures in a continuous flow system.

Table H-2 Cells immobilized on glass supports in column reactors of continuous flow system.

Column for sample	Time (hours)	Cell density of biofilm (cells per cm^2)			Standard deviation of biofilm (cells per cm^2)			Total immobilized cells on a glass support (cells on 186.1 cm^2)	Total immobilized cells on 5 glass supports (cells on 930.5 cm^2)	Normalization of total cells
		Live cells	Dead cells	Total cells	Live cells	Dead cells	Total cells			
Seed-BF	0	1.57E+05	5.87E+04	2.16E+05	4.34E+04	7.49E+03	3.60E+04	4.02E+07	2.01E+08	1.00
column-Y	11.25	3.30E+05	1.09E+05	4.39E+05	7.54E+04	9.23E+04	3.98E+04	8.17E+07	4.09E+08	2.03
column-Y	18	3.43E+05	3.67E+04	3.80E+05	1.15E+05	1.24E+04	1.10E+05	7.07E+07	3.53E+08	1.76
column-Y	26.7	2.78E+05	2.88E+05	5.67E+05	1.19E+05	2.69E+05	3.73E+05	1.05E+08	5.27E+08	2.62
column-Y	33	4.32E+05	8.07E+04	5.13E+05	1.67E+05	1.98E+04	1.71E+05	9.54E+07	4.77E+08	2.37
column-Y	39.5	5.59E+05	3.73E+05	9.32E+05	2.10E+05	2.09E+05	4.17E+05	1.74E+08	8.68E+08	4.32
column-X	51.3	6.78E+05	6.91E+05	1.37E+06	1.27E+05	2.61E+05	3.42E+05	6.37E+07	1.27E+09	6.34

Live cell % is assumed to be the same as for suspended cells in the same column.

Table H-3 Growth and viability of suspended cells in column-X of continuous flow system for biofilm culture.

Elapsed time (hours)	Cell density (cells/mL)			Standard deviation of cell density (cells/mL)			% of live cells in column-X	Total live cells in suspension of column-X (cells/441 mL)
	Live	Dead	Total	Live	Dead	Total		
0.25	1.34E+05	0.00E+00	1.34E+05	2.33E+05	0.00E+00	2.33E+05	100%	5.93E+07
11.25	9.41E+05	0.00E+00	9.41E+05	9.31E+05	0.00E+00	9.31E+05	100%	4.15E+08
18.00	4.84E+06	5.38E+05	5.38E+06	4.03E+05	4.66E+05	2.33E+05	90%	2.13E+09
26.70	6.05E+06	2.69E+05	6.32E+06	8.07E+05	2.33E+05	6.16E+05	96%	2.67E+09
33.00	5.92E+06	0.00E+00	5.92E+06	1.63E+06	0.00E+00	1.63E+06	100%	2.61E+09
39.50	3.76E+06	2.69E+05	4.03E+06	4.66E+05	4.66E+05	8.07E+05	93%	1.66E+09
51.3	3.50E+06	1.34E+05	3.63E+06	1.53E+06	2.33E+05	1.76E+06	96%	1.54E+09

Table H-4 Viable cells in column-X of continuous flow system.

Elapsed time (hours)	Live cells in suspension of column-X (%)	Live cells on biofilm applied from % of live cells in suspension of column-X (cells per cm^2)	Total live cells on biofilm on 5 glass supports applied from % of live cells in suspension of column-X (live cells on 930.5 cm^2)	Total live cells in suspension of column-X (cells/441 mL)	Total live cells of biofilm and suspended cells in column-X (live cells in column-X)	Live-immobilized cells in column-X (%)
0.25	100%	2.16E+05	2.01E+08	5.93E+07	2.60E+08	77%
11.25	100%	4.39E+05	4.09E+08	4.15E+08	8.24E+08	50%
18	90%	3.42E+05	3.18E+08	2.13E+09	2.45E+09	13%
26.7	96%	5.43E+05	5.05E+08	2.67E+09	3.17E+09	16%
33	100%	5.13E+05	4.77E+08	2.61E+09	3.09E+09	15%
39.5	93%	8.70E+05	8.10E+08	1.66E+09	2.47E+09	33%
51.3	96%	1.32E+06	1.23E+09	1.54E+09	2.77E+09	44%

Table H-5 Viability of suspended bacteria in column-Y of continuous flow system for biofilm culture.

Elapsed time (hours)	Cell density (cells/mL) Live	Dead	subtotal	# of glass supports in column-Y	Media volume in column-Y	Retention time at 1 mL/min. (hour)	% of live cells in column-Y	Total live cells in column-Y
0	0.00E+00	0.00E+00	0.00E+00	5	441	7.4	#DIV/0!	0.00E+00
11.25	4.03E+05	0.00E+00	4.03E+05	5	441	7.4	100%	1.78E+08
18	6.05E+06	4.03E+05	6.45E+06	4	460	7.7	94%	2.78E+09
26.7	4.44E+06	4.03E+05	4.84E+06	3	479	8.0	92%	2.13E+09
33	5.45E+06	2.02E+05	5.65E+06	2	498	8.3	96%	2.71E+09
39.5	6.25E+06	2.02E+05	6.45E+06	1	517	8.6	97%	3.23E+09
51.3	3.43E+06	2.02E+05	3.63E+06	0	536	8.9	94%	1.84E+09

(Sampled from only outlet)

Table H-6 Viable cells in column-Y of continuous flow system.

Elapsed time (hours)	Live cells in suspension of column-Y (%)	Live cells on biofilm applied from % of live cells in suspension of column-X (cells per cm^2)	Total live cells on biofilm on glass supports applied by % of live cells in suspension (cells / all glasses remained)	Total live cells in suspension of column-Y	Total live cells of biofilm and suspension in column-Y	Live-immobilized cells in column-Y (%)
0.0	98%	2.12E+05	1.93E+08	0.00E+00	1.93E+08	100%
11.3	100%	4.39E+05	4.09E+08	1.78E+08	5.86E+08	70%
18.0	94%	3.56E+05	2.48E+08	2.78E+09	3.03E+09	8%
26.7	92%	5.19E+05	2.66E+08	2.13E+09	2.39E+09	11%
33.0	96%	4.94E+05	1.77E+08	2.71E+09	2.89E+09	6%
39.5	98%	9.14E+05	1.67E+08	3.23E+09	3.40E+09	5%
51.3	95%	1.31E+06	0.00E+00	1.84E+09	1.84E+09	0%

(time "0" = % of seed BF container) (Sampled from only outlet of column-Y)

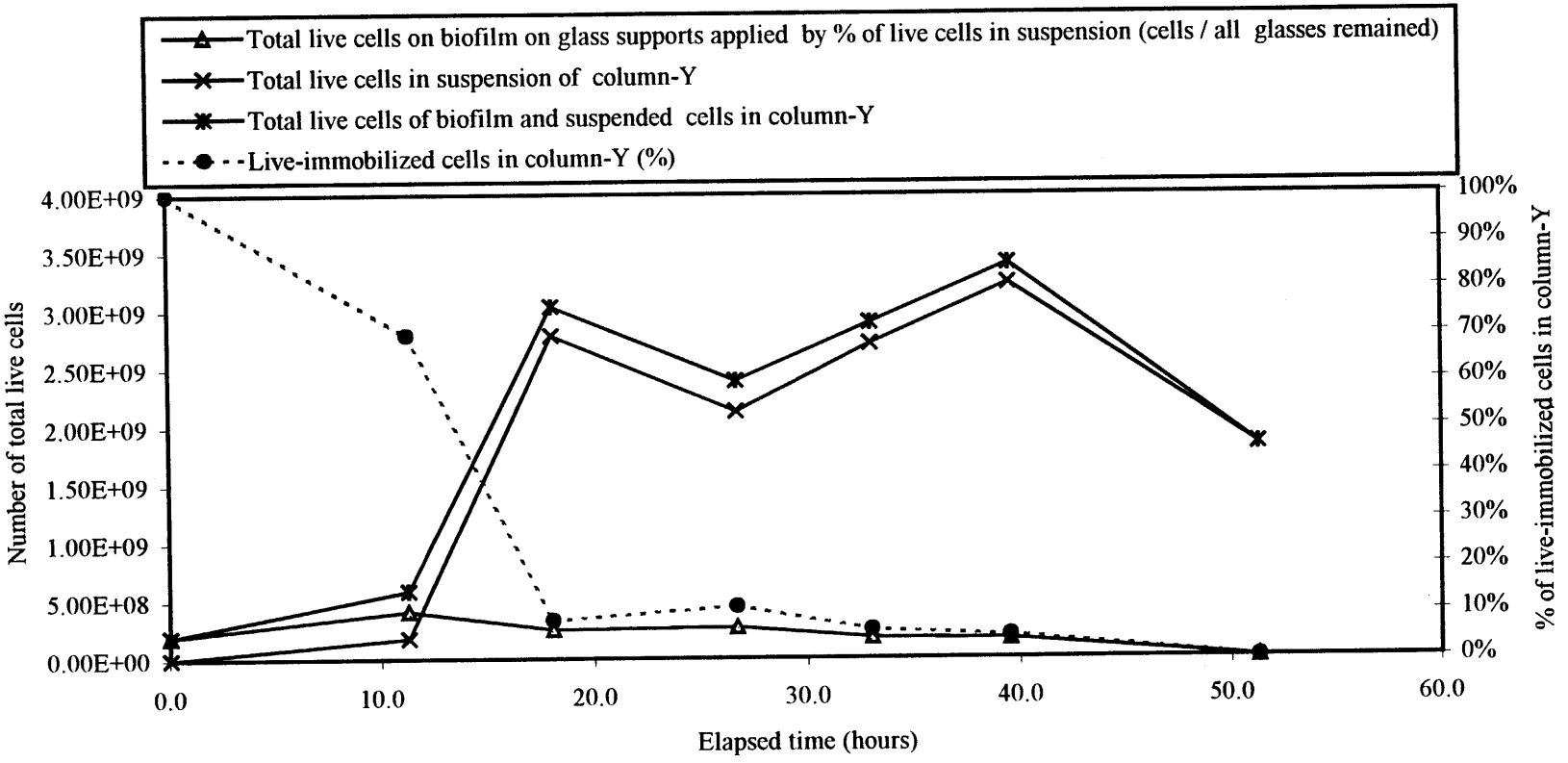


Figure H-2 Amount of live cells on biofilm and suspension in column-Y according to elapsed time in continuous flow system for biodegradation of naphthalene. (Media flow rate=1 mL/min.)

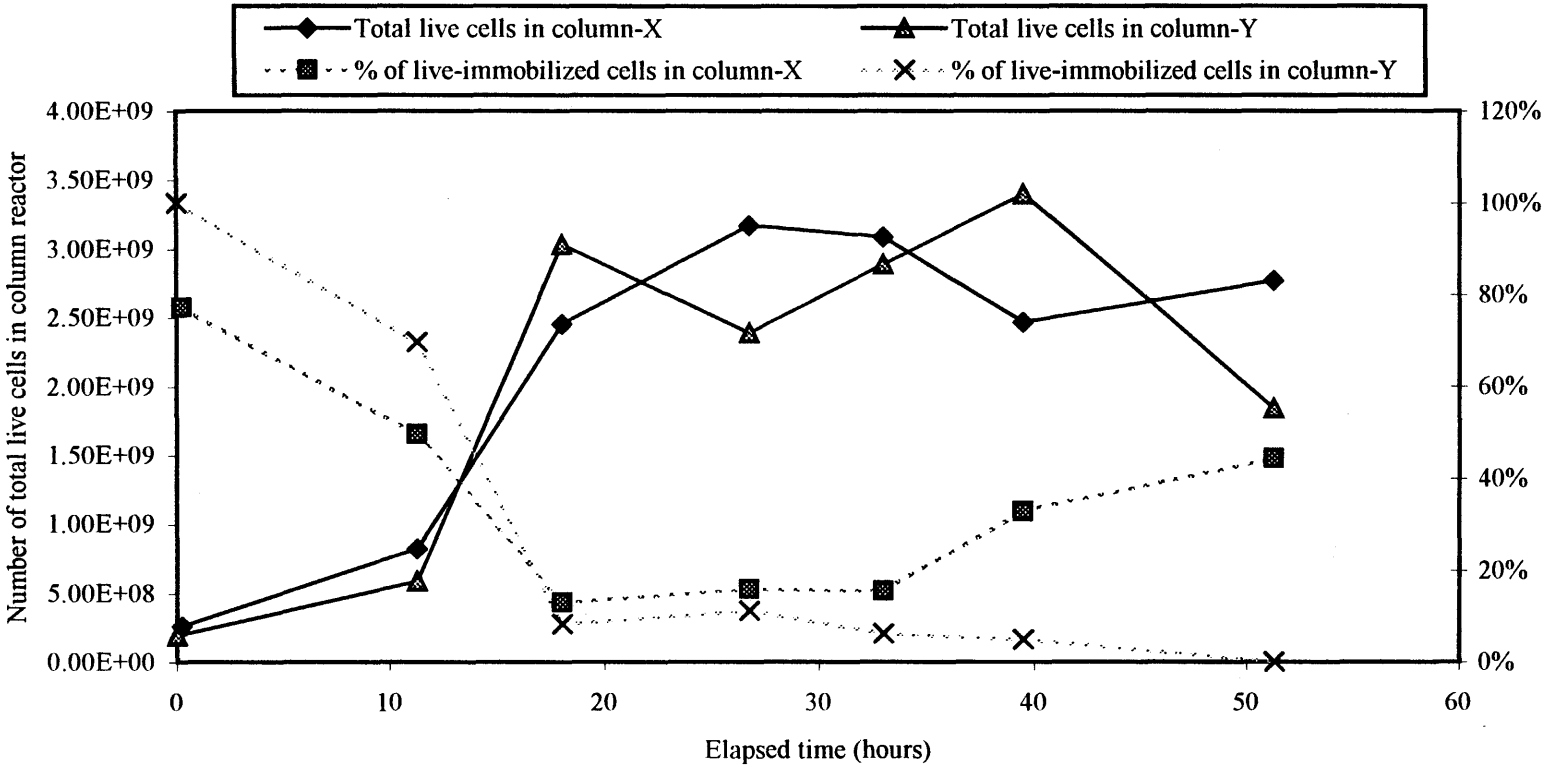


Figure H-3 Comparison of total live cells in column-X and -Y of continuous flow system for biodegradation of naphthalene by *Pseudomonas putida* .

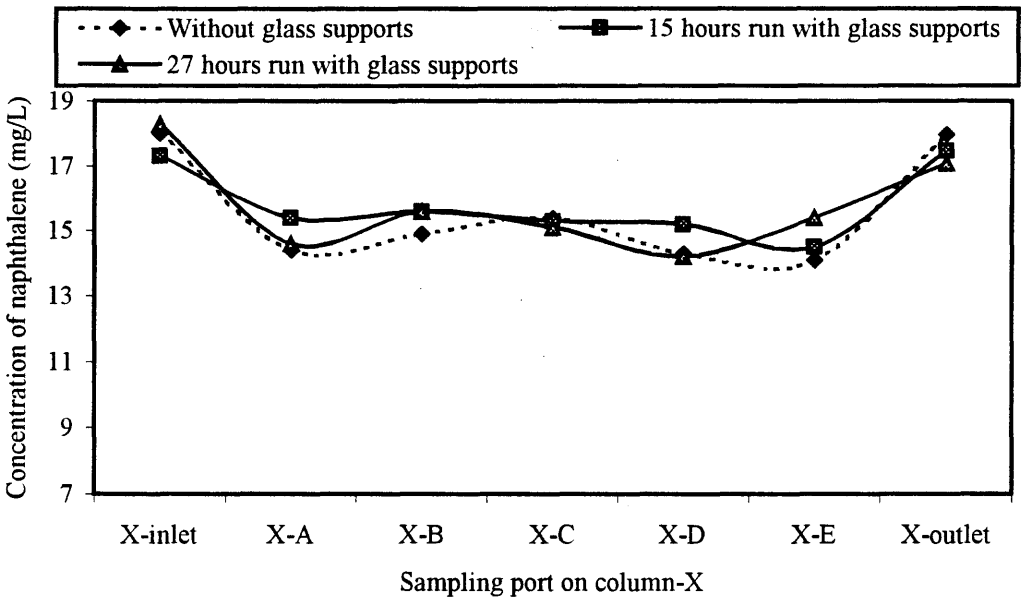


Figure H-4 Concentration profiles for control run on column-X.
(at 2, 7, 12, 17, 22 cm, inlet and outlet from the base of column)

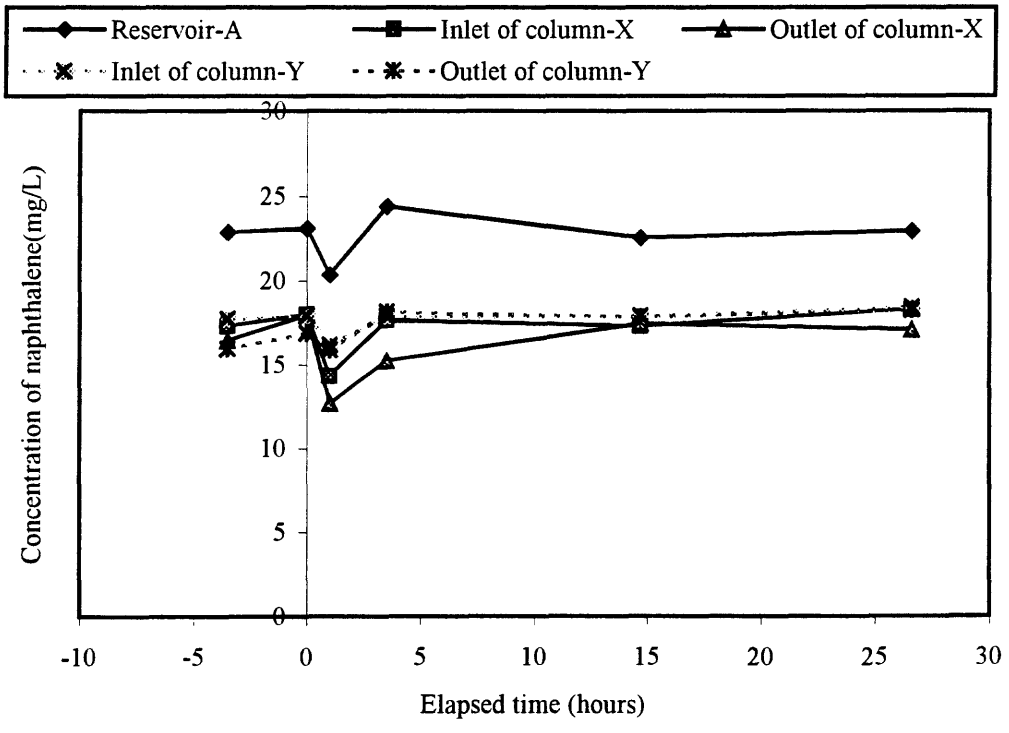


Figure H-5 Countrol runs with (column-X) and without (column-Y) glass supports without biofilm in a continuous flow system.
(Time "0" : starting time after installing glass supports on column-X; control run for about 27 hours)

Table H-7 Concentration profiles of naphthalene at different sampling ports of column-X in continuous flow system for biofilm cultures with *Pseudomonas putida* .

Time (hours)	Concentration of naphthalene on column-X (mg/L)				
	Inlet	X-A	X-B	X-C	Outlet
-11.2	19.10	18.82	19.02	18.39	18.73
-7.4	19.64	19.18	16.83	17.97	19.20
0	20.18	19.02	18.89	18.59	19.33
0.25	19.63	17.30	16.73	15.49	15.45
7.5	19.71	18.03	18.22	18.00	17.68
11.25	19.23	15.79	15.47	15.30	16.90
18	19.22	14.83	13.16	12.93	13.87
26.7	20.04	14.34	14.25	14.03	15.02
33	19.45	14.72	14.62	13.21	13.96
39.5	20.77	13.79	13.94	13.06	13.39
51.3	20.52	17.50	14.95	14.95	15.37

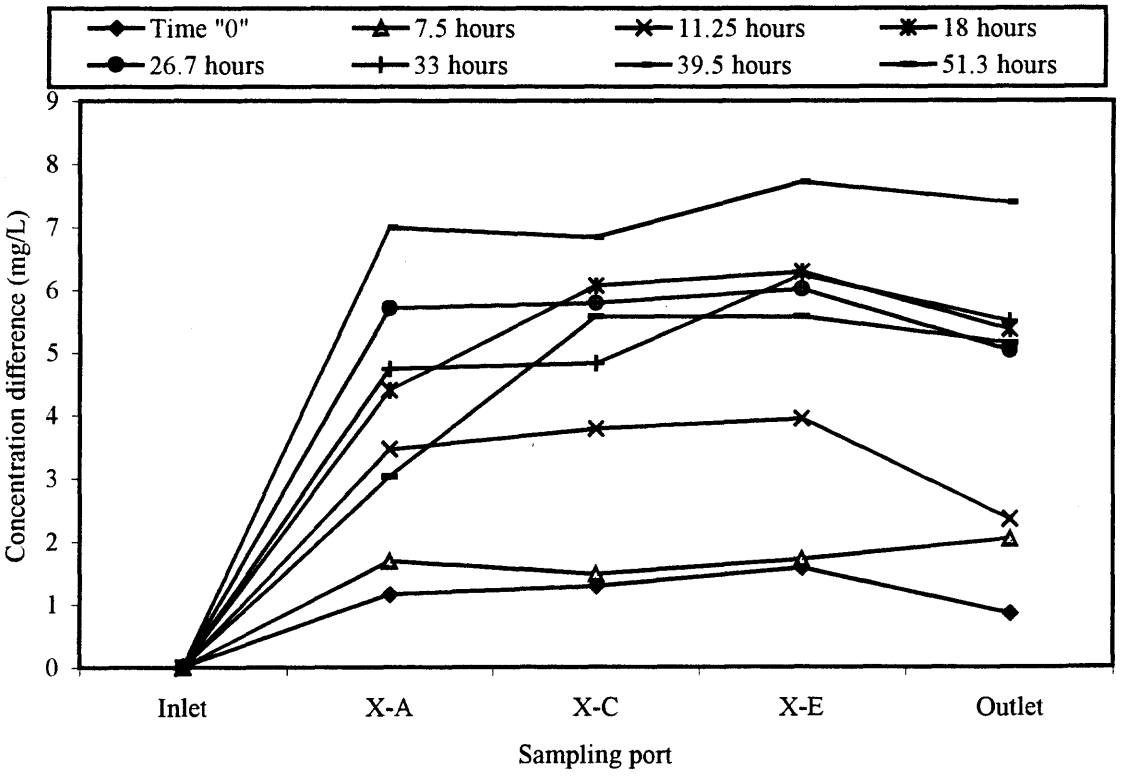


Figure H-6 Profiles of naphthalene consumption along with sampling ports in column-X of continuous flow system for biofilm cultures with *Pseudomonas putida.* (based on inlet concentration at each sampling time)

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