NANYANG TECHNOLOGICAL UNIVERSITY

MICROBIAL DEGRADATION OF PHTHALIC ACID AND ITS ESTERS BY AEROBIC GRANULES IN SEQUENCING BATCH REACTOR

ZENG PING

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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Microbial Degradation of Phthalic Acid and Its Esters by Aerobic Granules in Sequencing Batch Reactor

Zeng Ping

School of Civil and Environmental Engineering

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ABSTRACT

Phthalates, including phthalic acid (PA) and phthalic acid esters (PAEs), have wide application in industry and have become ubiquitous pollutants in the environment. However, the conventional activated sludge process cannot effectively treat recalcitrant phthalate wastewater. Bioaugmentation is one of the strategies for enhancement of the recalcitrant compounds degradation. A key ingredient for a successful bioaugmentation strategy is bioseeds selection.

Phthalic acid-degrading aerobic granules (PA-degrading aerobic granules) were found to be potential bioseeds for PA and PAEs degradation based on the study of the formation and characteristics of PA-degrading aerobic granules. First, they can be formed and matured in three weeks at an OLR of 3.0 kg COD m^{-3} d^{-1}. Second, they provided high concentration of biomass which was certified to be useful for the degradation of PA and PAEs. They also can sustain as the high PA concentration as 4000 mg l^{-1}. And their compact structures could provide protection for the functional cells in aerobic granules. For the first time, the PA-degrading aerobic granules were considered as bioseeds for enhancement of PA and PAEs' degradation. The rapid and easy formation of PA-degrading aerobic granules makes it feasible for further application.

The bioaugmentation strategy using PA-degrading aerobic granules as bioseeds has been successfully applied in phthalate degradation. The performances were investigated under different operation conditions, from the low organic loading rate (OLR) of 1.5 kg COD m^{-3} d^{-1} to the high OLR of12.4 kg COD m^{-3} d^{-1}, and from the biodegradable di-methyl phthalate (DMP) wastewater to the recalcitrant di-butyl phthalate (DBP) wastewater. The highest PA degradation OLR achieved by a PA-degrading aerobic granular system was 12.4 kg COD m^{-3} d^{-1}, with the total organic carbon (TOC) removal efficiency higher than 97.2%. When degrading DMP, the system innoculated with PA-degrading aerobic granules can reach steady state in 15 days at an OLR of 3.0 kg COD m^{-3} d^{-1}. The highest OLR of 6.0 kg COD m^{-3} d^{-1} can be achieved by PA-degrading aerobic granules with a DMP removal efficiency of 100%. For the recalcitrant compound DBP, the system innoculated

with PA-degrading aerobic granules could treat DBP at OLR of 1.5 kg COD m^{-3} d^{-1}, with a DBP removal efficiency of 100%. Such OLR has not been achieved by the conventional treatment processes in the DBP degradation. Thus, the PA-degrading aerobic granules exhibited good performance as bioseeds for bioaugmentation compared with the DMP acclimated activated sludge. The system innoculated with PA-degrading aerobic granules also exhibited a good performance in phthalates degradation compared with the conventional PAEs treatment processes. This application provides a better choice for the industrial phthalates wastewater treatment.

To probe deeper into the underlying reasons behind the successful application of PA-degrading aerobic granules as bioseeds, the variations of degradation rates and enzyme activities were traced in the DMP degradation process. The PA-degrading aerobic granules were found to originally possess constitutive enzymes catalyzing DMP and mono methyl phthalate (MMP) hydrolysis. They also have dioxygenase with high enzyme activity since they are cultivated with PA. During the DMP degradation process, the activity of esterase $_{(DMP)}$ increased from 1.01×10^{-3} to 0.112 U mg^{-1} protein. The activity of esterase $_{(MMP)}$ increased from 0.062 to 0.342 U mg^{-1} protein. A high activity of dioxygenase was maintained in the range from 0.097 to 0.133 U mg^{-1} protein. As a result, the reaction rates of DMP degradation changed from $k_1 > k_3 > k_2$ to be $k_3 > k_2 > k_1$, which makes the phthalate degradation proceed smoothly. Thus, it is quite important to keep the enzyme activity and the order of the reaction rates in $k_3 > k_2 > k_1$ in phthalates industrial wastewater treatment.

16 rDNA denaturing gradient gel electrophoresis (DGGE) and culture isolation methods were combined to assess the change of microbial community structure and the functionally important species in PA-degrading aerobic granules cultivated at an OLR of 3.0 kg COD m^{-3} d^{-1}. Although the microbial community structure of PA-degrading aerobic granules has changed during the DMP degradation process, some dominant species kept constant during the entire process. Further studied indicated that strain PA-02, a dominant species in PA-degrading aerobic granules,

possessed the ability to degrade both PA and PAEs. In the initial stage of DMP degradation, they were the critical DMP degraders, which contributed to the steady operation of the reactor. Kinetic studies revealed that PA-02 was robust under high concentrations of DMP and PA. Even when the PA concentration was increased to 1000.0 mg l^{-1}, the specific PA degradation rate was about 0.25 g-PA g^{-1} biomass h^{-1}. The corresponding value for DMP was 0.067 g-DMP g^{-1} biomass h^{-1} at 1000 mg l^{-1}. Thus the improvement of this functional bacteria ratio by seeding with PA-aerobic granules will enhance the degradation efficiency in the future application of this bioaugmentation strategy.

The storage experiment results showed that the 8 weeks storage had no significant influence on the structure of PA-degrading aerobic granules. However, more than 94% bioactivity of PA-degrading aerobic granules in terms of SOUR was lost after storage. In recovery experiments using storage aerobic granules as inocula, the TOC removal efficiency, PA degradation ability and bioactivity were completely resumed within a week. Thus, the storage aerobic granules also can be used as bioseeds to quickly start up an aerobic granular reactor. Besides fresh aerobic granules, the stored aerobic granules can be applied in industrial wastewater treatment as a source of bioseeds.

This study provided the information of phthalates wastewater treatment by the system inoculated with PA-degrading aerobic granules. Such information would be essential for the design of compact, high-rate aerobic granular systems for the treatment of industrial phthalates wastewater. This study probably for the first time shows that the PA-degrading aerobic granules could be used as a bioseeds for efficient phthalates removal from industrial wastewater.

Key Words:

Aerobic granules; Phthalate degradation; cell immobilization; Metabolism, Enzyme; Microbial community; Bioaugmentation; Kinetics; Isolation; Denaturing gradient gel electrophoresis(DGGE); Dominance

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NOMENCLATURE

APHA	American public health association
BOD	biological oxygen demand ($mg \cdot l^{-1}$)
C_0	initial concentration ($mg \cdot l^{-1}$)
C_e	equilibrium concentration ($mg \cdot l^{-1}$)
CFU	colony forming unit
CLSM	confocal laser scanning microscope
COD	chemical oxygen demand ($mg \cdot l^{-1}$)
DGGE	denaturing gradient gel electrophoresis
dNTPs	deoxynucleotide triphosphates
DO	dissolved oxygen ($mg \cdot l^{-1}$)
DW	dry weight
ECP	extracellular polymer
EPA	environmental protection agency
F/M	feed/microorganism
FISH	fluorescence in situ hybridization
HLR	hydraulic loading rate ($m \cdot day^{-1}$)
HRT	hydraulic retention time (hour or day)
IA	image analysis
ICP	inductively coupled plasma emission
k_d	endogenous metabolism or decay rate (h^{-1})
K_i	inhibition constant ($mg \cdot l^{-1}$)
K_s	half-saturation constant ($mg \cdot l^{-1}$)
MLSS	mixed liquor suspended solids ($mg \cdot l^{-1}$)
MLVSS	mixed liquor volatile suspended solid ($mg \cdot l^{-1}$)
NCBI	national centre for biotechnology information
OD	optical density
OLR	organic loading rate ($kg\ COD \cdot m^{-3} \cdot day^{-1}$)
OUR/OCR	oxygen uptake rate/oxygen consumption rate ($mg\ O_2 \cdot h^{-1}$)
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PN	proteins
PS	polysaccharides
PUM	phosphate urea magnesium
q	specific degradation rate ($mg \cdot mg^{-1}$ biomass $\cdot h^{-1}$)
Q	specific oxygen utilization rates (mg $O_2 \cdot g^{-1}$ biomass$\cdot h^{-1}$)
RDP	ribosmal database project
s	substrate concentration ($mg \cdot l^{-1}$)
s_0	initial substrate concentration ($mg \cdot l^{-1}$)
SBR	sequencing batch reactor
SDS	sodium dodecyl sulfate
SEM	scanning electron microscope
SLR	sludge loading rate (kgCOD$\cdot kg^{-1}$MLSS$\cdot day^{-1}$)
SOUR	specific oxygen uptake rate ($mgO_2 \cdot gMLVSS^{-1} \cdot h^{-1}$)
SRT	sludge retention time (hour)
SS	suspended solid concentration ($mg \cdot l^{-1}$)
SV	settling velocity ($m \cdot h^{-1}$)
SVI	sludge volume index ($ml \cdot g^{-1}$)
TOC	total organic carbon
TOUR	total oxygen utilization rate
TS	total solids ($mg \cdot l^{-1}$)
UASB	upflow anaerobic sludge blanket reactor
V	volume of liquid in the reactor (l)
VLR	volumetric loading rate (kg COD$\cdot m^{-3} \cdot day^{-1}$)
X	biomass concentration ($mg \cdot l^{-1}$)
X_0	initial biomass concentration ($mg \cdot l^{-1}$)
Y	true yield of microorganism (mg biomass$\cdot$ mg^{-1} substrate)
Y_c	theoretical yield of cell mass on phthalate (mg biomass$\cdot mg^{-1}$ substrate)
Y_E	yield of cell mass on substrate consumed for energy (mg biomass$\cdot$ mg^{-1} substrate)
Y_{obs}	observed biomass yield (mg biomass$\cdot mg^{-1}$ substrate)

ΔX	net growth of biomass ($mg \cdot l^{-1}$)
μ	specific growth rate (h^{-1})

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Zeng P., Benjamin, Y.-P. Moy, J.-H. Tay (Jan, 2008) **Biodegradation of Dimethyl Phthalate by *Sphingomonas* sp. Isolated from Aerobic Granules,** IWA Biofilm Technologies Conference, (8-10 January, 2008), *Singapore; (accepted)*

Zeng P., Stephen T.-L. Tay, J.-H. Tay (May, 2006) **Microbial Biodegradation of Dimethyl Phthalate by Phthalic Acid Aerobic Granules in Sequencing Batch Reactors,** *IWA 3rd biennial international young researchers conference (24-26 May, 2006), Singapore;* embodied by a book of IWA Water and Environmental Management Series, Editors: Stuetz Richard and Teik Thye Lim, IWA Publishing, London, pp. 485-492.

Zeng P., J.-H. Tay, Stephen T.-L. Tay (May, 2005) **Aerobic Granulation as a Novel Hybrid Biological Technology for Removal of High Concentration Phthalic acid Wastewater,** *NTU-Stanford symposium on the environment-technology challenges for water resources and the environment, Singapore*

JOURNAL PAPER

Zeng P., W.-Q. Zhuang, Stephen T.-L.Tay, J.-H. Tay (2007) **The influence of storage on the morphology and physiology of phthalic acid-degrading aerobic granules**, Chemosphere, 69(11): 1751-1757

J.-H. Tay**, Zeng P.,** Stephen T.-L. Tay (2006) **Aerobic granules as novel bioseeds for bioaugmentation of phthalate removal**, Applied Microbiology and Biotechnology (submitted)

CHAPTER 1

INTRODUCTION

1.1 Background

The twentieth century is the age of organic chemistry. The dramatic increase in the production of synthetic fibers, processed foods, pesticides, plastics, pharmaceuticals, and other materials has completely altered the immediate human environment and introduced a plethora of new materials (Giam et al., 1984).

As a result of this booming production of man-made chemicals, the natural environment has been transformed either intentionally or unintentionally. The enormous production of synthetic organic chemicals has resulted in the release of a wide variety of chemicals into the environment, which play a significant role in the natural life-cycle. During the past decades, extensive research has focused on the ecological impact of both controlled and uncontrolled discharges of these primarily anthropogenic (xenobiotic) compounds into the natural environment (Giam et al., 1984; Kleerebezem, 1999a).

Phthalates and corresponding esters are included in these primarily synthetic materials. As the main chemicals in industry, phthalates have been widely used as plasticizers to increase flexibility of plastics. A plasticizer is not bound covalently to the plastic resin and is therefore able to migrate into the environment, either leaching from products or through the non-bound application of phthalic acid esters (PAEs) as pesticide carriers, components of cosmetics, fragrances and insect repellents (Bauer and Hermann, 1997). It is reported that phthalates and PAEs have been commonly found in sediments, waters, and soils (Shelton et al., 1984; Fatoki et al., 1993; Huang et al., 1994).

Some phthalates and their degradation intermediates are suspected to be mutagens (Kozumbo et al., 1982), carcinogens (Huff et al., 1984), and their possible effects on the decreasing quality of sperm in the western world are also of concern (Jobling et al., 1995; Colon et al., 2000; Petrovic et al., 2001). Six of them, namely, di-methyl phthalate (DMP), di-n-butyl phthalate (DBP), di-noctyl phthalate (DOP), diethyl phthalate (DEP), di-isononyl phthalate (DINP), and di-(2-ethylhexyl) phthalate (DEHP), have been listed as priority pollutants by the US Environmental Protection Agency (US EPA, 1992). This has consequently led to the increasing public discussion concerning the application of these compounds in industry, especially as plasticizers in the manufacturing of polyvinyl chloride (PVC).

It is generally accepted that most PAEs come from the human environment (Giam et al., 1984; Ribbons et al., 1984). Due to their extremely long half-life in abiotic and biotic environments, PAEs may be regarded as highly persistent compounds (Wolfe et al., 1980a; Giam et al., 1984). Huang et al. (1994) also found that DBP and DEHP were the main refractory organic compounds in municipal wastewater which usually remained in the effluent of the conventional activated sludge plants. Wu et al. (2000) set up an integrated vertically constructed wetland system to treat DBP polluted wastewater. They reported that the removal efficiency was only about 79%, under such a low organic loading rate of 4.1 mg DBP l^{-1} d^{-1}.

The slow hydrolysis rate is one of the main reasons for the difficult degradation of PAEs (Xia et al., 2002). The more complex the structure of the ester moiety is, the more difficult is the hydrolysis (Wolfe et al., 1980a, b; Giam et al., 1984). At the same time, the decrease of pH value in the reactor due to the production of phthalic acid (PA) during the hydrolysis of PAEs can also cause difficult degradation of PAEs. The low pH value is inhibitory to the microorganisms for further degradation of PAEs. Besides, PAE degrading enzymes may also be susceptible to the low pH of

the medium in the reactor.

In order to improve the degradation of refractory compounds, one strategy is to utilize specific microbial cultures that are highly active in degrading recalcitrant compounds. However, isolating high-degradation-efficiency pure culture is an expensive process. Another strategy is to provide high concentration of biomass to treat them. In addition, Fan et al. (2001) reported the possibility to accelerate the degradation of the recalcitrant phthalate compounds. The addition of PA or PA-degrading microorganisms to PAE-containing wastewater could stimulate the complete biodegradation of PAEs due to the existence of bacteria and active enzyme system. Sivamurthy et al. (1989) also found that *Bacillus sp*. grown on terephthalate could use di-methyl terephthalate as a sole carbon source. Therefore, retention of these active microorganisms in the target environment is the key factor for successful application of these strategies.

Immobilization of biocatalysts (enzymes and cells) has received increasing interest in recent years. It offers a promising potential for the improvement of the efficiency of the bioprocess. Compared to free cells, immobilized cells have the advantages as follows: 1) the biodegradation rate can be increased through a higher cell loading; 2) the bioprocess can be controlled more easily; 3) the continuous process can take place at a high dilution rate without washout; and 4) the catalytic stability of biocatalysts, as well as the tolerance against toxic compounds, can be improved. Immobilized cells have been widely used to investigate the biodegradation of numerous toxic compounds such as pentachlorophenol (O'reilly et al., 1989), 4-chlorophenol (Balfanz et al., 1991), benzene derivatives and chlorobenzoates (Sahasrabudhe et al., 1988). Wang et al. (1997a) immobilized *Pseudomonas sp.* isolated from coke-plant wastewater treatment plant sludge into polyvinyl alcohol (PVA) beads. The degradation rate of the immobilized cells was higher than that of

free cells. Juneson et al. (2002) reported the treatment of di-methyl phthalate (DMP) using cultivated species in a biofilm reactor. The biofilm reactor exhibited better performance than an activated sludge system in DMP degradation.

Aerobic granulation, a new form of cell immobilization for exploitation in biological wastewater treatment, has attracted recent research attention (Beun et al., 2000; Tay et al., 2001b; Moy et al., 2002; Dulekgurgen, 2003; Arrojo et al., 2004; Mcswain et al., 2005). It has been reported that aerobic granulation systems could be used to efficiently treat wastewaters contaminated by diverse types of pollutants (Jiang et al., 2002; Moy et al., 2002; Kim et al., 2004; Li et al., 2004; Tay et al., 2005). Compared to conventional activated sludge systems, aerobic granulation systems have a number of advantages; for example, regular and strong structure, good settling ability, high biomass retention, and ability to withstand high organic loading rate and toxic compounds, less investment costs and lower operational costs (Jiang et al., 2002; Liu et al., 2002; De Bruin et al., 2004; De Kreuk et al., 2004a; b; Mcswain et al., 2005). Due to these characteristics, the aerobic granules cultivated with PA have the potential to be used as bioseeds for accelerating the degradation of recalcitrant phthalates (Tay et al., 2005; Zeng et al., 2006, 2007).

In this study, there is several research questions needed to be answered. Can the biological degradation of phthalate esters be enhanced by bioaugmentation of phthalate acid (PA)-degrading aerobic granules? Is it a useful strategy to treat recalcitrant phthalate compounds using PA-degrading aerobic granules as microbial seeds? What are the metabolic and enzyme changes during phthalate esters' degradation by PA-degrading aerobic granules? Are there changes of microbial ecology structure of the PA-degrading aerobic granules for PAEs' degradation, and what are the roles of these microbial populations on the degradation of PAEs? However, most of the studies hitherto were carried out using activated sludge or

anaerobic systems (Lau, 1978; Tur et al., 1997; Kleerebezem et al., 1999b). The performance of the system inoculated with aerobic granules in the degradation of phthalates and the influence of the bioaugmentation on the microbial diversity of aerobic granules, are not yet well understood.

1.2 Objectives of Study

The main research objectives are as follows:

1. To identify the possibility of the PA-degrading granules as bioseeds for bioaugmentation based on the study of PA-degrading aerobic granules' formation and characteristics;
2. To study the phthalate esters degradation, especially recalcitrant phthalate esters degradation by the PA-degrading aerobic granules;
3. To investigate the metabolic and enzymatic changes in PA-degrading granules when PAEs are used as substrates;
4. To examine the change of microbial community structure in PA-degrading aerobic granules during PAE degrading;
5. To identify the important microbial populations in PA-degrading aerobic granules being capable of phthalate esters degradation.

The research will be helpful in understanding the strategy of using aerobic granules as microbial seeds to enhance the degradation of industrial wastewaters containing phthalates, especially recalcitrant phthalates.

This thesis is organized into eight parts. Chapter 1 gives an overview of the research and describes the structure of the entire thesis. Chapter 2 is a detailed literature review discussing phthalate wastewater treatment and the phenomenon of aerobic granules. Chapter 3 describes the formation process and the property of

PA-degrading aerobic granules. The application of PA-degrading aerobic granules for the treatment of high strength PA wastewater, DMP and DBP wastewater is discussed in Chapter 4. Chapter 5 describes the metabolic and enzymatic variations during the process of PAE degradation by PA-degrading aerobic granules; whereas Chapter 6 explores the change of microbial community structure during DMP degradation by PA-degrading aerobic granules, the microbes in aerobic granules with both PA and PAEs degradation ability which contributed to the reactor's stable operation. Chapter 7 studies the influence of storage on the morphology and physiology of PA-degrading aerobic granules. Chapter 8 draws the important conclusions from the experiment results and outlines some recommendations for future works.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Phthalates, including phthalic acid (PA) and phthalate esters (PAEs), are primarily anthropogenic compounds (Kleerebezem et al., 1999b; Qiu et al., 2004). Phthalate esters (PAEs) are derivatives of PA with systematic name of 1, 2 benzenedicarboxylic acid (Fig. 2-1). There are two other benzenedicarboxylic acids, viz. the 1, 3 isomer (isophthalic acid) and the 1, 4-isomer (terephthalic acid) (Fig. 2-1). However, isophthalic acids, terephthalic acids and their derivatives have limited environmental distribution, and there is little information concerning the environmental significance of terephthalates and isophthalates (Giam et. al., 1984). Therefore, this review will be mainly focused on PA and their esters.

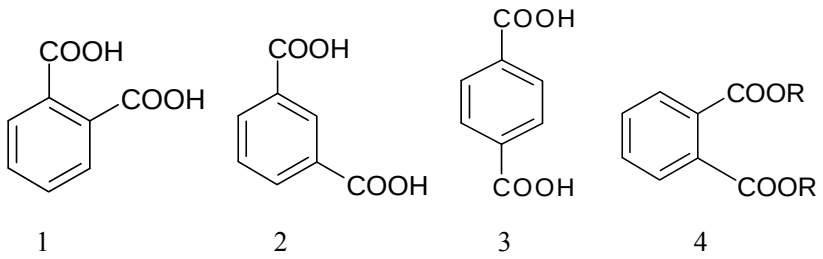


Fig. 2-1 Isomer and derivatives of Phthalic acid: 1, phthalic acid; 2, isophthalic acid; 3, terephthalic acid and 4, phthalate esters

2.1.1 Production of Phthalates and PAEs

The general structure of a di-alkyl phthalate is an aromatic ring with two flexible, often nonlinear, aliphatic side chains. The polar carboxyl group is buried in the middle of the molecule (except for small R groups such as methyl and ethyl) (Fig

2-2).

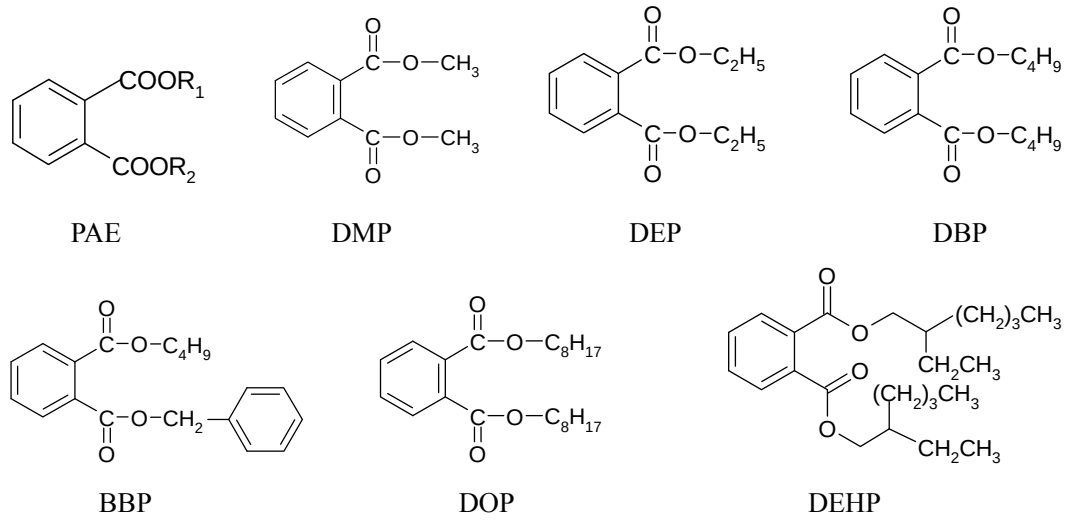
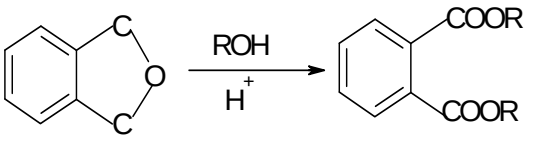
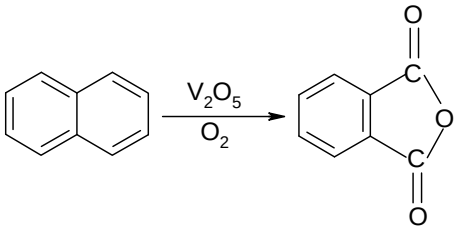


Fig. 2-2 A diagram of the chemical structures of typical PAEs in this study

Phthalate esters are synthesized from phthalic anhydride and the corresponding alcohol by the Fischer esterification (Eq. 2-1). Phthalic anhydride is produced by the gas phase oxidation of naphthalene (Eq. 2-2) obtained from coal tar or o-xylene (Eq. 2-3) over vanadium pentoxide (Weissermel et al., 1978; Wiseman, 1979) or the liquid phase oxidation of o-xylene with soluble salt catalysis(Eq. 2-3).



(2-1)



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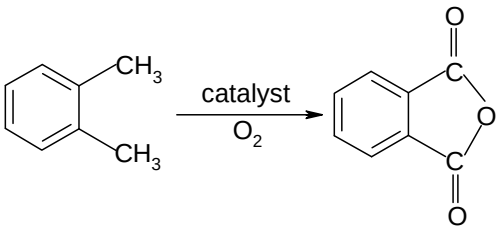


(2-3)

Worldwide production of phthalate was estimated to be 2.4 to 7.5 million metric tons per year (Bauer et al., 1997; Alatriste-Mondragon et al., 2003; GÓmez-Hens and Aguilar-Caballos, 2003). Most of it was used for PVC production. The phthalate plasticizers may make up to 67% by weight of the final product (Giam et. al., 1984; Kleerebezem et al., 1999b).

2.1.2 Application of Phthalates and Phthalate Esters

Because of the efficacies of stability, fluidity and low volatility, phthalate esters were introduced in the 1920's as plasticizers to overcome the problems caused by the excessive volatility and undesirable odor of camphor, which was used as a plasticizer in celluloid (Chang, 1997). Plasticizers are organic chemical substances added to a plastic resin that result in a more flexible and easier to fabricate product. Phthalates were later used in polyvinylchloride (PVC) with the commercial availability of PVC in 1931 (Chang, 1997; Kleerebezem, 1999a).With the boom of the plastic industry, phthalate esters became the predominant plasticizer produced in large quantities world wide. Plasticized PVC is used in a wide range of products such as floor and wall coverings, electrical insulation, clothing, plastic toys, medical tubing, and foot wear (Chang, 1997). In addition to their use as plasticizers in plastics, phthalates have also been utilized in manufacturing insect-repellents, pesticide carries, munitions, fragrance carriers and cosmetics (Chang, 1997; GÓmez-Hens and Aguilar-Caballos, 2003)

2.1.3 Chemical and Physical Properties of Phthalates and Phthalate Esters

A number of the important physicochemical properties of phthalate esters are listed in Table 2-1. The information on phthalate ester properties is helpful to understand their behaviors and fates in the environment.

Almost all the listed phthalates have melting points below 0 °C, except for DMP and MEHP with melting points of 2°C. Phthalate esters have boiling points varying from about 220°C to 384°C. The low melting point and high boiling point of these phthalate esters contribute to their application as plasticizers, heat transfer fluids, and carriers.

Water solubility is an important property that determines the biodegradation and bioaccumulation potential, as well as aquatic toxicity, of a chemical. It also controls the environmental distribution of phthalate esters and affects the extent of leaching of plastic products, the movement and chemical fates of dissolved phthalate esters in rivers, lakes, ground water and in drinking water supplies. Phthalate losses from wastewater treatment facilities, landfills and sludge-amended soils are partially because of aqueous solubility.

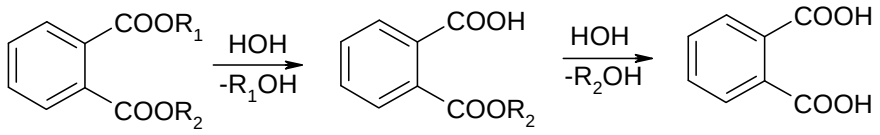
Vapor pressure plays an important role in the fate of fugitive emissions and other releases of phthalate esters to the atmosphere. Vapor pressure is typically determined by direct pressure measurement at elevated temperatures. With increasing alkyl chain length of phthalate esters, the volatilization of the phthalate ester decreases with decreasing vapor pressure.

Table 2-1 Chemical and physical properties of common phthalates

Compound	Abbreviation	mp(°C)	bp(°C)	Water solubility (mg l^{-1})		Vapor pressure (mmHg at 25 °C)		Log K_{ow}
Phthalic acid	PA	230		7000	(Chemfinder, 2005)	6.4×10^{-7}	(Chemfinder, 2005)	0.73
Di-methyl phthalate	DMP	2	284	4290	(Leyder and Boulanger, 1983)	3.08×10^{-3}	(Chemfinder, 2005)	1.53
				4320	(Wolfe *et al.*, 1981)			
Di-ethyl Phthalate	DEP	-3	298	928	(Leyder and Boulanger, 1983)	2.1×10^{-3}	(Weast, 1980)	2.35
				896	(Wolfe et al., 1981)			
Di-butyl phthalate	DBP	-35	340	10	(Leyder and Boulanger, 1983)	2.01×10^{-5}	(Staples *et al.*, 1997)	4.57
				13	(Wolfe et al., 1981)			
				20	(Giam *et al.*, 1980)			
Di-octyl phthalate	DOP		220[5]	3	(Wolfe et al., 1981)	1×10^{-7}	(Gray *et al.*, 1982)	8.01
Di-(2-ethylhexyl) phthalate	DEHP	-50	384	0.04	(Leyder and Boulanger, 1983)	1.42×10^{-7}	(Staples et al., 1997)	9.64
				0.4	(Giam et al., 1980)			
Butyl benzyl phthalate	BBP			3	(Leyder and Boulanger, 1983),	8.25×10^{-6}	(Gledhill et al., 1980)	4.91
Mono(2-ethylhexyl) phthalate	MEHP	2						
Di-isobutyl phthalate	DiBP		295.8	20	(Chemfinder, 2005)	6.65×10^{-3}	(Chemfinder, 2005)	4.11

The constant K_{ow} indicating equilibrium distribution of an organic chemical between water and octanol is an important physical constant for predicting the tendency of a chemical to partition to water, animal lipids, sediments, and soil organic matters. Octanol is believed to best imitate the fatty structures in plants and the aqueous phases of living tissues. So the distribution of a chemical in octanol is looked on as that in fatty materials. A high K_{ow} value means more distribution of the chemical in octanol than water. That indicates the greater hydrophobicity of the chemical. For phthalates, with increasing alkyl chain length, the log K_{ow} increases, so the long alkyl chain phthalates are more hydrophobic than short alkyl chain phthalates

Phthalates are stable chemicals in the environment. The principal chemical reaction of phthalate esters is hydrolysis (Eq. 2-4). This reaction is catalyzed by both acid and base. This reaction can undergo two hydrolysis steps, producing first the mono-ester and one free alcohol moiety and a second hydrolytic step creating PA and a second alcohol (Shelton et al., 1984; Staples et. al., 1997). The rate of ester hydrolysis is very slow, and acid hydrolysis of phthalate esters is estimated at four orders of magnitude slower than alkaline hydrolysis rate constants (Maybey et al., 1982). The hydrolysis rate is also strongly influenced by steric hindrance in the alcohol moiety. Hence, the esters derived from hindered alcohols would be more inert than those from straight-chain alcohols. For example, half-lives ranged from about 3 years for DMP to 2000 years for DEHP (Table 2-2). So hydrolysis is unlikely to be an important fate process for phthalate esters under typical environmental conditions (Schwartzenbach et al., 1992).



(2-4)

Photolysis is another important reaction for phthalate esters. Aqueous photolysis occurs through absorption of UV light from sunlight in the region of 290-400 nm. The

shorter wavelengths are attenuated by passage through the atmosphere and water columns. Longer wavelengths lack sufficient energy to break covalent bonds. The mechanism of photolysis may be either through direct absorption of UV radiation by the chemical or by absorption of UV radiation by water with the formation of activated species of singlet oxygen or hydroxy radicals that react with phthalate esters. Photolysis rates of phthalate esters are low. BBP has an aqueous photolysis half-life >100 days (Gledhill et al., 1980). Based on the data estimated by Wolfe et al. (1980a) and Howard (1991), the aqueous oxidation half-lives range from 2.4 to 12 years for DEP and DnBP and from 0.12 to 1.5 years for DEHP.

Table 2-2 Summary of abiotic degradation half-lives of phthalate esters (Staples et al., 1997)

Phthalate ester	Aqueous hydrolysis half lives (years)[a]	Atmospheric photooxidation half lives (days) [b]
DMP	3.2	9.3-93
DEP	8.8	1.8-18
DAP	--	0.04-0.4
DPP	--	0.9-9.0
DnBP	22	0.6-6,0
DIBP	--	0.6-6.0
BBP	>0.3	0.5-5.0
DHP	--	0.4-4.0
DnOP	107	0.3-3.0
BOP	--	0.4-4.0
DEHP	2000	0.2-2.0
DIOP	157	0.3-3.0
DINP	--	0.2-2.0
DIOP	--	0.2-4.0

a: Hydrolysis rate constant to monoester at pH=7, 25 ºC (Wolfe et al., 1980b), except for BBP (Gledhill et al., 1980) and DnOP (Howard, 1991).

b: Predicted half-lives obtained by the Atmospheric Oxidation Program (Atkinson, 1988)

The photolysis rates of phthalate esters in the atmosphere are higher than those in the aqueous environment. Reaction with hydroxyl radicals is generally the most important photodegradation process for organic chemical pollutants in the atmosphere (Atkinson, 1988; Kelly et al., 1994; Meylan et al., 1995). Photo-oxidation half-lives for phthalate esters based on hydroxyl radical attack were obtained from structure activity relationships contained in the Atmospheric Oxidation Program (AOP) (Meylan and Howard, 1993). Results indicated that, as alkyl chain length increases, the photo-oxidation rate of phthalate esters also increases (Table 2-2).

2.1.4 Environmental Contamination by Phthalates and Phthalate Esters

Phthalates and phthalate esters are among the most commonly used industrial chemicals and have become widespread in the environment, especially in lakes, rivers, streams, soils and sediments (Peterson et al., 1982). There were reports about the existence of naturally occurring phthalates, such as intermediates of polycyclic aromatic hydrocarbons (PAH) (Kleerebezem et al., 1999b). However, most of the phthalates in the environment are from man-made sources.

Since phthalates are not chemically bound in plastics, they can be lost from soft plastics over time. The release of phthalates into the ecosystem or wastewater effluent may occur largely during their production, distribution, while making final products or by leaching and volatilization from plastic products during their usage and/or after disposal (Turner, 1985; Psillakis et al., 2004). Table 2-3 shows the amounts of three phthalates released during three years from 1997 to 1999 in the USA. Table 2-4 shows the concentrations of DEHP in industrial wastewater. Another source of phthalates comes from the petroleum industry because it is an intermediate of phenanthrene and 2-naphthoate (Giam et al., 1984; Kleerebezem et al., 1999b). Furthermore, the exchange between these residues as water, air, soil, sediment and ecosystem could widen the distribution of phthalates. The distribution and concentration of some phthalates in nature are shown in Table 2-5.

Although most phthalates exist in the natural ecosystem at trace amounts, the concentrations in the area affected by human beings are very high. PA in industrial wastewater was reported to be 5000-7000 mg l^{-1}, with pH values below 3 (Zhang *et al.*, 2000). PAEs in industrial wastewater ranged from several to hundreds of mg l^{-1} (Table 2-4). Even in river water and tap water, some of them still existed at high levels. For example, several phthalate esters, dimethyl phthalate (DMP), diethyl phthalate (DEP), and di-n-butyl phthalate (DBP), were found present at levels of 10 mg l^{-1} to 1472 mg l^{-1} in river water and 91 mg l^{-1} to 1219 mg l^{-1} in tap water from city water treatment plants (Fatoki et al., 1993).

Table 2-3 Estimated releases of three phthalates during a 3-year period (Toxic Release Inventory, 2001)

Phthalate	Air (kg)	Land (kg)	Water (kg)	Underground injection (kg)	Off-site waste transfer (kg)
Di-n-butyl phthalate	51,819	9,258	302	299,640	1,207,640
Di-(2-ethylhexyl) phthalate	307,469	58,126	2,127	0	7,173,200
Dimethyl phthalate	389,882	7,922	940	3,995	398,996

Table 2-4 Selected DEHP industrial wastewater discharge data (Alatriste-Mondragon et al., 2003)

Industrial class	Number of industrial units in class	Average concentration (mg l^{-1})
Aircraft manufacturing, service and maintenance	12	114.9
Chemical manufacturing and packing	5	312.0
Etchers and engravers	1	456.0
Metal treating	14	135.4

Table 2-5 Summary of phthalate distribution (Hazardous Substances Data Bank, 2001)

Compound	Air (ng m^{-3})	Surface water (ug l^{-1})	Ground water (ug l^{-1})	Drinking water (ug l^{-1})	Sediment/soil (mg kg^{-1})
DMP	0.60 to 1.74 (Office building)	0.002 to 0.7 (Various rivers, USA & Europe)	0.1 (Wastewater treatment system , USA)	0.27 (Maximum concentration, municipal plant, USA)	2×10^{-4} to 0.150 (River, canal sediments, USA, UK& Asia)
DEP	3.7 ± 0.9 (Urban) 3.8 ± 2.8 (Urban) 1.60 to 2.03 (Indoor: home)	0.06 to 0.4 (Various rivers, USA, Europe & Japan)	4.1 (Near landfill, USA)	4.6 (Maximum in well water, USA) 0.011(Mean Concentration, Canada)	3.4×10^{-3} to 0.8 (Lake, river sediments, Canada, USA, Asia & Europe)
DBP		0.20 to 42 (Various rivers, USA, Europe, Japan)	0.4 to 2.38 (Near landfill, sewage contaminated groundwater, USA) Up to 450 mg l^{-1} (Sewage contaminated groundwater ,USA)	0.1 to 470 (Tap water samples of Japan, drinking water supplies in USA)	0.1 to 1100 (River sediments of USA, Europe, Asia & Canada, Estuary of UK)
DOP	Detected (Urban)	0.7 to 20 9 (Lake, rivers, USA & Asia)	100 to 200 (Near waste lagoons of Netherlands, agricultural plot of USA)		0.193 to 25 Largest concentration in sediment near a chemical company (USA), other river sediments (Asia)
	20 and 55 (Office buildings)		1.4 to 8 (Wastewater applied to site, hazardous waste site)	86 (Tap water, mean = 19.6 , USA)	
DEHP	3.6 to 132 (Urban)	0.06 to 80 (Various rivers, USA, Asia & Europe)			0.18 to 70 (River, coastal sediments, USA, Europe & Asia)
	0.77 to 3.6 (Rural/remote)		14 (Mean concentration near wood-treatment facilities, USA)	86 (Various cities, USA)	200 (Lake sediment, USA)
BBP		0.06 to 2.4 (Mississippi River)	2.0 (Well near a chemical plant, USA)	0.1 to 38 g l^{-1} (Various public water supplies, USA)	
DiBP	~9 (Urban)	0.05 to 1.1 (Rivers, Europe)	0.08 to 0.1 (USA & Spain)	0.59 (Drinking water plant, USA)	9×10^{-3} to 105×10^{-3} (River, lake sediments, Europe & Canada)

2.1.5 Toxicity of Phthalates and Phthalate Esters

Concern for the possible health effects of phthalates and phthalate esters has been reported for a number of years. The concern was initiated from their use in many items, such as blood transfusion bags and other medical materials, food bags and toys from which they could easily be transferred to human beings. In the past dozens of years, the toxicity of phthalate on environmental species such as soil organisms, aquatic species, plants and animals has been studied.

For aquatic organisms, BBP, DBP and DEHP have been detected in fish tissues (Mayer et al., 1972; Giam et al., 1978; Persson et al., 1978; Karara et al., 1984). The investigation about acute effect of phthalates in various species of fish showed that many phthalate esters, including BBP and DBP, were quite toxic to fish, but DEHP showed low toxicity (Woodward, 1988). In terms of long-term, chronic effects including reproductive effects, DBP has been proven to be toxic to embyo survival, hatchability and larval survival in the fathead minnow (Mccarthy and Witemore, 1985). In addition, DBP was the most toxic phthalate ester tested for potential effects on the hatching of brine shrimp (Sugawara, 1974)

There were reports about the influence of phthalates on plants. Some crops (tomato, cabbage and radish) planted in greenhouses were severely damaged by DBP and DIBP in the atmosphere released from PVC glazing. Photosynthesis in these plants was hindered by phthalates, especially when poor ventilation happened (Lokke et al., 1983). The Vitamin C and capsicum content in capsicum fruit decreased when capsicum plants were planted in soil contaminated by DBP (Yin et al., 2002)

Researchers started to pay attention to the possible toxicity of phthalates to human beings since Jaeger and Rubin's report was issued in 1970 (Jaeger et al., 1970; Chang, 1997). The acute toxicity, sub-acute toxicity, chronic toxicity, carcinogenic effects, teratogenic effects, mutagenicity, and other physiological effects of phthalates were

studied by many researchers (Woodward, 1988; Jobling et al., 1995; Chatterjee and Dutta, 2003). Although phthalates had a low order of toxicity when given orally or dermally, they were indeed toxic to human beings. Even at very low concentrations, they were suspected of interfering with reproductive systems and behavior in humans and wildlife, through disturbance of the endocrine system (Jobling et al., 1995; Petrovilc et al., 2001; Chatterjee and Dutta, 2003; Xu et al., 2005). As a result, several regulatory bodies, such as the US Environmental Protection Agency (1992), the European Union (1993) and the China National Environmental Monitoring Center (Wang et al., 1995) have listed phthalate esters as top priority pollutants for risk assessment. DEHP was also regulated by the FDA (Food and Drug Administration of USA) for packaging and storing high water content foods (Graham, 1973). Due to these concerns, regulations were set up for phthalates in water (Table 2-6) (Giam et al., 1984). Hence, it is necessary to removal phthalates from wastewater before discharge into natural environments.

Table 2-6 Regulation of phthalates and related compounds (Giam et al., 1984; Go'mez-Hens and Aguilar-Caballos, 2003)

Compound	Regulatory agency	Medium standard
Air standards [a]		
Phthalic anhydride	OSHA [b]	2 ppm
dimethyl phthalate	OSHA	5 mg m^{-3}
dibutyl phthalate	OSHA	5 mg m^{-3}
bis(2-ethylhexyl) phthalate	OSHA	5 mg m^{-3}
Water standards		
dimethyl phthalate	EPA	160 mg l^{-1}
Diethyl phthalate	EPA	60 mg l^{-1}
dibutyl phthalate	EPA	5 mg l^{-1}
Bis(2-ethylhexyl) phthalate	EPA	6 mg l^{-1}

[a] Time-weighted average concentrations

[b] Occupational Safety & Health Administration of US Department of Labor

2.2 Physical and Chemical Treatment of Phthalate Wastewater

The conventional approach in industrial wastewater treatment is by chemical–physical processes: adsorption, photocatalysis or chemical oxidation (Table 2-7). All these processes can achieve high removal efficiencies, but the adsorption treatments have the main disadvantage that they do not provide a real degradation of the compounds but only transfer them from a diluted to a concentrated stream to be ultimately treated or disposed of; on the other hand, chemical oxidation could produce intermediates characterized by a toxicity level similar to the original substance.

Table 2-7 Physical and chemical treatment of phthalates

Chemical	Treatment	Reagent	Removal rate or removal efficiency	Reference
DBP	Adsorption	Soil	40 mg g^{-1}	(Wang et al., 1997b)
DBP	Adsorption	Biomass	72%-79%	(Wang et al., 1994)
Phthalate	Adsorption	Activated carbon	98%	(Adhoum et al., 2004)
DBP	Photocatalysis	$Fe(ClO_4)_3 \cdot 9H_2O$	85%	(Bajt et al., 2001)
DEP	Photocatalysis	Fe(III)	85%	(Mailhot et al., 2002)
KHP*	Photocatalysis	TiO_2	100%	(Alhakimi et al., 2003)
DMP	Oxidation	Fenton reagent	81%	(Zhao et al., 2003)

* potassium hydrogen phthalate

2.3 Biological Treatment of Phthalate Wastewater

An alternative and promising approach is the application of biological treatments that could provide a complete biodegradation of the compound and at the same time are characterized by low investment and operation costs (Tchobanoglous and Burton, 1991; Eckenfelder, 2000). Biodegradation of chemicals has been studied since the 1940s, and the potential application of biological wastewater treatment plants for toxic compounds removal has been proposed since the 1980s. However, biological systems are extremely complex ecosystems which are difficult to operate due to high sensitivity of microorganisms to reaction environment conditions (Brock et al., 1991; Tchobanoglous and Burton, 1991; Grady et al., 1999). Thus, research activities are still required both in process and technology innovation in order to make the biological industrial wastewater treatment really competitive and for application on a larger scale (Brock et al., 1991; Grady et al., 1999).

2.3.1 Phthalate Degradation and Growth of Pure Culture

Phthalate can be degraded either by aerobic, anaerobic or facultative microorganisms (Shelton et al., 1984; Zhang et al., 1990; Wang et al., 1995). Aerobic degradation has been studied since the 1950s. *Pseudomonads* are the most easily isolated phthalate degraders among gram negative bacteria (Evans, 1955). More and more bacteria have been isolated by other researchers, including *P. testosterone* (Ono et al., 1970), *P. fluorescens* (Keyser et al., 1976; Wang et al., 1997a; Zeng et al., 2004), *Agrobacterium, Alcaligens, Bacillus, Micrococus* (Nomura et al., 1989), *Sphigomonas sp.* and *Corynebacterium sp.*(Chang et al., 2004), *Comamonas acidovoran* (Wang et al., 2003b). Although some individual microbes could completely mineralize PA, more efficient metabolism appeared to result from mixed microbial populations, which were typically found in the environment (Kurane, 1986; Staples et al., 1997). The study of anaerobic degradation of phthalate occurred later than for aerobic process. *Bacillus sp.*, *Pseudomonas testosterone* and denitrifying bacterium

Pseudomonas sp. were recently isolated as phthalate anaerobes (Nozawa et al., 1988; Kleerebezem et al., 1999b).

2.3.2 Phthalate Degradation Pathway

The aerobic degradation of PAE involves several transformation steps before the substrate becomes fully mineralized (Staples et. al., 1997). It starts with the hydrolysis of PAE by esterase to mono-alkyl phthalate and PPA. The hydrolysis of the PAE is initiated by the H^+ attacking the carboxyl bond. The smaller the alkyl chain, the barer the carboxyl bonds and the easier the PAEs' degradation are. For PAEs with longer alkyl chains or complex alkyl structures, it is difficult for the radical H^+ to get close to the polar carboxyl bonds and break down (Staples et. al., 1997). The PAEs molecular weight has a positive relation with the structure and longitude of alkyl chains. Thus the higher the PAEs' molecular weight, the more recalcitrant the PAE is (Xia et al., 2002)

Among all the reactions of PAE degradation, the rate-limited step was not related to the cleavage of the aromatic ring in the phthalate molecule, but related to the phthalate monoesters' hydrolysis (Chatterjee and Dutta, 2003). Roslev et al. (1998) also found that the cleavage of the ester bonds in metabolites of DEHP (e.g., mono-(2-ethyhexyl) phthalate was a biological bottle neck for further degradation. That meant the reaction rate of mono-alkyl phthalate hydrolysis was smaller than the rate of di-alkyl phthalate hydrolysis. The small degradation rate of mono-alkyl phthalate hydrolysis would cause the accumulation of mono-alkyl phthalate and PA, which were intermediates of PAE degradation. Accordingly, the pH in the reactor would decrease. Sometimes it decreased to a value inhibitory to the microorganisms for any further degradation of PAE.

Phthalate is further metabolized by two different dioxygenase-initiated pathways through the common intermediate, protocatechuate (3, 4-dihydroxybenzoate) (Fig.

2-3). Gram-negative bacteria (*Burkholderia cepacia*, *Comamonas testosteroni*, and *Pseudomonas sp.*.) transform phthalate through cis-4, 5-dihydroxy-4,5-dihydrophthalate and 4,5-dihydroxyphthalate to protocatechuate, while the gram-positive bacterium *Arthrobacter keyseri* (formerly *Micrococcus sp.*) converts phthalate to protocatechuate through cis-3,4-dihydroxy-3,4-dihydrophthalate and 3,4-dihydroxyphthalate.

Other pathways were also reported besides this main degradation pathway. Cartwright et al. (2000a) found that ethyl methyl phthalate, di-methyl phthalate and mono-methyl phthalate (MMP) were formed when DEP degraded in soil co-contaminated with methanol (Fig 2-4).

Some strains isolated from soil, such as *Arthrobacter sp*. were found without the ability to further degrade MMP, which was a kind of metabolism product. A mechanism of degradation of DMP was proposed with two ways: DMP to PA and DMP to MMP (Fig 2-5). The *S. paucimobilis strain* hydrolyses only MMP, and a co-culture of the two strains allowed a complete degradation of DMP (Vega et al., 2003).

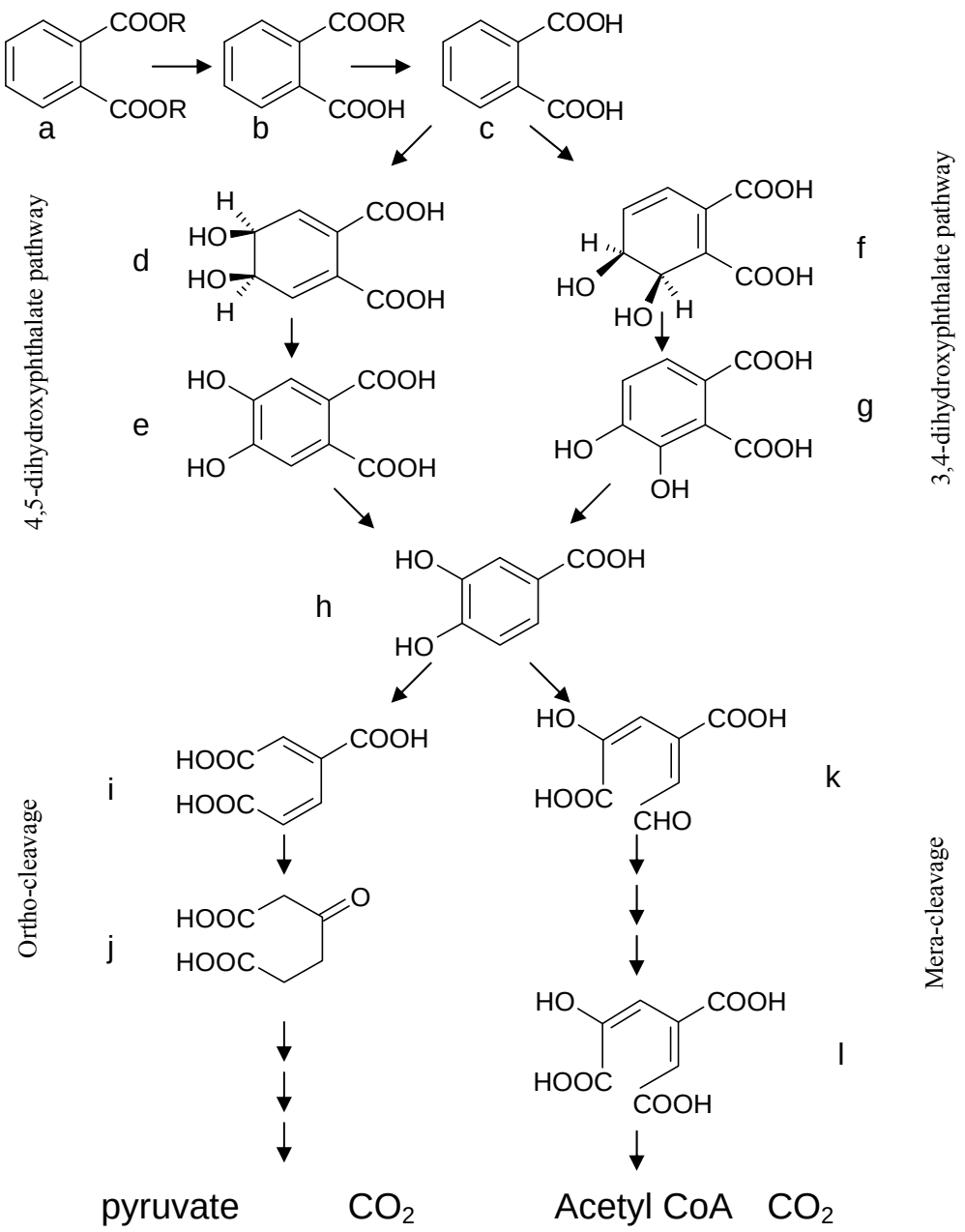


Fig. 2-3 Catabolism pathway of phthalate. a: di-ester, b: monoester, c: phthalate, d: 4, 5-dihydroxy-4, 5 dihydroxyphthalate, e: 4, 5 dihydroxyphthalate, f: 3, 4-dihydroxy-3, 4 dihydroxyphthalate, g: 3, 4 dihydroxyphthalate, h: protocatechuate, i: β-carboxy-cis,cis muconate, j: β-ketoadipate, k: 4-carboxy-2-hydroxymuconic semialdehyde, l: 4-carboxy-2- hydroxymuconate (Chang, 1997)

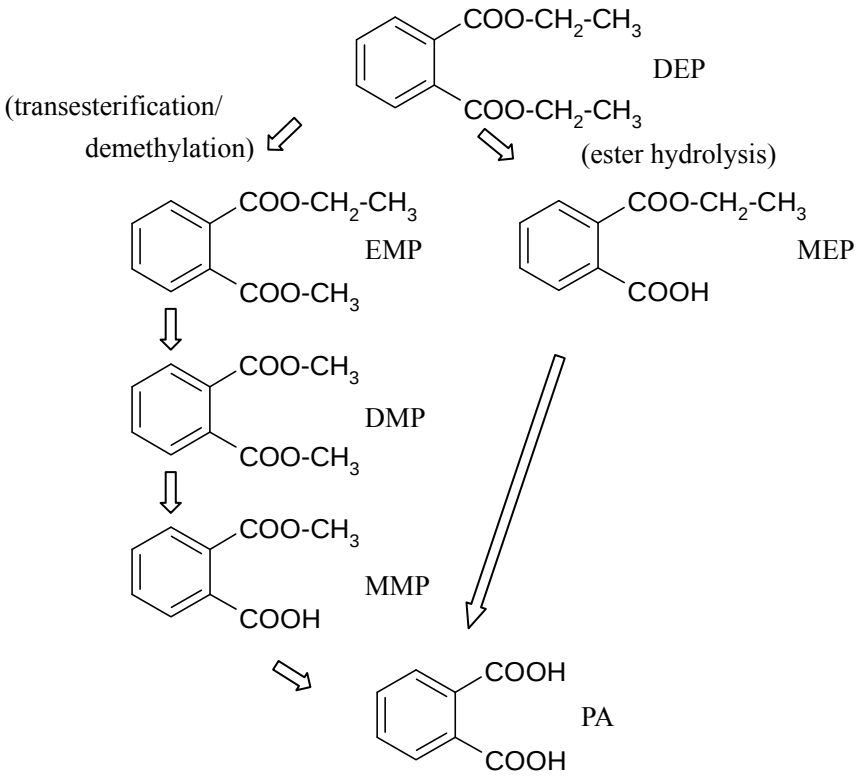


Fig. 2-4 Biodegradation of DEP by the indigenous soil microbial community in soil co-contaminated with methanol (MeOH). Published pathway proceeds by ester hydrolysis of the DEP, whilst proposed pathway involves a de-methylation or trans-esterification of the parent compound and the formation of ethyl-methyl phthalate (EMP), DMP and mono-methyl phthalate (MMP) (Cartwright et al., 2000a)

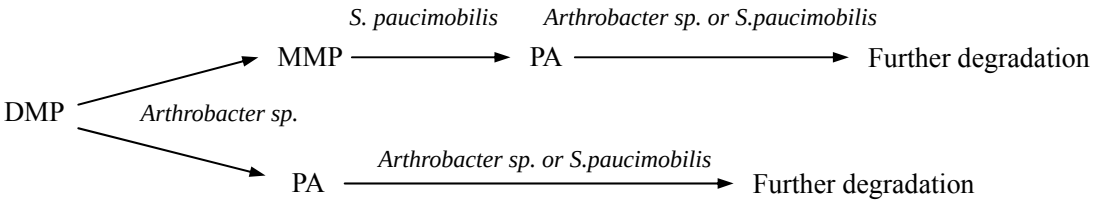


Fig. 2-5 A proposed degradation pathway scheme of DMP by two isolated strains (Vega et al., 2003)

Zeng et al., (2002) studied DEHP degradation by *Pseudomonas fluoresences*, which was isolated from activated sludge from a petrochemical factory, and proposed a new

pathway. It started from the monoester, and enzymatic degradation produced PA, benzoic acid, phenol and finally CO_2 and H_2O (Fig. 2-6).

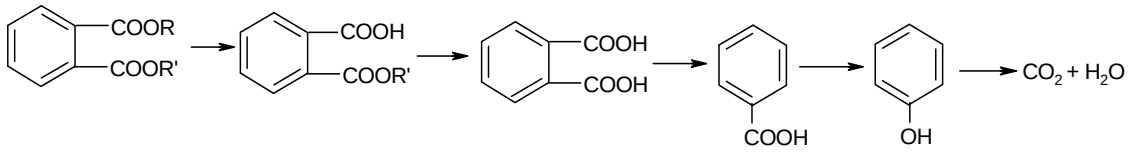


Fig. 2-6 The proposed phthalate degradation pathway by P. *fluorescens* (Zeng et al., 2002)

In conclusion, the alkyl phthalates transformation by microorganisms was a complex mechanism, with different microorganisms involved in different degradation pathways.

The anaerobic pathway from PA to benzoic acid is shown in Fig. 2-7 (Taylor et al., 1983). The anaerobic degradation reaction equations were listed in Table 2-9.

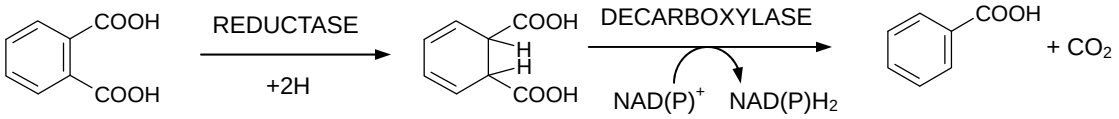


Fig. 2-7 Suggested mechanism for the conversion of o-PA to benzoic, with reduction to 1, 2-dihydrophthalic acid (3, 5-cyclohexadiene-1, 2-dicarboxylic acid) followed by oxidative decarboxylation (Taylor et al., 1983)

Table 2-8 Chemical reaction equations for the individual steps in mineralization of PA and benzoate and the standard Gibbs free-energy changes during the conversions, corrected for a temperature of 37 ºC (Kleerebezem et al., 1999b)

Reaction no.	Reaction	Equation	ΔG^0, 37 ºC (KJ reaction^{-1})
1	Phthalate fermentation	$C_8H_4O_4^{2-}+8H_2O\rightarrow 3C_2H_3O_2^-+3H^++3H_2+2HCO_3^-$	38.9
2	Benzoate fermentation	$C_7H_5O_2^-+7H_2O\rightarrow 3C_2H_3O_2^-+3H^++3H_2+HCO_3^-$	59.6
3	Hydrogenotrophic methanogenesis	$0.75HCO_3^-+0.75H^++3H_2\rightarrow 0.75CH_4+2.25H_2O$	-98.4
4	Acetoclastic methanogenesis	$3C_2H_3O_2^-+3H^+\rightarrow 3CH_4+3CO_2$	-97.8
5	Mineralization of phthalate	$C_8H_4O_4^{2-}+6.5H_2O\rightarrow 3.75CH_4+2HCO_3^-+2.25CO_2$	-157.3
6	Mineralization of benzoate	$C_7H_5O_2^-+5.5H_2O\rightarrow 3.75CH_4+HCO_3^-+2.25CO_2$	-136.6

2.3.3 Enzyme for Phthalate Degradation

The biodegradation of organic pollutants by microorganisms is composed of thousands of redox reactions. By performing redox reactions, the microorganisms generate energy to construct their components and maintain their lives. However, redox reactions are nearly always very slow. Catalysts are needed to fasten the essential reactions in microorganisms. Enzymes are biochemical catalysts which play the key role in the redox reactions. Without enzyme, some redox reactions even cannot be started (Rittmann and McCarty, 2002).

Constitutive enzyme and induced enzyme are two kinds of enzymes responsible for catalyzing the redox reactions for cell metabolism. Constitutive enzymes may be needed in about the same amounts under all growth conditions. They are generally key cellular enzymes required for growth under all nutritional conditions. Thus they are synthesized continuously in the growing cells. Induced enzyme can not be formed until the corresponding substrate is provided. Without the simulation of corresponding substrates, they would disappear again (Rittmann, & McCarthy, 2002).

The production of enzyme was controlled by two modes of regulation in the cell. One controlled the activity of preexisting enzyme and one controlled the amount (or even the complete presence or absence) of an enzyme. Regulation of the synthesis of an enzyme was a coarse level of control than regulating activity. The regulation of activity was typically very rapid, while the regulation enzyme synthesis was a relatively slow process. Thus, as a new enzyme needed to be synthesized, a quite long time was required before the enzyme was present in the cell in sufficient amount to affect metabolism (Rittmann, & McCarty, 2002).

The major enzymes which have been identified to metabolize phthalates include phthalate oxygenase, phthalate dioxygenase, phthalate dehydrogenase, and phthalate decarboxylase. The characteristics of the enzymes involved in phthalate degradation are listed in Table 2-9. Furthermore, the reports also indicate that the enzymes which mediate the catabolic pathway of phthalates are plasmid-encoded genes in some microorganisms (Chang and Zylstra, 1999). Although the enzymes of the two pathways (reductive dioxygenases, dihydrodiol dehydrogenases, and decarboxylases) catalyze similar reactions, the work described here demonstrates that they are not closely related.

Table 2-9 Characteristics of enzymes in phthalate degradation (Eaton and Ribbons, 1982)

Enzyme	Feature	Substrate	Production
Phthalate ester hydrolase	Constitutive	di-ester	monoester
Phthalate ester hydrolase	Constitutive	monoester	phthalate
Phthalate 3, 4 dioxygenase	inducible	phthalate	3,4-dihydoxyphthalate
Phthalate 4, 5 dioxygenase	inducible	phthalate	4,5-dihydoxyphthalate
3, 4-dihydroxyphthalate decarboxylase	Constitutive, slightly inducible	3,4-dihydoxyphthalate	protocatechuate
4, 5-dihydroxyphthalate decarboxylase	constitutive, slightly inducible	4,5-dihydoxyphthalate	protocatechuate
Protocatechate-3, 4-oxygenase	inducible	protocatechuate	β-ketoadipate
Protocatechuate-4, 5-oxygenase	inducible	protocatechuate	pyruvate and oxaloacetate

2.3.4 Molecular Analysis of Phthalate Degradation

Recently, a study about the molecular biology of degradative genes and their organization in bacteria was conducted (Nomura et al., 1990, 1992; Chang and Zylstra, 1998, 1999). *Burkholderia cepacia* DBO1 was employed in this study. Two overlapping cosmid clones containing the genes for phthalate degradation were isolated from this strain. Analysis of the nucleotide sequence of these two regions showed that the genes for phthalate degradation were arranged in at least three transcriptional units. The organization of genes for different metabolic steps was

presented. The map and diagram of the cloned and sequenced genes for phthalate degradation are shown in Fig 2-8 (Chang and Zylstra, 1999).

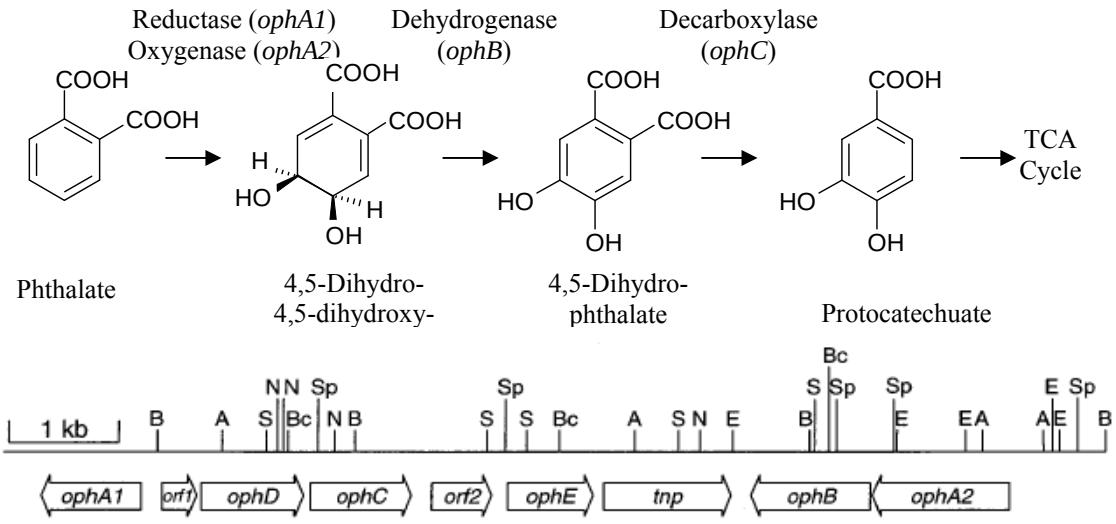


Fig. 2-8 Metabolic pathways for the degradation of phthalate and the synthesis of nicotinic acid mononucleotide from quinolinate and phosphoribosyl pyrophosphate. A (Chang and Zylstra, 1999).

2.3.5 Growth Kinetics

For non-inhibitory substrates, the specific growth rate (μ, h^{-1}) can be described by the commonly used Monod model (Equation 2-5) (Rittmann and McCarty, 2002)

$$\mu = \frac{\mu_m S}{K_S + S} \qquad (2\text{-}5)$$

Where μ = specific growth rate, d^{-1}

μ_m = maximum specific growth rate, d^{-1}

S = concentration of the rate-limiting substrate, mg l^{-1}

K_s = half-substrate constant, substrate concentration when the rate is one-half the maximum rate, mg l^{-1}

Many organisms will exhibit a threshold concentration at which a substrate will inhibit the heterotrophic and/or autotrophic organisms in the activated sludge process.

The specific growth rate for inhibitory substrates has been described by the Haldane equation (Equation 2-6) （Yang and Humphrey. 1975; Onysko et al., 2000）using Monod kinetics:

$$\mu = \frac{\mu_m S}{S + K_s + S^2 / Ki} \qquad (2\text{-}6)$$

Where μ = specific growth rate, d^{-1}

μ_m = maximum specific growth rate, d^{-1}

S = concentration of the rate-limiting substrate, mg l^{-1}

K_s = substrate concentration when the rate is one-half the maximum rate, mg l^{-1}

K_i = the Haldane inhibition coefficient

If substrate concentrations are low, the term S^2/K_i in equation (2-6) is usually less important than the term K_s, even for low values of K_i. Under these conditions, the inhibition function reduces to the Monod equation. Under high substrate concentrations present during the early stages of growth, the term S^2/K_i may be significant, even for higher values of K_i, resulting in a decrease in specific growth rate. The maximum specific growth rate attainable in the presence of inhibition occurs when $S^* = (K_s K_i)^{0.5}$ (Rittmann and McCarty, 2002).

2.3.6 Conventional Activated Sludge Process to Treat Phthalate Wastewater

The activated sludge process is the most widely used biological wastewater treatment process today (Grady et al., 1999). It could be operated under a continuous or sequencing batch reactor mode, where a dense microbial suspension is vigorously mixed with the wastewater under aerobic conditions. In the presence of adequate nutrients, oxygen, and a good flocculant, the organic matter in the wastewater can be utilized for the biosynthesis of more microorganisms, and the production of oxidized end products such as CO_2, NO_3^-, SO_4^{2-} and PO_4^{3-}. In addition, the flocs could

remove suspended colloidal and ionic matter in the wastewater by adsorption and agglomeration, and promote rapid and efficient separation of sludge from treated effluent during sedimentation (Tchobanoglous and Burton, 1991).

Traditionally, phthalate wastewaters are treated using the activated sludge process (Lau, 1978). Both batch and continuous modes were adapted to the treatment process. For batch experiments, 4000 mg l^{-1} phthalate was degraded to 40 mg l^{-1} in five days, and the efficiency could be 99% (Fan et al., 2001). For large-scale experiments, the two-stage activated sludge process was used to treat high concentration phthalate wastewaters. The efficiency of the system was 85% (Zhou et al., 2000).

Advantages of aerobic treatment of PA wastewater are high purification efficiencies (>90%), high process stability and rapid aerobic biodegradability of all compounds present in the wastewater. Disadvantages are high hydraulic retention times due to the high strength of the wastewater, high nutrient requirements due to the absence of nutrients in the raw wastewater, high surplus sludge production and vulnerability to shock loading (Kleerebezem et al., 1997). In addition, low pH and high strength aromatic compounds had inhibitory effects on the microorganisms (Goldstein et al., 1985; Blum et al., 1991). That would affect the treatment efficiency of the system.

2.3.7 Anaerobic Process to Treat Phthalate Wastewater

Due to the lower nutrient and energy requirements and lower surplus biomass production, anaerobic pre-treatment might represent an attractive alternative for, or contribution to, conventional aerobic treatment (Shelton et al., 1984; Battersby et al., 1989; Liangming et al., 1991; Macarie et al., 1992).

Several technology studies have been conducted to assess the feasibility of anaerobic pre-treatment of PA and PAE wastewater in lab-scale as well as full-scale reactors (Kleerebezem, 1999a, Kleerebezem et al., 1999b, c, Kleerebezem and Lettinga, 2000;

Wang et al., 2000a). The up-flow anaerobic sludge blanket reactor (UASB), down-flow fixed film reactor (DFF), anaerobic hybrid reactor, anaerobic fluidized bed reactor and anaerobic fixed film reactor were employed in these studies. Some of the operational data are listed in Table 2-10.

Table 2-10 Operational data from anaerobic bioreactors treating PA wastewater as described in literature (Kleerebezem et al., 1997)

Reference	Full/lab scale	Reactor type[1]	VLR[2] (kg COD m^{-3} d^{-1})	SLR[3] (kg COD kg VSS^{-1} d^{-1})	Efficiency (%)	Degradation of terephthalat
(Liangming et al., 1991)	lab	UASB	15	1.40	90	NA
	lab	hybrid	22	0.73	90	NA
(Macarie et al., 1992)	lab	UASB	2.6	0.29	46	NA
	lab	DFF	1.9	0.09	74	NA
(Pereboom *et al.*, 1994)	full	UASB	10	1.10	55	_
	lab	UASB	4.5	0.40	80	+
(Van Duffel, 1993)	full	DFF	4.0	NA	80	+

1) UASB: Upflow Anaerobic Sludge Blanket reactor; DFF: Downflow Fixed Film reactor, Hybrid: UASB reactor with packing material in top of the reactor instead of a three phase separator.

2) VLR: Volumetric Loading Rate

3) SLR: Sludge Loading Rate

4) NA: data not available, -: no degradation observed, +: significant degradation obsenved.

However, the degradation rates found in anaerobic bioreactors were low. Tur and Huang (1997) reported 0.81-0.85 g COD g VSS^{-1} d^{-1} at a loading of 26 g COD l^{-1} d^{-1}. And the lag phases prior to degradation of the PA were long, ranging from 1 to 3

months in batch studies (Kleerebezem and Lettinga, 2000) to more than 1 year in full-scale reactors (Pereboom et al., 1994).The treatment efficiency ranged from 78% to 47.7% when the PA loading increased from 26.7 to 46.3 g COD l^{-1} d^{-1} (Tur and Huang, 1997). In addition, the aromatic by-products, such as benzoate, acetic and formic acid, inhibited the role of PA fermentation.

2.4 Bioaugmentation

The large diverse microbial communities present in the environment can transform a wide range of organic chemicals. However, many synthetic organic compounds, although biodegradable, might persist in nature for a long time because the populations of degrading microorganisms are not large or active enough. One way to enhance breakdown of these chemicals is bioaugmentation (Dejonghe et al., 2000).

Bioaugmentation is the inoculation of selected microbial biomass to improve certain biological properties of a particular ecosystem. Indigenous or allochthonous wild-type or genetically modified organisms were inoculated to accelerate the removal of undesired compounds from contaminated hazardous waste sites or bioreactors (Van Limbergen et al., 1998; Boon et al., 2000). This provides promising methods in dealing with the problems of bacterial acclimatization, toxicity of compounds, and the restart of the treatment system (Kennedy et al., 1990; Wilderer et al., 1991; Belia et al., 1997; Rai et al., 2005). A few cases have reported the success of bioaugmentation (Mcclure et al., 1991; Rittmann and Whiteman, 1994; Selvaratnam et al., 1997), and some commercial products are also available. However, there are numerous reports of bioaugmentation failure, and the real efficiency of the procedure remains highly controversial (Stephenson et al., 1992; Tchelet et al., 1999; Boon et al., 2000; Bouchez et al., 2000a, b;), therefore this technology is not yet widely applied.

The reasons for the failure of bioaugmentation are numerous and complex. The concentration of contaminant sometimes is too low to support microbial metabolism

of the inocula. The growth rate of the degrading organism is usually lower than the cell removal rate by predation or wash-out. Furthermore, the failure of bioaugmentation may also be due to the fact that the introduced species out-competed by indigenous species. Therefore it is difficult for the inoculated microorganisms to adapt to the targeted ecosystem. Other factors that also could affect the success of bioaugmentation include (1)sufficiency of inocula introduced into the target ecosystem; (2) microbial inhibitors which are present in the environment; (3) inocula may use substrates other than the target pollutant; and (4) the organism may physically fail to reach the pollutant or the pollutant may not be bioavailable due to sorption (Goldstein et al., 1985; Ramadan et al., 1990; Watanabe et al., 1998; Boon et al., 2002; Van Der Gast et al., 2002).

Successful bioaugmentation depends mainly on the behavior of the inoculated strain in the environment where it is introduced. Therefore, a first criterion is strong survival and retention of the strain in the system. The origin and the type of the inoculated strain could play an important role in the survival of the strain. The second criterion for successful bioaugmentation is the activity of the inoculums. Thus, the selection of suitable bioseeds is one of the important factors for bioaugmentation.

Enrichment pure cultures, mixed cultures, acclimated activated sludge, and anaerobic granules have been selected as inocula for bioaugmentation (Wilderer et al., 1991; Guiot et al., 2000; Aulenta et al., 2003; Wang et al., 2004). It was difficult to maintain sufficient inoculated microorganisms in the target ecosystem, although the selected inocula were highly efficient in the removal of the xenobiotic targets under laboratory conditions. Under natural conditions, these laboratory strains, which were cultured from the targeted ecosystem, have to compete with the established microbial community, resulting in a decrease of the amount of inoculated cells (Boon et al., 2000).

Inocula selection is therefore a challenge to the successful application of the

bioaugmentation strategy. To increase the success rate of bioaugmentation strategies, bacterial or fungal (yeast) cells immobilized in a carrier have been selected as bioseeds for bioaugmentation. These carrier materials, such as alginate, agarose, polyurethane, Lentikats, or hollow fibers, are used to provide a temporary protective environment to the inoculum (Vanveen et al., 1997, Wang et al., 1999b; Loh et al., 2000; Boon et al., 2002).The results showed that bioaugmentation was enhanced by the immobilization procedure (Bouchez et al., 2000b; Quan et al., 2003).

Compared with the immobilization by carrier, self-immobilization is a better strategy to meet with the successful bioaugmentation criterions. The aerobic granules were a novel form of self-immobilization which has excellent settling ability, dense microbial structure, and high biomass retention. These characteristics can facilitate the important species to be maintained in the reactor and be protected from toxic or inhibitory substrates (Jiang et al., 2002).

2.5 Aerobic Granulation

Successful wastewater treatment depends upon the selection of metabolically capable microorganisms and the efficient separation of those organisms from the treated effluent. Much research has focused on reducing the settling time required for activated sludge by forming dense flocs or by using biofilm reactors. Biogranules are a kind of condensed bofilm formed through self-immobilization. Biogranulation can be classified as aerobic or anaerobic granulation. These granules are dense microbial consortia packed with different bacterial species and typically contain millions of organisms per gram of biomass.

Formation of anaerobic granules has been extensively studied and is probably best recognized in the upflow anaerobic sludge blanket (UASB) reactor. Anaerobic granulation technology has already been applied in many wastewater treatment plants (Yan and Tay, 1997; Alves et al., 2000; Lettinga et al., 2001; Kassam et al., 2003). In

granular sludge reactors, the anaerobic granules with high density settle rapidly, which reduces the separation time of the treated effluent from the biomass. On the other hand, anaerobic granular sludge technology is suitable for high-strength wastewater treatment. However, the anaerobic granulation technology has some drawbacks, such as a long start-up period, a relatively high operation temperature and unsuitability for low strength organic wastewater and nutrient removal. In order to overcome those weaknesses, research has been devoted to the development of aerobic granulation technology.

2.5.1 Aerobic Granulation in Sequencing Batch Reactors

The aerobic granular sludge technology started in the early nineties (Mishima and Nakamura, 1991). An increase of the research intensity on fundamentals and applications of aerobic granulation was studied from the end of nineties. The research on formation of storage polymers resulted in growing aerobic granules without carrier materials in sequencing batch reactors (Morgenroth et al., 1997; Beun et al., 1999; Peng et al., 1999).

The sequencing batch reactor (SBR) was developed in the U.S. in the late 1960's and became widely used during the 1980s and 1990s (Tchobanoglous and Burton, 1991). Compared to continuous microbial culture, SBR is a fill-and-draw process that is fully mixed during the batch reaction step. The sequential steps of aeration and clarification in a SBR occur in the same tank. The key feature of SBR technology is the change between the feast and famine periods during the reactor cycle. As currently used, all anaerobic SBR systems have five steps in one reaction cycle: fill, aerate, settle (sedimentation /clarification), draw and idle (Tchobanoglous and Burton, 1991). The operation of nearly all aerobic granular sludge SBR systems comprises four steps: feeding, aeration, settling and discharge. Compared to operation of a suspended sludge SBR (Tchobanoglous and Burton, 1991), there is no idling phase during the operation of aerobic granular sludge SBR.

Morgenroth et al. (1997) was the first research group who reported the formation of aerobic granules in sequencing batch reactors. The SBR was operated with a very short sedimentation and draw phase, resulting in the washout of low settling biomass. After 40 days of operation, granules were the dominant form of biomass in their reactor.

2.5.2 Aerobic Granulation in Continuous Flow Reactors

Aerobic granulation in continuous flow systems has also been reported in an aerobic upflow sludge blanket (AUSB) reactor (Mishima and Nakamura, 1991; Shin et al., 1992), a fluidized-bed reactor (De Beer et al., 1993) and an airlift biofilm reactor (Tijhuis et al., 1995).

Unlike the formation of aerobic granules in SBR, granulation occurs under very low shear forces in AUSB, attributed to the vertical stresses supplied from the upflowing liquid and horizontal stresses from agitation at a rate of 1-6 rpm. The granules form in about 5 days (Shin et al., 1992), or three weeks (Mishima and Nakamura, 1991) after start up. However, the AUSB reactor system is complex, and the requirements for pure oxygen, i e. 100% O_2, and a dissolved oxygen tank which is used for dissolving oxygen in a water solution might limit the widespread application of AUSB reactor.

As for two-phase fluidized-bed reactors, the relatively low shear forces and self-aggregation were possible reasons for the formation of nitrifying granules. Nitrifying granules can also form in airlift reactors, forming from broken biofilm fragments. The oxygen depletion that occurred inside the nitrifying biofilm caused the breakup of biofilm, and the broken biofilm fragments were kept in the reactor due to their high density. In general, a small amount of carrier was needed to stabilize the system.

2.5.3 Mechanism of aerobic granulation

The mechanism of microbial aerobic granulation is still a topic of considerable discussion, owing to the complexity of aerobic granulation. Currently there are three mechanisms proposed to explain the biological assemblies. Based on experiments in an aerobic upflow sludge blanket (AUSB), Mishima and Nakamura (1991) hypothesized that, similar to the anaerobic granulation process, filamentous bacteria tangled with each other to form aerobic granules. Beun et al. (1999) proposed a model in which aerobic granulation could be started with fungi. As shown in Fig 2-9 (Beun et al., 1999), fungi easily formed pellets, which settled very fast and could be retained in the reactor. When the pellets grew up to a diameter of 5-6 mm, they lysed, probably due to oxygen limitations in the inner part of the pellets. Then, the pellets broke up and only the colonies that were dense enough could settle. The colonies eventually grew out to form new granules.

Tay et al. (2001b) proposed the model of gradual formation of aerobic granules from seed sludge to compact aggregates, then to granular sludge and finally to mature granules with the sequential operation proceeding. During the granulation process, a significant shift in the microbial diversity was demonstrated by amplified ribosomal DNA restriction analysis (ARDRA) (Yi et al., 2003). Comparing with the initial stage and death stage of aerobic granules, the bacterial community of mature granules showed the least diversity in terms of richness, evenness and Shannon-Wiener index. Some specific microorganisms were suggested to play an important role in the development of aerobic granules.

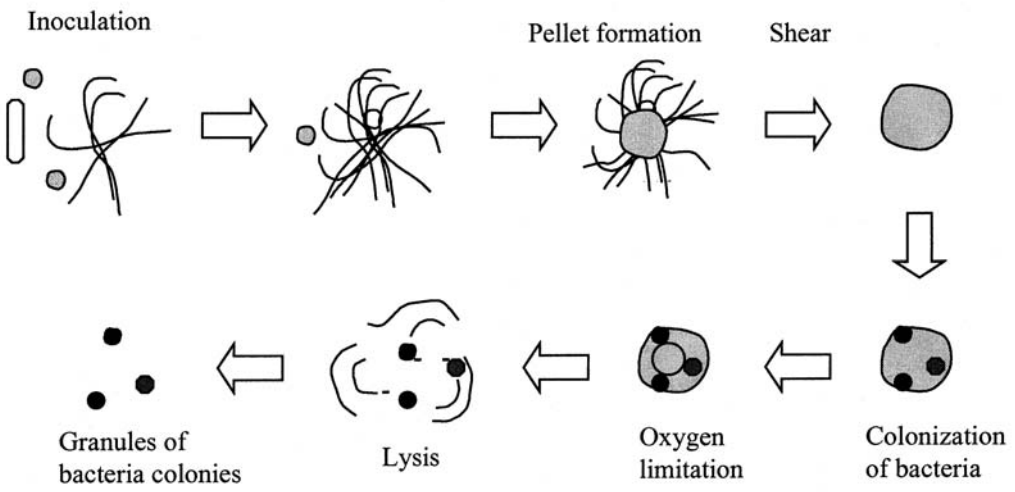


Fig. 2-9 Proposed mechanism of aerobic granulation in SBR (Beun et al., 1999)

Recently, Liu et al. (2004c) proposed that hydraulic pressure is a decisive parameter in the formation of biogranulation, and cell hydrophobicity contributes to the formation of granules. In addition, the formation of aerobic granules was the result of cooperative effects among different functional groups, as well as the interaction between these functional groups and the environment. It has been disclosed that bacteria can sense a large number of environment signals and process this information into specific transcriptional responses in recent molecular studies (Loo et al., 2000). Quorum sensing is one example of social behavior in bacteria, as signal exchange among individual cells allows the entire population to choose an optimal way of interacting with the environment. Intercellular communication and multi-cell coordination are known to contribute to the organization of bacteria into spatial structures (Liu et al., 2004b). So it is reasonable to assume that interaction of both environmental signals and signals between individual cells play an important role in aerobic granules formation and spatial structure organization.

2.5.4 Characteristics of Aerobic Granules

Aerobic granular sludge is completely different from floc like sludge. Characteristic of aerobic granular sludge are described as follows (Etterer and Wilderer, 2001; Moy

et al., 2002; Tay et al., 2002a, b, c, d; 2003a, b; Cai et al., 2004; Tsuneda et al., 2004a, b; Hu et al., 2005; Zheng et al., 2005)

- Round and regular shape with a clear and smooth outer surface;
- Dense and compact microbial structure;
- Large enough to be visible as separate entities in the mixed liquor during mixing and settling phase;
- Excellent settleability to ensure a fast and easy liquid-solid separation;
- High biomass retention;
- Ability to withstand high organic loading rates;
- Ability to resist the toxicity of recalcitrant chemicals and heavy metals in wastewater.

Detailed information about aerobic granular sludge is given in Table 2-11

Table 2-11 Summary of characteristic data about aerobic granular sludge (Etterer and Wilderer et al., 2001; Moy et al., 2002; Tay et al., 2002c, 2003b; Cai et al., 2004; Tsuneda et al., 2004b; Hu et al., 2005; Zheng et al., 2005)

Parameter	Diameter (mm)	SVI (ml g^{-1})	MLSS (g l^{-1})	Specific gravity	Settling velocity (m h^{-1})	SOUR (mg O^2 $gVSS^{-1}$ h	Strength (%)
Range	0.33-8.00	14-100	0.7-30	1.004-1.065	30-70	18-160	81.2-99.0
Average value	1.75	64	7	1.037	55	82	NA

SOUR: specific oxygen utilization rate

The size of aerobic granules varies from 0.3 mm to 7 mm (Morgenroth et al., 1997; Beun et al., 1999; 2000; Peng et al., 1999; Tay et al., 2001a, b, c), depending on the operational condition and the substrate used. The average density of aerobic granules ranged from 1.044 g ml^{-1} to 1.048 g ml^{-1} (Etterer and Wilderer et al., 2001), which is

significantly denser than activated sludge flocs. The average granule porosity was reported to be between 65% and 72% (Etterer and Wilderer, 2001).

The aerobic granules also had a layered structure. Peng et al. (1999) observed that there were three levels in the aerobic granules. In the granules that were fed with acetate, the living cells, lysed cells, non-degradable cell debris and influent solids formed the first level, which was between 0.5 to 5 μm thick. The second level consisted of individual aggregates that were encapsulated in the clearly defined polymeric matrices to form microcolonies, which were roughly spherical. These microcolonies were 5 to 50 μm in size. The numerous microcolonies were embedded within extracellular polymer mats to form the third level. Tay et al. (2002a) detected a variety of biological layers by using oligonucleotide probes, specific fluorochromes, and fluorescent microspheres. The channels were observed in the granule matrix, which penetrated into the depths of 900 μm. Furthermore, a layer of obligate anaerobic bacteria existed in the region of 800 μm below the granule surface. Dead cells were also observed in the granule interior.

2.5.5 Structure of Aerobic Granule

The aerobic granules can be looked upon as spherical biofilms which are composed of microorganisms embedded in extracellular polymers (ECP) matrix. Several researchers have found the layered structure of aerobic granules using Confocal laser-scanning microscopy (CLSM) (Meyer et al., 2003; Tay et al., 2002a, 2003b; Toh et al., 2003). There is a layer of dead microbial cells located at a depth of 800 to 1000 μm below the surface of aerobic granules (Toh et al., 2003), and the anaerobic bacterium *Bacteroides spp*. is also detected at a depth of 800 to 900 μm from the granule surface. Polysaccharide formation peaked at a depth of 400 μm below the granule surface (Tay et al., 2002e). Aerobic granules contained channels and pores that penetrated to a depth of 900 μm below the granule surface. The porosity peaked at depths of 300 to 500 μm from the granule surface (Tay et al., 2002a, 2003b). These

channels and pores would facilitate the transport of oxygen and nutrients into, and metabolites out of, the granules. Influenced by the distribution of pores and channel layers, the distribution of active biomass in granules was governed by different granule diameters.

2.5.6 Diversity of Aerobic Granules

To better understand the aerobic granulation process and to improve aerobic granulation system design and performance, the microbial diversity of aerobic granules has been studied by molecular biotechnology techniques (Tay et al., 2002a; Meyer et al., 2003; Tsuneda et al., 2003; Yi et al., 2003; Jiang et al., 2004b) . Heterotrophic, nitrifying, denitrifying, P-accumulating bacteria and glycogen-accumulating bacteria have been identified in aerobic granules developed under different conditions and fed with different culture media (Tay et al., 2002a; Jiang et al., 2003; Lin et al., 2003; Liu et al., 2003a; Meyer et al., 2003; Tsuneda et al., 2003; Yang et al., 2003; Yi et al., 2003).

2.5.7 Factors Affecting Formation and Structure of Aerobic Granule

Shear force

In SBR, shear force is mainly described by the upflow air velocity. A high shear force appears to favor the formation of aerobic granules (Shin et al., 1992; Beun et al., 1999; Tay et al., 2001a). Tay et al. (2001a) found that only fluffy flocs were observed in an SBR operated at a low superficial air upflow velocity of 0.8 cm s^{-1}, while regular-shaped granules were successfully developed at a high shear superficial air velocity of 2.5 cm s^{-1}. Beun et al. (1999) also observed the same phenomena. The granule density and strength were also proportionally related to the shear force applied (Tay et al., 2004a). These results may imply that shear force strongly affects formation of aerobic granulation and determines the structure of aerobic granules. In addition, Tay et al. (2001a) reported that the production of cellular polysaccharides

was closely associated with the shear force and the stability of aerobic granules; this was found to be related to the production of cellular polysaccharides. Thus, the shear force contributed to the formation of aerobic granules through promoting and stimulating the production of cellular polysaccharides.

Settling time

The settling time acts as a major hydraulic selection pressure on the microbial community during the aerobic granulation process. Short setting times are needed not only for the wash out of slow settling biomass and the retention of fast setting granules, but also for the effective functioning and economic operation of biological systems. Qin et al. (2004) reported that aerobic granules were successfully cultivated and became dominant only in an SBR operated at a settling time of 5 minutes. Settling times higher than 5 min resulted in mixtures of aerobic granules and suspended sludge. Generally, the settling time was set at 1-4 minutes (Morgenroth et al., 1997; Beun et al., 1999; 2000). Thus, choice of an optimal settling time is very important in aerobic granulation.

Organic loading rate

Although a high organic loading rate (OLR) facilitates the formation of anaerobic granules in UASB systems, it is unfavorable for the aerobic granulation process because of excessive growth of suspended forms. Aerobic granules can form when the COD loading rates range from 0.42 to 15 kg COD $m^{-3}\cdot day^{-1}$ (Mishima and Nakamura, 1991; Morgenroth et al., 1997; Moy et al., 2002; Liu et al., 2003b; Tay et al., 2004c). It seems the OLR has no significant effect on the formation of aerobic granules, but the physical characteristics of aerobic granules are closely related to the OLR (Toh et al., 2003). The mean size of aerobic granules increased with an increase of OLR (Moy et al., 2002; Liu et al., 2003b). Morphology in terms of roundness, dry biomass density, specific gravity and sludge volume index (SVI) have not been shown to have a close relationship with the applied OLR. On the other hand, the physical strength of aerobic granules decreased with an increase of OLR. This is because of the fact that

high biomass growth rates caused by high OLR reduce the strength of the 3-dimentional structure of the microbial community (Liu et al., 2003a, 2003b).

Substrate composition

Aerobic granules have so far been cultivated with a variety of substrates including glucose, acetate, ethanol, phenol, yeast extract, particulate organic matter-rich wastewater and other synthetic wastewater (Shin et al., 1992; Beun et al., 1999; Peng et al., 1999; Moy et al., 2002;; Jiang et al., 2003, 2004a; Schwarzenbeck et al., 2004; Tay et al., 2001b, 2004a, b, d; Yang et al., 2004). Nitrifying and phosphorus-accumulating granules have also been developed (Lin et al., 2003; Tsuneda et al., 2003, 2004a, b). However, microbial diversity, microstructure and elemental composition of mature aerobic granules might be influenced by substrate composition (Tay et al., 2001b; Liu et al., 2003b). With acetate as substrate, the aerobic granules formed a compact microstructure with rod-like bacteria in a radial arrangement, while glucose granules consisted mainly of cocci-type bacteria in the internal part of the granules and filamentous bacteria tangle together with rod bacteria on the surface (Tay et al., 2001b)

Hydraulic retention time

Hydraulic retention time (HRT) is related to the SBR cycle time and volume exchange ratio. It is defined as the volume of effluent discharged divided by the working volume of the SBR. HRT can serve as a main hydraulic selection pressure on the granulation by suppressing the growth of dispersed sludge. A suitable selection of HRT would favor the granulation process. Too short an HRT would result in great sludge loss without compensation, while too long an HRT would make bioflocs be dominant in the system. The seeded aerobic granules were kept stabilized with an HRT from 2 to 12 h (Pan et al., 2004), while an HRT of 12 and 24 h would be favored by nitrifying bacteria (Tay et al., 2002d).

Dissolved oxygen

Dissolved oxygen (DO) concentration is an important parameter in the operation of an aerobic wastewater treatment system. However, DO concentration is not a decisive variable in the formation of aerobic granules. Aerobic granules can form at DO concentrations as low as 0.7 to 1.0 mg l^{-1} in a SBR (Peng et al., 1999). They are also successfully developed at DO concentrations higher than 2.0 mg l^{-1} (Moy et al., 2002; Tay et al., 2002c).

Aerobic starvation

There are two phases in one SBR sequencing cycle time: a degradation phase in which the substrate is depleted to a minimum, followed by an aerobic starvation phase in which the external substrate is no longer available. Thus microorganisms in the SBR are subject to a periodical starvation. Periodic starvation was reported to trigger microbial adhesion and aggregation by affecting cell hydrophobicity (Tay et al., 2001c; Mcswain et al., 2004). However, the assumption has still not directly confirmed by experiment although the relationship between starvation and cell hydrophobicity has been widely studied.

Trace elements

Trace elements enhance aerobic granulation. Jiang et al. (2003) reported adequate calcium shorten the period required for formation of aerobic granules by approximately 50%. The Ca^{2+}-augmented aerobic granules also showed better settling and strength characteristics and had higher polysaccharides contents. Ca^{2+} was proposed as a bridge to promote bacterial aggregation by binding negatively charged groups present on bacterial surfaces and extracellular polysaccharide molecules. Tsuneda et al. (2004a) found that feeding Fe caused, to accumulate at the central part of nitrifying granules in an aerobic upflow fluidized bed (AUFB) reactor. In addition, Fe pre-aggregated sludge could promote nitrifying granules formation.

2.5.8 Application of Aerobic Granulation Technology

To treat high-strength organic wastewater efficiently, a high biomass concentration and high microbial degradation rate are expected for biological systems. In aerobic granulation SBRs, a high biomass concentration of 6.0 to 12.0 g l^{-1} has been accumulated in reactors because of the compact and dense structure of the granules (Tay et al., 2002b, c). The feasibility of applying aerobic granulation technology for the treatment of high-strength organic wastewaters was demonstrated by Moy et al. (2002). Aerobic granules were able to sustain a maximum organic loading rate of 15.0 kg COD m^{-3} day with glucose as substrate, while removing more than 92% of the COD.

With their compact structure and high degradation efficiency, aerobic granules show excellent ability in degradation of toxic compounds, such as phenol (Jiang et al., 2002; 2004a). For an influent phenol concentration of 500 mg l^{-1}, a stable effluent phenol concentration of less than 0.2 mg l^{-1} was achieved in an aerobic granular sludge reactor (Jiang et al., 2002, 2004a). Aerobic granules possess high tolerance to phenol because much of the biomass in the granules is not exposed to the same high concentration as present in the wastewater.

Aerobic granules may prove powerful bioagents for removing other inhibitory and toxic organic compounds from high strength industrial wastewaters. Recent studies also demonstrated that aerobic granules can be used for phosphate and ammonia removal (Beun et al., 2001; Dulekgurgen et al., 2003; Liu et al., 2003a; Tsuneda et al., 2003)

2.6 Summary

Phthalate wastes exist in effluents in high concentrations from phthalate production process. Compared with bulk removal of phthalate by physical or chemical methods,

biological degradation is generally preferred due to lower costs and the possibility of complete mineralization.

A wide range of microbes have the ability to utilize phthalate as carbon and energy sources at low concentrations. Phthalates are aerobically biodegraded by two metabolic pathways, initiated by ester bond hydrolysis. The key intermediates are monoester, PA and protochatechuate. The benzene ring is broken by ortho or meta cleavage. The limiting degradation step was monoesters' hydrolysis.

Phthalates are biodegradable. However, the efficiency of biological treatment is low with the high organic loading rates. It seemed more difficult if the target compounds were recalcitrant with high molecular weight and complex structure. The conventional activated sludge process cannot effectively treat phthalate wastewater with effluent containing dozens to thousands of mg l^{-1} phthalates. However, immobilizations of mix cultures in/on various supports were able to completely degrade phthalate at higher loadings. The use of supports increased investment dramatically. Therefore, the self-immobilization of mix cultures in environmental conditions without the use of supports, like granular sludge is suggested. Upflow anaerobic sludge blanket (UASB) reactors with anaerobic granules could be operated at a steady state condition under the phthalate loading rate of 26.7 to 46.3 g COD l^{-1} d^{-1}. However, the removal efficiency was only 47% due to the low efficiency of anaerobic degradation.

Aerobic granules are self-immobilized bacterial aggregates from activated sludge flocs. Compared to conventional activated sludge flocs, aerobic granules have denser and more compact microbial structure, better settling ability, and higher biomass retention. Aerobic granulation can provide large quantities biomass for refractory compounds and create a more favorable microenvironment for microorganisms under unfavorable conditions. In addition, aggregation of activated sludge cells into aerobic granules helps to establish syntrophic relationships. Because of these characteristics, aerobic granules have potential to treat high-loading refractory industrial wastewaters.

In addition, addition of PA-degrading microorganisms was reported to accelerate phthalates degradation (Fan et al., 2001). PA-degrading aerobic granules possessed the characteristics of aerobic granules. At the same the time, they also provided PA-degrading microorganisms for phthalates degradation. Thus they have the potential to be bioaugmentation seeds for enhancement of phthalates degradation.

However, there is little information about the performance of phthalates degradation when PA aerobic granules are used as bioseeds for bioaugmentation. The main objective of this study was to investigate the degradation of phthalates, especially recalcitrant PAEs by the system which was seeded with PA-degrading aerobic granules. The metabolic and enzymatic changes in PA-degrading aerobic granules during phthalates degradation will be studied. The change of microbial community structure of PA-degrading aerobic granules will also be surveyed, and the role of functional species in PA aerobic granules will be evaluated.

CHAPTER 3

FORMATION AND CHARACTERISTICS OF PHTHALIC ACID-DEGRADING AEROBIC GRANULES

3.1 Introduction

Many synthetic organic compounds might persist in nature for a long period of time because of shortage of degrading microorganisms. One way to enhance the degradation of these chemicals is bioaugmentation. At present, bioaugmentation is not yet widely applied because of the controversial results of bioaugmentation experiment. To make sure a successful bioaugmentation, the selected bioseeds with economically and easily cultured, fast growing properties are expected. And the bioseeds with high bioactivity are also hoped to have good survival and retention ability in the target ecosystems.

There are several reports that bioaugmentation was enhanced by the immobilization procedure (Bouchez et al., 2000b; Quan et al., 2003). Aerobically grown microbial granules are self-immobilized aggregates of bacteria cultivated in sequential batch reactors (SBRs). The aerobic granules has been successfully applied in diverse types of pollutant treatment systems (Jiang et al., 2002; Moy et al., 2002; Kim et al., 2004; Li et al., 2004; Tay et al., 2004d). However, little is known about the possibility of using aerobic granules as bioseeds for bioaugmentation. The main objectives of this study were therefore to cultivate PA-degrading aerobic granules. The characteristics of aerobically grown microbial granules were evaluated to estimate the possibility of aerobic granules as inocula of bioaugmentation.

3.2 Materials and Methods

3.2.1 PA-degrading Aerobic Granules Cultivation

Experiments were performed in column-type sequencing batch reactors (SBRs) (5 cm diameter and 120 cm height) with a working volume of 2.4 l. The configuration of the reactor is shown in Fig. 3-1. Reactors were operated sequentially in 4-h cycles (5 min of influent filling, 226 min of aeration, 5 min of settling and 4 min of effluent withdrawal) with a hydraulic retention time (HRT) of 8 h. The reactor was housed in a temperature-controlled room at 25 ºC. Effluent was discharged at a volumetric exchange ratio of 50%. Fine air bubbles for aeration were supplied through a dispenser at the bottom of the reactor with an airflow rate of 3.5 l min^{-1}. PA was fed as the sole carbon source with an organic loading rate (OLR) of 3.0 kg COD m^{-3} d^{-1}.

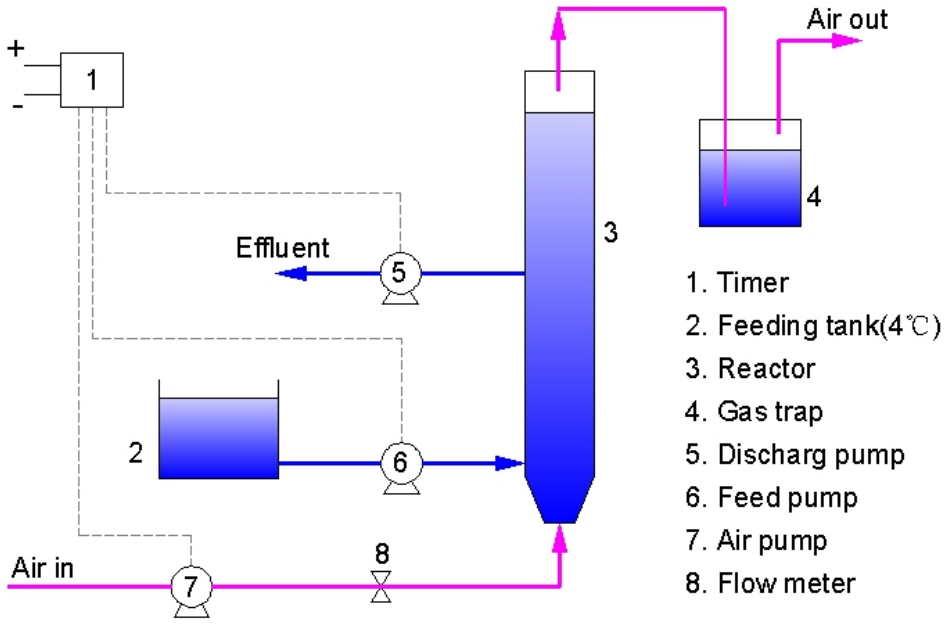


Fig.3-1 Scheme of experiment system

3.2.2 Seeding Sludge

The seeded activated sludge was taken from SembCorp Utilities Industrial Wastewater Treatment Plant in Singapore. Pre-cultivation and acclimation of the seed sludge were conducted in the reactor using a fill and draw operation mode for a period of one week. PA was provided as sole carbon and energy source at concentration of 500.0 mg l^{-1}. The enrichment was considered to be completed when neither lag phase for PA degradation nor accumulation of the intermediate metabolites was observed. About 5.8 g enriched activated sludge was used as the inoculum for the aerobic granules cultivation experiments, which gave the biomass concentration of 2.4 g l^{-1} in the reactor. The SVI and the mean biomass size of inoculated activated sludge were 147 ml g^{-1} and 0.12 mm, respectively.

3.2.3 Medium

The reactor was fed with PA as the sole carbon source using a synthetic wastewater. A stock solution of PA was prepared at a concentration of 50,000 mg l^{-1}, and neutralized with 55,000 mg l^{-1} sodium bicarbonate to give a pH of 7.17. The influent PA solution in the experiment with a concentration of 692 mg l^{-1} was diluted from the stock solution.

A synthetic wastewater was composed of PA (692 mg l^{-1}), mineral salt medium (MSM) and trace solution 1 mg l^{-1}, which gave a total COD of 1000 mg COD l^{-1}. PA was used as sole carbon and energy source.

The MSM had the following composition: NH_4Cl, 200 mg l^{-1}; K_2HPO_4, 45 mg l^{-1}; $CaCl_2 \cdot H_2O$, 30 mg l^{-1}; $MgSO_4 \cdot 7H_2O$, 25 mg l^{-1}; $FeSO_4 \cdot 7H_2O$, 20 mg l^{-1}. The trace solution contained: H_3BO_3, 50 mg l^{-1}; $ZnCl_2$, 50 mg l^{-1}; $CuCl_2$, 30 mg l^{-1};

$MnSO_4 \cdot H_2O(NH_4)_6$, 50 mg l^{-1}; $Mo_7O_{24} \cdot 4H_2O$, 50 mg l^{-1}; $AlCl_3$, 50 mg l^{-1}; $CoCl_2 \cdot 6H_2O$, 50 mg l^{-1}; and $NiCl_2$, 50 mg l^{-1} (Yan and Tay, 1996).

3.2.4 Analytical Methods

Wastewater and biomass samples in the reactors were collected periodically. The influent was collected in influent filling period, the effluent was collected in effluent withdraw period and the biomass samples were taken at aeration period, around 10 min before the start of settling period. All the samples were analyzed for pH, COD, TOC, suspended solids (SS), volatile suspended solids (VSS), sludge volume index (SVI) and specific oxygen utilization rate (SOUR) using standard methods (APHA, 1998). For SOUR determinations, samples with known VSS concentrations were filled to overflowing in standard BOD (biochemical oxygen demand) bottles. DO levels were measured with a DO meter (YSI 5000; YSI Incorporated, Yellow Springs, OH, USA) at time intervals of 15 s over a 15 min period, or until DO became limiting, whichever occurred earlier. The oxygen consumption rate in mg l^{-1} min^{-1} was determined from the slope of the line of best fit of a plot of DO against time. SOUR was calculated by dividing the oxygen consumption rate by the VSS concentration.

Concentrations of PA were determined by high-pressure liquid chromatography (HPLC, Perkin-Elmer, series 2000, Perkin-Elmer Corporation, Norwalk, USA). Liquid samples were filtered through 0.2-μm-pore-size PVDF Acrodisc Minispike (Pall Gelman Laboratory, Ann Arbor, Michigan) syringe filters. Separation of the PA was obtained by using a chromospher C18 column (100 by 3 mm). The mobile phase was a mixture of methanol and a 1% acetic acid water solution in a 40/60 (V/V) ratio. The applied flow rate was 0.3 ml min^{-1}. The separated components were detected using a UV spectrophotometer (Spectroflow 773) at a wavelength of

230 nm. The typical retention time for PA was 5 min (Kleerebezem et al., 1999b). The calibration curves of low range (0-100 mg l^{-1}) and high range (100-800 mg l^{-1}) PA concentrations were established, with R^2 higher than 0.999.

Extracellular polymers (ECPs) in the granules were extracted by the cold aqueous extraction method (Zhang et al., 1998). About 2.3 mg biomass samples were harvested by centrifuge at 14,000 rpm for 5 minutes. The harvested biomass was then re-suspended in 2 ml 8.5% NaCl solution containing 0.22% formaldehyde. The contents were then shaken with a Mini-Beadbeater (Biospec Products, USA) for 5 min at 4800 rpm. The mixture was chilled in ice and was homogenized for 3 minutes. The supernatant was recovered by high speed centrifugation at 12,000 rpm for 30 minutes for determination of ECP. The carbohydrate content in the ECPs was measured using the phenol-sulfuric acid method (Dubois et al., 1956), and the protein content was measured using the bicinchoninic acid protein assay (Stoscheck, 1990). No significant cell lysis was detected during ECP extraction, as confirmed by measurements of DNA content in the extracts (Zhang et al., 1999).

Biomass size was measured by either a laser particle size analysis system (Malvern Mastersizer 2600) or an image analysis system (Quantimet 500 image analyzer, Leica Cambridge Instrucments) (Yan and Tay, 1997). The Mastersizer operates on the principle of laser diffraction, and the range of measurable particle sizes is 0.5-1880 μm, whereas the Image Analyzer is used to measure larger particles. With the Image Analyzer, samples of well-mixed granules were placed in a Petri dish, and granule samples were analyzed with a minimum of 100 granules per sample. The granules were photographed and analyzed with an image system using image analyzing software (Quantimet 500).

Granule microstructure was observed by scanning electron microscopy (SEM)

using a Stereoscan 420 (Leica Cambridge Instruments). Granules were prepared for SEM by washing with a phosphate buffer and fixing with 2% glutaraldehyde overnight at 4 ºC. Fixed granules were washed with 0.10 mol l^{-1} sodium cacodylate buffer, dehydrated by successive passages through 25, 50, 75, 80, 90, 95 and 100% ethanol and dried with a critical point dryer (Polaron E3000, VG Microtech). Dried samples were sputter-coated with gold at 20 mA in a high vacuum (2.8×10^{-6} Torr) and then viewed with a SEM at 25 KV.

3.2.5 Kinetic Study of PA Biodegradation

Specific PA biodegradation rates were determined by incubating biomass samples at predefined PA solution (concentrations from 10.0 to 4000.0 mg l^{-1} with pH at 7.0±0.1). The MSM used in the batch incubations had the same composition as that described in Section 3.2.3. Fresh activated sludge, PA acclimated activated sludge and PA-degrading aerobic granules were used for the batch experiment. For each batch, the total inoculum concentration was 1.0 g suspended solids. The inoculum biomass was harvested from the reactor during the aeration period, washed twice, re-suspended in 500 ml of fresh MSM and added to 1 l batch reactor which was located in a temperature-controlled room at 25 ºC. Killed controls contained 2.9 g l^{-1} sodium azide. Air stripping was provided by an air stone in the bottom of the batch reactor with air flow rate of 1 l min^{-1}, which provided a superficial air velocity of 3.0 cm s^{-1}. That was the same superficial air velocity as in the reactor. The PA concentrations in the batch reactor were assayed periodically. All experiments were performed in triplicate, and completed biomass samples were then centrifuged and washed, and returned to the reactor. A kinetic analysis of the degradation data was performed based on Haldane's formula for an inhibitory substrate.

3.2.6 Volatilization

For some xenobiotic pollutants, air stripping or volatilization is known to be a high efficiency removal mechanism. Although PA and corresponding esters are poorly volatile chemicals based on the log P value, tests to determine the volatilizing potential for PA were still carried out at the selected aeration rate. Batch studies and lab scale studies were carried out separately. The batch studies were conducted on a 1 l cylinder with PA concentrations from 200-4000 mg l^{-1}. Air stripping was provided by an air stone in the bottom of the cylinder with air flow rate of 1 l min^{-1}, which provided a superficial air velocity of 3.0 cm s^{-1}. That was the same superficial air velocity as in the reactor.

The duration of the batch study was 24 h. The lab scale tests were conducted at a 2.4 l working volume reactor with a PA concentration of 1000 mg l^{-1}. The operational conditions were similar to the conditions for the PA degradation, except for the absence of biomass or inoculum in the reactor. The duration of the volatilization test was 4 h, the cycle time for the PA degradation. The PA samples were withdrawn at regular intervals from the reactor throughout the test period. Comparisons between these samples gave the information as to whether volatilization happened. Under such conditions, any reduction of PA would be contributed to air stripping from aeration.

3.2.7 Kinetic Analysis of PA Degradation

According to Rittmann and MaCarty (2002) and Wang et al. (1998), the substrate inhibition model proposed by the Haldane（Yang and Humphrey. 1975; Onysko et al., 2000）was used in these cases.

$$V = \frac{V_{\max} \times S}{K_s + S + S^2 / K_i} \qquad (3\text{-}1)$$

Where V represents the specific degradation rate (g g VSS^{-1} d^{-1}) of substrate (S, mg l^{-1}), V_{max} represents the maximum specific degradation rate (g g VSS^{-1} d^{-1}), K_s is the substrate concentration when the rate is one-half the maximum rate (mg l^{-1}), and K_i is the Haldane inhibition constant.

The minimum inhibition substrate concentration can be calculated from equation (3-1), by setting the first derivative of the Haldane model equal to zero

$$\frac{dV}{dS} = 0 \qquad (3\text{-}2)$$

the following relation is obtained

$$S^* = \sqrt{K_i \times K_s} \qquad (3\text{-}3)$$

which gives the PA concentration （S*） at the highest attainable degradation rate.

The corresponding specific degradation rate can be calculated as below

$$V = \frac{V_{max}}{1 + 2 \times \sqrt{K_s / K_i}} \qquad (3\text{-}4)$$

3.3 Results

3.3.1 Volatilization

Although chemicals of the phthalate family have low log P values, air stripping tests were carried out at different PA concentrations and in a reactor to confirm the low volatilization of PA. The data in Figs 3-2 and 3-3 show that there was no stripping of PA, whether in the reactor or in the cylinder, even with the concentration as high as 4200 mg l^{-1}. This confirmed that the pathway for PA removal in the reactor was completely through biodegradation.

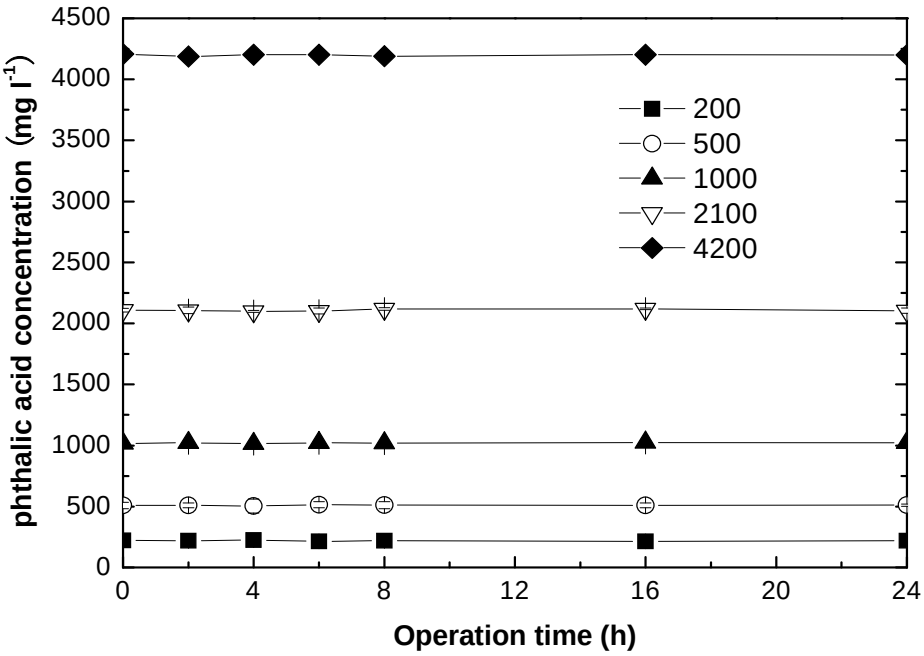


Fig. 3-2 PA concentrations versus time in volatilization batch experiment

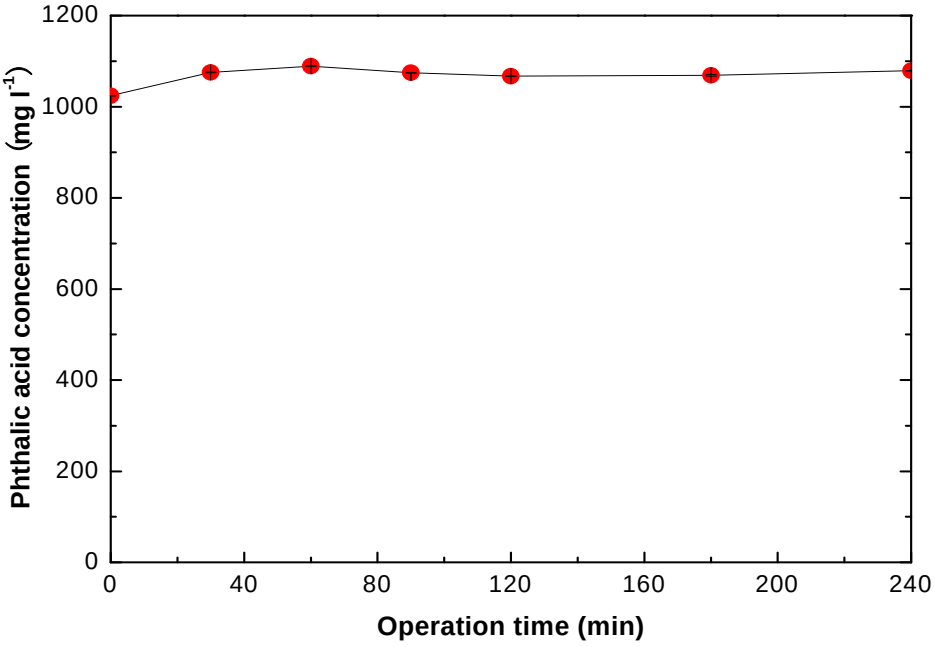


Fig. 3-3 PA concentrations versus time in volatilization lab scale experiment

3.3.2 The Formation and Characteristics of PA-degrading Aerobic Granules

The reactor was started up using acclimated activated sludge as inocula, which had a mean biomass size of 0.12 mm and a SVI value of 147 ml g^{-1}. To avoid excessive washout of the acclimated biomass, settling time was initially imposed at 20 min, and subsequently reduced to 15 min on day 4, 10 min on day 7, and then 5 min from day 11 to the end of reactor operation. Granules first appeared on day 7 by which time the mean biomass size exceeded 0.25 mm and the SVI value decreased approximately to 41 ml g^{-1}, as shown in Fig. 3-4.

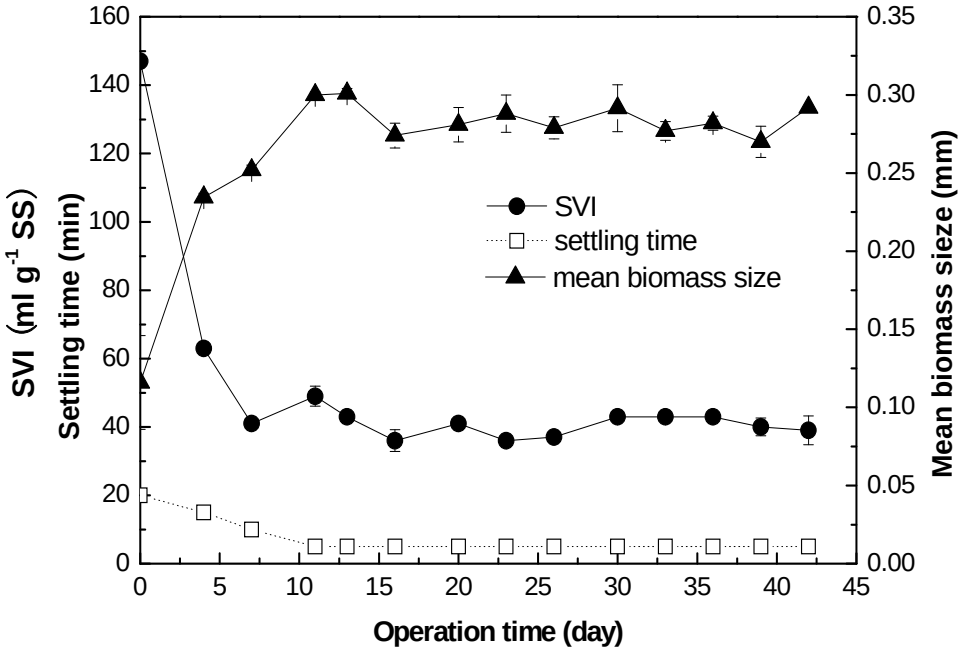


Fig. 3-4 Profiles of sludge volume index (SVI) and the mean biomass size of PA-degrading aerobic granules

Image analysis of the time dependent development of the granules, extending from seed sludge to granules, is shown in Fig. 3-5. The granules eventually displaced the activated sludge flocs to become the dominant form of biomass within the reactor.

As the granules grew, the mean biomass size gradually increased, reached a steady value of 0.28 mm from day 16. At steady state, 90% of the granular biomass was between 0.27 mm and 0.29 mm. The step-wise decreases in the settling time selected for granules with fast settling characteristics resulted in a concomitant increase of biomass concentration, from 2.4 to 4.6 g l^{-1} in the reactor (Fig. 3-6). The influent PA concentration was maintained at 692.0 mg l^{-1}. Most of the PA fed into the reactor was successfully removed, with approximately 75% PA removal on day 0 and effluent PA concentrations reaching to zero level after 23 day of reactor operation (Fig. 3-6).

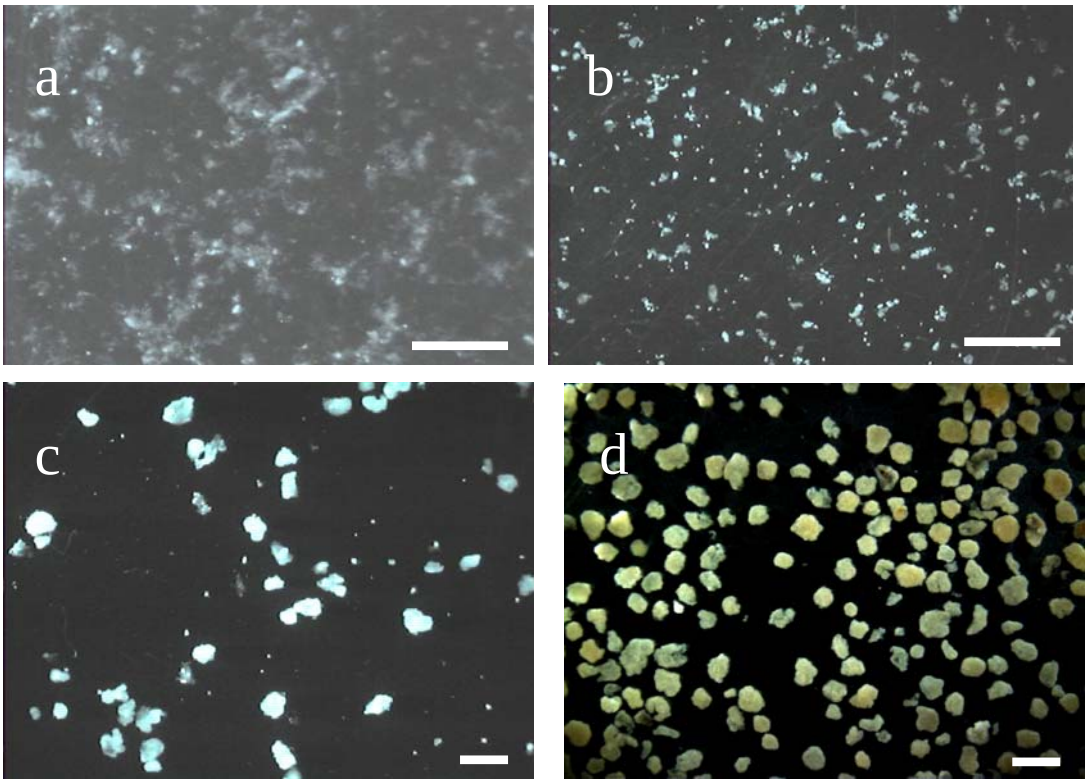


Fig. 3-5 Image analysis of biomass at different operation time (a) inoculated activated sludge (bar: 1 mm); (b) day 7 (bar: 1 mm); (c) day 16 (bar: 0.5 mm); (d) day 42 (at end of formation) (bar: 0.5 mm).

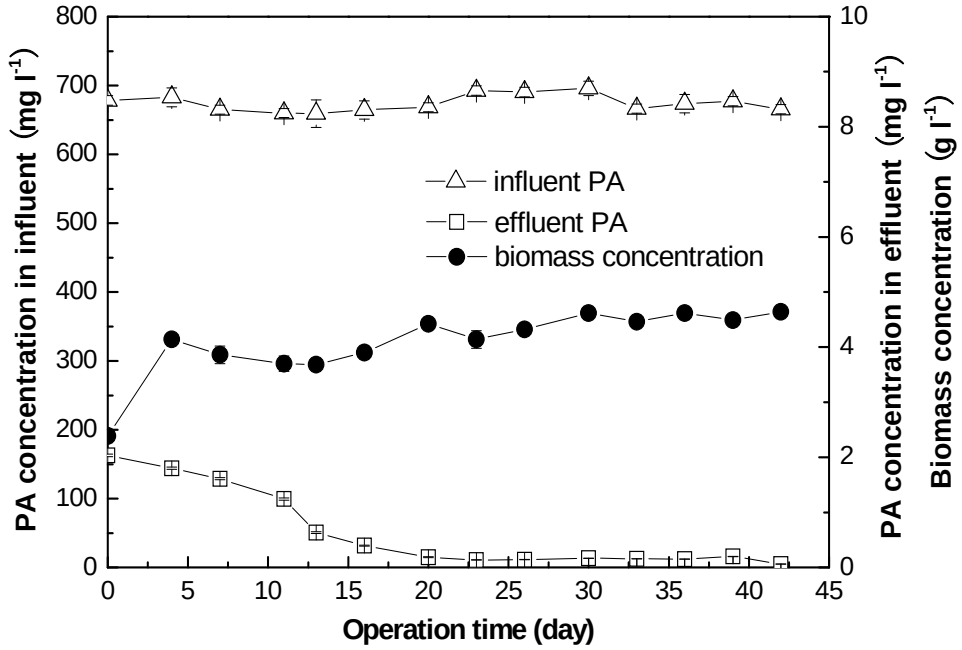


Fig. 3-6 Profiles of biomass concentrations and influent and effluent PA concentrations

Specific oxygen utilization rate (SOUR) of the biomass was measured to monitor changes in biomass activity during the experiment. The initial SOUR value of the biomass was 86 mg DO g VSS^{-1} h^{-1}. It decreased gradually during settling time with step-wise decreasing periods. 15 days later, it began to increased and stabilized at 73 mg DO g VSS^{-1} h^{-1} at the end of the experiment, approximately 86% of the initial value of 86 mg DO g VSS^{-1} h^{-1} (Fig. 3-7).

The total oxygen utilization rate (TOUR) of the reactor (Fig. 3-7) was estimated by the SOUR values multiplied by the corresponding amount of biomass in the reactor, as was described in Jiang et al. (2002). The decrease of TOUR values during the settling time step-wise decreasing periods was due to wash out of the biomass and decrease of SOUR, but TOUR increased sharply after granules were formed and stabilized after day 30 (Fig. 3-7). TOUR values at the end of the experiment were

311 mg DO h^{-1}, which increased approximately two-fold of the initial values (187 mg DO h^{-1}), resulting from the incremental change in biomass concentrations and SOUR values.

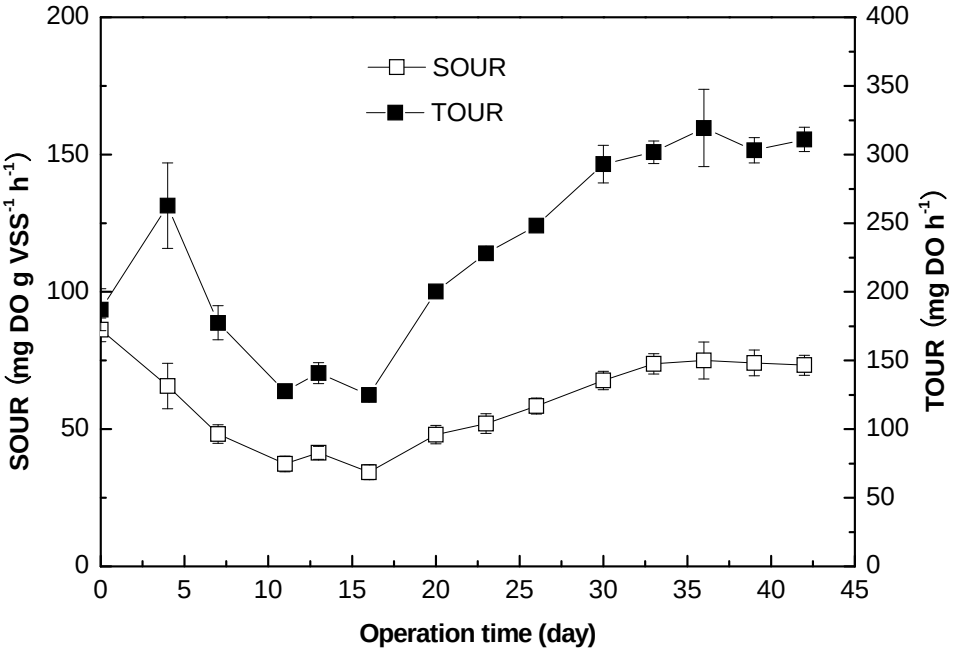


Fig. 3-7 Profiles of SOUR and TOUR for PA-degrading aerobic granules

The change of the extracellular polymer (ECP) concentrations during aerobic granules formation is listed in Table 3-1. Polysaccharides (PS) in inoculated activated sludge were 36 mg g VSS^{-1} and gradually increased to 70 mg g VSS^{-1} after mature aerobic granules were formed. Protein (PN) kept a steady value of around 21 mg g VSS^{-1} during granules formation process. The ECP concomitantly increased from 57 mg g VSS^{-1} in acclimated activated sludge to 92 mg g VSS^{-1} in aerobic granules during the immobilization process. Thus, the secretion of ECPs increased during the aerobic granules formation process.

Table 3-1 Profiles of polysaccharides (PS), proteins (PN) and extracellular polymers (ECPs) of PA-degrading aerobic granules

Day	PS (mg g VSS^{-1})	PN (mg g VSS^{-1})	ECPs (mg g VSS^{-1})
0	36 (±3)	21 (±1)	57 (±3)
4	38 (±2)	18 (±1)	57 (±2)
11	50 (±2)	22 (±2)	72 (±3)
16	62 (±3)	24 (±1)	86 (±3)
23	59 (±1)	23 (±1)	82 (±4)
30	66 (±3)	21 (±2)	87 (±3)
42	70 (±4)	22 (±1)	92 (±4)

3.3.3 Kinetic Study of PA Degradation by Aerobic Granules

To evaluate the ability of the different microbial consortia to degrade PA, the PA degradation rates were measured at different concentrations in batch experiments. The specific PA degradation rate increased with PA concentrations and declined with further increases in PA concentrations as substrate inhibition effects became important (Figs. 3-8, 3-9 and 3-10).

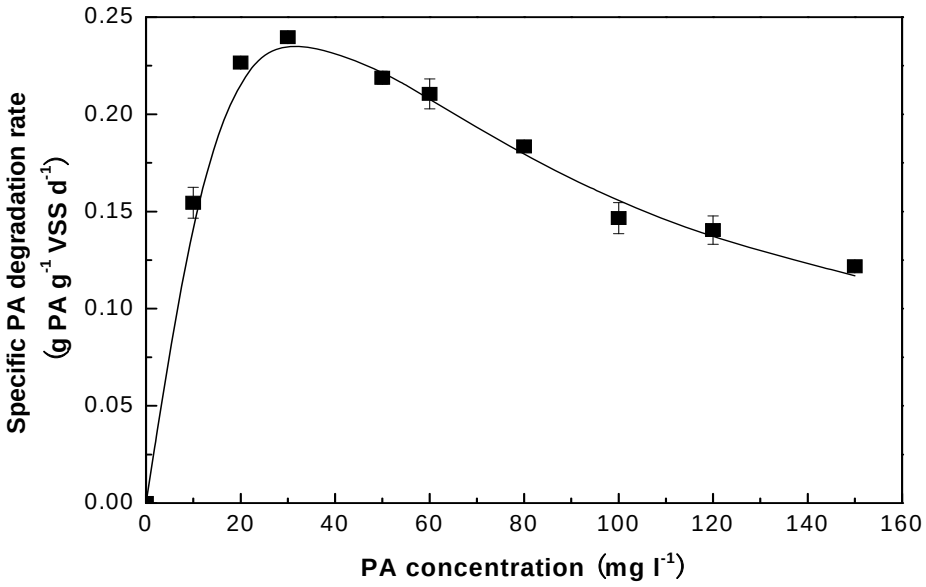


Fig. 3-8 Specific PA degradation rates of fresh activated sludge at different PA concentrations

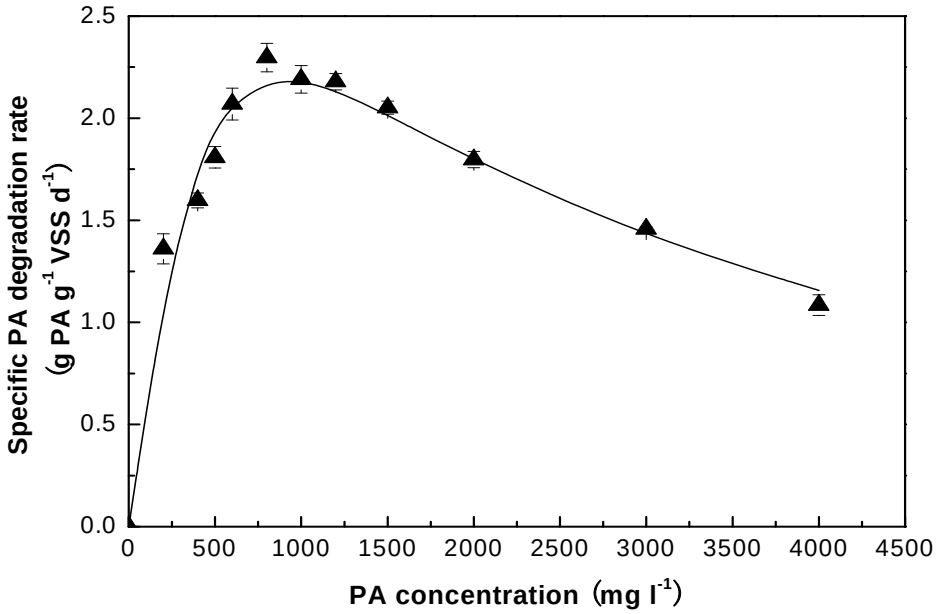


Fig. 3-9 Specific PA degradation rates of acclimated activated sludge at different PA concentrations

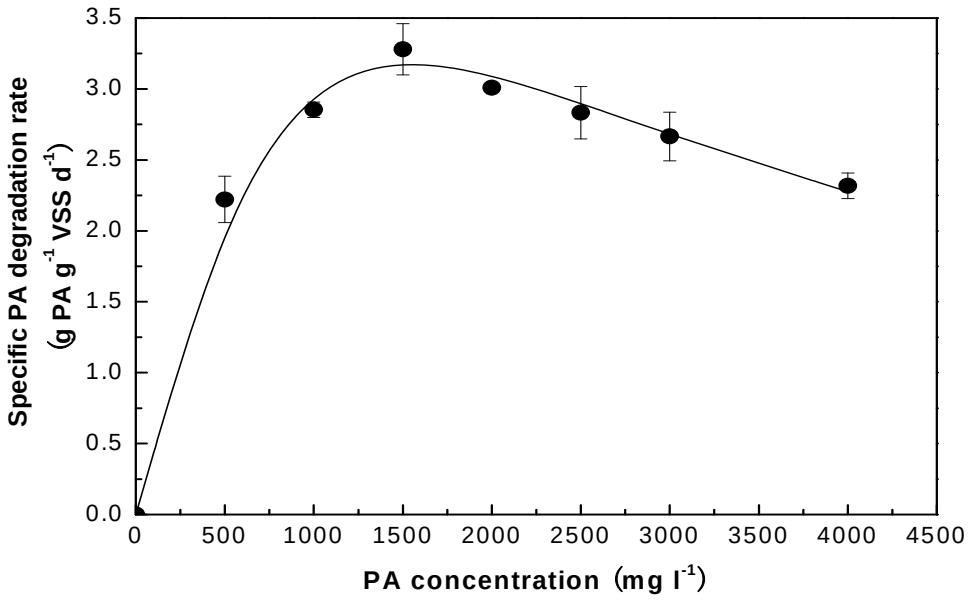


Fig. 3-10 Specific PA degradation rates of aerobic granules at different PA concentrations

The Haldane and least squares model were used to simulate the curves (Nakhla et al., 2006; Adav et al., 2007). The kinetic parameters are calculated and given in Table 3-2. Compared with fresh and acclimated activated sludge, aerobic granules possessed the highest K_s and K_i value.

Table 3-2 Kinetic parameters of PA biodegradation

Item	V_{max} (g PA g VSS^{-1} d^{-1})	K_s (mg l^{-1})	K_i (mg l^{-1})	R^2
Fresh activated sludge	0.9 (±0.03)	45.7 (±2.4)	21.6 (±0.9)	0.996
Acclimated activated sludge	6.9 (±0.3)	1000.0 (±21)	834.0 (±37)	0.973
Aerobic granule	13.3 (±0.5)	2310.0 (±34)	934.8 (±52)	0.992

The minimum PA inhibition concentrations and the highest attainable degradation

rates are calculated according to equations (3-3) and (3-4). The results are listed at Table 3-3. The minimum PA inhibition concentration and attainable degradation rate of aerobic granules were the highest in the three kinds of biomass.

Table3-3 PA concentration and the highest attainable degradation rate

Item	S* (mg l^{-1})	V (g PA g VSS^{-1} d^{-1})
Fresh activated sludge	31.4 (±2.2)	0.23 (±0.03)
Acclimated activated sludge	913.2 (±14.1)	2.10 (±0.10)
Aerobic granule	1469.5 (±32.3)	3.20 (±0.12)

From the results, PA was found to exert a great inhibition effect on the unacclimated activated sludge: the specific PA degradation rate increased when the PA concentration increased from 0 to 31.4 mg l^{-1}, peaked at 0.23 g PA g VSS^{-1} d^{-1}, and decreased after further increases of PA concentration (Fig. 3-8, Table 3-3). For the acclimated activated sludge, the maximum degradation rate was 6.9 g PA g VSS^{-1} d^{-1} and the minimum inhibition concentration was 913.2 mg l^{-1} (Fig. 3-9). However, the aerobic granules facilitate the tolerance of PA at an even higher concentration. The minimum inhibition concentration for aerobic granules increased to 1469.5 mg l^{-1} and the maximum degradation rate increased to 13.3 g PA g $VSS^{-1}.d^{-1}$ (Fig. 3-10, Table 3-2).

3.4 Discussion

In biological wastewater treatment, bioaugmentation is a good strategy to speed up the treatment of toxic/recalcitrant compounds. However, the experiments results of bioaugmentation remain controversial. In order to achieve success in bioaugmentation, inocula with high activity, good survival rate and good retention in the system are expected (Van Limbergen et al., 1998, Bouchez et al., 2000b). In

addition, selection of the easily cultivated inocula with fast growing rates is also an essential factor for bioaugmentation. Study had shown that PA-degrading aerobic granules have the specific traits to meet these requirements.

3.4.1 PA-degrading Aerobic Granule, an Easily Produced Bioseeds Source

Aerobically grown microbial granules were successfully cultivated in a reactor maintained at an OLR of 3.0 kg COD m^{-3} d^{-1}. The granules first appeared in the reactor on day 7 after inoculation with acclimated activated sludge, which grew and became the dominant form of biomass in the reactor. The SVI of the biomass decreased gradually and eventually stabilized at around 41 ml g^{-1} from day 16 to the end of the study on day 42 (Fig. 3-4). The granules were formed in one week and matured within three weeks.

Generally, pure culture and engineered bacteria have been selected as inocula for bioaugmentaion (Andreoni *et al.*, 1998; Abeysinghe *et al.*, 2002; Backman *et al.*, 2004; Bathe et al., 2004a, b; 2005). These bacteria are expected to possess high degradation ability for the target compounds. To find a robust and suitable strain for bioaugmentation, enrichment, isolation, identification, even the recombination of special degradation genes in the chromosome are needed. Thus the pure culture and engineered bacteria production are time consumable and expensive. Comparing with that, the process of enhancing the quantity and activity of microorganisms that are naturally growing on the contaminant is more economical and easy.

The aerobic granules could be formed with any kind of growth substrate whatever it is degradable, recalcitrant or toxic. The formation period varied from 2 weeks to several months (Peng et al., 1999; Tay et al., 2002a, 2005; Yi et al., 2006). It only

took 3 weeks for PA-degrading aerobic granules formation from activated sludge. The indigenous bacteria with PA degrading ability were enriched in the granules formation process. No special procedures or conditions were required during this process. Compared with other pure cultures or engineered bacteria, PA-degrading aerobic granules could be formed in comparable short time that acetate aerobic granules formed. If they are considered as bioseeds source for bioaugmentation, it is easy and fast to be produced.

3.4.2 High Degradation Ability of Aerobic Granules for PA

Although the decrease of bioactivity might be caused by the mass–transfer limitation after aerobic granules formation (Tijhuis et al. 1995; Liu et al., 2005), the SOUR of PA-degrading aerobic granules still maintained a high SOUR value of 70 mg DO g VSS^{-1} h^{-1} (Fig. 3-7). In addition, the granules had significantly better SVI values than the seed flocs, and this allowed a large amount of granules to be retained in the reactor (Figs. 3-4 and 3-5). The significant biomass retention and high SOUR value provided a high TOUR value of 311 g DO h^{-1}, which was almost twice the TOUR of activated sludge. Thus, the overall PA degrading capability of the reactor was enhanced (Fig.3-7). Complete PA removal gives the evidence of the high degradation ability of the aerobic granules (Fig. 3-6).

As compared with fresh activated sludge and acclimated activated sludge, where minimum PA inhibition concentrations occurred at 31.5 and 913.2 mg l^{-1} respectively (Figs 3-8 and 3-9), the minimum PA inhibition concentration for aerobic granules were 1469.5 mg l^{-1} (Fig. 3-10). The simulation of PA-degrading aerobic granules had higher K_s and K_i values than fresh and acclimated activated sludge, which indicated higher affinity and low inhibition of PA on the PA-degrading aerobic granules than the other two kinds of sludge. Thus an effective

PA degradation system has been developed (Fig. 3-10). In addition, the significant rates of PA degradation were still attained at the PA concentrations as high as 4000.0 mg l^{-1}, although the specific PA degradation rate in the granules declined slightly at the PA concentrations above 1469.5 mg l^{-1}. This high tolerance of microbial granules to PA can be exploited to be bioseeds for the treatment of industrial wastewaters containing high PA concentrations. Further more, the PA-degrading aerobic granules could be used as the source of PA-degrader. According to the study of Fan et al. (2001), the DMP (PAEs) degradation could be enhanced by these PA-degraders.

Inocula with high degradation ability for the target compound are desired when selecting bioseeds for bioaugmentation. Considering this aspect, PA-degrading aerobic granules with high PA degradation capability are an eligible resource of bioseeds for phthalate degradation.

3.4.3 Immobilization Provides Protection for Inocula

Generally, immobilization or cell adhesion (as biofilms) provides protection for cells against inhibitory chemicals in the environments. Heipieper (1991) reported that cells embedded in alginate had higher tolerance ability than free cells to high phenol concentrations. The hollow fibre membrane held up the effects of substrate inhibition for the cells entrapped in it (Loh et al., 2000). The biofilms protected cells against biocide agents and antibiotics (LeChevallier et al., 1988). And the aerobic granules showed great tolerance against inhibition by phenol at high concentrations (Jiang et al., 2002).

In the sequencing batch reactor, the suspended PA acclimated activated sludge was aggregated to form aerobic granules with a strong and compact structure (Fig. 3-7). In the process, the inoculated PA-degrading microorganisms were incorporated into

the aerobic granules, and ECPs increased almost 2 times as compared to the activated sludge (Table 3-1). This immobilization, together with the production of ECPs, could form a protective environment for the bacteria against adverse influences, such as predatory protozoa or wash out of the system (Wingender et al., 1999). The predatory protozoa were found to be one the most important factors causing failure of bioaugmentation inoculated with free cells (Barcina et. al., 1997; Van Limbergen et. al., 1998).

Similar to the mechanism of aerobic granules preventing microorganisms from phenol inhibition, the aggregation of microbial cells into compact granules was more likely to provide protection against the high PA concentrations because of the normally existing diffusion resistance from the surface to the inner layer of aerobic granules. As a self-immobilization form, aerobic granules were more attractive bioseeds than the free cells which were immobilized on carriers for bioaugmentation. The cost of the carrier was reduced. Also, the immobilization made sure the high bioactivity of microorganisms and the survival of active microorganisms in targeted ecosystem.

3.5 Summary

This chapter provides information on PA-degrading aerobic granules as bioseeds based on the study of the formation and characteristics of the formed PA-degrading aerobic granules. The PA-degrading aerobic granules with a strong, compact microbial structure were formed within one week and matured within three weeks. They possessed good settling ability, as indicated by the low SVI value of approximately 41 ml g^{-1} and a high biomass concentration of 4.6 g l^{-1} maintained in the reactor. (The biomass concentration of the PA acclimated activated sludge under the same operation conditions was 1.5 g l^{-1}). As a result, an overall PA degradation

capability of the granules was almost improved two-fold over that provided by inoculated activated sludge. They are also robust to endure high PA concentration which was promising in PA and PAEs degradation. In addition, the mount of ECPs produced during the aerobic granules formation was almost twice that in inoculated activated sludge. These components not only provided support for a strong and compact structure, but also protect the functional cells that are immobilized in the aerobic granules from grazing by protozoa. Based on the information provided in this study, PA-degrading aerobic granules were proposed as bioseeds for bioaugmentation in the phthalates degradation which was carried out in next chapter.

CHAPTER 4
PHTHALIC ACIDS AND THEIR ESTERS DEGRADATION BY PHTHALIC ACID-DEGRADING AEROBIC GRANULES

4.1 Introduction

Phthalic acid esters (PAEs) are primarily synthetic compounds mainly used as plasticizers. Some of them are suspected to be mutagens, carcinogens and endocrine disruptor (Kozumbo et al., 1982; Huff et al., 1984; Jobling et al., 1995; Colon et al., 2000; Petrovic et al., 2001). It is necessary to removal of PAEs from wastewater. However, some phthalates are recalcitrant, especially those with high molecular weight and complex structure. Conventional activated sludge processes cannot treat recalcitrant phthalate wastewater effectively (Huang et. al., 1994).

As one of the strategies for recalcitrant compound degradation, bioaugmentation offers the benefits of high populations of degrading microorganisms and highly active species to maintain the high efficiency of the system. In addition, bioaugmentation of PA-degrading microorganisms could enhance the degradation of PAEs (Fan et al. 2001; Wang et al., 2003a). It is hypothesized that PA-degrading microorganisms existing in PA-degrading aerobic granules could enhance the degradation of PAEs by degrading the intermediates. At the same time, the granular sludge could make sure the retention of PA-degrading bacteria in the reactor. Therefore PA-degrading aerobic granules were selected as bioseeds for the degradation of PAEs.

Although PA-degrading aerobic granules possess the special traits to meet the requirements for bioseeds, little is known about the performance of a wastewater treatment system when it is bioaugmented with PA-degrading aerobic granules. This

study was aimed at evaluating the performance of phthalates degradation by the system which was inoculated with PA-degrading aerobic granules. The effects of the bioaugmentation on the morphology and physiology of PA-degrading aerobic granules were also investigated.

4.2 Materials and Methods

4.2.1 Experiment Design and Set-up

Experiments were performed in four identical column-type sequencing batch reactors (SBRs). The configuration of the SBRs was described in section 3.2.1 (page 50). Phthalic acid (PA), di-methyl phthalate (DMP) and di-butyl phthalate (DBP) were fed as the sole carbon and energy source. The operating conditions are listed in Table 4-1.

Table 4-1 Overview of operating conditions.

Reactor	**R1**	**R2**	**R3**	**R4**
Inocula	PA-degrading aerobic granules	PA-degrading aerobic granules	PA-degrading aerobic granules	DMP AS[a]
Concentrations of inocula (mg l^{-1})	4.5	2.4	2.4	2.4
Substrate	PA	DMP	DBP	DMP
Influent substrate concentration (mg l^{-1})	692-2849	577-1154	223	577-1154
Influent COD concentration (mg l^{-1})	1000-4117	1000-2000	500	1000-2000
OLR (kg COD m^{-3} d^{-1})	3.0-12.4	3.0-6.0	1.5	3.0-6.0
Aeration rate (l min^{-1})	3.5	3.5	3.5	3.5
HRT (hr)	8	8	8	8

[a] Activated sludge

4.2.2 Seeding Sludge

The seeding sludge and their concentrations in R1 to R4 are shown in Table 4-1. All the seeding sludge was cultivated at the organic loading rate (OLR) of 3.0 kg COD m^{-3} d^{-1}.

4.2.3 Medium

The substrates feed into the four reactors and the corresponding OLRs are shown in Table 4-1. The nitrogen source, mineral salts and trace solution in the synthetic wastewater were the same as described in Chapter 3.

4.2.4 Analytical Methods

The samples collection and the analysis of pH, TOC, SS, VSS, SVI, SOUR, biomass size and microbial structure observations has been described in Chapter 3. The concentrations of DMP and DBP are measured by a PerkinElmer Series 200 high-pressure liquid chromatography (PerkinElmer, Wellesley, USA). The pretreatment of water samples has been described in Chapter 3. Separation of the DMP and DBP was obtained by using an Inertsil C8-3 (5 μm, 150×4.6 mm I. D.). A mixture of acetonitrile and water in a 70/30 ratio was used as the mobile phase. The applied flow rate was 0.3 ml min^{-1}. The separated components were detected by a UV detector (Spectroflow 773) at a wavelength of 275 nm. Typical retention time for DMP and DBP was 3.1 min and 8.0 min respectively (Kleerebezem et al., 1999b; Fan et al., 2001). The measurement of PA has been described in Chapter 3. Each determination was performed in triplicate.

The volumetric ratio and weight ratio of aerobic granules to whole biomass was measured by the method which was described by Wang et al. (2000b).

4.3 Results

4.3.1 PA Degradation by PA-degrading Aerobic Granules

Reactor R1 was started up using mature PA-degrading aerobic granules as bioseeds, with a mean biomass size of 0.26 mm and a SVI value of 39 ml g^{-1}. The substrate OLR was stepwise increased from 3.0 kg COD m^{-3} d^{-1} to 5.8 kg COD m^{-3} d^{-1} and finally to 12.4 kg COD m^{-3} d^{-1} to evaluate the performance of the aerobic granular reactor with the increment of OLR.

4.3.1.1 PA Degradation at OLR of 3.0 kg COD m^{-3} d^{-1}

From day 0 to day 29, the reactor R1 was operated at an OLR of 3.0 kg COD m^{-3} d^{-1} which was the same OLR cultivated the PA-degrading aerobic granules. R1 reached its steady state operation within a week. The PA and TOC removal efficiency were 99.9% and 98.2%, respectively. Aerobic granules showed the compact structure (Figs. 4-1 to 4-3 and Fig. 4-4a) with an average SVI of 40 ml g^{-1}. The volumetric ratio of aerobic granules to whole biomass was 97.0 %, and the weight ratio was above 97.8% (Fig. 4-5a and Table 4-2).

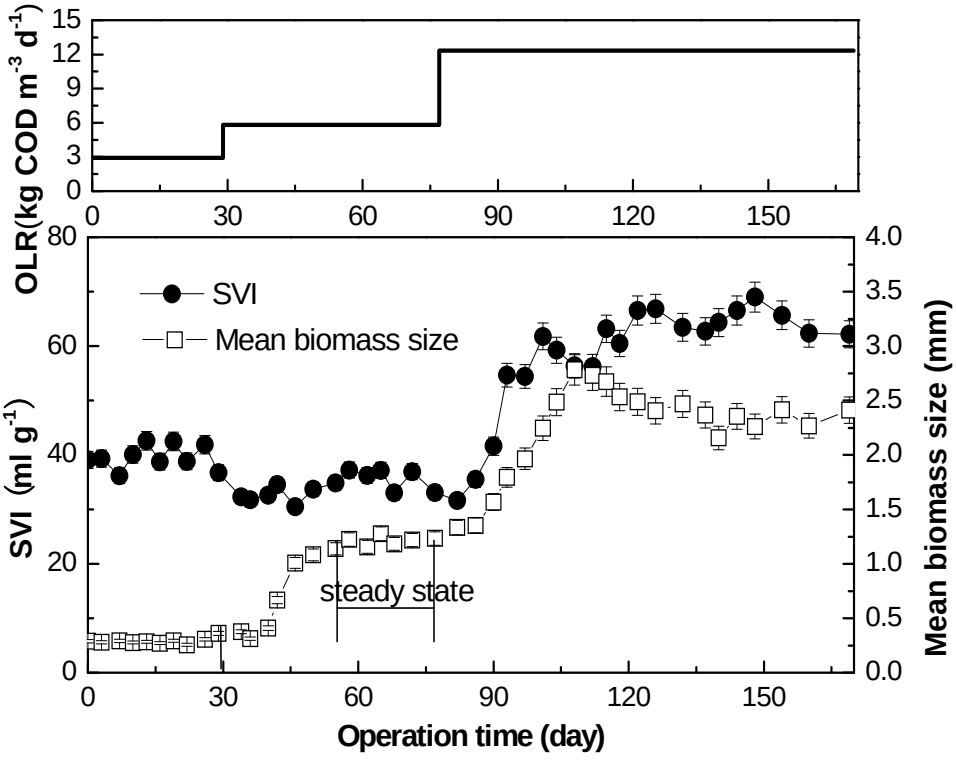


Fig. 4-1 Profiles of sludge volume index (SVI) and mean biomass size of aerobic granules in R1.

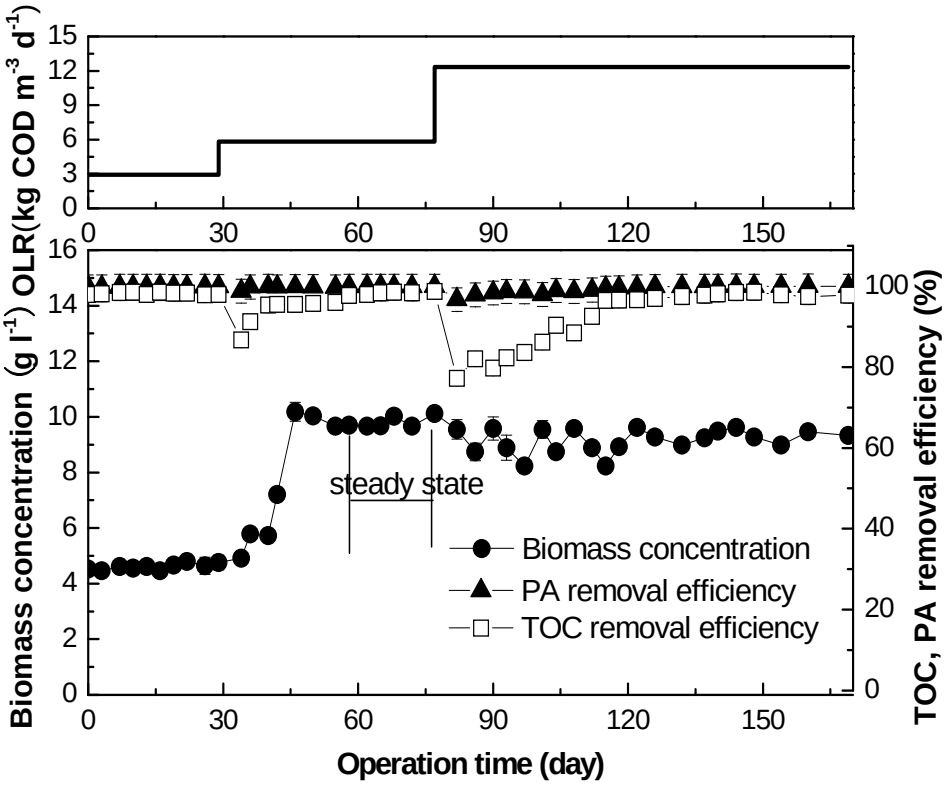


Fig. 4-2 Profiles of biomass concentration and PA, TOC removal efficiency in R1.

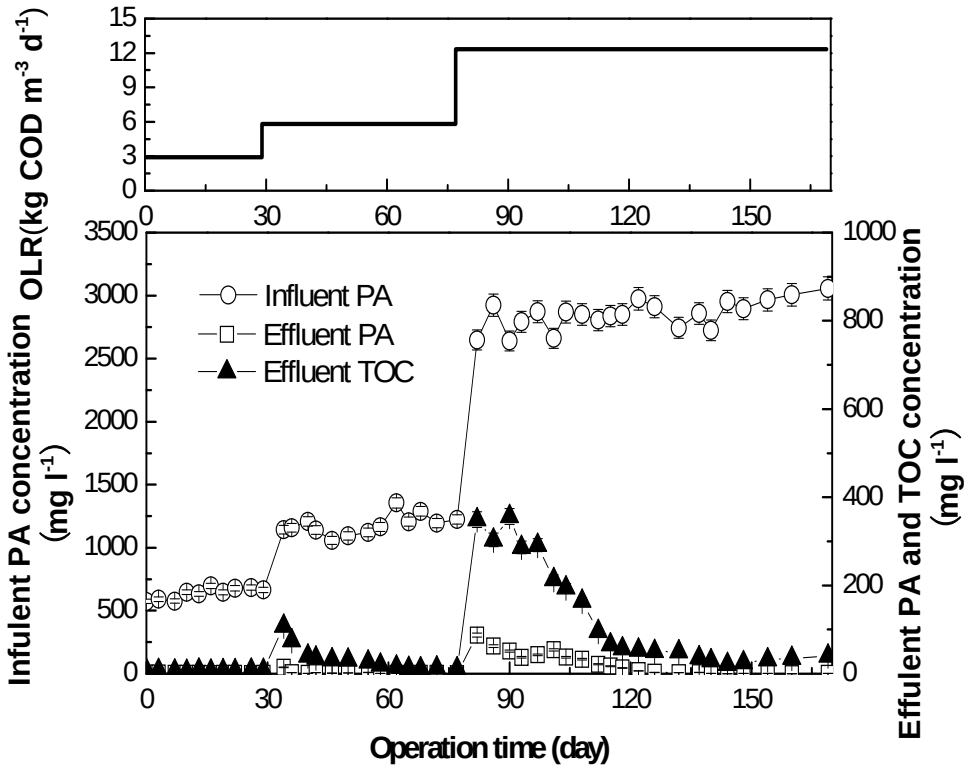


Fig. 4-3 Profiles of influent PA concentrations, effluent PA concentrations, and TOC concentrations in R1.

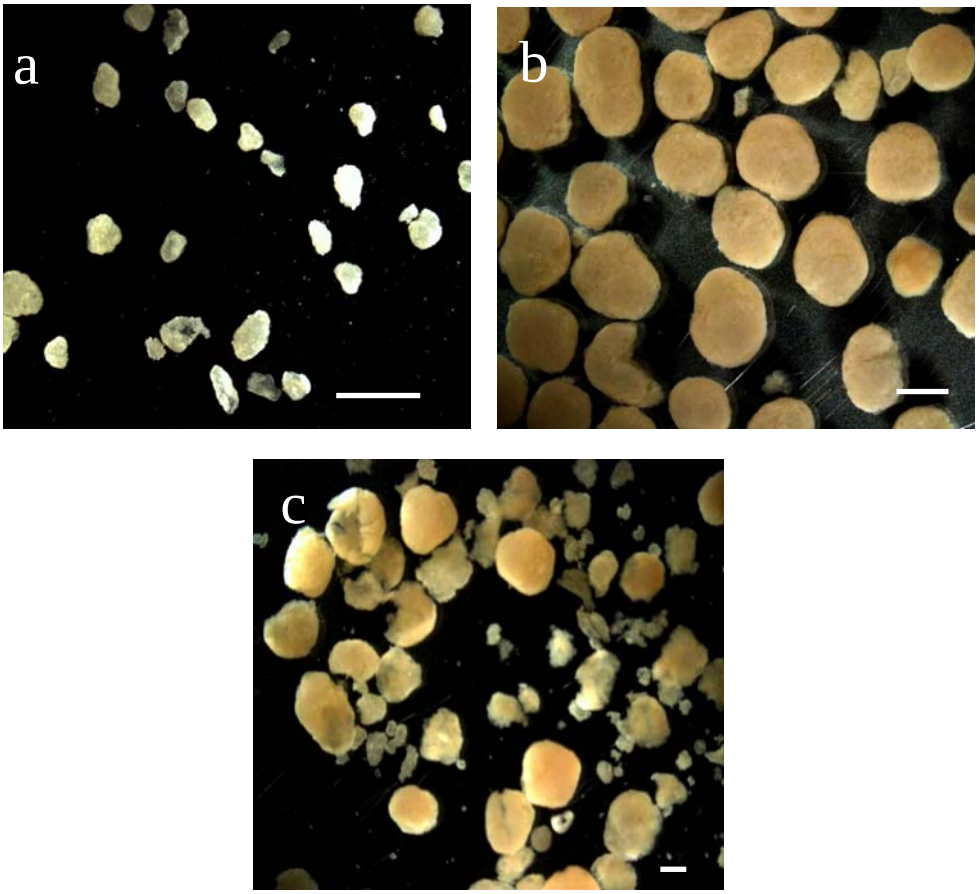


Fig. 4-4 Morphology of aerobic granules in R1 at OLR of (a) 3.0 kg COD m^{-3} d^{-1} (bar: 1 mm), (b) 5.8 kg COD m^{-3} d^{-1} (bar: 1 mm) and (c) 12.4 kg COD m^{-3} d^{-1} (bar: 1 mm).

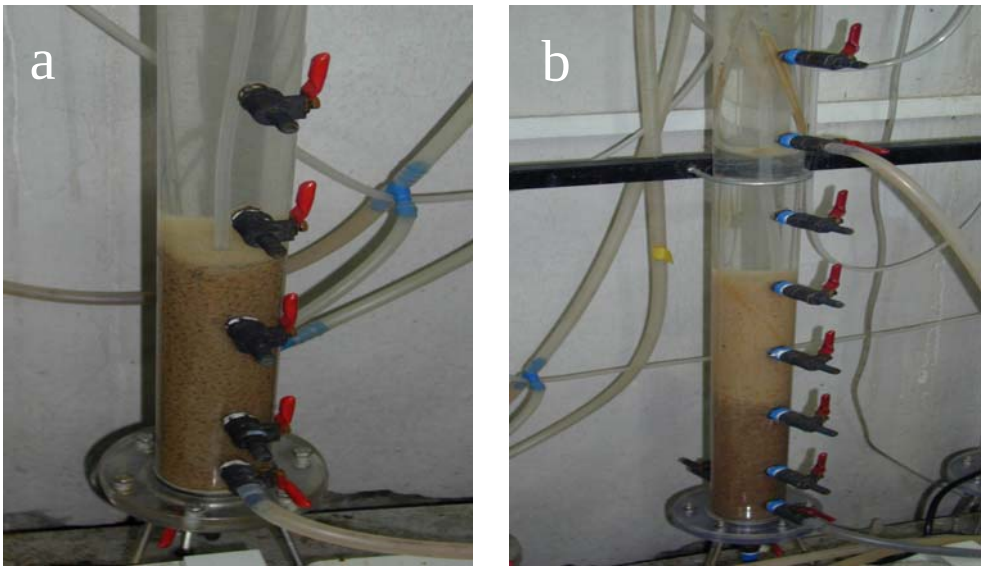


Fig. 4-5 Aerobic granules in R1 at OLR of (a) 3.0 kg COD m^{-3} d^{-1} and (b) 12.4 kg COD d^{-1} m^{-3}.

Table 4-2 Experimental results in R1 at different OLRs

OLR (kg COD m^{-3} d^{-1})	**3.0**	**5.8**	**12.4**
Average influent TOC (mg l^{-1})	390.1 (±3.0)	776.8 (±8.1)	1647.2 (±12.3)
Average effluent TOC (mg l^{-1})	6.5 (±0.7)	20.0 (±2.5)	39.6(±1.8)
TOC removal efficiency (%)	98.3	96.6	97.7
Aerobic granules, Xa (g l^{-1})	4.4 (±0.1)	9.2 (±0.3)	7.4 (±0.1)
Compacted biomass*, Xs (g l^{-1})	0.1 (±0.0)	0.5 (±0.1)	2.1 (±0.1)
Total biomass, Xt (g l^{-1})	4.5 (±0.1)	9.7 (±0.4)	9.5 (±0.2)
Weight ratio of aerobic granules to total biomass, Xa/Xt (%)	97.8	94.9	78.4
Volumetric ratio of aerobic granules to total biomass (%)	97.0	93.3	68.2
SOUR of compacted biomass, (mg DO g^{-1} VSS h^{-1})	127 (±12)	134 (±10)	131 (±11)
SOUR of aerobic granules, (mg DO g^{-1} VSS h^{-1})	67 (±5)	42 (±10)	47 (±8)
TOUR of compacted biomass, (mg DO h^{-1})	30 (±3)	157 (±12)	629 (±36)
TOUR of aerobic granules, (mg DO h^{-1})	668 (±26)	859 (±38)	750 (±33)
TOUR of reactor, (mg DO h^{-1})	698 (±32)	1018 (±23)	1379 (±35)

*The detached aerobic granules, co-existed with aerobic granules in granular reactor, had high setting ability and compact structure. Hereafter, they were defined as compact biomass.

The PA concentrations, TOC concentrations and pH values were measured during one cycle time in R1 at its steady state with an OLR of 3.0 kg COD m^{-3} d^{-1} (Fig. 4-6). Time zero coincided with the start of one cycle. Since the volumetric exchange ratio for the reactor was 50%, PA and TOC concentrations in the reactor at time zero were approximately half of the influent concentrations. The pH value ranged between 6.0 and 8.0. It took 20 min to completely remove PA/TOC, and aerobic granules were under famine conditions for the rest of the cycle time (Fig. 4-6). After 20 min of the cycle time, PA was completely degraded to zero while the TOC level still remained approximately at 10.0 mg l^{-1}, which was probably due to the existence of non-biodegradable microbial products.

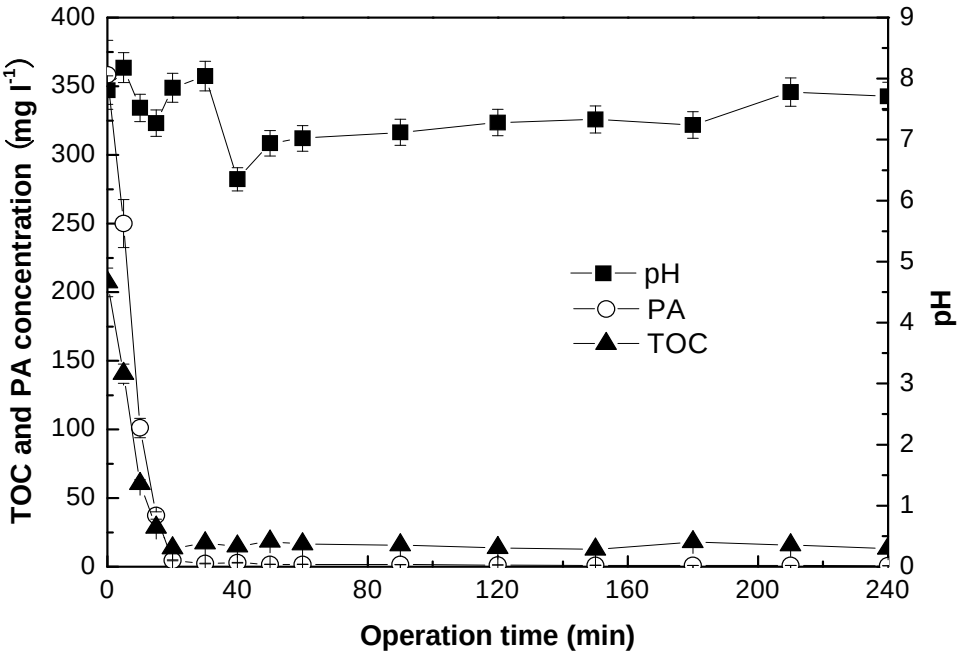


Fig. 4-6 Profiles of PA and TOC removal in one cycle at steady state at an OLR of 3.0 kg COD m^{-3} d^{-1}.

4.3.1.2 PA Degradation at OLR of 5.8 kg COD m^{-3} d^{-1}

From day 30 to day 77, the OLR was increased to 5.8 kg COD m^{-3} d^{-1} by increasing the influent PA concentration to 1180.0 mg l^{-1}. The reactor reached its steady state in two weeks, which was evidenced by stable TOC removal efficiencies and biomass concentrations. Fig. 4-1 shows that the increment of OLR did not significantly affect the settling ability of granules, as the SVI values were maintained at between 25 and 45 ml g^{-1}. The mean biomass size increased above 1.20 mm and biomass concentration increased greatly from 4.5 to 9.7 g l^{-1} (Fig. 4-2 and Table 4-2). PA removal efficiency decreased to 98.7% in the first 5 days after the increment of OLR and then increased back to 99.9%. TOC removal efficiency in the reactor also dropped to 86.7% in the first 5 days and then increased to 96.6% (Fig. 4-2). The effluent TOC concentration in the rector was less than 13.0 mg l^{-1} (Fig. 4-3). The volumetric ratio of aerobic granules to whole biomass was 93.3% and the weight ratio was 94.9% (Table

4.3.1.3 PA Degradation at OLR of 12.4 kg COD m^{-3} d^{-1}

From day 77 onwards, the OLR was increased to 12.4 kg COD m^{-3} d^{-1} by increasing the influent PA concentration to 2849.0 mg l^{-1}. The SVI increased immediately after OLR was increased and then stabilized at approximately 64 ml g^{-1} (Fig. 4-1). The mean biomass size increased from 1.20 mm to 2.70 mm after the OLR increased to 12.4 kg COD m^{-3} d^{-1}. However, part of the aerobic granules detached and became small pieces when the mean biomass size increased to 2.70 mm, which resulted in the mean biomass size finally dropping and stabilizing at 2.40 mm (Fig. 4-1 and 4-4c). The biomass concentration in R1 had no significant change after the OLR increased to 12.4 kg COD m^{-3} d^{-1}(Fig. 4-2), but the volumetric ratio of aerobic granules to total biomass decreased to 68.2% and the weight ratio decreased to 78.4% (Fig. 4-5b and Table 4-2).

The increase of OLR to 12.4 kg COD m^{-3} d^{-1} did not diminish the PA degrading capability. The PA removal efficiency decreased to 95.8% and the TOC removal efficiency decreased to 78.3% on the first day with the OLR at 12.4 kg COD m^{-3} d^{-1} (Fig. 4-2). After the reactor reached its steady state operation, the PA concentrations in the discharge effluent were less than 1.0 mg l^{-1}, while TOC concentrations were less than 40.0 mg l^{-1}. The PA and TOC removal efficiencies were 99.9% and 97.7%, respectively (Fig. 4-3 and Table 4-2).

The pH value, PA and TOC concentrations were measured during one cycle time when the reactor reached steady state at an OLR of 12.4 kg COD m^{-3} d^{-1} (Fig. 4-7).

The pH value was between 7.6 and 8.3. 60 min were required for complete removal of the PA and TOC at the OLR of 12.4 kg COD m^{-3} d^{-1} (Fig. 4-7).

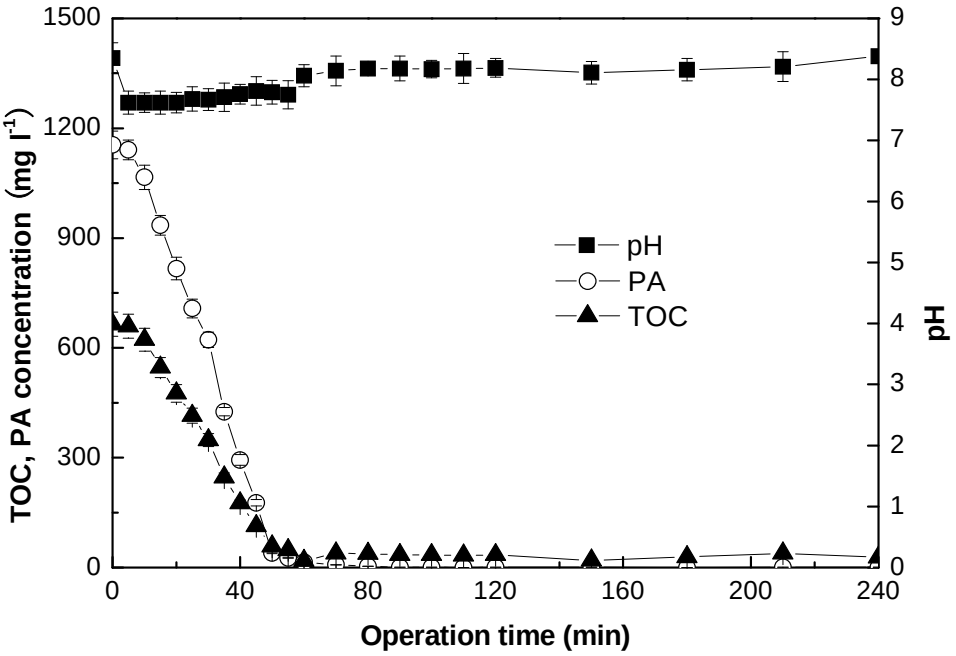


Fig. 4-7 Profiles of PA and TOC removal in one cycle at steady state at an OLR of 12.4 kg COD m^{-3} d^{-1}.

When the OLR increased to 12.4 kg COD m^{-3} d^{-1}, some aerobic granules was broken and converted to smaller size aerobic granules or small pieces of scarps which is defined as compact biomass (Fig. 4-4c). Most of these compact biomass were retained in reactor. The granules and compact biomass were observed by SEM (Fig 4-8). These granules and compact biomass both had compact structures, but cracks or holes can be observed.

The SOUR of the compact biomass was three-fold that of aerobic granules (Table 4-2). About 45.6% of total TOUR of R1 provided by the compact biomass when the OLR increased to 12.4 kg COD m^{-3} d^{-1}. The SOUR of aerobic granules had no significant change, but the TOURs of aerobic granules dropped to 750 mg DO h^{-1} resulting from the decrease of aerobic granular biomass in R1 (Table 4-2). However, the TOUR

provided by the compact biomass compensated for the loss caused by the decrease of aerobic granules' SOUR. Therefore, the TOUR of the total biomass in R1 at the OLR of 12.4 kg COD m^{-3} d^{-1} was higher than that for 5.8 kg COD m^{-3} d^{-1}(Table 4-2).

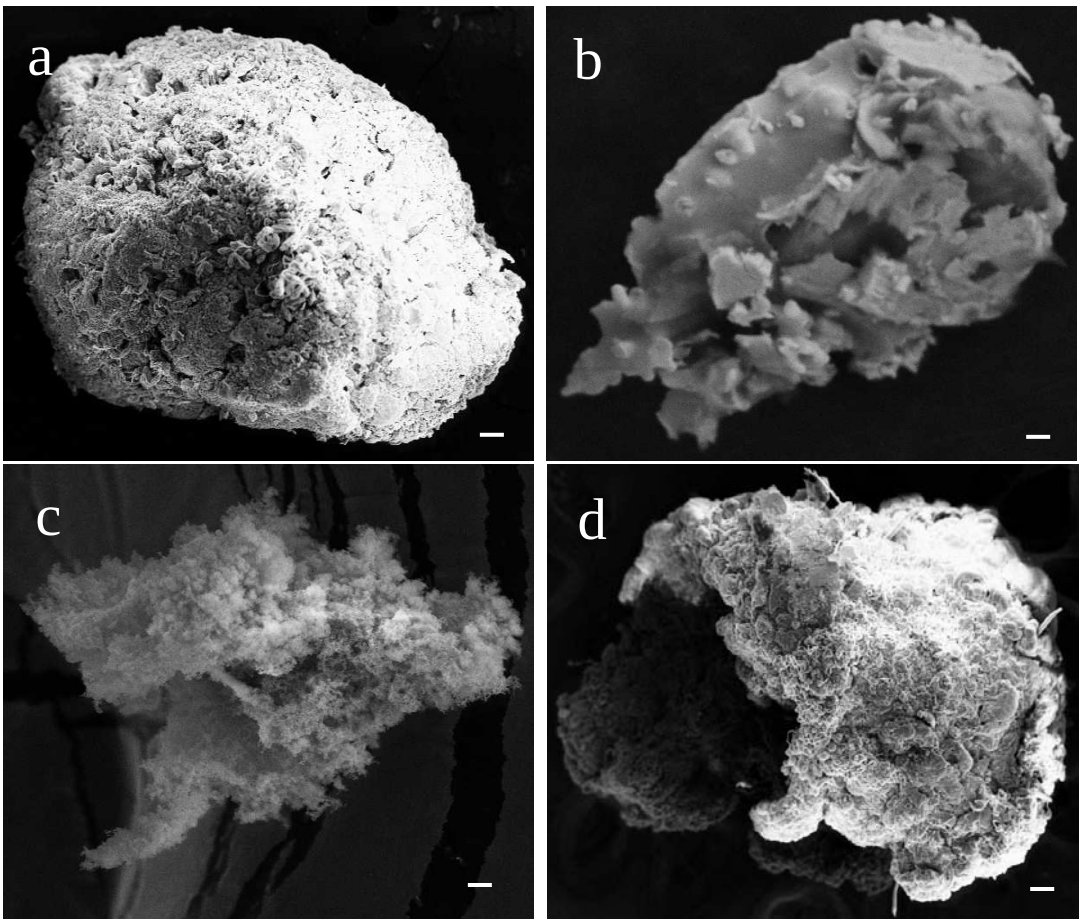


Fig. 4-8 Microstructure of aerobic granules at 12 kg COD m-3 d-1 observed by SEM. (a) whole granule (bar: 20 μm). (b) Compact biomass with condense structure (bar: 400 nm) (c) Compact biomass with condense structure (bar: 1 μm) (d) Compact biomass with condense structure (bar: 20 μm).

4.3.2 DMP Degradation by PA-degrading Aerobic Granules

4.3.2.1 Reactor Operation at 3.0 kg COD m^{-3} d^{-1}

Reactor R2 was operated by feeding DMP as the sole carbon and energy source at 3.0 kg COD m^{-3} d^{-1}, which corresponded to an influent DMP concentration of 577.0 mg

l^{-1}. PA-degrading aerobic granules were used as the inocula with mean biomass size of 0.37 mm and a SVI value of 41 ml g^{-1}.

The mean biomass size of aerobic granules increased from 0.37 to 1.00 mm after 4 days operation and was kept at a steady value of 1.10 mm from day 4 to day 39 at an OLR of 3.0 kg COD m^{-3} d^{-1} (Fig. 4-9). The SVI value of the aerobic granules had a slight decrease in the first 10 days' operation. Gradually, the SVI reached steady value of 30 ml g^{-1} at an OLR of 3.0 kg COD m^{-3} d^{-1}.

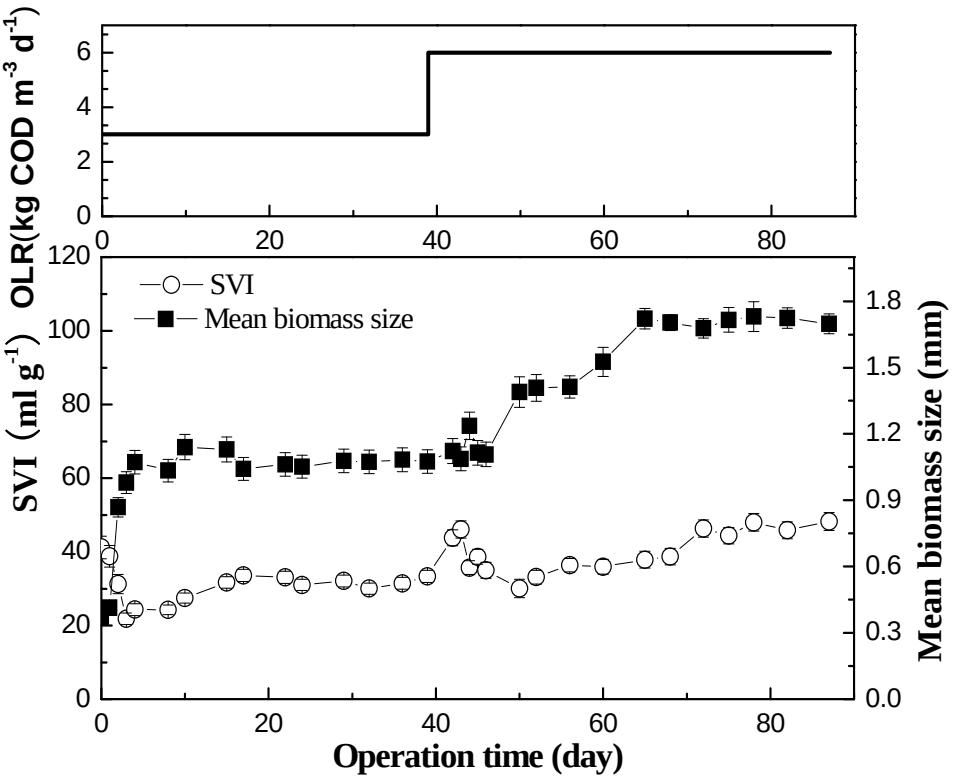


Fig. 4-9 Profiles of SVI and mean biomass size in R2.

The biomass concentration of R2 increased when the substrate changed from PA to DMP on day 0, even though it was under the same OLR operation. After 15 days, the biomass concentration reached 6.0 g l^{-1}, and maintained at this concentration from day 15 to 39 (Fig. 4-10). The influent pH value was controlled between 7.1 and 7.5 during the entire process by the addition of $NaHCO_3$ solution. The effluent pH value dropped

to 6.5 on days 3 and 9 after the DMP was fed into R2. It soon increased to 6.9 on day 4 and 7.0 on day 15, without extra dosage of $NaHCO_3$. After that, the effluent pH value increased gradually. When the reactor reached steady state, the effluent pH value finally increased to above 7.0 (Fig. 4-10).

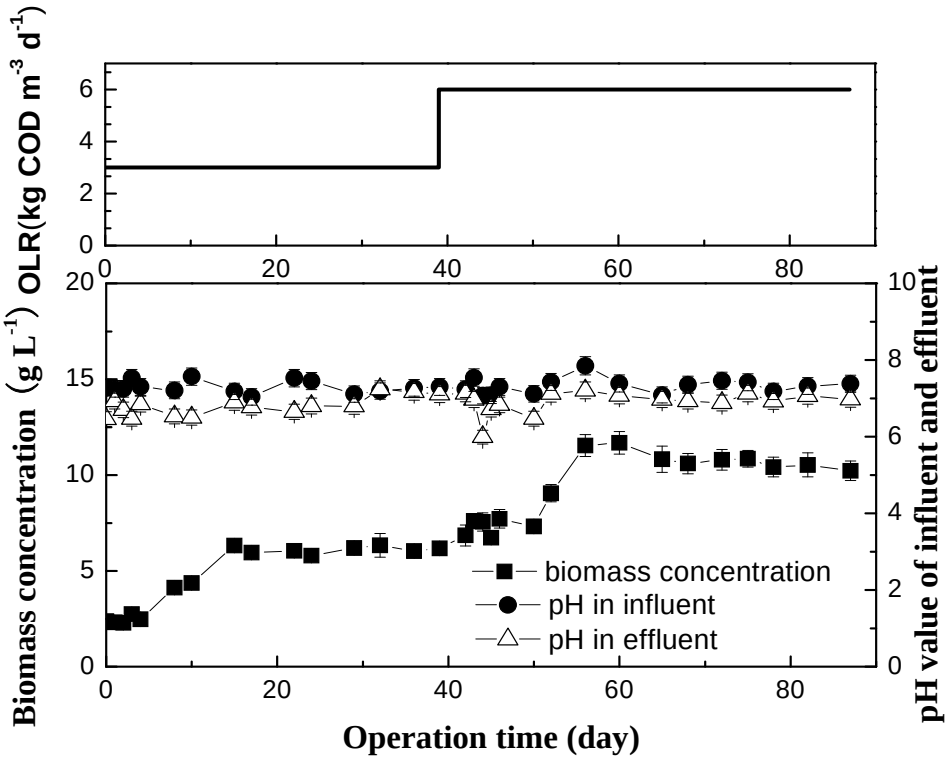


Fig.4-10 Profiles of biomass concentration, influent and effluent pH value in R2.

Although there was no acclimation process during the start up, the inoculated PA-degrading aerobic granules successfully removed all of the DMP fed into the reactor. At an OLR of 3.0 kg COD m^{-3} d^{-1}, the DMP removal efficiency was 100% in the entire process except on day 0 with a DMP removal efficiency of 98.5% (Fig. 4-11). The TOC removal efficiency was only 45.6% on day 0 and reached 90.3% on day 4. When the reactor reached its steady state, the TOC removal efficiency had increased to 98.5% (Fig 4-11).

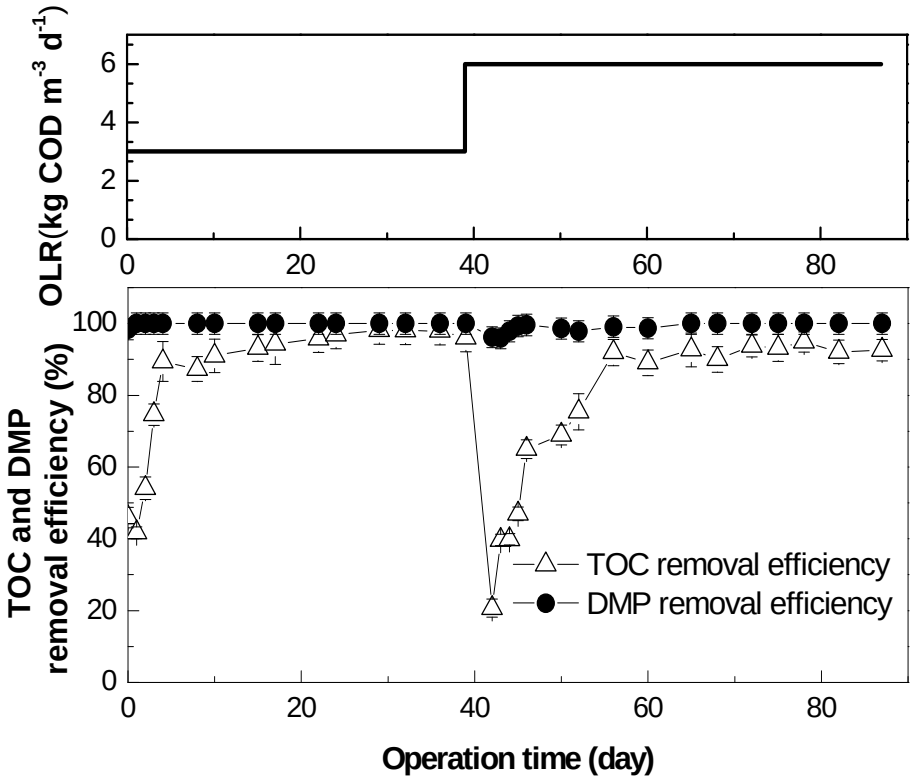


Fig. 4-11 Profiles of DMP and TOC removal efficiency in R2.

DMP, PA and TOC concentrations were also measured during one cycle time in the reactor on day 0 and day 39 at an OLR of 3.0 kg COD m^{-3} d^{-1}. The DMP and TOC concentrations in the reactor at time zero were approximately half of the influent DMP and TOC concentrations for the same reasons mentioned before. It took 180 and 40 min to completely remove DMP on day 0 and day 39, respectively. The DMP degradation ability improved greatly (Figs. 4-12 and 4-13).

PA is the intermediate of DMP degradation. During one cycle time on day 0, PA appeared at 10 min with a concentration of 23.2 mg l^{-1}, and then quickly decreased to 1.6 mg l^{-1} at 15 min. At 20 min, the PA accumulated to 9.3 mg l^{-1} and it was exhausted at 40 min (Fig. 4-12). During one cycle time on day 39, PA appeared at 5 min with a concentration of 25.8 mg l^{-1} and was exhausted at 10 min. It accumulated again at 20 min to be 9.5 mg l^{-1} and was completely removed at 30 min (Fig. 4-13).

On day 0, TOC could not be completely degraded in one cycle time. Approximately 40.0% TOC remained at the end of the cycle. On day 39, 98.0% TOC was removed within 50 min. Owing to the presence of some microbial products, TOC concentration cannot decrease to zero level. The change of pH value was controlled between 7.0 and 8.0 for the two cycles on day 0 and day 39, respectively (Figs. 4-12 and 4-13).

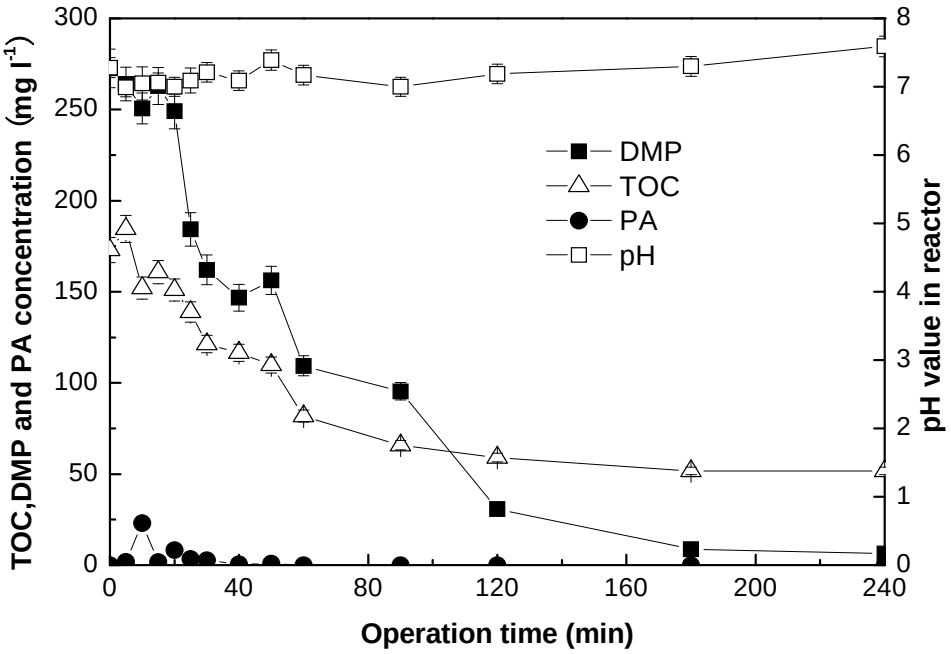


Fig. 4-12 Concentration profiles of DMP, TOC, PA and pH value during one cycle time in R2 on day 0 at an OLR of 3.0 kg COD^{-3} d^{-1}.

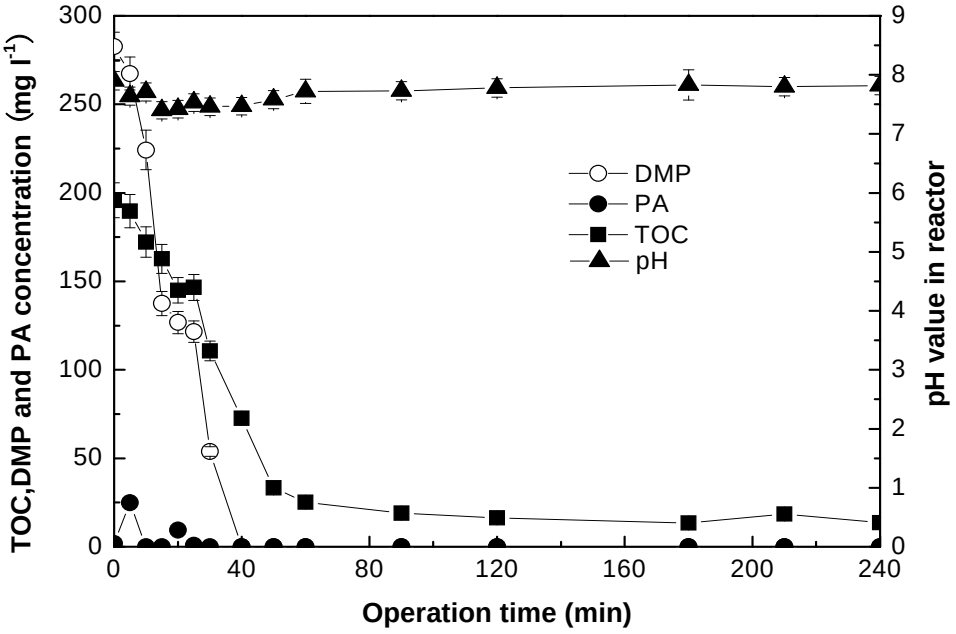


Fig. 4-13 Concentration profiles of DMP, TOC, PA and pH value during one cycle time in R2 on day 39 at an OLR of 3.0 kg COD^{-3} d^{-1}.

The initial DMP concentration of 290.0 mg 1^{-1} in the reactor was used in the SOUR assays with a biomass concentration of 0.3-0.4 g VSS l^{-1}. SOUR values increased from 47 to 68 mg DO g^{-1} VSS h^{-1} in the first 3 days operation and quickly stabilized at 45 mg DO g^{-1} VSS h^{-1} after 10 days operation (Fig. 4-14). TOUR values increased sharply with the increase of biomass concentration. After 3 weeks' operation, TOUR values stabilized at around 272 mg DO h^{-1}. The SOUR value on day 39 had no obvious change as compared with the initial SOUR values of PA-degrading aerobic granules, while TOUR values were approximately three-fold higher than the initial one because of the increasing biomass (Fig. 4-14).

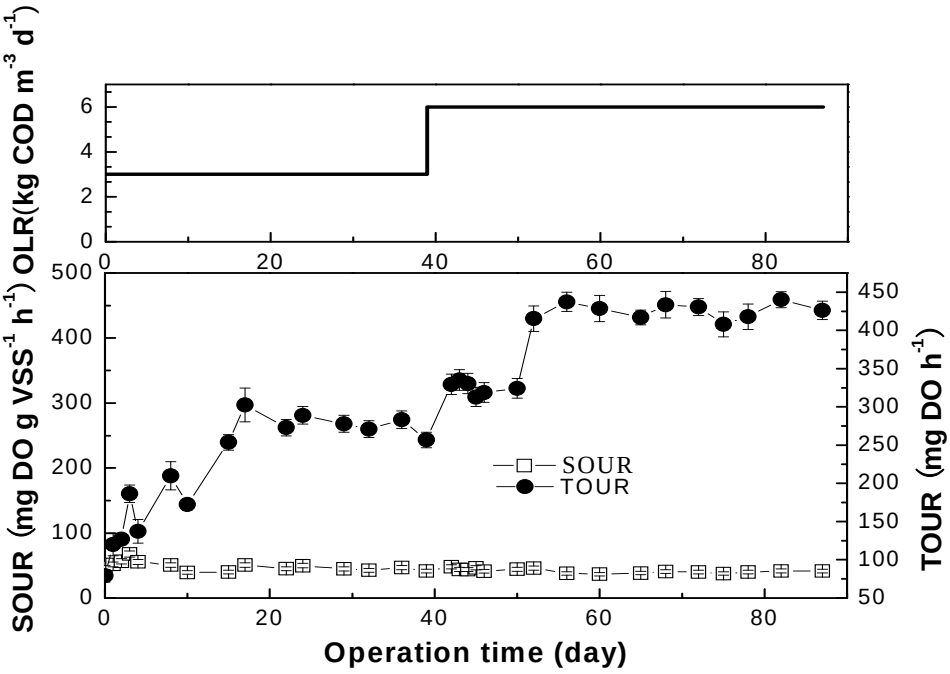


Fig. 4-14 Profiles of SOUR and TOUR in R2.

The morphology of aerobic granules became more compact after the substrate changed from PA to DMP (Fig. 4-15a, b). SEM images revealed that the inoculated PA-degrading aerobic granules and DMP-degrading aerobic granules had a cauliflower-like appearance at the surface (Fig. 4-16a, c). Bacterial cells were tightly clustered together and embedded within the extracellular polymeric matrix. In PA-degrading granules, non-filamentous bacteria, such as rods and cocci, dominated the microbial community residing in the granules. For DMP-degrading aerobic granules, filamentous bacteria appeared in the microbial community besides the rods and cocci (Fig. 4-16b, d).

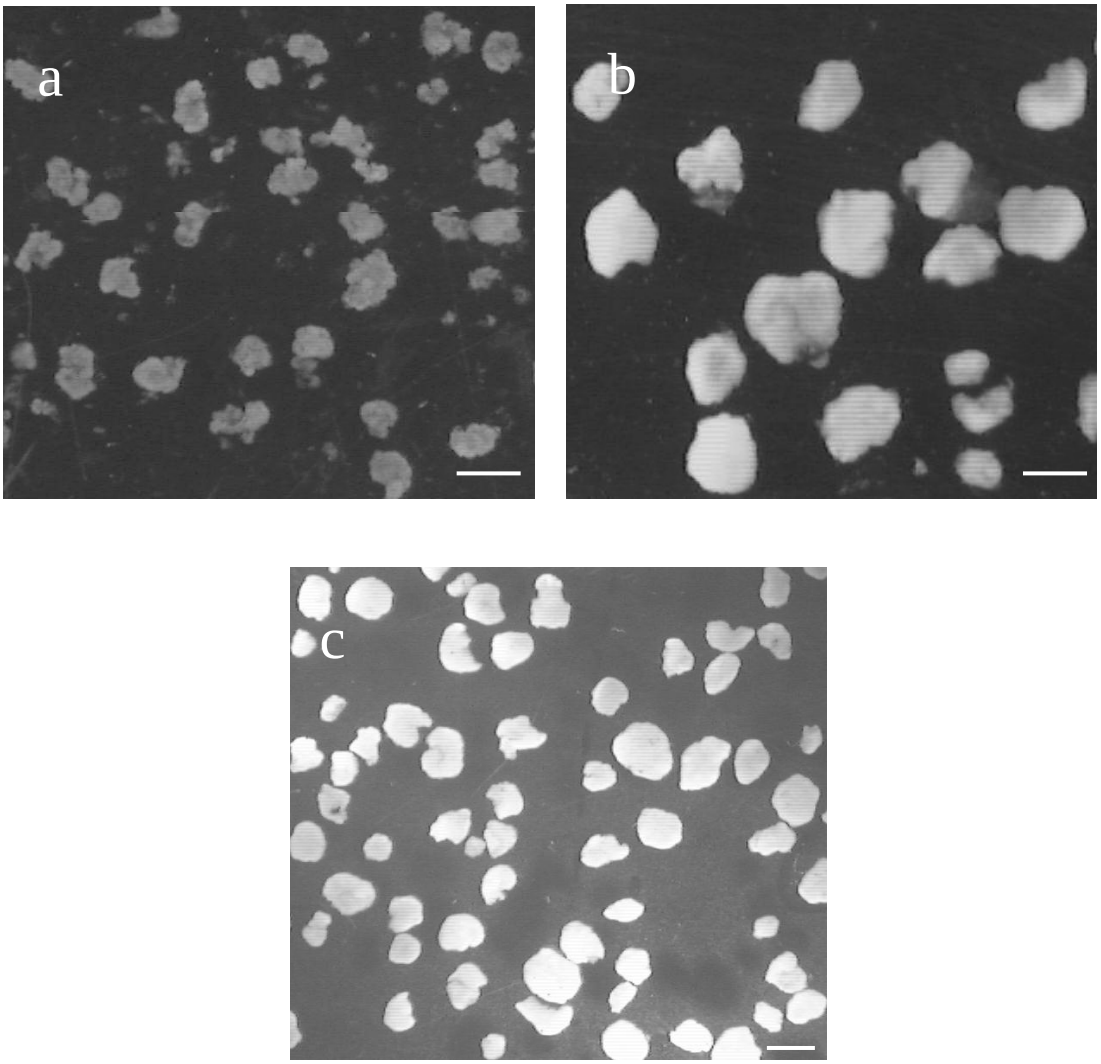


Fig. 4-15 Image analysis of aerobic granules (a) PA-degrading aerobic granules (bar: 0.5 mm); (b) DMP-degrading aerobic granules in R2 at an OLR of 3.0 kg COD m^{-3} d^{-1} (bar: 1 mm) (c) DMP-degrading aerobic granules in R2 at an OLR of 6.0 kg COD m^{-3} d^{-1} (bar: 2 mm).

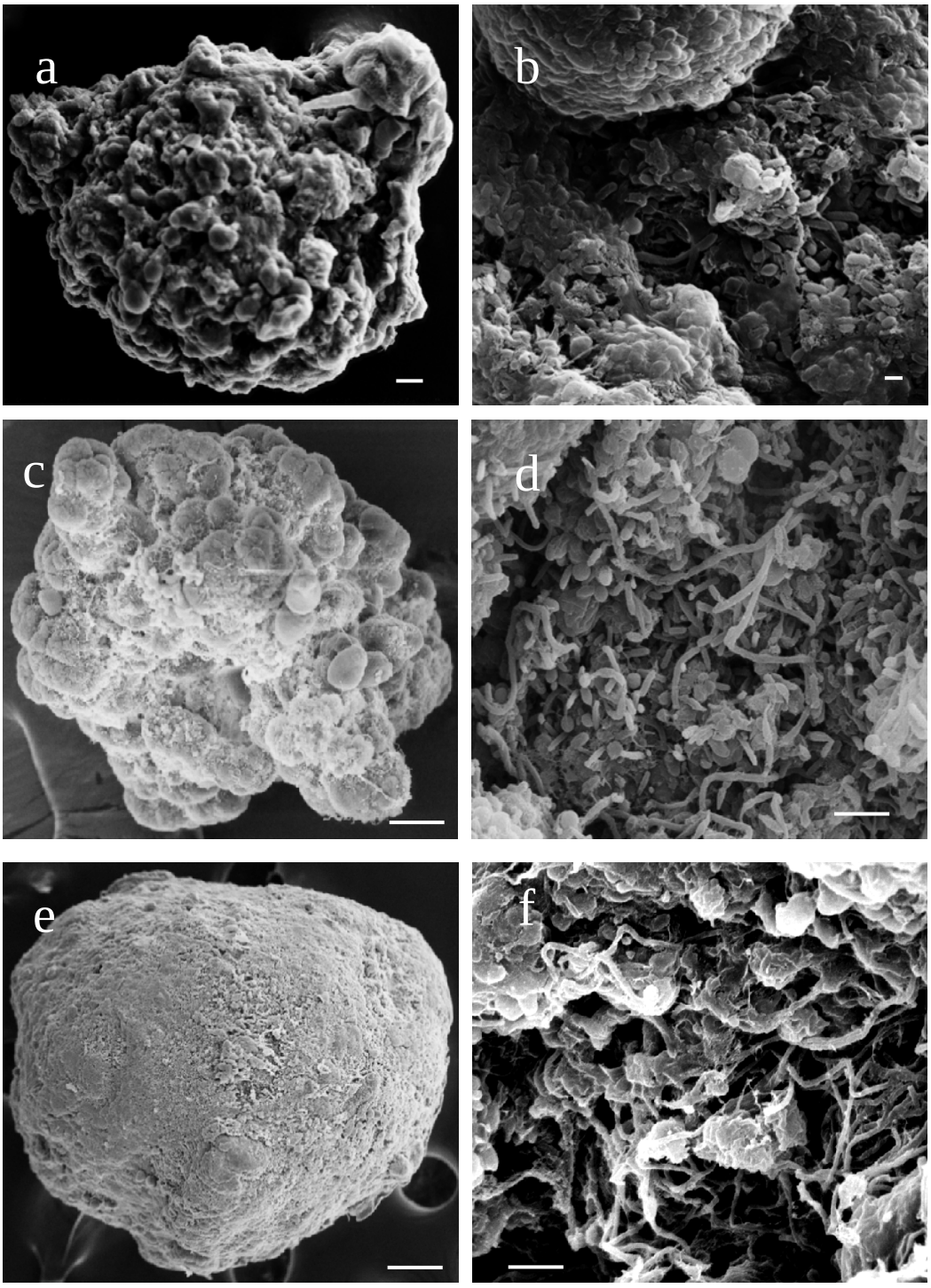


Fig. 4-16 Scanning electron micrographs of aerobic granules, (a) PA-degrading aerobic granules (bar: 20 μm); (b) Surface of PA-degrading aerobic granules (bar: 1 μm); (c) DMP-degrading aerobic granules at 3.0 kg m^{-3} d^{-1} (bar: 30 μm) (d): Surface of DMP-degrading aerobic granules at 3.0 kg m^{-3} d^{-1} (bar: 3 μm); (e) DMP-degrading aerobic granules at 6.0 kg m^{-3} d^{-1} (bar: 100 μm) (f): Surface of DMP-degrading aerobic granules at 6.0 kg m^{-3} d^{-1} (bar: 3 μm).

4.3.2.2 Reactor Operation at OLR of 6.0 kg m^{-3} d^{-1}

From day 40 onwards, the OLR of R2 increased to 6.0 kg COD m^{-3} d^{-1}. The SVI of aerobic granules immediately increased to 76 ml g^{-1}. After several days' operation, the SVI decreased gradually and was stable at 46 ml g^{-1} after 33 days' of operation (Fig. 4-9). The mean biomass size increased sharply when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. At steady state, the mean biomass size increased to 1.70 mm, 1.5 times that at an OLR of 3.0 kg COD m^{-3} d^{-1}(Fig. 4-9).

The biomass concentration increased with the increase of OLR and was steady at 10.6 g l^{-1} after 26 days' operation (Fig. 4-10). Similar to the previous operation, the influent DMP pH value was controlled between 7.0 and 8.0 by adding $NaHCO_3$ when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. The effluent pH value decreased to 6.0 on day 44 and decreased to 6.5 on day 50 because of intermediates accumulation. However it returned to 6.7 on day 45 and 7.1 on day 52 without adding extra buffer solution. After that the pH of the effluent was maintained at above 7.0 when the reactor reached steady state (Fig. 4-10).

The increase of OLR had little influence on the DMP removal efficiency. The DMP removal efficiency decreased to 95.9% when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. Two days later, the DMP removal efficiency increased to 99.3%. At steady state, the DMP was completely removed (Fig. 4-11). The TOC removal efficiency of R2 decreased to 20% when the OLR was increased from 3.0 to 6.0 COD m^{-3} d^{-1} in reactor R2 on day 40. From day 42 onwards, the aerobic granules resumed their degradation ability, with TOC removal efficiency recovered to above 90% 15 days later. At steady state, the average TOC removal efficiency was about 93.1% (Fig. 4-11).

DMP, PA and TOC concentrations in one cycle time were measured at steady state when operated at an OLR of 6.0 kg COD m^{-3} d^{-1}. 60 min were required for complete removal of DMP. TOC was degraded slowly in the first 25 min because of the

accumulation of PA, and it was completely degraded within 120 min. PA appeared at 5 min with a concentration of 13.1 mg l^{-1}; it subsequently increased to 57.7 mg l^{-1} at 10 min and then dropped to zero at 15 min. At 30 min, it accumulated to 44.8 mg l^{-1} again and was completely degraded at 90 min. The pH value in the reactor varied between 7.4 and 8.0. The lowest pH value of 7.4 during a cycle time appeared at 20 min (Fig. 4-17).

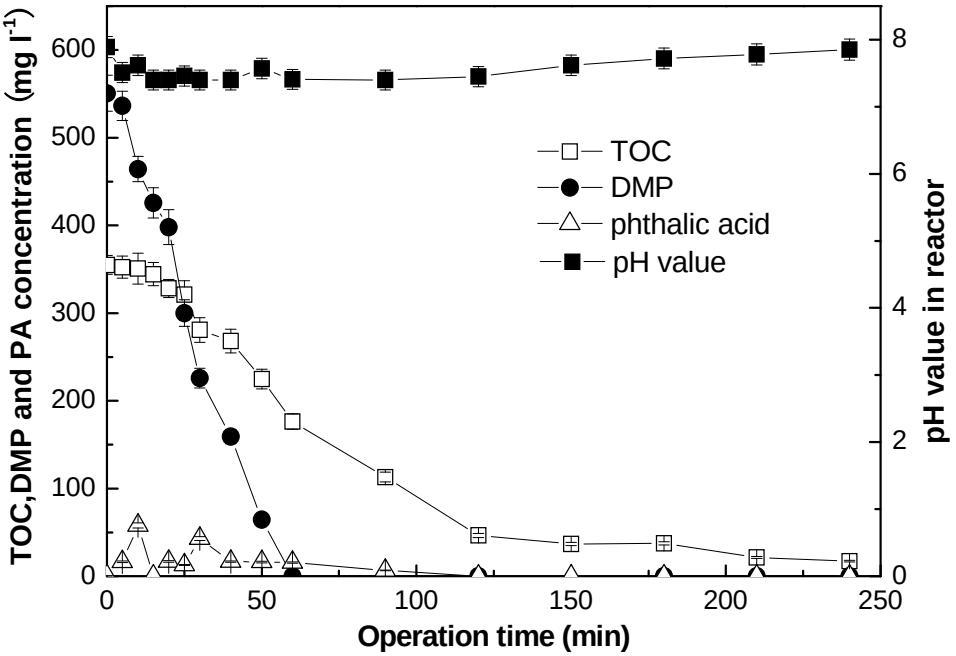


Fig. 4-17 Profiles of DMP, TOC, PA concentrations and pH value in one cycle time of R2 at an OLR of 6.0 kg COD m^{-3} d^{-1}.

The increasing OLR had little influence on the aerobic granules' SOUR in R2. SOUR values slightly decreased and stabilized at 40 g DO VSS^{-1} h^{-1} after 26 days' operation (Fig. 4-14). TOUR values increased sharply with the increasing OLR and stabilized at 425 g DO VSS^{-1} h^{-1} after 2 weeks operation. TOUR values were approximately 1.5 times that at an OLR of 3.0 kg COD m^{-3} d^{-1} because of the increase of biomass (Fig. 4-14).

The structure of aerobic granules in R2 was maintained after the OLR increased to 6.0

kg COD m^{-3} d^{-1} (Fig. 4-15c). SEM images revealed that granules at 6.0 kg COD m^{-3} d^{-1} had a smooth outer surface with a compact and regular appearance. A close examination of the granule surface revealed the presence of diverse microbial morphotypes, but filaments were the domain population. These microorganisms were embedded in an extracellular polymeric matrix (Fig. 4-16e, f).

4.3.3 Di-n-butyl Phthalate Degradation by PA-degrading Aerobic Granules

Reactor R3 was seeded with mature PA-degrading aerobic granules with a mean biomass size of 2.44 mm and SVI value of 41 ml g^{-1}. Di-n-butyl phthalate (DBP) was fed as the sole carbon source and energy source. Considering the recalcitrance and toxicity of DBP (Huang et al., 1994, Wang et al., 1998), the OLR was set at 1.5 kg COD m^{-3} d^{-1}.

The SVI value of the aerobic granules had a sharp increase from 41 ml g^{-1} on day 0 to 81 ml g^{-1} on day 8 after the PA-degrading aerobic granules were fed with DBP as the sole carbon source. After that, the SVI decreased gradually and leveled off at 51 ml g^{-1}. The mean biomass size slightly increased from 2.44 mm to 2.50 mm from day 0 to day 8. After that, the granules broke into small parts; the mean biomass size decreased to 1.12 mm and then leaved off until the end of the experiment (Fig. 4-18).

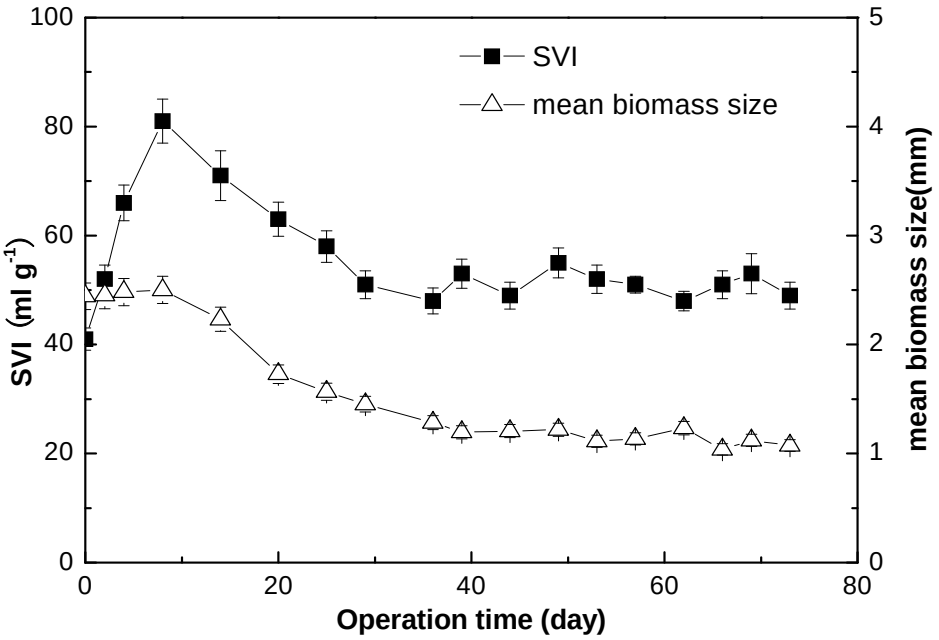


Fig. 4-18 Profiles of SVI and mean biomass size in R3.

The biomass concentration of R3 decreased from 2.4 to 2.1 g l^{-1} in the first two weeks operation after the DBP was fed as the substrate. After that, the biomass concentration increased to approximately 4.4 g l^{-1}, and kept this level till steady state was reached (Fig. 4-19). The influent pH value was controlled between 7.0 and 7.5 by adding $NaHCO_3$ in the entire process. While the effluent pH value varied with operation, it dropped to 6.2 on day 8 after the DBP was fed into R3 and increased to 6.4 on day 14. After that, the effluent pH value increased gradually to above 7.4 when the reactor reached steady state (Fig. 4-19).

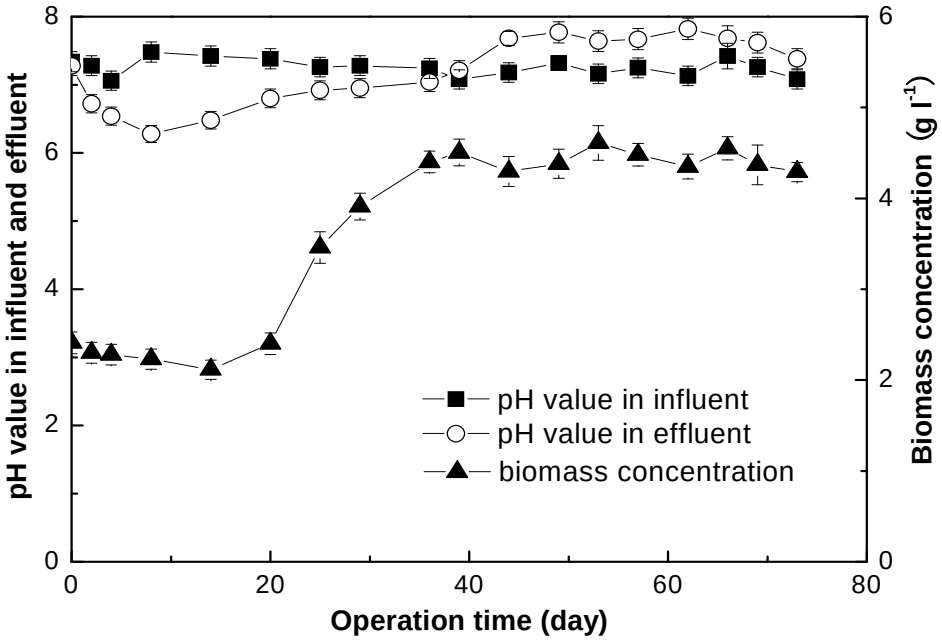


Fig. 4-19 Profiles of biomass concentration, pH value of influent and effluent in R3.

The PA-degrading aerobic granules successfully removed most of the DBP fed into the reactor, even though there was no acclimation process. The DBP removal efficiency was 95.3% on day 0. 25 days later, DBP removal efficiency increased to 100% till the end of experiment (Fig. 4-20). The TOC removal efficiency was only 51.4% on day 0 and increased gradually with the operation. When the reactor reached steady state, the TOC removal efficiency leveled off at around 92.7 % (Fig. 4-20).

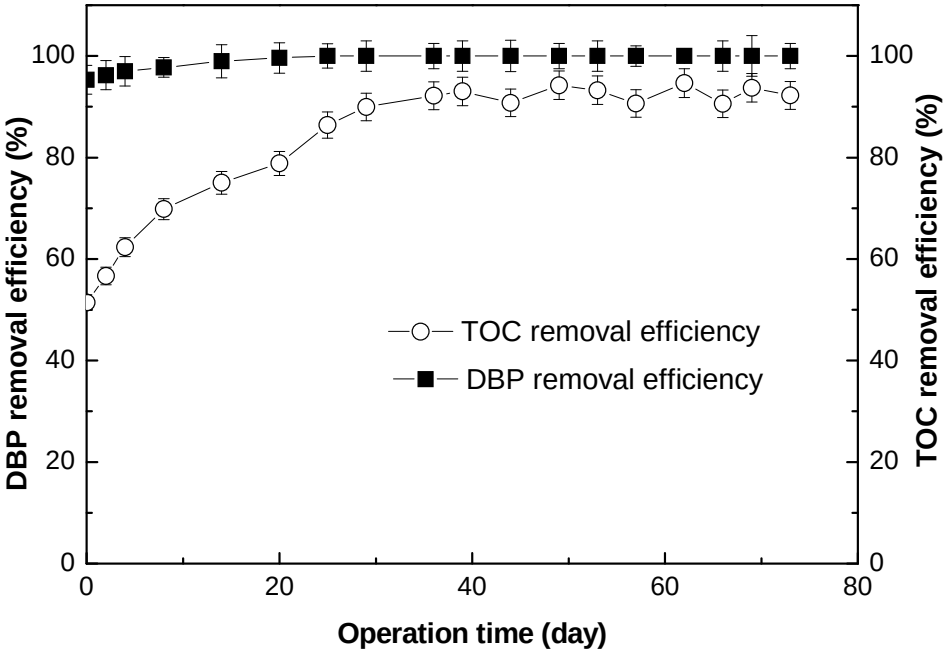


Fig. 4-20 Profiles of DBP and TOC removal efficiency in R3.

DBP, PA, TOC concentrations and pH values were measured during one cycle time when the reactor reached steady state. It took 60 min for complete removal of DBP. About 90 min were required for the completely removal of TOC (Fig. 4-21). As the intermediate of DBP degradation, PA appeared at 10 min and leveled up to 18.4 mg l^{-1} at 20 min. It decreased to 3.3 mg l^{-1} at 30 min and was exhausted at 50 min (Fig. 4-21). The pH value decreased from 7.7 at 0 min to 7.5 at 20 min and increased to 7.8 with the degradation of intermediates (Fig. 4-21).

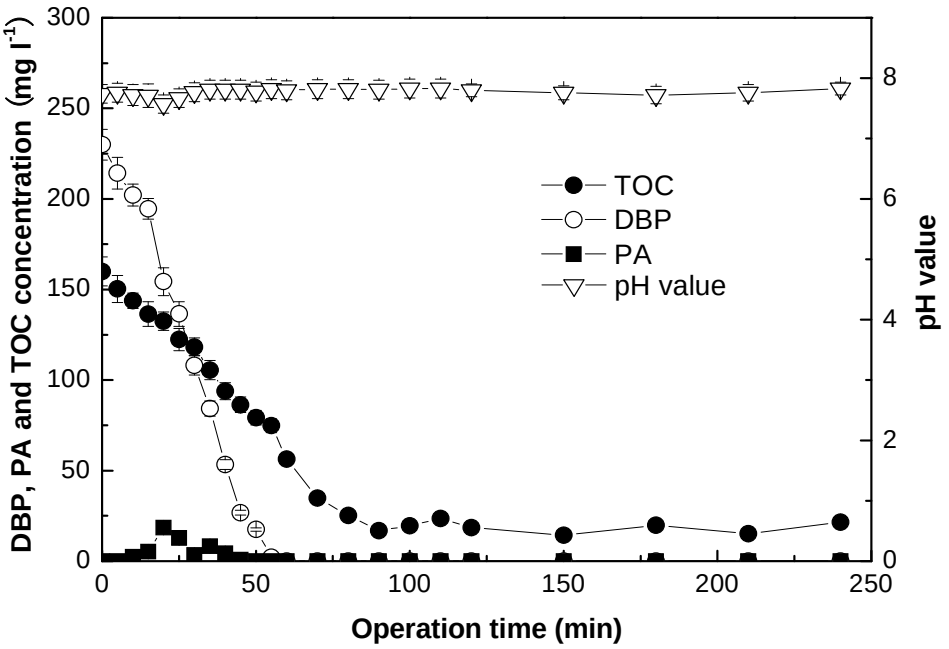


Fig. 4-21 Concentration profiles of DBP, TOC, PA during one cycle time in R3.

The activities of DBP and PA degraders were described by the specific DBP oxygen utilization rate $(SOUR)_{DBP}$ and the specific PA oxygen utilization rate $(SOUR)_{PA}$ respectively. The changes of $(SOUR)_{DBP}$ and $(SOUR)_{PA}$ are shown in Fig. 4-22. The initial $(SOUR)_{PA}$ was 35 mg DO g^{-1} VSS h^{-1} and decreased to 19 mg DO g^{-1} VSS h^{-1} on day 8 after DBP was fed as the sole carbon source. Then, it increased gradually and leveled off at 33 mg DO g^{-1} VSS h^{-1} which was almost the same level as the initial value. The initial $(SOUR)_{DBP}$ was 10 mg DO g^{-1} VSS h^{-1} which did not change in the first 20 days' operation. It increased gradually from day 20 onwards and increased to approximately 24 mg DO g^{-1} VSS h^{-1} at steady state, which was almost two times that of the initial value.

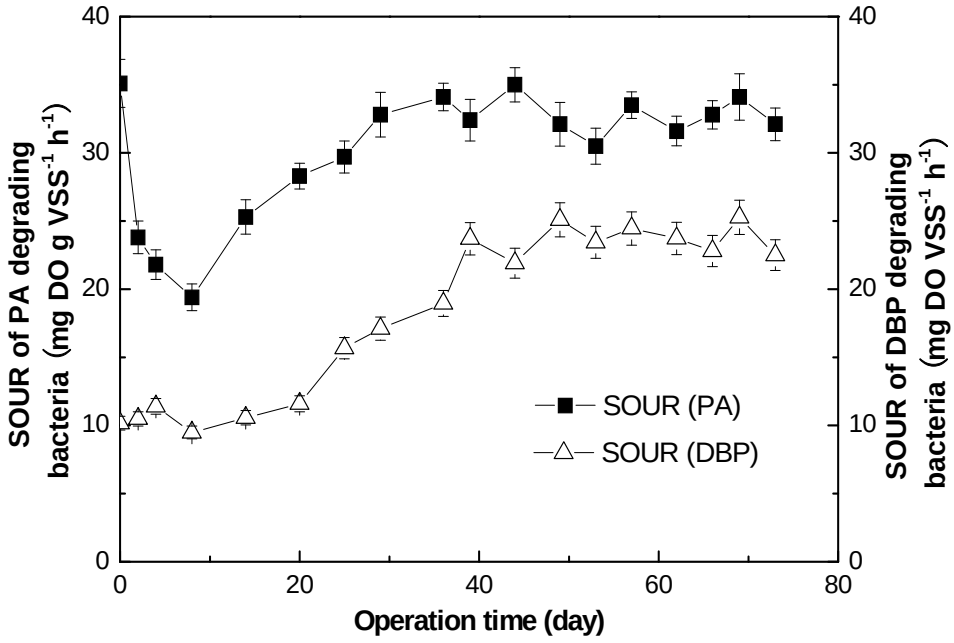


Fig. 4-22 Profiles of SOUR of PA-degrading bacteria and DBP-degrading bacteria in R3.

The PA-degrading aerobic granules selected as bioseeds had a compact structure and regular shapes (Fig. 4-23a). Some broken granules appeared on day 8 after DBP was fed as the sole carbon source (Fig. 4-23b). On day 20, the integrated granules and small parts granules co-existed in the same reactor (Fig. 4-23c). At the end of experiment, the granules in the reactor had irregular appearances and the small parts of granules re-formed into granules with a compact structure after 54 days operation (Fig. 4-23d). SEM images revealed that DBP-degrading aerobic granules had a fluffy out appearance with folds and crevices (Fig. 4-24a). Filamentous bacteria were the dominant population in DBP-degrading aerobic granules. Bacterial cells were tightly clustered together and embedded within the extracellular polymeric matrix (Fig. 4-24b).

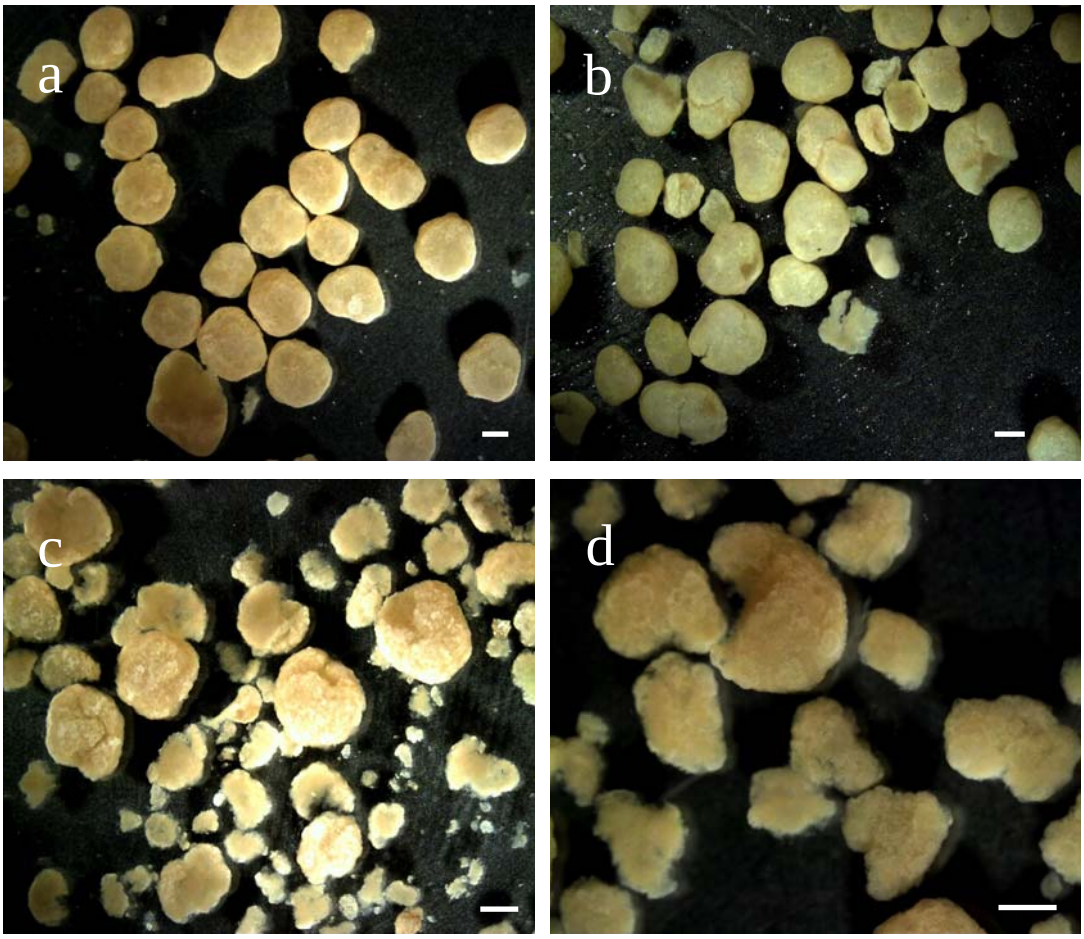


Fig. 4-23 Image analysis of aerobic granules (a) day 0 (bar: 1 mm); (b) day 8 (bar: 1mm) (c) day 20 (bar: 1 mm) (d) day 74 (bar: 1 mm).

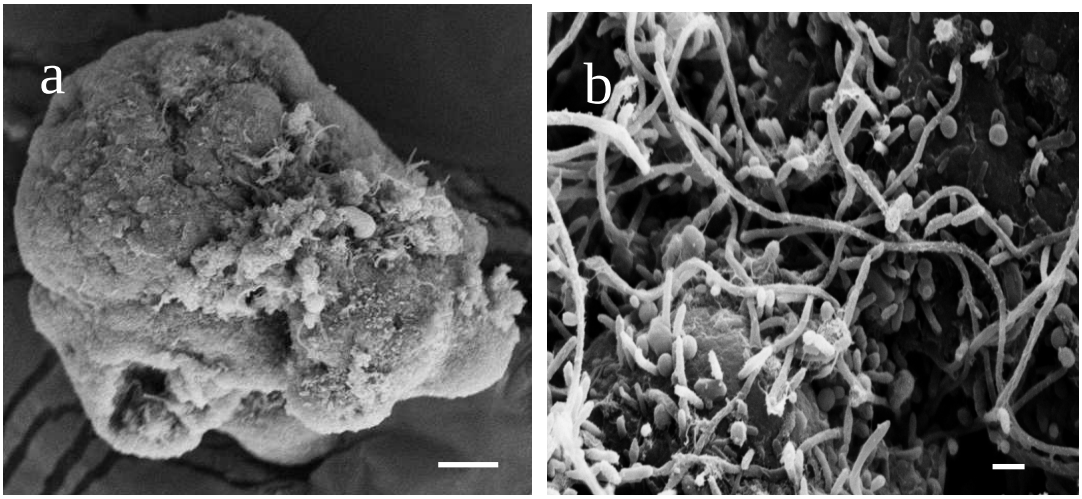


Fig. 4-24 Scanning electron micrographs of aerobic granules, (a) DBP-degrading aerobic granules (bar: 30 μm); (b) Surface of DBP-degrading aerobic granules (bar: 1 μm).

4.3.4 DMP Degradation by DMP-degrading Aerobic Granules

4.3.4.1 Reactor Operation at OLR of 3.0 kg m^{-3} d^{-1}

Reactor R4 was started up using 2.3 g l^{-1} DMP acclimated activated sludge as the inocula, which had a mean biomass size of 0.07 mm and a SVI value of 134 ml g^{-1}. The stepwise decreasing of the settling time was similar to the operation in Chapter 3.

The influent DMP pH value was adjusted to between 7.0 and 7.5. There was no extra sodium bicarbonate addition in this operation process. The effluent pH value was 7.3 to 7.6 during the first 13 days' operation. Then, the pH value decreased with the accumulation of DMP degradation intermediates until it reached 4.3 on day 26 (Fig.4-25). The initial biomass concentration was 2.4 g l^{-1}. Owing to the decreasing pH value, the biomass concentration continued to decrease to 0.5 g l^{-1} on day 26. Most of the biomass was washed out of the reactor because of the low pH in R4 (4.0 to 5.0). The operation using DMP activated sludge as seeds failed (Fig.4-25).

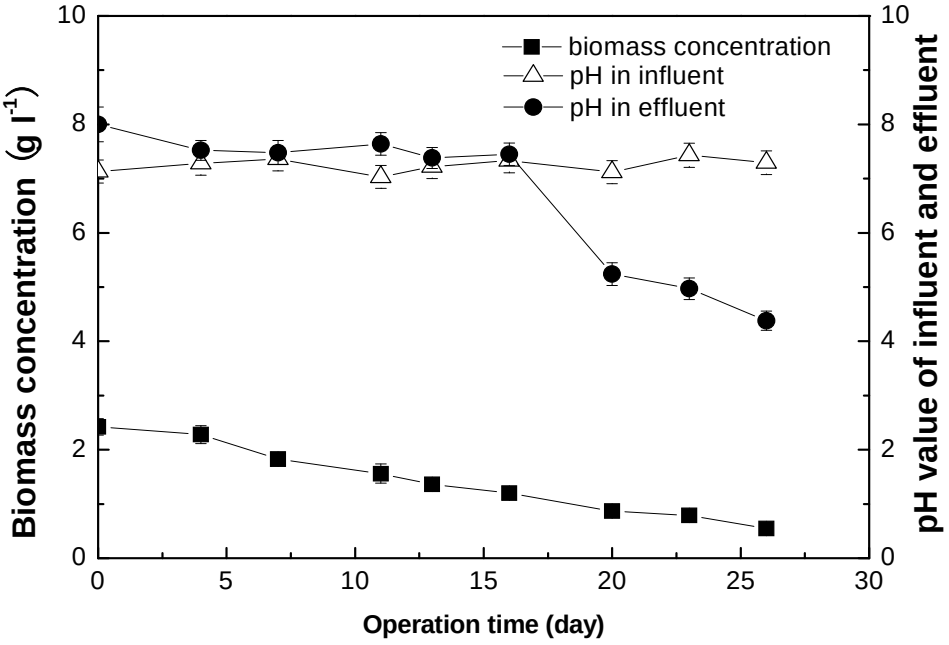


Fig. 4-25 Profiles of biomass concentration, influent and effluent pH in R4 in the first round operation.

Reactor R4 was re-started with DMP acclimated activated sludge as inocula. The operation was the same as the previous operation. Aerobic granules first appeared on day 26 of reactor operation, by which time the mean biomass size was around 0.25 mm and the SVI value decreased to less than 35 ml g^{-1} (Fig. 4-26). However, the operation was not steady as the granules size gradually reduced to 0.19 mm (between floc and granule) in the next 20 day's operation. At the same time, the SVI fluctuated between 35 and 78 ml g^{-1}. After 54 days' operation, the reactor reached steady state, as evidenced by stable TOC removal efficiencies and biomass concentrations. The mean biomass size was about 1.14 mm with an average SVI value of 58 ml g^{-1} (Fig. 4-26 and 4-28).

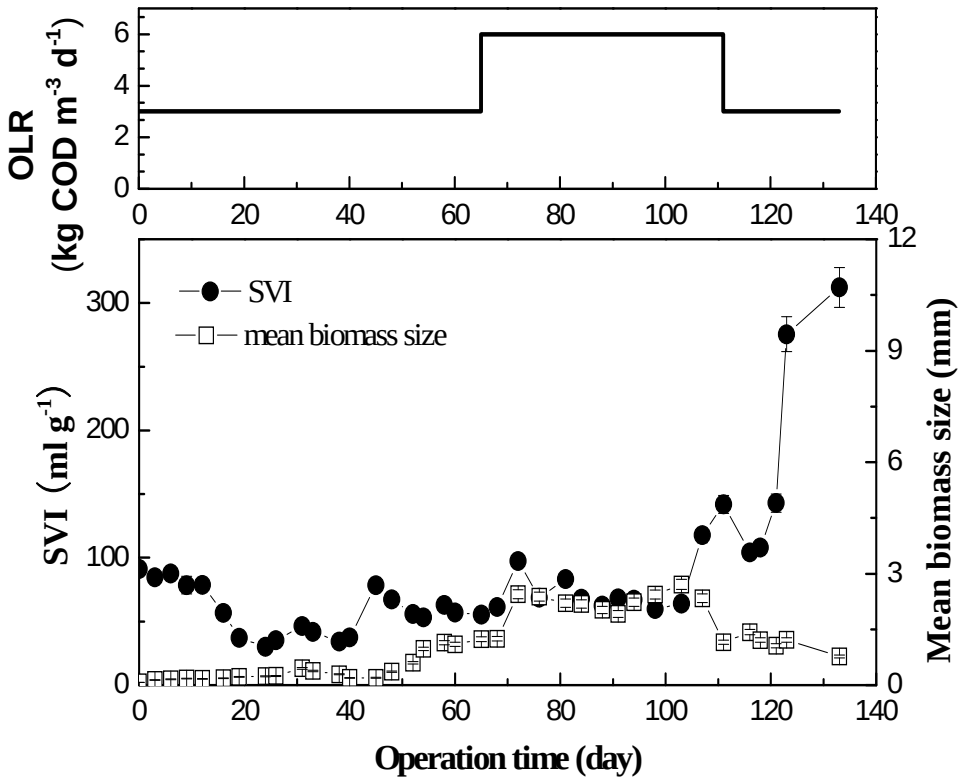


Fig. 4-26 Profiles of SVI and mean biomass size of aerobic granules in R4.

The influent pH value was adjusted between 7.0 and 7.5 by adding $NaHCO_3$. The initial effluent pH value was 7.2. Because of the intermediates accumulation, the pH

value of the effluent decreased to 6.1. To avoid reactor operation failure caused by low pH value, extra $NaHCO_3$ was added to increase the buffer capacity. In the next two days of operation, the effluent pH value was higher than 7.2. On day 12, the effluent pH value decreased to 6.2, and extra $NaHCO_3$ was added again. After that, the effluent pH value increased to around 7.2 to 7.6. At steady state, the effluent pH value was kept between 6.8 and 7.4. The seeding biomass concentration was 2.4 g l^{-1}. It fluctuated frequently, even after formation of aerobic granules. When aerobic granules matured, the biomass concentration was steady at 2.8 g l^{-1} (Fig. 4-27).

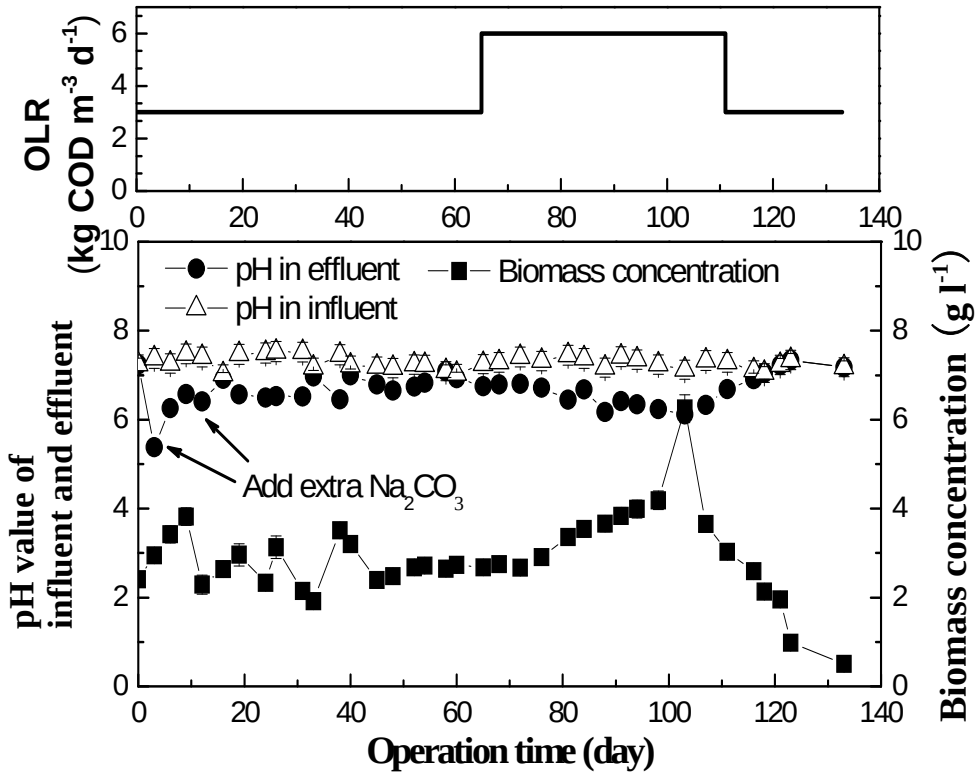


Fig. 4-27 Profiles of biomass concentration, influent and effluent pH in R4.

R4 was fed with the same DMP synthetic wastewater which was fed to R2, at an OLR of 3.0 kg COD m^{-3} d^{-1}. Since the biomass in R4 was acclimated before it was inoculated into R4, the DMP removal efficiency of R4 was 100% on day 0 and maintained the removal efficiency for 65 days. The TOC removal efficiency was 82.8 % on day 0, and increased to 90% when the reactor reached steady state (Fig.

4-28).

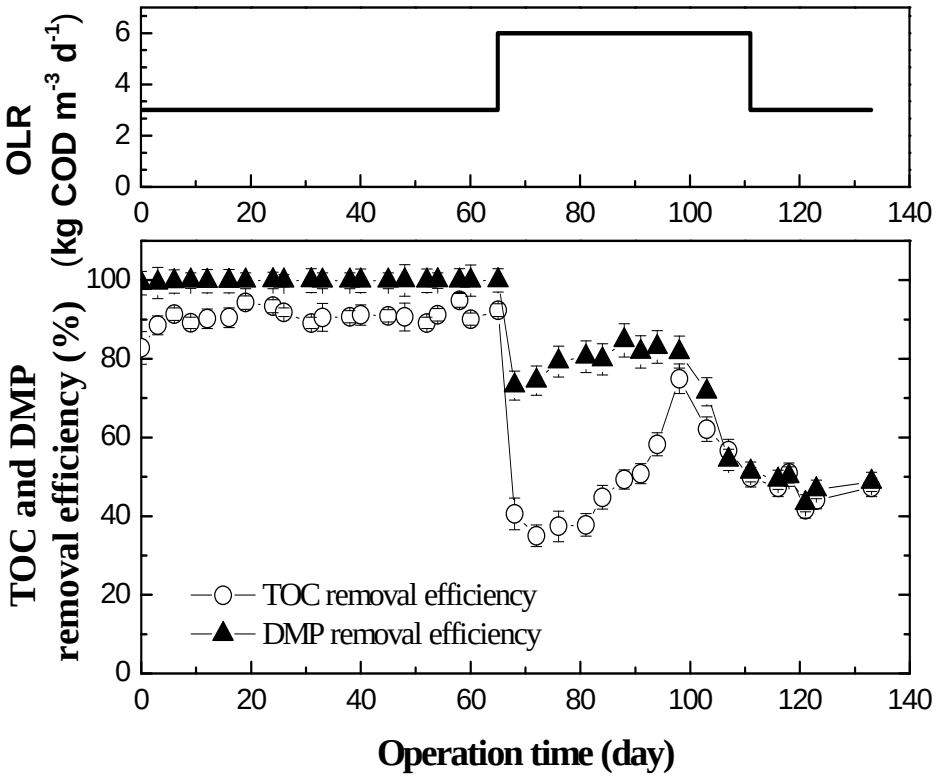


Fig. 4-28 Profiles of DMP and TOC removal efficiency in R4.

The degradation profiles of DMP, PA and the TOC concentration in R4 in one cycle time were compared on day 0 and on day 65 at the OLR of 3.0 kg COD m^{-3} d^{-1}. Since inoculated activated sludge had been acclimated with DMP, the DMP could be completely degraded within 60 min on day 0, while 50 min were required on day 65 (Figs. 4-29 and 4-30). On day 0, 90.8 mg l^{-1} PA was accumulated in 5 min, and 90 min was needed to completely degrade it (Fig. 4-29). On day 65, PA appeared at 5 min with a concentration of 15.0 mg l^{-1}, which quickly was exhausted in 5 min. At 25 min, it accumulated to 10.5 mg $l^{-1}$and then was completely removed before 40 min (Fig. 4-30). On day 0, 120 min were required for the TOC complete removal, while it took 60 min to completely remove TOC on day 65 (Figs. 4-29 and 4-30). During the cycle on day 0, the pH value decreased from 7.4 at time zero to 6.3 at 20 min because of PA accumulation. It gradually increased to 7.1 after 100 min operation. On day 65, the pH

value decreased from 7.3 at time zero to 6.5 at 15 min, and increased to 7.0 at 50 min.

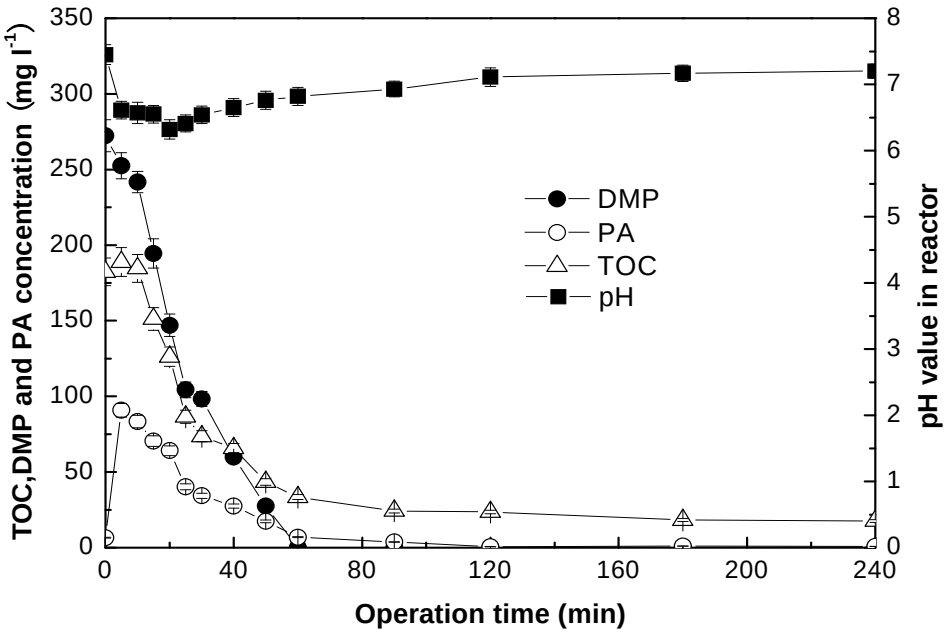


Fig. 4-29 Concentration profiles of DMP, TOC, PA and pH value during one cycle time in R4 on day 0 at an OLR of 3.0 kg COD^{-3} d^{-1}.

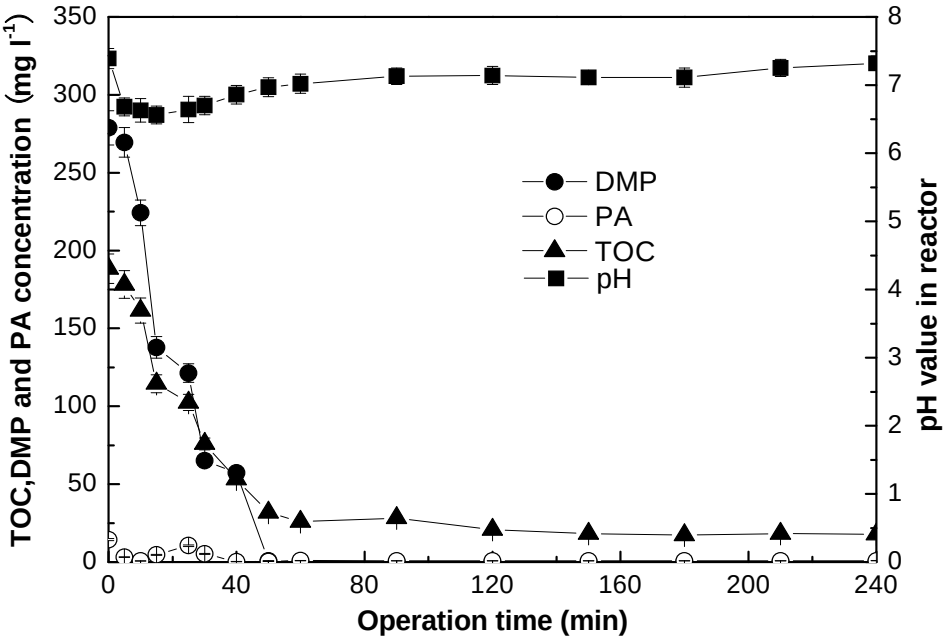


Fig. 4-30 Concentration profiles of DMP, TOC, PA and pH value during one cycle time in R4 on day 65 at an OLR of 3.0 kg COD^{-3} d^{-1}.

The SOUR of the biomass was measured to monitor changes in biomass activity during the experiment. An initial DMP concentration of 290.0 mg 1^{-1} in the reactor was used in the SOUR assays, with a biomass concentration around 0.3 g VSS l^{-1}. The initial SOUR values of the DMP acclimated activated sludge was 87 mg DO g^{-1} VSS h^{-1}; SOUR values fluctuated between 44 and 102 mg DO g^{-1} VSS h^{-1} during granules formation and stabilized at 64 mg DO g^{-1} VSS h^{-1} when aerobic granules were formed and the reactor reached steady state. TOUR was used to estimate the total potential degrading ability of the whole reactor. Before the reactor reached steady state, the TOUR fluctuated between 112 and 390 mg DO h^{-1} because of the change of SOUR and biomass concentration. After 51 days operation, it stabilized at 182 mg DO h^{-1} (Fig. 4-31).

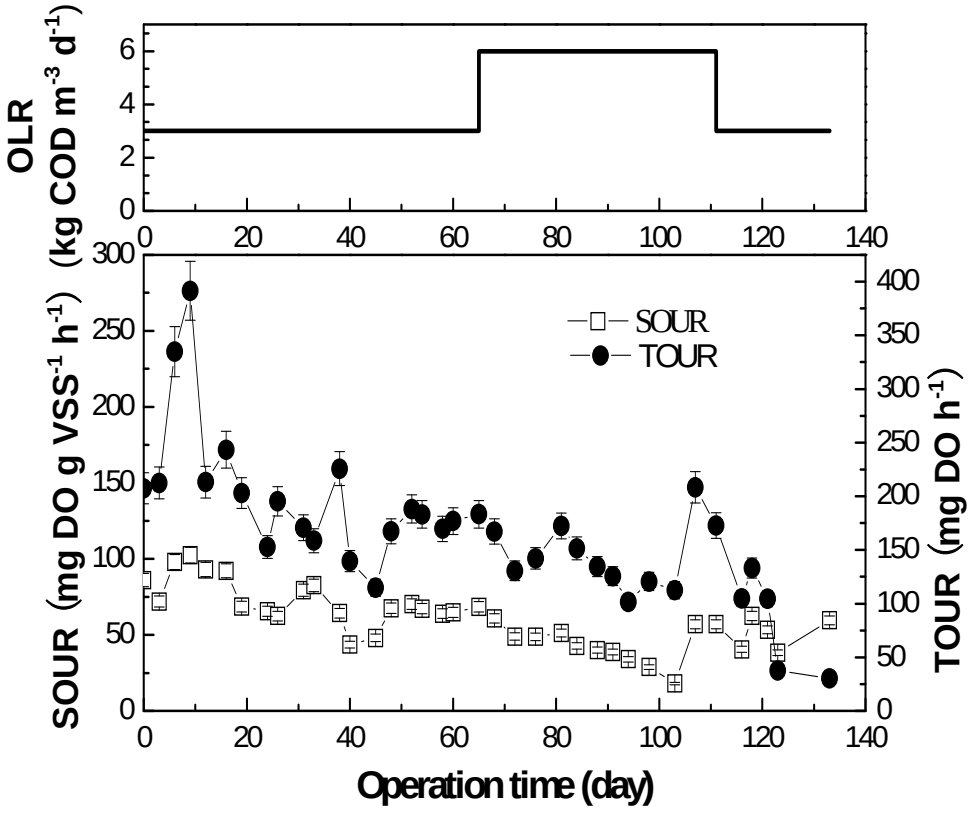


Fig. 4-31 Profiles of SOUR and TOUR in R4.

Aerobic granules in R4 were developed from a loose structure, flurry seed sludge.

Under the same operation conditions as R2, 54 days were required for the formation of aerobic granules, with a compact structure as the dominant biomass in R4 (Fig. 4-32a, b). SEM images revealed that DMP granules in R4 also had a cauliflower-like appearance (Fig. 4-33a). Bacterial cells were tightly clustered together and embedded within the extracellular polymeric matrix. In these aerobic granules, bacteria such as short rods, long rods and cocci dominated the microbial community residing in the granules. There filamentous bacteria were not the dominant form in this community (Fig. 4-33b).

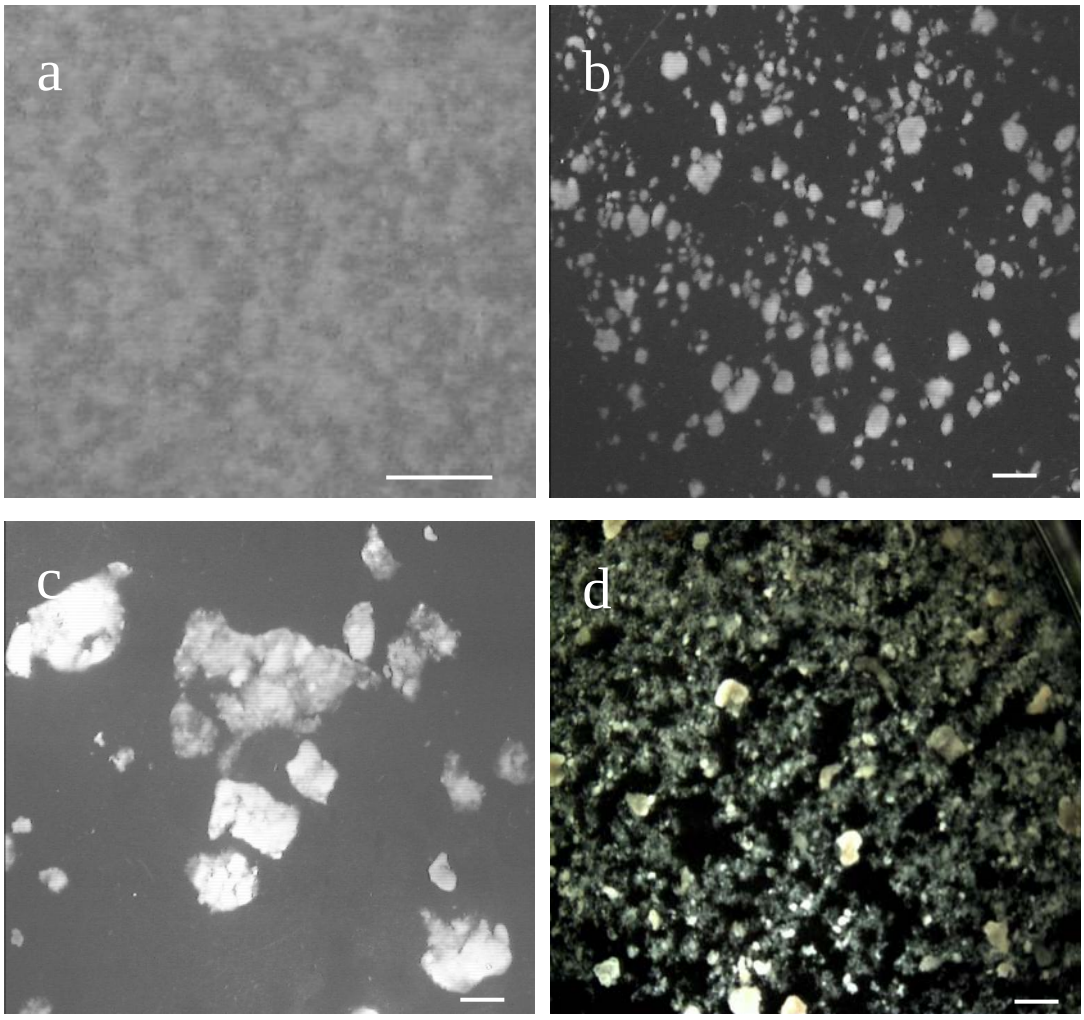


Fig. 4-32 Image analysis of biomass in R4 (a) acclimated activated sludge (bar: 1 mm); (b) DMP-degrading aerobic granules on day 26 at 3.0 kg COD m^{-3} d^{-1} (bar: 0.5 mm) (c) DMP-degrading aerobic granules at 6.0 kg COD m^{-3} d^{-1} (bar: 1 mm) (d) Disintegrated aerobic granules at 6.0 kg COD m^{-3} d^{-1} (bar: 1 mm).

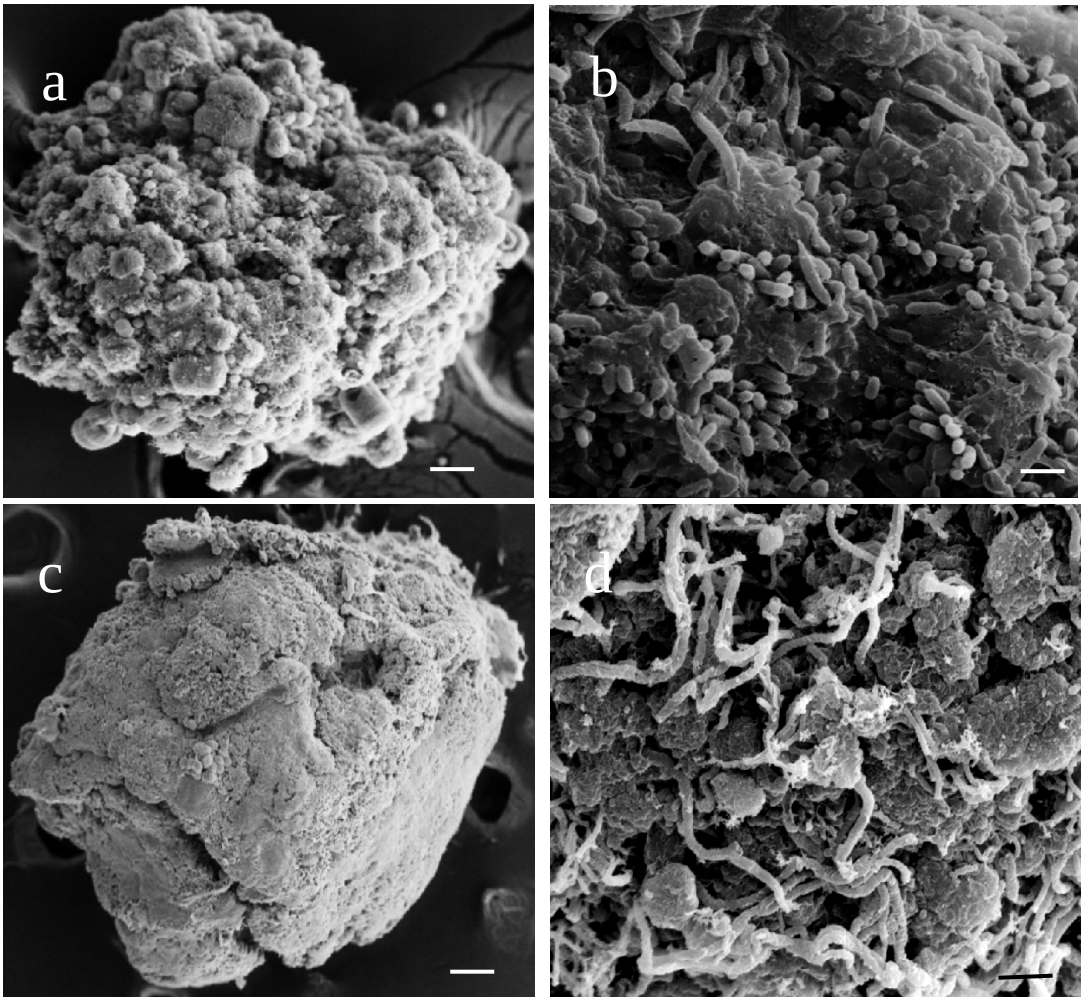


Fig. 4-33 Scanning electron micrographs of aerobic granules, (a) DMP-degrading aerobic granules in R4 at an OLR of 3.0 kg COD m^{-3} d^{-1} (bar: 30 μm); (b) DMP-degrading aerobic granules in R4 at an OLR of 3.0 kg COD m^{-3} d^{-1} (bar: 2 μm) (c) DMP-degrading aerobic granules in R4 at an OLR of 6.0 kg COD m^{-3} d^{-1} (bar: 100 μm) (d) DMP-degrading aerobic granules in R4 at an OLR of 6.0 kg COD m^{-3} d^{-1} (bar: 4 μm).

4.3.4.2 Reactor Operation at OLR of 6.0 kg m^{-3} d^{-1}

When the OLR of R4 increased to 6.0 kg COD m^{-3} d^{-1}, the SVI increased to 96 ml g^{-1} immediately. It gradually decreased to 66 ml g^{-1} after 34 days' operation. However, it increased suddenly to 117 ml g^{-1} on day 107 and continued to 144 ml g^{-1} on day 112 because of the granules' disintegration. The OLR was manually adjusted to 3.0 kg

COD m^{-3} d^{-1} when the granule disintegration happened. The settling time for aerobic granules maintained in the reactor increased. The SVI continuously increased to 312 ml g^{-1} on day 133. That almost caused all of the biomass to be washed out of R4. The mean biomass size increased sharply to 2.44 mm when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. From day 72 to 102, the mean biomass size was in the range of 1.95 to 2.76 mm. When granule disintegration happened, the mean biomass decreased to 1.20 mm in 7 days. When most biomass was washed out, the mean biomass size of the remaining biomass was only 0.77 mm (Fig. 4-26).

When the OLR of R4 increased to the OLR of 6.0 kg COD m^{-3} d^{-1}, the pH value of the effluent changed significantly in this process. Although the pH value of the influent was adjusted to between 7.0 and 7.5 by adding $NaHCO_3$, the pH value of the effluent had decreased continuously to 5.8 on day 103 (Fig. 4-27). The biomass concentration increased from 3.0 to 6.2 ml g^{-1} from days 65 to 103 with the OLR increasing. After that, the biomass concentration decreased sharply because most of the biomass was washed out of the reactor caused by the aerobic granules disintegrating and increasing the SVI. On day 133, the biomass decreased to 0.5 m g^{-1}, indicating the failure of the reactor operation.

The DMP removal efficiency immediately decreased to 72.5% when the OLR in R4 increased to 6.0 kg COD m^{-3} d^{-1}. It had increased to above 80% after 30 days operation. However, the washing out of biomass caused a sharp decrease in the DMP removal efficiency to 48.3% on day 133. The TOC removal efficiency also decreased to 34.6% when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. It had increased to 74.8% on day 98. However, it decreased to 46.6% on day 133 with the wash out of the biomass. Although the OLR decreased to 3.0 kg COD m^{-3} d^{-1} from day 103 onwards, the damaged system in R4 could not recover the DMP degradation ability (Fig. 4-28).

The SOUR of granules in R4 decreased when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. The mass transfer difficulty caused by the mean biomass size increasing should

contribute to the decreasing SOUR. On day 103, the SOUR decreased to 18 mg DO g^{-1} VSS h^{-1}. From day 103 onward, the SOUR increased to approximately 60 mg DO g^{-1} VSS h^{-1} because aerobic granules were broken. The TOUR had the similar variation trends as the SOUR in the entire process. It increased to 208 mg DO h^{-1} on day 107. However it quickly decreased to 30 mg DO h^{-1} because the biomass was washed out (Fig. 4-31).

Image analysis showed that the aerobic granules in R4 possessed an irregular morphology when the OLR increased to 6.0 kg COD m^{-3} d^{-1}. Some broken granules can be observed (Fig. 4-32). Close observation by SEM revealed that granules had a hairy appearance with a loose microbial structure. Some cracks and channels can be observed on the surface of the granules, and filamentous bacteria were the dominant population in the granules (Fig. 4-33c, d). When the granules disintegrated, the biomass had a similar structure as activated sludge, except some small aggregates existed in the biomass (Fig. 4-32d).

4.4 Discussion

Bioaugmentation has been widely applied in the degradation of recalcitrant compounds. However, the seeded inocula seem to have difficulty in surviving in the target ecosystem (Mueller et al., 1992; Alexander, 1999; Bouchez et al., 2000a, b). Selection of inocula is one of the important factors for a successful bioaugmentation. The PA-degrading aerobic granules were selected as bioseeds for PA and PAEs degradation because they possess these potential traits for bioaugmentation which have been demonstrated in Chapter 3. The experimental results in this chapter showed that PA-degrading aerobic granules possessed high degradation ability for PA and PAEs.

4.4.1 PA degradation by PA-degrading Aerobic Granules

PA is low toxicity compounds. At an OLR of 1.1 kg COD $m^{-3}.d^{-1}$, PA removal efficiency by conventional activated sludge was 99.0% (Fan et al., 2001), while at an OLR of 5.0 kg COD $m^{-3}.d^{-1}$, the PA biodegradation efficiency dropped to 45% by conventional activated sludge (Zhou et al., 2000). A high OLR is desirable in biological wastewater treatment systems as it is required by the treatment of high-strength wastewaters. For industrial PA wastewater, the concentrations were usually between 7,000 and 15,000 mg l^{-1} (Zhou et al., 2000). Anaerobic treatments were commonly employed to treat PA wastewater (Chapter 2, section 2.3.7), but the results were not as good as expected (Pereboom et al., 1994; Tur and Huang, 1997; Kleerebezem and Lettinga, 2000;).

In biological wastewater treatment systems, a high biomass concentration in the reactor is expected to maintain high wastewater treatment capacity (Wang et al., 2000b). To improve the biomass concentration in the reactor, few attempts including biomass immobilization technology have been adopted (Hamoda and Abd-El-Bary, 1987; Nicol et al., 1988; Wanner et al., 1988; Borja et al., 1996). Biomass immobilization technology could improve the biomass concentration in the reactor and accordingly improve the degradation capacity of system. However, macrocarriers used in immobilization technology occupy a large portion of the reactor volume, which would reduce the work volume of reactors (Wang et al., 2000b). Aerobic granulation is a self-immobilization technology with high biomass retention in the reactor and no extra volume occupied by the carriers.

In this study, the highest biomass concentration which was maintained in the reactor was 9.5 mg l^{-1} at OLR of 12.4 kg COD m^{-3} d^{-1}. This high biomass concentration provided a high PA degradation ability which was evidenced by TOC removal efficiency of 97.7%. This high OLR sustainable ability and high removal efficiency demonstrated by PA-degrading aerobic granules were consistent with the previous

studies (Moy et al., 2002; Liu et al., 2003b; Tay et al., 2004b, c). The highest OLRs recently reported were 15 kg COD m^{-3} d^{-1} and 9 kg COD m^{-3} d^{-1} when glucose and acetate were fed as substrate, respectively (Moy et al., 2002). When degrading the toxic compound phenol, aerobic granules also exhibited the higher removal ability than activated sludge. The compact aerobic granules with good phenol degradation ability were maintained at the loading rates up to 2.0 kg phenol m^{-3} d^{-1} (Jiang et al., 2004a).

The influence of OLR on the PA degradation was investigated. When the OLR increased form 3 kg COD m^{-3} d^{-1} to 5.8 kg COD m^{-3} d^{-1}, the total biomass increased from 4.5 mg l^{-1} to 9.7 mg l^{-1}. Accordingly, the aerobic granules increased from 4.4 mg l^{-1} to 9.2 mg l^{-1}. The TOC removal efficiencies were 98.3% and 96.6% respectively. The OLR increase almost had no effect on the performance of TOC removal. Among the total biomass, major part of PA was degraded by aerobic granules according to TOUR values.

When the OLR increased to 12.4 kg COD m^{-3} d^{-1}, the disintegration of aerobic granules happened in this stage. Part of disintegrated biomass was discharged with the effluent and part of them with compact structure was kept in the reactor. Thus the total biomass was composed of two parts: aerobic granules of 7.4 mg $l^{-1}$and compact biomass of 2.1 mg l^{-1}. The TOC removal efficiency was 97.7%. The total biomass concentration and TOC removal efficiency at OLR of the 12.4 kg COD m^{-3} d^{-1} kept in the comparable levels at the OLR of 5.8 kg COD m^{-3} d^{-1}. The substrate degraded by aerobic granules and compact biomass was calculated based on the method described by Wang et al. (2000b). About 45.6% of PA was removed by compact biomass. This observation was different from the previously reported studies, in which the granules were completely disintegration and washed out of the reactor when OLR increased higher than 8 kg COD m^{-3} d^{-1} (Tay et al., 2003a). The possible reason of the compact biomass was kept in the reactor was that the compact biomass still possessed compact structure.

The compact biomass was also an important part of the aerobic granular system in R1 at a high OLR. The characteristics and structure of the compact biomass in R1 were between suspended biomass and aerobic granules, but they had a lower SVI value and a more compact structure than did activated sludge. Thus they could stay in the reactor within the same settling times of aerobic granules. Compared with aerobic granules, the compact biomass had a smaller size and an irregular surface characterized by folds, crevices and depressions. The irregular structure allowed better penetration of nutrients into the interior through shorter diffusion distances compared with spherical-shaped granules (Toh et. al., 2003). Thus, the biomass had the higher substrate diffusion efficiency than aerobic granules.

In conclusion, the high biomass concentration and high biomass activity provided by aerobic granules and compact biomass in the aerobic granular system improved the PA degradation ability at a high OLR.

4.4.2 Comparison of DMP Degradation by the System Inoculated with Activated Sludge and PA-degrading Aerobic Granules

In this study, DMP was degraded by two systems in two reactors. One was innoculated with PA-degrading aerobic granules in reactor R2 and the other was innoculated with DMP acclimated activated sludge in reactor R4. The comparisons of the two systems operation are listed in Table 4-3.

Table 4-3 The comparison of DMP degradation by R2 and R4

System	R2		R4
Innocula	PA-degrading aerobic granules		DMP acclimated activated sludge
OLR (kg COD m^{-3} d^{-1})	3.0	6.0	3.0
The time for the system to get steady (day)	15	30	54
Biomass concentration (mg l^{-1})	6.0	10.6	3.0
DMP removal efficiency (%)	100	100	100
TOC removal efficiency (%)	98.0	93.1	90.0
SVI (ml g^{-1})	30	46	58
Reagent to maintain neutral	No	No	Extra $NaHCO_3$ addition

4.4.2.1 The rapid stable operation inoculated with PA-degradation aerobic granules

Generally, the ecosystem would be disturbed if a new substrate was fed into a system without acclimation. For the PA-degrading aerobic granular systems, no disturbance on the ecosystem was observed after DMP was fed to R2. Although PA-degrading aerobic granular system did not acclimated with DMP, the DMP and TOC removal efficiency in the first 4 days was 100% and 90.3%, respectively. It only took 15 days for reactor R2 to reach steady state (Fig. 4-11). The biomass concentration was increased to approximately 6.0 g l^{-1} (Fig. 4-10), the DMP and TOC removal efficiency in the first 4 days was 100% and 98.0% at steady state (Fig. 4-11). In the entire process, the aerobic granules had low SVI value of approximately 30 ml g^{-1} (Fig. 4-9).

In contrast, it took more time for reactor R4, which inoculated with DMP acclimated activated sludge, to reach steady state. About 26 days passed when the DMP aerobic granules appeared in R4, and the system did not reached steady state until day 54 (Fig. 4-26). The biomass concentration increased from 2.4 to 3.0 g l^{-1} and SVI stabilized at 58 ml g^{-1} when the reactor reached steady state (Figs. 4-26 and 4-27). Although the

inoculated DMP acclimated activated sludge had achieved 100% of DMP removal efficiency and 90.0% of TOC removal efficiency, the time was consumed to induce the enzyme for PA and other intermediates degradation. Thus more time was required for reactor R4 to reach steady state (Fig. 4-28).

In DMP degradation, the bioaugmentation of PA-degrading aerobic granules would facilitate the reactor to achieve its steady reactor system operation in a shorter period and produce stable DMP-degrading granules with good settling ability, good biomass retention and good metabolic activity which indicated by the high TOC removal efficiency.

4.4.2.2 The high tolerance to high OLR of ecosystem inoculated with PA-degradation aerobic granules

For reactor R2 which was inoculated with PA-degrading aerobic granules, it only took 15 days to reached steady state with high DMP, TOC removal efficiency and high biomass retention at OLR of 3.0 kg COD m^{-3} d^{-1}. When the OLR increased to 6.0 kg COD m^{-3} d^{-1}, about 30 days were spent when the aerobic granular system reached steady state. At steady state, the SVI, biomass concentrations, DMP removal efficiency and TOC removal efficiency were 46 ml g^{-1}, 10.6 g l^{-1}, 100%, 93.1%, respectively (Figs 4-9, 4-10 and 4-11).

As contrast, rector R4 which was inoculated with DMP acclimated activated sludge cannot form aerobic granules if no extra sodium bicarbonate was used for adjusting the pH at neural range. Even pH of system was maintain at neural value, it took 54 days for R4 to reached steady state at 3.0 kg COD m^{-3} d^{-1}. When OLR of R4 increased to 6.0 kg COD m^{-3} d^{-1}, the structure was destroyed by the accumulation of degradation intermediate. As a result, the aerobic granules was disintegrated, the SVI increased to 144 ml g^{-1}, biomass concentration decreased to 0.5 m g^{-1} and DMP removal efficiency and TOC removal efficiency decreased to 48.3% and 46.6%, respectively. The

operation of R4 was failed. Thus, the system inoculated with DMP acclimated activated sludge could not sustain the OLR of 6.0 kg COD m^{-3} d^{-1}.

From the results, the system inoculated with PA-degrading aerobic granules possessed the capability to sustain high OLR.

4.4.2.3 The protection of PA-degradation aerobic granules for the functional microorganisms.

PA-degrading aerobic granules provided protection for the microorganisms in aerobic granules from two aspects. Firstly, PA-degrading aerobic granules could provide a benign environment for all the microorganisms of the aerobic granules. Generally, DMP was degraded into CO_2 and water through monomethyl phthalate (MMP) and PA (Staples et. al., 1997). If the intermediates could not be degraded completely, they would hinder the overall DMP mineralization. At the same time, the accumulation of intermediates, especially PA, would decrease the pH value of the medium in the reactor, which would in turn influence the activity of the DMP-degrading enzymes (Fan et al., 2001). PA-degrading aerobic granules possessed high PA degrading ability. The PA produced in the DMP degradation could be degraded quickly and pH value of reactor could be kept at neural range. This advantage was confirmed by fast PA degradation and low accumulation of PA during the DMP degradation process (Fig. 4-12 and 4-13).

Secondly, the compact structure and high content of ECPs in the aerobic granules provided protection for the microorganisms even when some adverse environment appeared. For example, when OLR increased to 6.0 kg COD m^{-3} d^{-1}, the aerobic granules changed from a cauliflower-like external appearance to a smooth, compact and regular appearance. The phenomena were similar to that exhibited by phenol degrading aerobic granules (Jiang et al., 2002). This structure was reported to provide protection for the microorganisms against toxic phenol. The DMP was reported to be

toxic to microorganisms, since it caused disruption of the cell membrane (Cartweight et al., 2000b). Therefore, the more compact structure in aerobic granules under high OLR was a response to the high toxicity loading and could provide protection for the microorganisms in aerobic granules (Fig. 4-16c, e).

Without the protection of PA-degrading aerobic granules, extra sodium bicarbonate must be provided for the DMP acclimated activated sludge in the granules formation process. The DMP acclimated activated sludge possessed higher DMP hydrolysis ability and a lower PA degradation ability. That would lead to the accumulation of intermediates and a low pH condition in the reactor during the DMP degradation process. Thus the more PA was accumulated in the reactor and more time was spent for complete degradation of PA (Figs. 4-29 and 4-30).

In conclusion, the reactor seeded with PA-degrading aerobic granules showed high degradation ability, good resistance to high OLR and a short time requirement to reach steady state as compared with the reactor seeded with DMP acclimated activated sludge.

4.4.2.4 PA-degradation aerobic granules is good choice of DMP industrial wastewater treatment

Previously, the biological DMP degradation has been carried out for many years. In shake flask studies, about 90% degradation of DMP in a garden soil by a natural microflora occurred in 10 days (Shankar et al., 1985), and 86% degradation of DMP in aqueous medium by an acclimated culture in 28 days (Sugatt et al., 1984). In a semi-continuous activated sludge system, greater than 80% degradation of DMP was observed in 24 h at low DMP (1 to 3 mg l^{-1}) concentrations (O'Grady et al., 1985). However, most of these studies involved low phthalate concentrations, low OLR and required long degradation times. The biodegradation of DMP in a packed-bed immobilized cell bioreactor with high DMP loading and removal rates was reported

(Juneson et al., 2002). An average DMP loading rate of 5.2 kg m^{-3} d^{-1} and 100% removal rate were achieved. The results of the DMP degradations are summarized in Table 4-4.

Table 4-4 The comparison of DMP degradation

System	Innocula	DMP removal efficiency	Reaction time	Reference
Activated sludge	natural microflora	90%	10 d	Shankar et al., 1985
Activated sludge	Acclimated culture	86%	28 d	Sugatt et al., 1984
Activated sludge	Acclimated sludge	Greater than 80%	24 hr	O'Grady et al., 1985
Immobilized biofilm	*Pseudomonas putida, P. fluorescens* and *Micrococcus halobius.*	53%-100%	8-24 hr	Juneson et al., 2002
PA-degrading aerobic granules	No acclimated cultures	100%	4 hr	This study

The OLR achieved by aerobic granules in this study was 6.0 kg COD m^{-3} d^{-1}. The OLR achieved by the packed-bed immobilized cell bioreactor seemed a bit higher than that in the aerobic granular reactor. However, the microorganisms in the packed-bed immobilized cell bioreactor were mixed cultures of *Pseudomonas putida, P. fluorescens* and *Micrococcus halobius.* They were three predominant pure cultures isolated from acclimated activated sludge and possessed high DMP degradation ability than mixed cultures. In our study, mix cultures of PA-degrading aerobic granules without DMP acclimation were seeded into reactor. In addition, the hydraulic retention time (HRT) of the packed-bed immobilized cell bioreactor ranged from 8 to 24 h, which was the two to six times that of the 4 h HRT applied in the aerobic granular reactor. Considering the aspects of prevalent seeds selection and short HRT, the reactor bioaugmented with PA-degrading aerobic granules would be the first

choice in the application for industrial DMP wastewater treatment.

4.4.3 DBP Degradation by the System Innoculated with PA-degrading Aerobic Granules

Similar to the operation of R2, DBP was fed as the sole carbon and energy source to PA-degrading aerobic granules in R3 without acclimation in this study. At an OLR of 1.5 kg COD m^{-3} d^{-1}, the DBP removal efficiency was 100% from 25 days onwards. TOC removal efficiency was 92.7% at steady state after 25 days' operation (Fig. 4-20). The mean biomass size was stable at around 1.12 mm, and the SVI decreased to 51 ml g^{-1} at steady state (Fig. 4-18). Due to the complete DBP degradation and high settling ability of the aerobic granules, the biomass concentration increased to 4.4 g l^{-1} (Fig. 4-19). At steady state, 60 min were needed for complete removal of DBP and 90 min were required for complete removal of TOC. The highest concentration of accumulated PA during one cycle time was 18.4 mg l^{-1}, and it was completely removed after 50 min operation (Fig. 4-21). The DBP was successfully removed by the bioaugmented PA-degrading aerobic granules.

The biodegradation of DBP has been reported by several researchers (Sugatt et al., 1984; Walker et al, 1984; Wang et al., 1995, 1999a).These studies focused on the biodegradability of DBP as well as the degradation pathway and most of the experiments were carried out in shake flasks or batch reactors. It is concluded from their studies that DBP can be degraded.

However, Huang et al. (1994) found that some PAEs, such as DBP and DEHP, were part of the main refractory organic compounds in municipal wastewater which were difficult to degrade. In conventional activated sludge plants, a large part of these organic pollutants pass through the treatment facilities unaltered. Even in the experiments with low phthalate concentration, a low OLR, and a quiet long time was required for DBP degradation. For example, DBP was degraded by the isolated

denitrifying bacterium. 100 h was required to completely degrade 1.25 mM DBP (Wang et al., 1999a). 100 mg l^{-1} DBP could be degraded completely within 100 h by acclimated activated sludge in batch reactors (Wang et al., 2004). Wu et al. (2000) set up an integrated vertical constructed wetland system to treat DBP polluted wastewater. The removal efficiency was only about 79%, even under a low OLR of 4.1 mg DBP l^{-1} d^{-1}.

Compared with previous studies, the aerobic granular system showed promising properties when degrading recalcitrant pollutants such as DBP. The DBP removal efficiency was 100% under an OLR of 1.5 kg COD m^{-3} d^{-1}, which was higher than previous studies. The HRT was 8 h, much shorter than that in previous reports.

Although, the bioaugmentation of PA-degrading aerobic granules improve the DBP degradation capability, the characteristics of PA-degrading aerobic granules were fluctuated with the feeding of DBP, which is a toxic and recalcitrant compound. During the first several days of DBP fed to PA-degrading aerobic granules, the SOUR of PA-degrading aerobic granules decreased from 35 to 19 mg DO g VSS^{-1} h^{-1} (Fig. 4-22). The decrease of bioactivity led to the accumulation of PA. The pH value of the effluent decreased to 6.2 on day 8 (Fig. 4-19). The structure of aerobic granules was affected by the environment with the low pH value. Some broken aerobic granules appeared and led to the decreasing mean biomass size (Fig. 4-18). The settling ability of aerobic granules decreased, which was evidenced by increasing SVI value. Thus part of broken aerobic granules were washed out and led to the decrease of biomass concentration. However, the microorganisms maintained in the reactor were protected by aerobic granules. When they adapted to the new substrate, the $SOUR_{(PA)}$ recovered to 33 mg DO g VSS^{-1} h^{-1}, at the same time, the $SOUR_{(DBP)}$ increased to 24 mg DO g VSS^{-1} h^{-1} (Fig. 4-22). The TOC removal efficiency improved with the increase of aerobic granular bioactivity. The pH value in the reactor increased and was maintained at around 7.0 when the reactor reached steady state. Finally, the aerobic granules were stabilized in the reactor.

4.5 Summary

In this study, the strategy of enhancing PA and PAE degradation by bioaugmentation of PA-degrading aerobic granules was studied. The results showed that the strategy was successful for accelerating the degradation of PA and PAEs.

In R1, The highest OLR achieved in PA degradation by the PA-degrading aerobic granular system was 12.4 kg COD m^{-3} d^{-1}. Under an OLR of 5.8 kg COD m^{-3} d^{-1}, aerobic granules kept good structure and more than 90% of the TOC removal was contributed by aerobic granules. While under an OLR of 12.4 kg COD m^{-3} d^{-1}, some of aerobic granules were broken into small pieces of compact biomass. This biomass, with high settling ability, can be maintained in the reactor and contribute 46% TOC removal. It played an important role in granular reactor at high OLRs.

DMP was fed to the PA-degrading aerobic granules in R2 and the DMP acclimated activated sludge in R4. PA-degrading aerobic granules were a more appropriate inoculum than DMP acclimated activated sludge for DMP degradation. The reactor inoculated with PA-degrading aerobic granules stabilized fast than DMP acclimated activated sludge. It only took 15 days to reach steady state at an OLR of 3.0 kg COD m^{-3} d^{-1}. The system seeded with PA-degrading aerobic granules could sustain a higher OLR as 6.0 kg COD m^{-3} d^{-1}, which caused the failure operation of the system seeded with DMP acclimated activated sludge. The good structure of the aerobic granules was maintained within the whole DMP degradation process. The compact structure of the granules afforded sufficient protection against the toxicity caused by DMP and minimized sludge washout, thus facilitating the rapid and high efficiency DMP degradation. Of all the systems, PA-degrading aerobic granules are better choice for industrial DMP wastewater treatment.

DBP, a refractory compound, was fed to the PA-degrading aerobic granules in R3. 38

days were required for the reactor to reach steady state. PA-degrading aerobic granules could handle the DBP with an OLR of 1.5 kg COD m^{-3} d^{-1}, which has not been reported for conventional treatment. The DBP removal efficiency was 100% and the TOC removal efficiency was 92.7%. The structure of the aerobic granules was affected by the DBP in the initial operation stage and some granules were broken during operation. At steady state, DBP-degrading aerobic granules with a compact structure were regained after 38 days of operation.

CHAPTER 5
METABOLIC AND ENZYMATIC ANALYSIS OF PHTHALATE DEGRADATION BY PHTHALIC ACID-DEGRADING AEROBIC GRANULES

5.1 Introduction

Phthalic acid and its esters (including DMP and DBP) have been successfully degraded by the system inoculated with PA-degrading aerobic granules (Chapter 4). However, the detail information about the enhancement of DMP degradation by PA-degrading aerobic granules is still unknown.

Generally, metabolism is the sum of all the biochemical reactions by which microorganisms synthesize cellular components from carbon source and obtain energy. Enzymes are triggers to initiate the cell metabolism reactions (Rittmann and McCarty, 2002). Thus the main objective of this study was to explore the metabolism of DMP degradation by PA-degrading aerobic granules and the influence of enzymes on the DMP degradation. A deeper view would be provided by studying the change of reaction rate and enzyme activity in the DMP degradation by PA-degrading aerobic granules. The optimum operations would be adopted to accelerate the PAEs degradation when the inner reasons of the enhancement of DMP degradation by inoculation with PA-degrading aerobic granules were explored.

5.2 Materials and Methods

5.2.1 Reactor Operation

The experiment was repeated as described in Chapter 4, reactor R2.

5.2.2 Kinetic Study

To monitor the change of DMP and its intermediates' degradation rates, kinetic tests were carried out in the reactor. The reactor operation was described in Section 5.2.1. During one cycle time, samples of 5 ml suspended liquor at different time intervals were collected throughout the whole experiment process. Samples were analyzed to evaluate the DMP and PA concentrations according to the methods previously described in Chapter 4, section 4.2.4 and Chapter 3, section 3.2.4, respectively. The concentrations of mono-methyl phthalate (MMP) was analyzed by HPLC using the same Inertsil C8-3 column the same mobile phase as DMP which has been described in Chapter 4, section 4.2.4. The mobile phase flow rate was 1 ml min^{-1}, and they were detected at a wavelength of 235 nm. Typical retention time was 3.5 min (Fan et al., 2001).

5.2.3 Preparation of Cell Extracts for Enzyme Activity Measurement

The aerobic granule samples for enzyme analysis were collected 30 min after start a cycle operation. The aerobic granules were harvested by centrifuging at 3000 g for 10 min. They were washed twice with 10 volumes of cold potassium phosphate buffer (0.1 M, pH 7.5) before re-suspension in one volume of the same buffer.

First, the aerobic granules were grinded into small particles with a size below 0.3 mm. Then the biomass was broken by a Mini BeadBeater (Scientific Support Inc., Hayward, CA) at 4600 rpm for 30 s. Every 10 s the tube with biomass was put into an ice bath to keep it at a low temperature. Then the cells were centrifuged (13000 rpm for 30 min), and the supernatant was used as the crude enzyme solution.

5.2.4 Enzyme Activity Analyses

Enzyme activity (esterase and dioxygenase) was analyzed according to the method reported by Niazi et al. (2001). The reaction solution consisted of a potassium phosphate buffer of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 (pH 8.0), 50.0 mg l^{-1}

DMP/ 50.0 mg l^{-1} MMP/ 50.0 mg l^{-1} PA in a final reaction volume of 2.0 ml and incubated with an appropriate amount of enzyme for 5 min at 30 ºC with suitable controls. The residue of DMP/MMP/PA was analyzed by HPLC. One unit of enzyme activity (U) was defined as 1 µmol of DMP/MMP/PA consumed per min.

Protein concentration was estimated according to the traditional Bradford method (Bradford, 1976) with bovine serum albumin (Sigma-Aldrich Corporation, St Louis, MO, USA) as the standard.

5.3 Results

5.3.1 DMP Degradation and Degradation Rate

To monitor the profiles of DMP degradation by PA-degrading aerobic granules in the entire experiment process, DMP residue concentrations and the concentrations of its degradation intermediates, mono-methyl phthalate (MMP) and phthalic acid (PA), were measured during a single cycle in the reactor. The DMP degradation on day 21 was selected as a typical model to describe the process of the DMP degradation by PA-degrading aerobic granules (Fig 5-1). The initial DMP concentration in the reactor was 330.0 mg l^{-1}. It took 75 min to completely remove DMP. MMP and PA appeared at 5 min with concentrations of 42.2 mg l^{-1} and 13.0 mg l^{-1}, respectively. The MMP accumulated to 49.1 mg l^{-1} at 10 min, and decreased to zero at 75 min., The PA accumulated to 31.1 mg l^{-1} at 15 min and decreased to zero at 50 min.

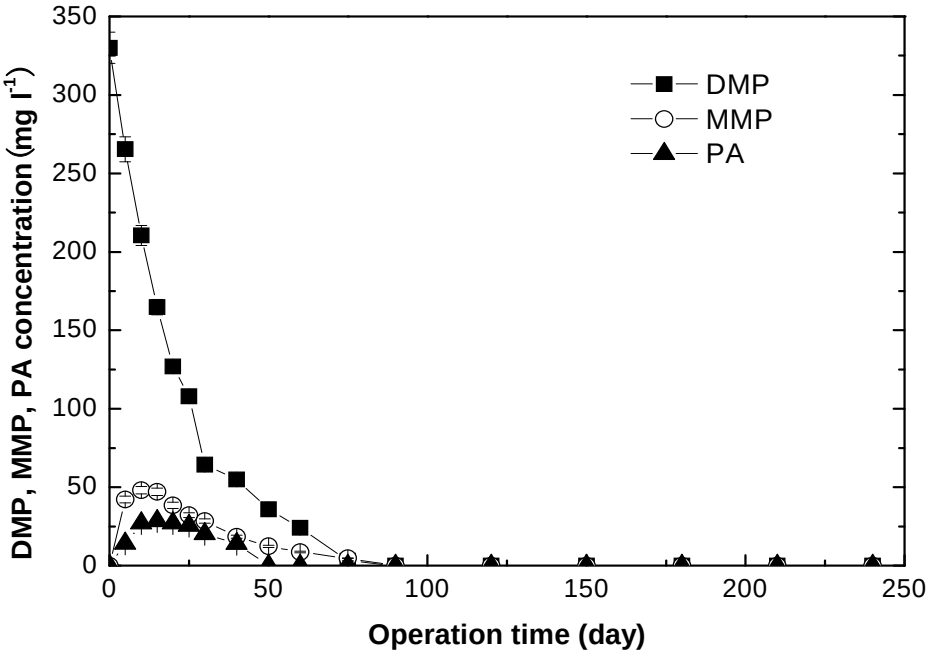


Fig. 5-1 Typical pattern of DMP and its intermediates degradation in one cycle.

A number of models have been applied to describe the biodegradation of organic pollutants, and first-order kinetics is frequently used as a convenient way to describe the phthalates degradation process (Wang et al., 1997b; Madsen et al., 1999; Gavala et al., 2003).

The simulation of the DMP degradation data followed the assumptions that:

1) The aerobic degradation of DMP involves several steps of transformation before the substrate's complete mineralization (Shelton et al., 1984; Staples et al., 1997). The microbial metabolism of DMP was initiated by an initial ester hydrolysis to form MMP and methanol (Equation (5-1)), and was followed by a further hydrolysis of MMP to PA and methanol (Equation (5-2)). The third step is that PA is mineralized to CO_2 and water (Equation (5-3)).

$$DMP + H_2O \xrightarrow{k_1} MMP + CH_3OH \qquad (5\text{-}1)$$

$$MMP + H_2O \xrightarrow{k_2} PA + CH_3OH \quad (5\text{-}2)$$

$$PA + 9.5O_2 \xrightarrow{k_3} 8CO_2 + 3H_2O \quad (5\text{-}3)$$

2) The hydrolysis of PAE and the mineralization of PA follow first-order kinetics according to Equations (5-4 to 5-6).

$$-\frac{dC_{DMP}}{dt} = k_1 C_{DMP} \quad (5\text{-}4)$$

$$-\frac{dC_{MMP}}{dt} = k_2 C_{MMP} - k_1 C_{DMP} \quad (5\text{-}5)$$

$$-\frac{dC_{PA}}{dt} = k_3 C_{PA} - k_2 C_{MMP} \quad (5\text{-}6)$$

Assume that when the degradation begins, t = 0, the concentrations of DMP, MMP, PA are C_0, 0 and 0, respectively. The concentrations of DMP, MMP, PA at t were calculated as follows:

$$C_{DMP} = C_0 e^{-k_1 t} \quad (5\text{-}7)$$

$$C_{MMP} = \frac{k_1 C_0}{k_2 - k_1}\left(e^{-k_1 t} - e^{-k_2 t}\right) \quad (5\text{-}8)$$

$$C_{PA} = k_1 k_2 C_0 \left(\frac{e^{-k_1 t}}{(k_2 - k_1)(k_3 - k_1)} - \frac{e^{-k_2 t}}{(k_3 - k_2)(k_2 - k_1)} + \frac{e^{-k_3 t}}{(k_3 - k_1)(k_3 - k_2)}\right) \quad (5\text{-}9)$$

Simulation of the results was made using a modified nonlinear least-squares fitting method that is based on converting the differential mass balances to algebraic, upon discretization of time. The EXCEL software was used to formulate the optimization process. The least-squares objective was to select the best kinetic parameter values so as to minimize the squared residuals between the experimentally measured and the respective predicted concentrations (Gavala et al., 2001; Li et al., 2005).

The simulation of a typical experimental data on day 21 is shown in Figs. 5-2, 5-3 and 5-4. The high values of the correlation coefficients ($R^2 > 0.90$) revealed that the degradation of DMP by PA granules was conformed to a first-order reaction kinetic equation.

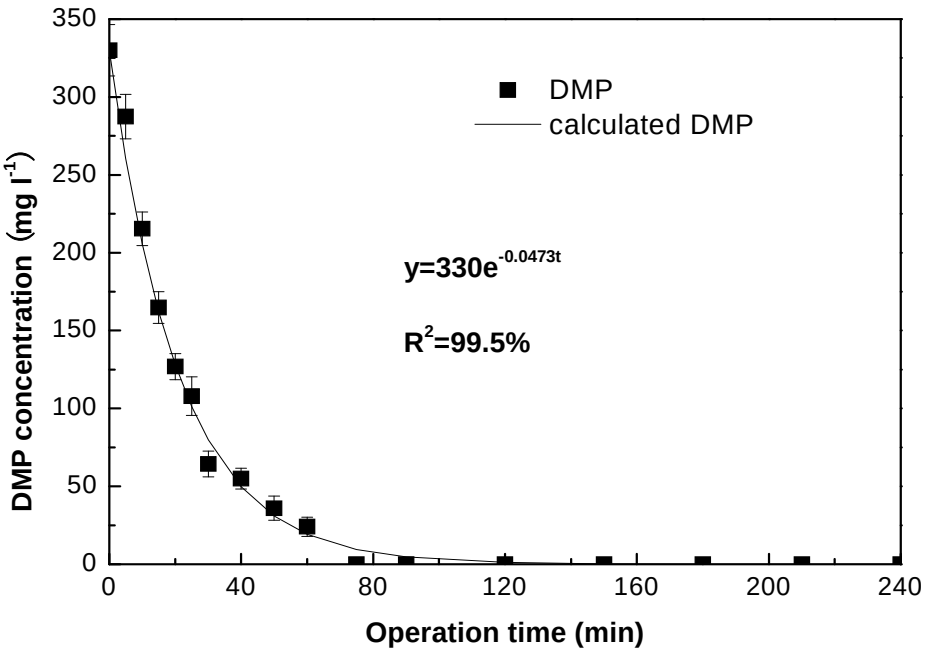


Fig. 5-2 Simulation of DMP degradation in one cycle.

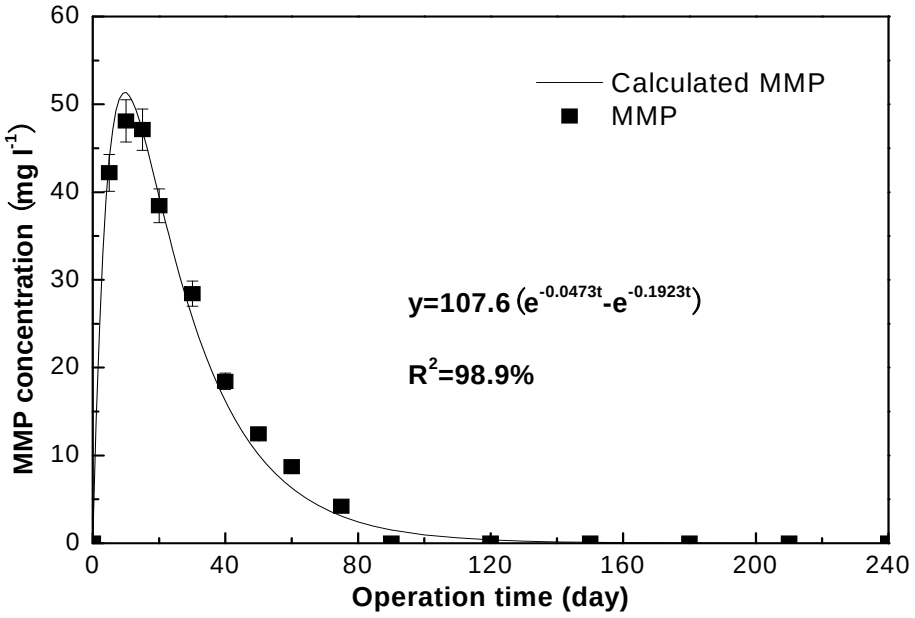


Fig. 5-3 Simulation of MMP degradation in one cycle.

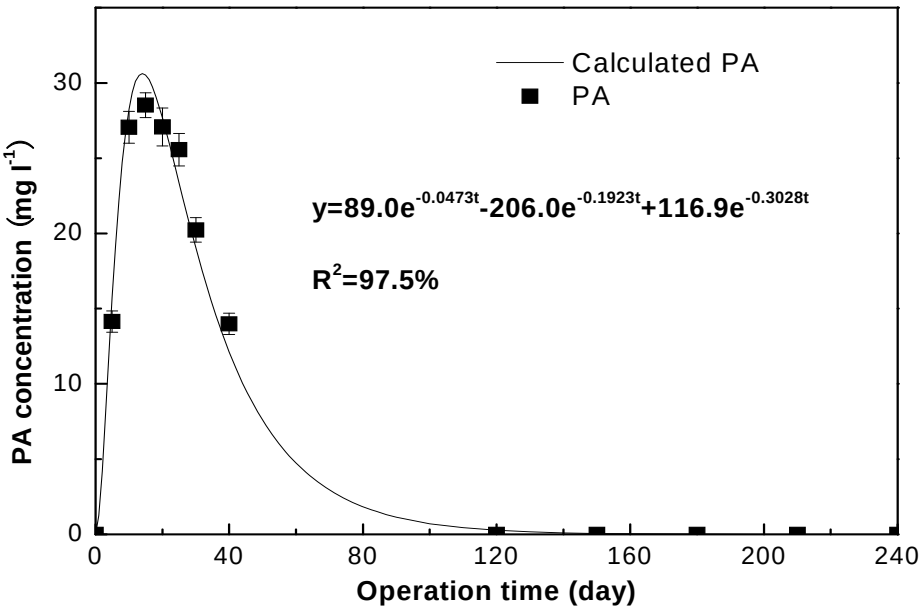


Fig. 5-4 Simulation of PA degradation in one cycle.

The kinetic equations of DMP degradation are summarized in Tables 5-1, 5-2 and 5-3. A Comparing the degradation rates on the same day, they are found to be

$k_3 > k_2 > k_1$ (Tables 5-4). The specific DMP and its intermediates' degradation rates for the three degradation reactions were defined as K_1, K_2, K_3, and they were calculated from the kinetic equations, are shown in Figs. 5-5, 5-6 and 5-7.

Table 5-1 The kinetic equations of DMP degradation.

Operation time (day)	Kinetic equation	k_1 (h^{-1})	R^2 (%)
1	$C_{DMP}=247e^{-0.0239t}$	0.0239	98.5
2	$C_{DMP}=248e^{-0.0240t}$	0.0240	99.4
3	$C_{DMP}=242e^{-0.0466t}$	0.0466	95.5
4	$C_{DMP}=272e^{-0.0522t}$	0.0522	98.5
5	$C_{DMP}=277e^{-0.0454t}$	0.0454	98.8
7	$C_{DMP}=290e^{-0.0475t}$	0.0475	94.3
10	$C_{DMP}=284e^{-0.0477t}$	0.0477	98.5
12	$C_{DMP}=320e^{-0.0416t}$	0.0416	97.8
16	$C_{DMP}=310e^{-0.0439t}$	0.0439	98.7
21	$C_{DMP}=330e^{-0.0473t}$	0.0473	99.5
26	$C_{DMP}=320e^{-0.0451t}$	0.0451	98.9
33	$C_{DMP}=321e^{-0.0489t}$	0.0489	99.8

Table 5-2 The kinetic equations of MMP degradation.

Operation time (day)	Kinetic equation	k_2 (h^{-1})	R^2 (%)
2	$C_{MMP} = 64.5(e^{-0.0239t}-e^{-0.116t})$	0.116	96.8
3	$C_{MMP} = 128.0(e^{-0.0466t}-e^{-0.1346t})$	0.1346	96.2
4	$C_{MMP} = 223.7(e^{-0.0522t}-e^{-0.1156t})$	0.1156	96.8
5	$C_{MMP} = 128.5(e^{-0.0454t}-e^{-0.1431t})$	0.1431	97.4
7	$C_{MMP} = 125.0(e^{-0.0475t}-e^{-0.1576t})$	0.1576	97.7
10	$C_{MMP} = 88.4(e^{-0.0477t}-e^{-0.2007t})$	0.2007	95.7
12	$C_{MMP} =99.8(e^{-0.0416t}-e^{-0.1751t})$	0.1751	98.9
16	$C_{MMP} = 94.9(e^{-0.0439t}-e^{-0.1859t})$	0.1859	98.3
21	$C_{MMP} = 107.6(e^{-0.0473t}-e^{-0.1923t})$	0.1923	98.9
26	$C_{MMP} = 90.6(e^{-0.0451t}-e^{-0.2044t})$	0.2044	97.6
33	$C_{MMP} =95.8(e^{-0.0452t}-e^{-0.1967t})$	0.1967	98.8

Table 5-3 The kinetic equations of PA degradation.

Operation time (day)	Kinetic equation	k_3 (h^{-1})	R^2 (%)
2	$C_{PA}= 15.5\ e^{-0.0240t}-19.2e^{-0.116t}+3.646e^{-0.5081t}$	0.5081	96.6
3	$C_{PA}= 46.4\ e^{-0.0466t}-60.8e^{-0.1346t}+14.4e^{-0.4177t}$	0.4177	97.8
4	$C_{PA}= 97.8\ e^{-0.0522t}-128.6e^{-0.1156t}+42.7e^{-0.3166t}$	0.3166	91.3
5	$C_{PA}= 70.8\ e^{-0.0454t}-113.6e^{-0.1431t}+23.80e^{-0.3049t}$	0.3049	97.2
7	$C_{PA}= 78.0\ e^{-0.0475t}-138.3e^{-0.1576t}+60.3e^{-0.3001t}$	0.3001	94.9
10	$C_{PA}= 71.5e^{-0.0477t}-186.5e^{-0.2007t}+115.1e^{-0.2958t}$	0.2958	94.1
12	$C_{PA}=67.9\ e^{-0.0416t}-142.9e^{-0.1751t}+76.5e^{-0.2731t}$	0.2731	93.5
16	$C_{PA}= 66.3\ e^{-0.0439t}-56.90e^{-01859t}+30.47e^{-0.3094t}$	0.3094	99.2
21	$C_{PA}= 89.0\ e^{-0.0473t}-206.0e^{-0.1923t}+116.9e^{-0.3028t}$	0.3028	97.5
26	$C_{PA}= 64.4\ e^{-0.0451t}-144.5e^{-0.2044t}+80.0e^{-0.3326t}$	0.3326	98.4
33	$C_{PA}= 68.4\ e^{-0.0452t}-151.7^{-0.1967t}+83.3e^{-0.3210t}$	0.3210	98.8

Table 5-4 Comparison of the reaction rates in DMP degradation process.

Operation time (day)	k_1 (h^{-1})	k_2 (h^{-1})	k_3 (h^{-1})
1	0.0239		
2	0.0240	0.116	0.5081
3	0.0466	0.1346	0.4177
4	0.0522	0.1156	0.3166
5	0.0454	0.1431	0.3049
7	0.0475	0.1576	0.3001
10	0.0477	0.2007	0.2958
12	0.0416	0.1751	0.2731
16	0.0439	0.1859	0.3094
21	0.0473	0.1923	0.3028
26	0.0451	0.2044	0.3326
33	0.0489	0.1967	0.3210

Although the PA-degrading aerobic granules were not acclimated by DMP, the specific DMP degradation rate K_1 by PA-degrading aerobic granules was 0.282 g-DMP g VSS^{-1} h^{-1} on day 1 (Fig. 5-5). It increased to 0.449 g DMP g VSS^{-1} h^{-1} on day 2 and to 0.861 g-DMP g VSS^{-1} h^{-1} on day 4. After that, the specific DMP degradation rate reached a steady value between 0.722 and 0.860 g-DMP g VSS^{-1} h^{-1}.

Figure 5-5 also reveals that specific DMP degradation rate by PA-degrading aerobic granules increased rapidly in the first four days. The specific DMP degradation rate increased to the highest value of 0.861 g-DMP gVSS^{-1} h^{-1} in only 4 days and kept this value for the rest of the operation.

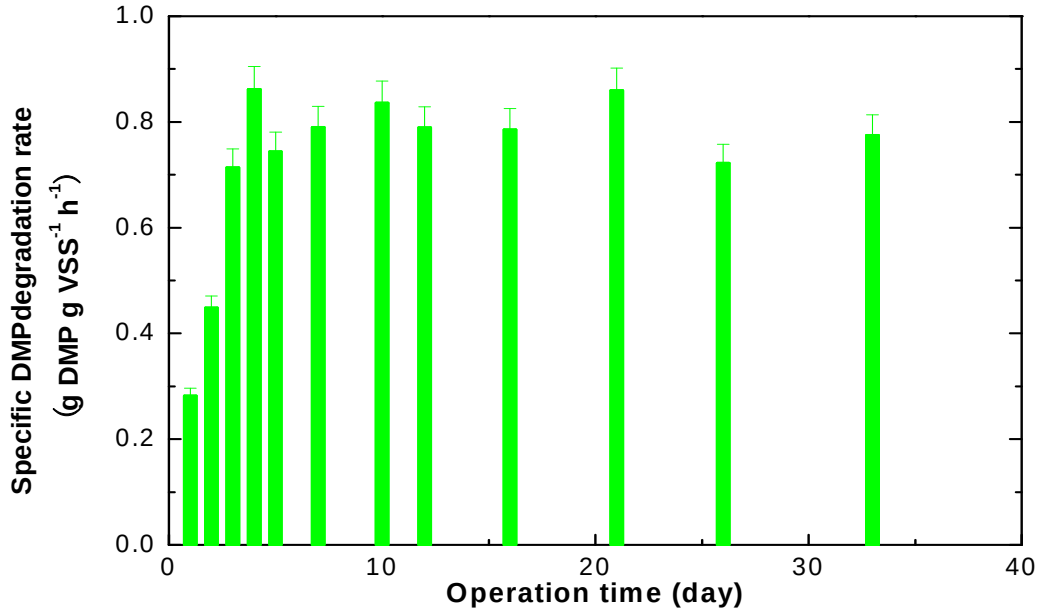


Fig. 5-5 Profiles of specific DMP degradation rates.

The change of specific MMP degradation rate K_2 over the operation time is shown in Fig. 5-6. No MMP intermediates appeared on day 1, so the specific MMP degradation rate for day 1 could not be calculated. From day 2 onwards, the MMP intermediates began to accumulate in the reactor. The specific MMP degradation rate was 2.175 g-MMP g VSS^{-1} h^{-1} on day 2, and it increased gradually to 3.517 g-MMP g VSS^{-1} h^{-1} on day 10. After that, the specific MMP degradation rates were maintained between 3.205 and 3.527 g-MMP g VSS^{-1} h^{-1}.

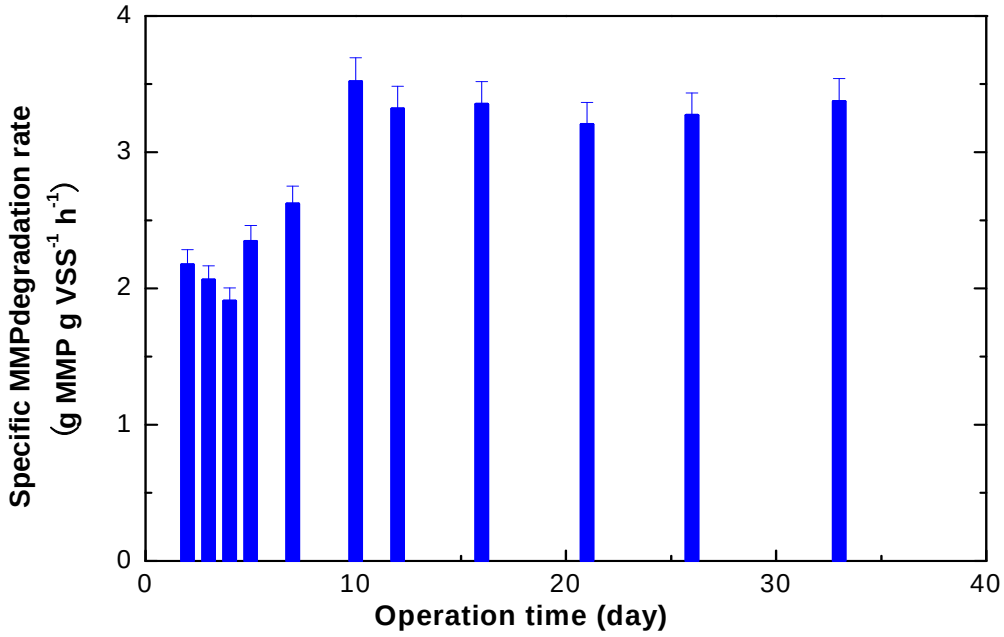


Fig. 5-6 Profiles of specific MMP degradation rates.

The change of specific PA degradation rates K_3 is shown in Fig. 5-7. Similar to MMP, there was no PA degradation on day 1. Since the PA aerobic granules had a low DMP hydrolysis rate on day 1 (Fig. 5-5) and high PA degradation rates, the produced PA during DMP hydrolysis was consumed rapidly by the PA-degrading aerobic granules. Thus, no PA intermediates appeared on day 1. The specific PA degradation rate for day 1 could not be calculated. From day 2 onwards, PA, the intermediates of DMP degradation, appeared in the reactor. The highest PA concentration was around 29.1 mg l^{-1}. The specific PA degradation rate decreased from 9.509 g-PA g VSS^{-1} h^{-1} on day 2 to 5.229 g-PA g VSS^{-1} h^{-1} on day 4. From day 4 onwards, it was steady between 4.998 and 5.576 g-PA g VSS^{-1} h^{-1}.

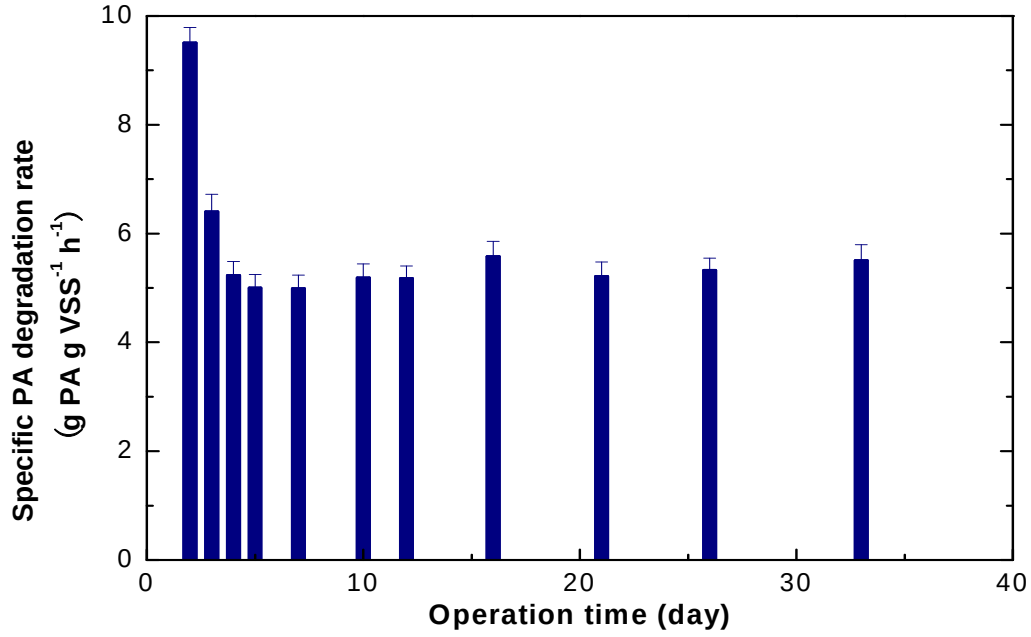


Fig. 5-7 Profiles of specific PA degradation rates.

5.3.2 Enzyme Activity of DMP Degradation by PA-degrading Aerobic Granules

5.3.2.1 Enzyme Activity of Esterase for DMP Hydrolysis

The profiles of enzyme activity of esterase for DMP hydrolysis are shown in Fig. 5-8. , The crude enzyme extract was extracted from the PA-degrading aerobic granules on day 0, before the PA-degrading aerobic granules were fed with DMP as the sole carbon and energy source. The enzyme activity in the crude enzyme extract on day 0 was measured to be 1.01×10^{-3} U mg^{-1} protein. It indicated that the PA-degrading aerobic granules possessed the enzyme for DMP hydrolysis on day 0, although there was no DMP for induction of the esterase. The feeding of DMP as substrate accelerated the enzyme activity of esterase for DMP hydrolysis. On day 2, the enzyme activity of esterase $_{(DMP)}$ slightly increased to 2.63×10^{-3} U mg^{-1} protein. On day 4, it sharply increased to 0.081 U mg^{-1} protein. From day 10 onwards, it fluctuated between 0.098 and 0.112 U mg^{-1} protein, and the average value was 0.105 U mg^{-1} protein.

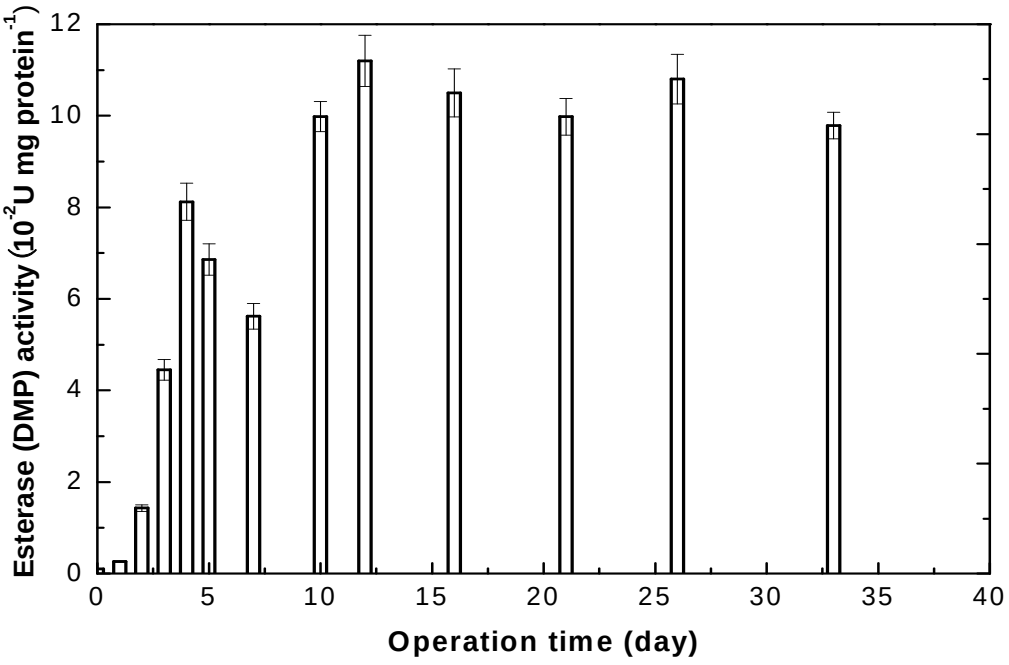


Fig. 5-8 Profiles of enzyme activity of DMP hydrolysis

5.3.2.2 Enzyme Activity of Esterase for MMP Hydrolysis

The profiles of enzyme activity of esterase for MMP hydrolysis are shown in Fig. 5-9. Similar to the esterase for DMP hydrolysis, the esterase activity of PA-degrading aerobic granules for MMP hydrolysis was tested on day 0, and found to be 0.062 U mg^{-1} protein. Thus the PA-degrading aerobic granules possessed the enzyme for MMP degradation, although there was no MMP provided for enzyme induction. Just like esterase for DMP hydrolysis was induced by DMP, the esterase for MMP hydrolysis was induced by MMP produced in DMP hydrolysis. On day 1, the enzyme activity of esterase $_{(MMP)}$ was 0.140 U mg^{-1} protein, which was two times that of day 0. On day 5, it increased to 0.342 U mg^{-1} protein. From day 5 onwards, it was tested between 0.274 and 0.342 U mg^{-1} protein (average value of 0.308 U mg^{-1} protein).

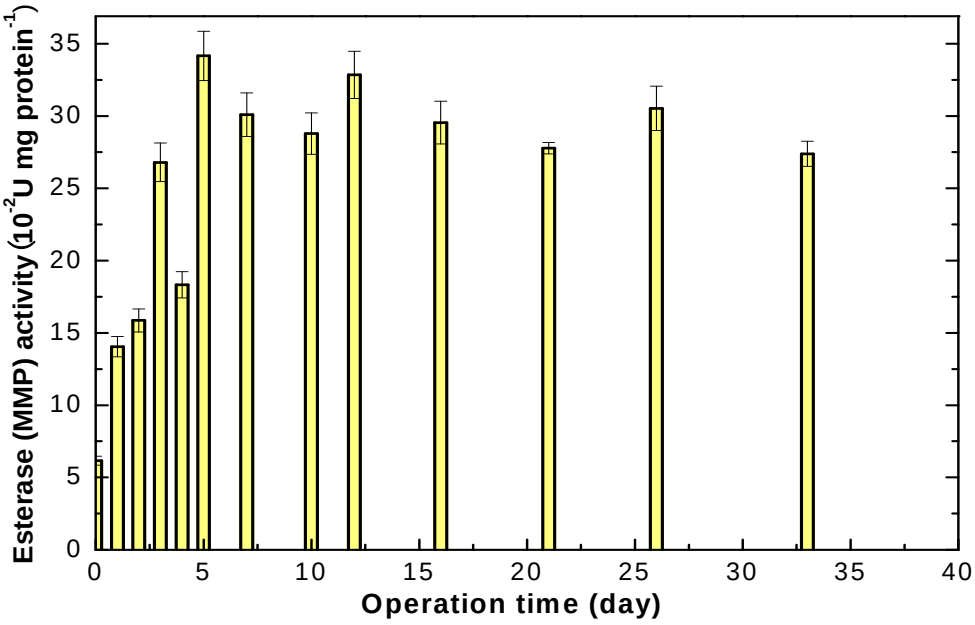


Fig. 5-9 Profiles of enzyme activity of MMP hydrolysis.

5.3.2.3 Enzyme Activity of Dioxygenase for PA Degradation

The profiles of enzyme activity for PA degradation are shown in Fig. 5-10. Since the PA-degrading aerobic granules had been cultivated using PA as the sole carbon and energy source, the PA-degrading aerobic granules possessed the highest enzyme activity of 0.133 U m^{-1}g protein in the beginning of the experiment. When the DMP was fed to PA-degrading aerobic granules as sole carbon and energy source, the intermediate PA of DMP hydrolysis was degraded by existing dioxygenase in the PA-degrading aerobic granules, which was the enzyme responsible for PA degradation. The enzyme activity slightly decreased from 0.133 U mg^{-1} protein on day 0 to 0.113 U mg^{-1} protein on day 4. From day 4 onwards, the enzyme activity for PA degradation was kept between 0.097 and 0.115 U mg^{-1} protein (average value of 0.106 U mg^{-1} protein).

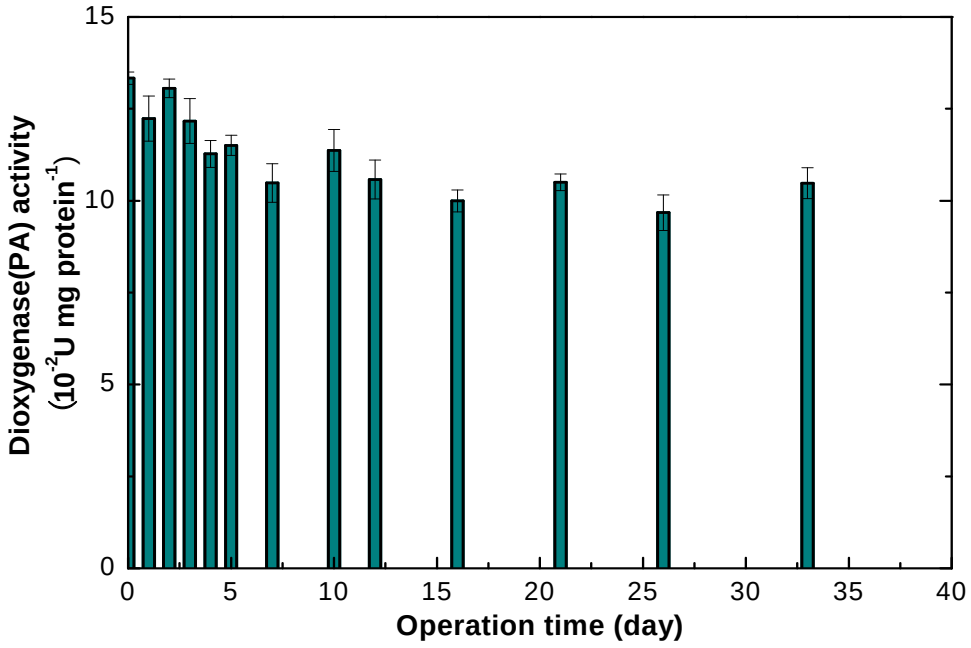


Fig. 5-10 Profiles of enzyme activity of PA degradation.

5.4 Discussion

5.4.1 The Influence of PA-degrading Aerobic Granules on DMP Degradation Rates

In the study, the DMP was degraded by the system innoculated with PA-degrading aerobic granules. The reaction rates at different reaction steps during the DMP degradation were found to be changed compared with the system without the PA-degrading aerobic granules inoculation.

When DMP was degraded by DMP acclimated microorganisms (without PA-degrading aerobic granules inoculation), the first step from DMP to MMP was carried out smoothly because the system already possessed the DMP degradation ability. However, the degradation rates for MMP and PA degradation were slow because there were not enough bacteria or the bioactivities were not strong enough for the biodegradation of the produced intermediates, MMP and PA. Thus, k_1 was

higher than k_2 and k_3. Further degradation of DMP was hindered by the intermediates accumulation (Fan et al., 2001; Wang et al., 2003a).

The innoculated PA-degrading aerobic granules changed the relations among the reaction rates during DMP degradation (Table 5-4). From day 1 to day 33, the degradation rates was observed to be $k_3>k_2>k_1$ on the same day. Since PA-degrading aerobic granules possessed a high PA degradation rate, the produced MMP and PA was consumed completely on the first day. There was no transient appearance of intermediates. With the reactor operation, the specific DMP degradation rate k_1 increased, which resulted in the transient appearance of two intermediates. MMP was produced as a major intermediate, as the concentration of MMP had once been 48.4 mg l^{-1} (Fig. 5-3). Then it was degraded during further operation. Finally, it could not be detected at the end of operation. A small amount of PA was also concomitantly produced when the MMP appeared. The largest mount of PA produced during DMP degradation was 28.5 mg l^{-1} (Fig. 5-4). Almost all the intermediates disappeared in a short period of time when DMP was completely degraded (Fig. 5-1). The degradation rates maintained the trend of $k_3 > k_2> k_1$ until the reactor reached steady state.

In conclusion, the produced PA from DMP degradation was consumed quickly because of the introduced PA-degrading microorganisms in PA-degrading aerobic granules. In turn, the quick removal of PA accelerated the conversion of MMP to PA. As a result, there were no intermediates accumulated in the reactor. Thus, the degradation rates changed to be $k_3 > k_2> k_1$, which indicated the bioaugmentation of PA-degrading aerobic granules could stimulate the completely biodegradation of DMP.

5.4.2 The Influence of PA-degrading Aerobic Granules on the DMP degradation Enzyme System

The biodegradation of organic pollutants by microorganisms is composed of thousands of redox reactions. Enzymes are biochemical catalysts which speed up

the essential reactions in microorganisms (Rittmann and McCarty, 2002). Complete aerobic degradation of PA and PAEs (e.g., DMP, DBP) involves several enzymes, including hydrolases (esterase), dehydrogenases, decarboxylases, and dioxygenases (Ribbons et al., 1984). Among them, esterase and phthalate dioxygenase are the key enzymes to decide the accumulation of monoester and PA.

On day 0, the PA-degrading aerobic granules showed enzyme activity on the DMP and MMP hydrolysis. This result indicated that the enzymes for DMP and MMP hydrolysis could be constitutive. The enzyme activity for DMP and MMP hydrolysis increased after DMP fed to the system innoculated with PA-degrading aerobic granules. This result indicated that the enzymes for DMP and MMP hydrolysis were inducible.

In the report of other researchers, whether the esterase is constitutive or inducible is still a topic under discussion. Esterase hydrolyzing di- and mono-alkyl phthalates from *Nocardia* isolates were reported to be induced by phthalate esters. While the mono-alkyl phthalate esterase of *Pseudomonas* and *Arthrobacter* isolates were reported to be induced by free PA (Chatterjee and Dutta, 2003). In contrast, esterase involved in the phthalate ester catabolic pathway in *Arthrobacter keyseri* 12B was found to be constitutive in nature (Eaton, 2001). It seemed that for different pure cultures, the esterase showed different properties. PA-degrading aerobic granules were composed of all kinds of bacteria. Different bacteria may produce different esterase with inducible or constitutive properties. The crude enzyme extract of PA-degrading aerobic granules contained esterase from all kinds of bacteria. Thus, it showed constitutive and inducible properties for the degradation of DMP and MMP.

The constitutive enzymes played an important role in the DMP degradation by PA-degrading aerobic granules. Because of the constitutive enzyme, the PA-degrading aerobic granules can degrade DMP without acclimation. When a novel substrate was fed to the microorganisms, there generally existed a lag phase for the microorganisms to adapt to new environments (Rittmann and McCarthy, 2002). Because of the constitutive enzyme in PA-degrading aerobic granules, the lag phase

was completely depleted when DMP was degraded by PA-degrading aerobic granules in this study. In addition, the enzyme was also an inducible enzyme. Thus the enzyme activity improved with the reactor operation. Accordingly, the DMP and MMP hydrolysis rate increased with the increase of enzyme activity. As a result, DMP and MMP were degraded smoothly and the reactor operated steadily.

Dioxygenase, which catalyzed PA degradation, is an inducible enzyme (Fan et al., 2001). It is induced by the PA which is produced in the hydrolysis of DMP. During DMP degradation, dioxygenase was a new enzyme which was needed to be synthesized. The production of dioxygenase was controlled by two modes of regulation in the cell. Before the dioxygenase existed, a quite long time was required to synthesize the enzyme in the cell to a sufficient amount to affect metabolism.

Besides the slow production of new enzymes for the degradation of intermediates, the DMP degradation was also affected by a mechanism called feedback inhibition. In the initial stage of DMP degradation, MMP and PA were accumulated because of no sufficient dioxygenase to provide a high degradation rate. The accumulated PA which possesses a similar structure to DMP competed with DMP to get the activity position in the enzyme of DMP degradation. If the PA degradation microorganisms were bioaugmented to the DMP degradation systems, the PA produced in the DMP degradation could be consumed rapidly to avoid feedback inhibition.

PA-degrading aerobic granules were good sources of PA degradation microorganisms. PA-degrading aerobic granules possessed dioxygenase before they were used for DMP degradation. They had a strong PA degradation ability and PA could be consumed quickly by them. Without accumulation of MMP and PA in the reactor, there was no feedback inhibition. Thus the whole DMP degradation process proceeded smoothly and the DMP could be degraded completely.

In conclusion, the PA-degrading aerobic granules provided enzymes for DMP, MMP and PA degradation. As a result, the reactor operated steadily in the initial

operation period and the substrate feedback inhibition was avoided by degrading DMP degradation intermediates.

5.4.3 Enzyme Activity and Reaction Rate

The principle reactions in DMP degradation included three steps. The specific reaction rates of the three reactions were K_1, K_2, and K_3, and the esterase $_{(DMP)}$, esterase $_{(MMP)}$ and dioxygenase were the enzymes catalyzing the three reactions, respectively. When DMP was degraded by PA-degrading aerobic granules, the variations of the three enzyme activities were consistent with the changes of DMP degradation rates. These results indicated that the metabolism of phthalate was controlled by the related enzyme. In this study, the DMP degradation enzyme activity was improved by the bioaugmentation with PA aerobic granules. The increasing enzyme activity accelerated the DMP and its intermediates degradation rates.

5.5 Summary

The degradation rates and enzyme activities during the process of DMP degraded by PA-degrading aerobic granules were studied to find the underlying reason behind the strategy that PAEs' degradation was enhanced by the system inoculated with PA-degrading aerobic granules.

In PA-degrading aerobic granules, the enzymes catalyzing DMP and MMP hydrolysis were constitutive and inducible, while dioxygenase for PA degradation was inducible. The inoculated PA-degrading aerobic granules provided esterase for DMP and MMP hydrolysis and dioxygenase for PA degradation on the first day fed with DMP. The DMP degradation was initiated by these enzymes.

With the induction of DMP, the enzyme activity of esterase $_{(DMP)}$ increased from 1.01×10^{-3} U mg^{-1} protein on day 0 to 0.105 U mg^{-1} protein when the reactor reached steady state. For esterase catalyzing MMP hydrolysis, the enzyme activity increased

from 0.062 to 0.308 U mg^{-1} protein. The enzyme activity of dioxygenase maintained a high value, ranging from 0.097 to 0.133 U mg^{-1} protein.

As a result, the Specific reaction rate K_1 increased from a value of 0.282 g-DMP g VSS^{-1} h^{-1} on day 0 to 0.841 g-DMP g VSS^{-1} h^{-1} on day 10 when the reactor reached steady state. The specific reaction rate K_2 also increased from 2.175 g-MMP g VSS^{-1} h^{-1} on day 2 to 3.527 g-MMP g VSS^{-1} h^{-1} on day 10 at steady state. The specific PA degradation rate K_3 was maintained in the range of 4.998 g-PA g VSS^{-1} h^{-1} to 9.509 g-PA g VSS^{-1} h^{-1} in the entire DMP degradation process. Compared the reaction rates on the same day, the reaction rates in DMP degradation maintained $k_3 > k_2 > k_1$ after seeding with PA-degrading aerobic granules. Thus, the reaction bottle neck was broken by the bioaugmentation of PA-degrading aerobic granules. The DMP mineralization process was carried out smoothly.

CHAPTER 6

MICROBIAL STRUCTURE AND POPULATION SHIFT OF MICROBIAL COMMUNITY IN PHTHALIC ACID-DEGRADING AEROBIC GRANULES

6.1 Introduction

In biological wastewater treatment, the microbial ecosystems could respond in a very dynamic manner with changes of the environment, especially changes that stress community (Rittman and MaCarty, 2002). For example, a newly appeared substrate which was a stress to the community, the microbial ecosystems responded at a combination of different mechanisms, such as gene transfer or mutation, enzyme induction and population changes (Spain and VanVeld, 1983; Madigan et al., 2003). Similarly, the dynamic changes occurred in PA-degrading aerobic granules when the system innoculated with PA-degrading aerobic granules successfully degraded phthalates (Chapter 4). However, the detail information of the changes is still unknown. The further study on the genetic information and microbial ecology of PA-degrading aerobic granules could lead to better understanding of the mechanisms of the enhancement of phthalates degradation. The information would assist in improving the system design and performance (Clote, 1997).

Traditionally, the classical microbiological techniques, such as isolation, were used for the study of microbiology. With the advancement of science and technologies, microbiologists recognized that only a small fraction of all bacteria have been isolated and characterized (Ward et al., 1990; Amann et al., 1995). Thus, the application of molecular biological techniques to detect and identify microorganisms

by certain markers, such as 16S rRNA, is now used frequently for microbial diversity and microbial community structure analysis (Olsen et al., 1986; Amann et al., 1995).

However, cultivation-based methods such as isolation can not be totally supplanted. Ecological roles of microorganisms often can not be inferred from a comparison of their 16S rRNA sequences (Heuer et al., 1999). Especially, the successful isolations and studies of the functionally important populations in the environment can facilitate a better understanding of their physiologies. In addition, it also bears a potential bias by only using molecular biotechnological techniques to make microbial diversity analysis. The combination of both culture-independent and culture-dependant techniques can provide very useful and complementary information about the structure of microbial communities. In addition, functional redundancy and functional niche complementarity usually contribute to process performance and stability. Thus, recognizing the diversity and relationship within each functional group of a system would facilitate modeling diversity and function as well as improving process stability and treatment capacity (Hulot et al., 2000; Kasai et al., 2002; Van Der Gast et al., 2003).

In this study, denaturing gradient gel-electrophoresis (DGGE) and culture isolation were combined to study the microbial community of PA-degrading aerobic granules and identify the functional organisms in aerobic granules. Physiological and biochemical diversity of the isolates were characterized, and some physiological features which were helpful for maintenance and growth of organisms in aerobic granules were discussed. One dominant strain with high PA degradation ability was isolated, and its important role in PA and PAE degradation was also analyzed. The objective of this study was to better understand the microbial community of aerobic granules and hence to improve the degradation ability of aerobic granular systems.

6.2 Materials and Methods

6.2.1 Sampling of PA-degrading Aerobic Granules

The operations of the two SBRs R1 and R2 were presented in section 4.2.1 and section 3.2.1. R1 was fed with DMP as substrate after PA-degrading aerobic granules matured. The granules samples were taken out of R1 for DGGE analysis until day 39, when reactor R1 reached steady state. R2 was used as a control reactor with no change of substrate. PA-degrading aerobic granular samples were taken out of the reactors R2 for isolation and DGGE analysis at the same day of R1 collecting samples.

6.2.2 Isolation Procedure

The mineral salt medium (MSM) with PA or DMP used for isolation and growth consisted of the following chemicals (mg l^{-1}): $(NH_4)_2SO_4$ 1000, KH_2PO_4 800, K_2HPO_4 200, $MgSO_4 \cdot 7H_2O$ 500, $FeSO_4$ 10, $CaCl_2$ 50, $NiSO_4$ 32, $Na_2BO_7 \cdot H_2O$ 7.2, $(NH_4)_6Mo_7O_{24} \cdot H_2O$ 14.4, $ZnCl_2$ 23, $CoCl_2 \cdot H_2O$, 21, $CuCl_2 \cdot 2H_2O$ 10 and $MnCl_2 \cdot 4H_2O$ 30 (Wang et al., 2003a). PA (500.0 mg l^{-1}) or DMP (100.0 mg l^{-1}) were supplied as the sole carbon and energy source. The medium was autoclaved for 20 min at 121 ºC. PA and DMP solutions were sterilized by sterile filtration (0.2 μm) and added to the medium after autoclaving.

About 200 mg wet weight of granule samples were aseptically added to 20 ml of MSM, and were mixed in a sterilized beaker in order to detach granules. All operations were performed in a biohazard flow cabinet (ESCO, Singapore). The supernatant was then diluted with MSM (10^{-1} to 10^{-7}-fold dilutions), and 100 μl of

each dilution was spread onto a MSM (supplied with 500.0 mg l^{-1} PA or 100.0 mg l^{-1} DMP) agar plate solidified with 1.5% (W/V) Bacto Agar (Difco, Detroit, Mich). Plates were incubated in a 30 ºC incubator (Sanyo, Japan), and monitored for over 1 week. Visible colonies appeared after 24 to 48 h of incubation. Pure cultures of PA-degrading or DMP-degrading bacteria were isolated by cycles of re-plating onto MSM/PA or MSM/DMP agar plates. Purity was confirmed by microscopic examination with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM) (Stereoscan 420, Leica Cambridge Instruments) as described previously in Chapter 3. Pure cultures were stored in 50 mM KH_2PO_4 : K_2HPO_4 buffer (pH 7.2) containing 20% (V/V) glycerol at -70 ºC.

6.2.3 Morphological and Phenotypic Characterizations

Cells were observed with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM). Gram-stain testing was performed as previously described (Smibert et al., 1994).

6.2.4 Repetitive Extragenic Palindromic-polymerase Chain Reaction (REP-PCR)

Repetitive extragenic palindromic (REP)-PCR was done as described by Versalovic et al. (1991) to distinguish identical isolates. The primer used for the REP-PCR reaction was BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic et al., 1991). DNA amplification was carried out in a thermocycler (Mastercycler, Eppendorf, Germany) using approximately 100 ng of genomic DNA. The amplification program involved an initial denaturation cycle for 7 min at 95 ºC, followed by 35 cycles of 1min denaturation at 94 ºC, 1 min annealing at 53 ºC, 8 min

extension at 65 ºC, and 16 min final extension at 65 ºC. The reaction mixture contained 1.5 U Tag DNA polymerase (Promega Corporation, Madison, USA), 300 ng μl^{-1} of primer, 0.875 mM of each dNTP, 1×reaction buffer (Invitrogen) and 6 mM $MgCl_2$ (Invirogen), in a final volume of 25 µl. The PCR product underwent 1.5% agarose gel electrophoresis for 18 h under constant 70 V in 0.5×TAE, and was visualized under UV light after being stained with ethidium bromide (0.5 µg ml^{-1}). Fragments were photographed with an EDAS 290 gel imaging system (Eastman Kodak Company, Rochester, NY) and analyzed using Kodak 1D image Analysis software.

6.2.5 Direct-lysis PCR Amplification Method

A whole cell direct lysis PCR amplification method was used to amplify 16S rDNA as described previously (Maszenan et al., 1997). Cells were cultured on MSM/PA or MSM/DMP agar plates at 30 ºC for 1-2 days. A single colony was aseptically collected with a sterile pipette tip and smeared onto the bottom of a PCR tube. 10 µl of 100 mM Tris-HCl (pH 8.3), 8 µl 25 mM $MgCl_2$, 5 µl 1% NP40 and 64 µl sterilized Milli-Q water were added, and heated at 98 ºC for 30 min in a thermal cycle (Mastercycler, Eppendorf, Germany) for cell lysis to occur. The nearly full-length 16S rRNA gene was amplified by PCR with forward primer Eubac27F and reverse primer Universal 1492R (Lane, 1991). Thermal cycling was carried out as follows: 30 cycles consisting of 1.5 min at 94 ºC, 1.5 min at 62 ºC, and 2 min at 72 ºC. The last step of the last cycle was continued for 10 min and followed by cooling at 4 ºC. The PCR products were purified with a Qiagen PCR purification Kit (Qiagen, Germany) according to the manufacturer's instructions, and stored at -20 ºC before sequencing. The volume of purified PCR product selected as template for subsequent sequencing was determined by estimating its concentration after gel electrophoresis and comparing its brightness with concentrations of DNA standards

under UV light.

6.2.6 16S rRNA Gene Sequencing

The nucleotide sequences of 16S rDNA from the isolates were determined using the dideoxy chain termination chemistry and the ABI PRISM BigDyeTM Terminator Ready Reaction Cycle Sequencing Kit (version 3.0 Applied Biosystems, PerkinElmer) was used as specified by manufacturer. The double stranded 16S rDNA fragments from isolates were sequenced using the primer listed in Table 6-1. These target the conserved region of the 16S rDNA of the domain Bacteria (Lane, 1991).

Table 6-1 The sequences of primers used for sequencing 16S rDNA of isolates in the domain bacteria (Lane, 1991).

Primers	**Sequence (5'---------->3')**
27F	AGA GTT TGA TCM TGG CTC AG
357F	CTC CTA CGG GAG GCA GCA G
530F	GTG CCA GCM GCC GCG G
800R	CAT CGT TTA CGG CGT GGA C
907R	CCG TCA ATT CMT TTR AGT TT
1492 R	TAC GGY TAC CTT GTT ACG ACT T

Note: Where Y= C:T; M= C:A; R= A:G in equal proportion

6.2.7 Phylogenetic analyses of 16S rDNA data

The 16S rDNA sequences were aligned by using the BioEdit program (Tom Hall, Department of Microbiology, North Carolina State University [http://jwbrown.mbio.ncsu.edu/Bioedit/bioedit.html]), and checking the overlapping positions between sequences obtained from different primers and previously aligned sequence date sets. The probable identity of the query sequence was determined

using a BLAST search (Altschul et al., 1990) program from the database maintained by the National Centre for Biotechnology Information (NCBI) (National Institutes of Health, USA), which compared the query sequence with all other sequences available in the NCBI database and ranked them from the highest to lowest using similarity scores. A data set containing sequences of closely related organisms obtained from the Ribosomal Database Project (RDP) (Maidak et al., 2001) or GenBank was established, and re-aligned according to the secondary structure of the 16S rRNA molecular using BioEdit program.

6.2.8 DNA Extraction from Seeding Activated Sludge, Aerobic Granules, and Isolates

The DNA was extracted from seeding activated sludge and aerobic granular samples by a modified physical disruption of cells involving a direct-lysis procedure (Eichner et al., 1999). Approximately 200-300 mg (wet weight) activated sludge samples or aerobic granular samples were suspended in 800 µl sterile MilliQ water in a 2 ml microcentrfuge tube. 800 mg sterile baked glass beads and 50 µl of 20% (W/V) SDS (200g SDS in 1 liter distilled water pH 7.2 [Sigma, USA]) were added into the suspension. The tube was nearly topped up with saturated phenol (0.1 M Tris-HCl, pH 8.0 [Sigma, USA]). The suspension was shaken for 5 min with a Mini BeadBeater (Biospec products, USA) at room temperature. Then the suspension was supplemented with nucleic acids that were precipitated with 0.1 volume sodium acetate (pH 4.5-5.2, 20%)

6.2.9 DGGE-PCR Amplification

PCR primers P2 and P3 (containing 40 bp of GC clamp, the sequence of P2 is 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CA-3′ and the P3 is 5′-ATT ACC GCG GCT GCT GG -3′)

were used to amplify the variable V3 (Muyzer et al., 1993) region of bacterial 16S rRNA gene (corresponding to positions 341–534 in the Escherichia coli sequence) (Brosius et al., 1981).

Amplification was performed with a Mastercycler gradient (Eppendorf AG, Hamburg, Germany) using a 50 µl (total volume) mixture containing 1.25 U of Taq polymerase (Promega), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 200 µM deoxynucleoide triphosphate (dNTP), 10 pmol of each primer, and 1 µl of DNA solution (20 ng μl^{-1}) extracted from activated sludge, mature granules, or isolated strains. Touchdown PCR was employed (Muyzer *et al.*, 1993) with a Mastercycler Gradient thermal cycler (Eppendorf A. G., Hamburg, Germany), which involves 10 min of activating the polymerase at 94 ºC, 1 min at 65 ºC, and 2 min at 72 ºC. The annealing temperature was subsequently decreased by 1°C for every second cycle until it reached 55 ºC, at which point 20 additional cycles were carried out, finally, a 10 min extension step at 72 ºC was performed. The amplicons obtained were confirmed by electrophoresis through 2% agarose gel in 1µ TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM Na_2-EDTA, pH 8.0) stained with ethidium bromide.

6.2.10 Denaturing Gradient Gel-electrophoresis (DGGE)

The PCR-amplified fragments were separated by DGGE with a DCode system universal mutation detection system (Bio-Rad Laboratories, USA) using a modified method by Muyzer et al. (1993). The 25 ml of 30 to 70% urea-formamide denaturant gradient gel (10% [wt/vol] acrylamide solution [40% acrylamide: bisacrylamide, 37.5:1 stock solution, Bio-Rad Laboratories] in TAE buffer) was covered by a 4 ml acrylamide stacking gel (10%) without denaturant. 40 µl PCR amplicons from the DNA of biomass samples or 20 µl of PCR amplicons from the DNA of pure cultures were loaded with 20 µl loading dye in each well. The gel was placed in TAE buffer

and run at 40V at 60 ºC for 30 min and then at 85V at 60 ºC for 14 h. After electrophoresis, the gel was stained with ethidium bromide for 30 min, and viewed and photographed with an EDAS 290 gel imaging system (Kodak, USA).

6.2.11 PA and PAE Biodegradation Kinetic Study

The DMP/PA growth cells at mid-exponential phase were used in the kinetic study. Experiments were performed in triplicate in 500 ml serum bottles. Each bottle contained 200 ml of MSM solution with an initial PA concentration of 350.0 mg l^{-1} or a DMP concentration of 250.0 mg l^{-1}. The individual strain was then introduced, giving the liquid solution on optical density (OD) around 0.05, and all reagent bottles were capped immediately. The culture medium was incubated at 30 ºC on an orbital shaker at 150 rpm. PA or DMP concentrations were assayed at regular intervals of incubation. Cell growth was monitored by OD measurement using a spectrophotometer (Perkin-Elmer, USA) at a wave-length of 600 nm. The linear relationship between OD values and the biomass weight was confirmed using strain PA-02. The specific growth rates were calculated from cell growth measurements during the initial period.

6.2.12 Plate Counting Method

The isolates which were identified to be different from each other by rep-PCR were cultivated on PA/MSM or DMP/MSM agar plates. For each assay, 1 ml of cell suspension was sampled and serially diluted (10^{-3} to 10^{-9}). 100 µl of each dilution was spread on a marine 2216 agar plate. The plates were incubated for three days at 30 ºC and then counted. All experiments were performed in triplicate.

6.2.13 Analytical Methods

All biodegradation experiments were conducted in triplicate. Periodically, 2 ml of culture samples were withdrawn aseptically from flasks by syringe and stored at -20 ºC in a glass vial until analyzed. Sterile controls were prepared by autoclaving for 20 min on three successive days before introduction of the substrate through a 0.2-µm-pore-size membrane filter fitted to a syringe (Pall Gelman Laboratory, Ann Arbor, Michigan). The pretreatment and analysis of water samples have been described in Chapters 3 and 4.

The microbial biomass was determined spectrophotometrically by optical density measurements at 600 nm (OD600) using an UV 1201 (Shimadzu Co., Kyoto, Japan). The culture was mixed intensively before sampling if flocs were observed. The samples were diluted when the OD600 value was greater than 0.4.

6.2.14 Calculation of Biomass Cellular Yield

According to Rittmann and McCarty (2002), the growth rate of microbial cells is mathematically expressed as

$$\frac{dX}{dt} = Y(\frac{-dS}{dt}) - k_d X \qquad (6\text{-}1)$$

where dX/dt represents the net growth rate (mg l^{-1} h^{-1}) of active organisms (X, mgl^{-1}), dS/dt represents the rate of disappearance (mg l^{-1} h^{-1}) of substrate (S, mg l^{-1}), k_d is the endogenous metabolism or decay rate (h^{-1}) of the organism, and Y is the true yield of microorganisms (mg biomass mg $substrate^{-1}$).

Equation (6-1) can be rearranged as follows:

$$\frac{dX}{Xdt} = Y(\frac{-dS}{Xdt}) - k_d \qquad (6\text{-}2)$$

Since μ (specific growth rate, h^{-1}) is defined as $\frac{dX}{Xdt}$, and $\frac{-dS}{Xdt}$ is defined as q (specific PA or DMP degradation rate, mg substrate mg $biomass^{-1}$ h^{-1}), then

$$\mu = Yq - k_d$$

that is

$$Y = \frac{\mu}{q} + \frac{k_d}{q} \qquad (6\text{-}3)$$

Assuming the observed biomass yield $(Y_{obs}) = \frac{\mu}{q}$, then

$$Y = Y_{obs} + \frac{k_d}{q} \qquad (6\text{-}4)$$

In this case, if k_d is close to zero, or $<< \mu$, then $Y = Y_{obs}$.

6.3 Results

6.3.1 Detection of Change of Microbial Community Structure by DGGE

In this study, PCR-DGGE of 16s rDNA was employed to produce genetic fingerprints that could provide information on the composition and diversity of

microbial communities over time after the PA-degrading aerobic granules were fed with DMP. DNA was extracted from the biomass that was sampled once every few days from day 0 to 39. Activated sludge and acclimated activated sludge were studied as a control of aerobic granules. PCR amplification and DGGE analysis of community 16S rRNA genes were performed in replicate on pooled aliquots of extracted nucleic acids. Identical fingerprints were obtained for replicate samples. Figure 6-1 shows the representative DGGE profiles of the amplified 16S rRNA genes from activated sludge, acclimated activated sludge and aerobic granules at different operating times.

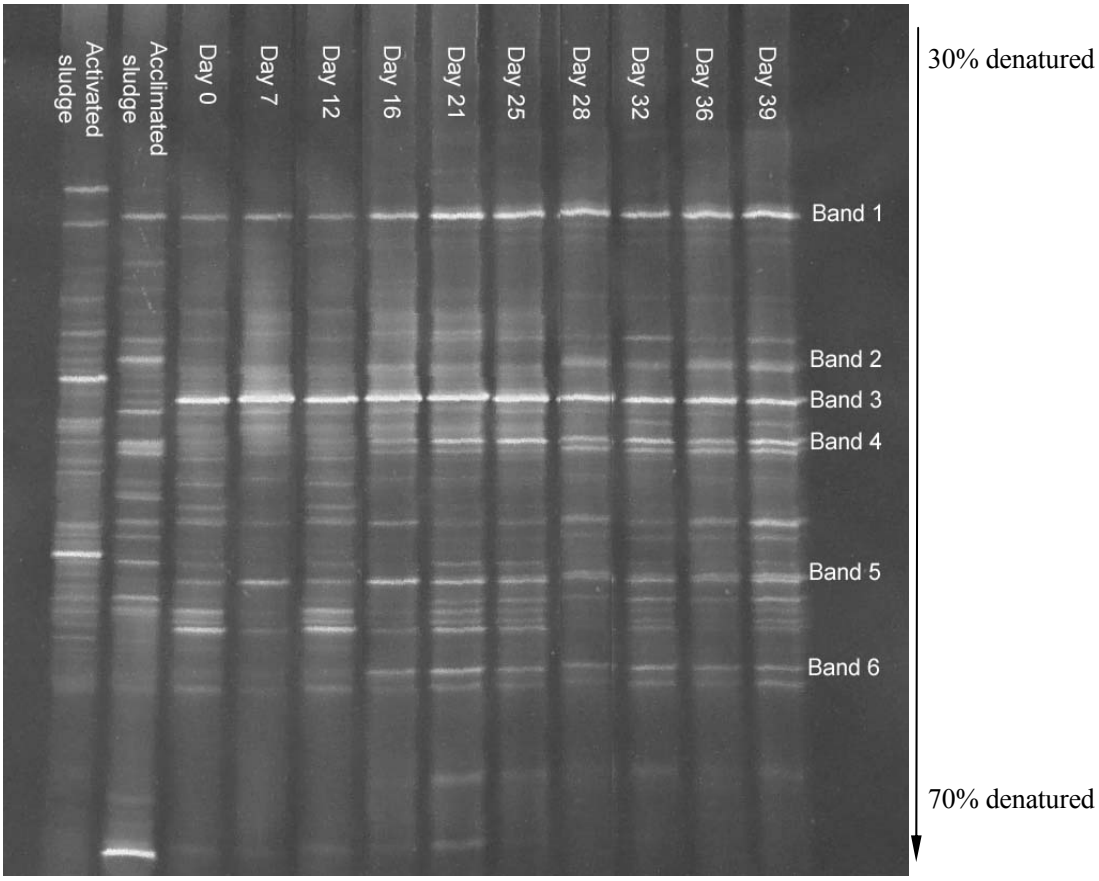


Fig. 6-1 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30%-70%) with DGGE profiles of the fragments of 16S rDNA after PCR amplification from activated sludge, acclimated activated sludge, matured aerobic granules in R1 at different operation time. The designations of the gel patterns are indicated above the lanes.

The seeding activated sludge was collected from a wastewater treatment plant. Because of abundant and complete nutrients provided for the seeding activated sludge, the diversity of the microbial community was high. More than 10 dominant populations were present. After the seeded activated sludge was acclimated to PA, there were still more than 10 dominant populations in acclimated activated sludge, although the microbial community had changed. Some dominant populations disappeared after the acclimation, and some appeared in the acclimated activated sludge, although they could not be seen in the original activated sludge. After one month operation, the mature PA-degrading aerobic granules were formed. A high diversity of the microbial community with more than 10 dominant populations was still kept in mature PA-degrading aerobic granules. Similar patterns between PA-degrading granules and acclimated activated sludge were observed, which probably due to being fed the same substrate.

The fingerprint patterns of acclimated activated sludge were different for the granule samples collected from R1 at different operation times, which indicates the microbial community structure changed during the operation process. The bacterial species represented by band 1 was the dominant population in the acclimated activated sludge and aerobic granules from day 0 to day 39. Bacteria represented by band 2 were the dominant population of the activated sludge but a minor population in acclimated activated sludge samples. The increasing density of this band in aerobic granules indicated that their population increased after PA-degrading aerobic granules were fed with DMP. There were two possibilities to stimulate the increasing density of band 2. First, DMP was the favorable substrate for species represented by band 2. Second, this species was more competitive in the DMP degrading environment than other species. Band 3 can not be seen in the activated sludge sample and was a weak band in the acclimated activated sludge sample. However it

became a strong band after the acclimated sludge was aggregated and formed granules. In the granulation process, most of the suspended bacteria with a slow settling velocity in the reactor would be washed out by the short settling time. Some bacteria with strong aggregation abilities would be kept in the reactor. As a result, they became the dominant species after granulation.

Bacteria represented by band 4 were present in the activated sludge sample. They became one of the dominant populations after the activated sludge was acclimated by PA. The population almost completely disappeared in the granulation process and increased gradually after DMP was fed to the reactor. Hence, DMP facilitated the growth of bacteria of band 4. Bacteria expressed by band 5 had the same shift pattern as band 3, but it had a lower population density as compared to band 3. Band 6 was a minor population in activated sludge, acclimated activated sludge and PA-degrading aerobic granules on day 0. The population density increased after the reactor was fed with DMP, indicating that this species might prefer DMP over PA. They survived after two kinds of selections, which were PA acclimation and granulation.

It is worth pointing out that bacteria represented by band 1, 3 and 5 had the highest population density throughout the 39-day operation. This indicated that these species possessed the ability to degrade both PA and DMP. Because of the existence of such bacteria, the reactor could maintain steady operation, even at the beginning when the substrate changed from PA to DMP.

At the same time, R2 was operated as the control of R1, with PA as substrate. Similar to R1, DNA was extracted from PA-degrading aerobic granules that were collected on the same day as R1 through the 39-day operation. The diversities and changes of microbial community structure in aerobic granules were monitored by DGGE during the 39-day operation. Figure 6-2 shows the representative DGGE profiles of the

amplified 16S rRNA genes from aerobic granules at different operation times. At least 10 different bands were detected on the polyacrylamide gel. The fingerprint patterns were similar among different granules' samples, indicating a highly stable microbial community. Biomass characteristics and reactor performance also showed little variation during this period.

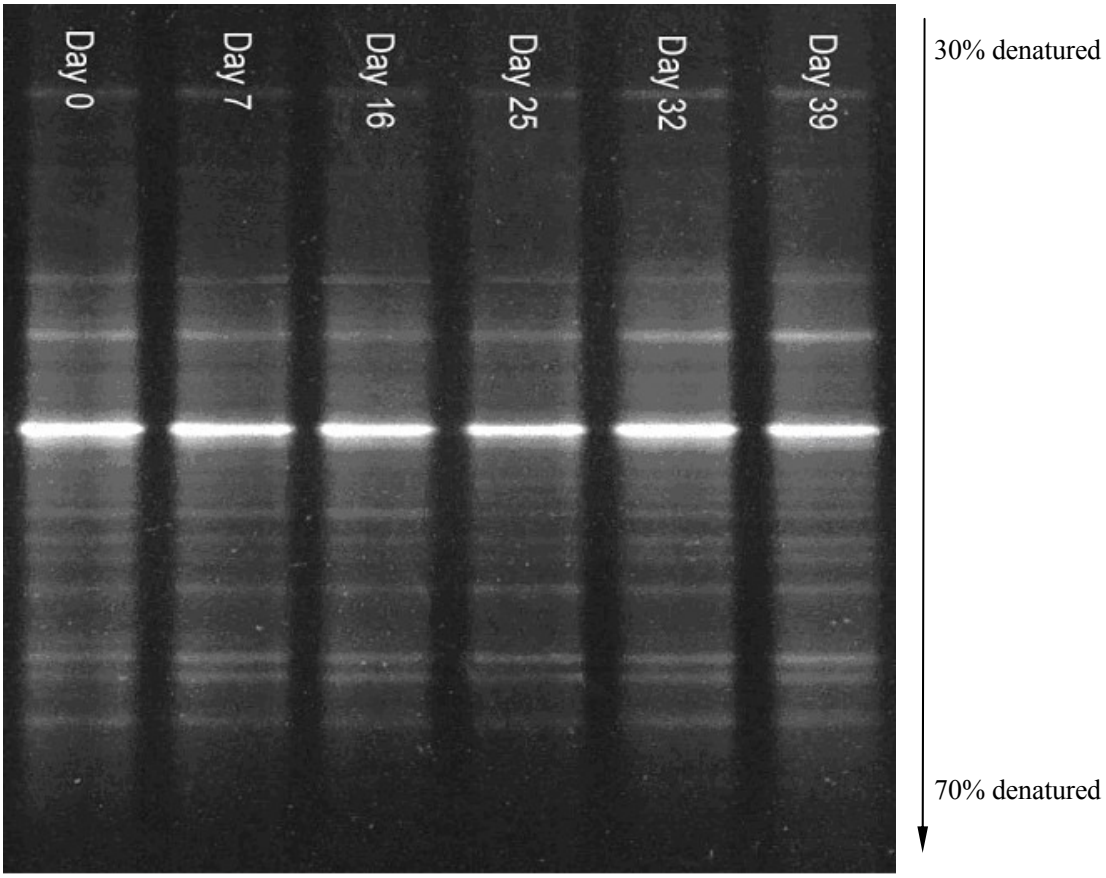


Fig. 6-2 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30%-70%) with DGGE profiles of the fragments of 16S rDNA after PCR amplification from matured aerobic granules in R2 at different operation time. The designations of the gel patterns are indicated above the lanes.

6.3.2 Isolation of Bacteria from Aerobic Granules

In the isolation process, 100 µl of supernatant from detached PA-degrading aerobic granules was spread onto PA-MSM agar plates and DMP-MSM agar plates,

respectively. A total of 22 different colonies based on colony morphology, color and size were isolated from the PA-degrading aerobic granules by direct isolation techniques on PA-MSM (supplanted with 500.0 mg PA l^{-1}) agar plates. All of them were further spread on DMP-MSM medium (supplanted with 100.0 mg DMP l^{-1}) agar plates. Only 3 out of 22 isolates appeared on DMP-MSM agar plates. The second round isolation was made on DMP-MSM agar plates. A total of 14 different colonies based on colony morphology, color and size were isolated from the PA-degrading aerobic granules. All of 14 isolates can also grow on PA-MSM agar plates.

All of the 36 isolates were subjected to REP-PCR analysis. The isolates with the same fingerprint patterns were considered to be the same species. Then the screened isolates were subjected to partial 16S rDNA sequencing for identification. 7 distinct REP-PCR patterns were obtained from all colonies, as shown in Fig. 6-3. The representative isolates were given the designation PA-01 to PA-07. Image analysis, epifluorescence microbiology and scanning electronic microscopy (SEM) were used to examine the morphotypes. All isolates showed different colony phenotypes with different sizes. 1 to 5 days were needed for them to grow on PA-MSM agar plates (Table 6-2). PA-02 and PA-04 were the fastest growing isolates on PA-MSM agar plates.

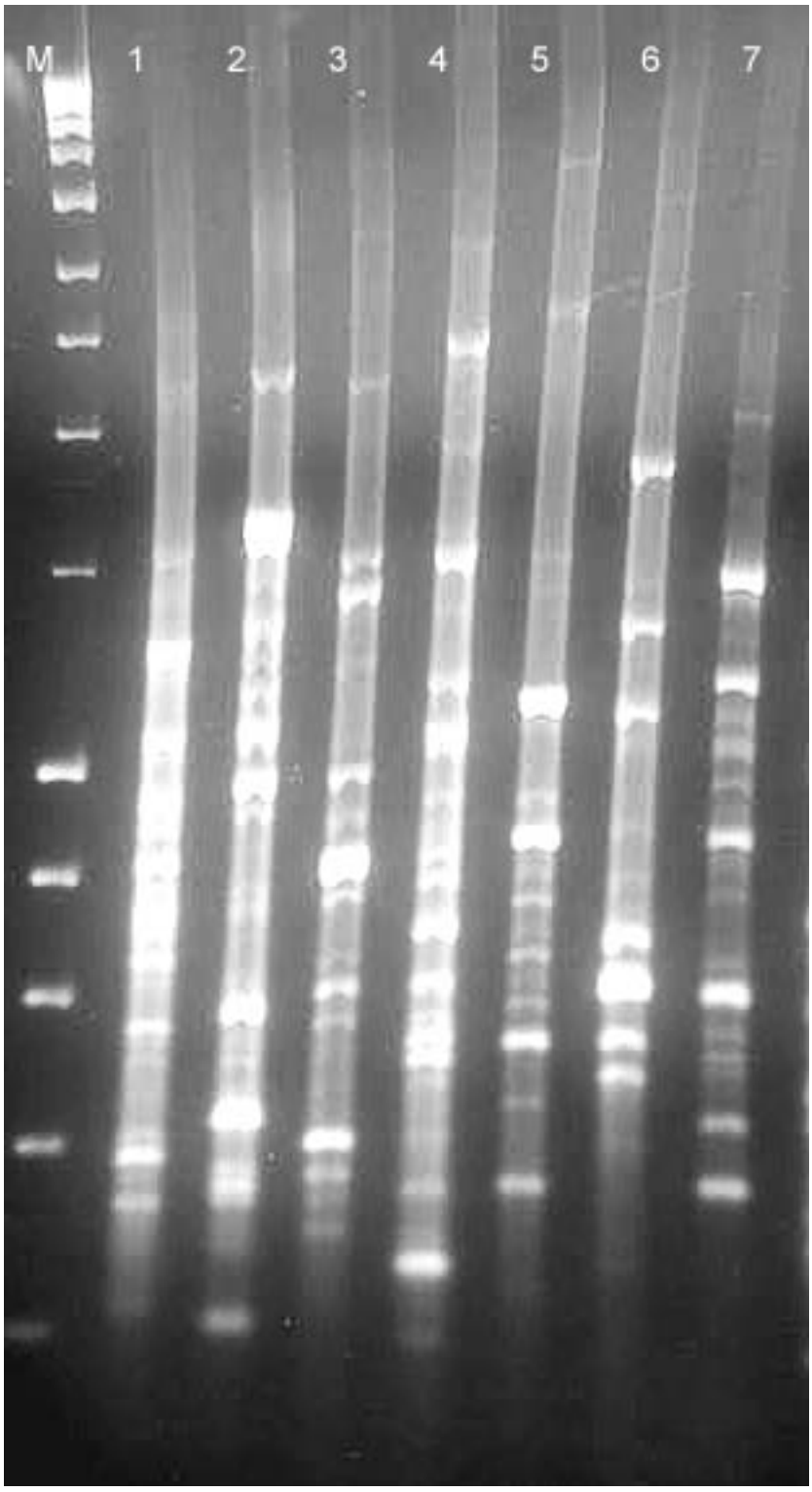


Fig. 6-3 REP-PCR profiles of bacterial strains isolated from PA-degrading aerobic granules. Lane M: 1 kb DNA ladder; lane 1-7: PA-01, PA-02, PA-03, PA-04, PA-05, PA-06, and PA-07.

Table 6-2 Isolate-colony morphology and time taken for colony to form on MSM plate.

Isolates	Isolation method	Colony phenotype	Diameter of colony (mm)	Time taken for colony to form on DMP-MSM plate
PA-01	Direct isolation	Light, brown	2-4	2 days
PA-02	Direct isolation	Yellow, round	2-5	1 day
PA-03	Direct isolation	White, tiny	1-2	2 days
PA-04	Direct isolation	White	3-4	1 days
PA-05	Direct isolation	White, tiny	<2	5 days
PA-06	Direct isolation	Light, yellow	1-2	2 days
PA-07	Direct isolation	Yellow	2-3	2 days

Four isolates (PA-01, PA-02, PA-03, PA-05,) were rod-type bacteria, while the other three were cocci-type (Table 6-3). All isolate colonies appeared on plates with dilution factors higher than 10^3 times. PA-02 colonies appeared on the highest dilution factor of 10^7 times, indicating PA-02 had the highest number of active culturable cells in PA-degrading aerobic granules compared to the other six isolates. Isolate PA-02 was estimated to be present in PA-degrading aerobic granules at a concentration of $3.39\pm1.54\times10^{10}$ cells g VSS^{-1} of granules, suggesting that strain PA-02 constituted a significant fraction of the total bacteria residing in the granules.

Table 6-3 Phenotypic characterization of isolates, CFU, colony forming unit.

Isolates	No. of dilution plate of colony appearance	Minimum cell density (CFU g VSS^{-1})	Morphology	Gram stain[a]
PA-01	10^5	$6.94\pm0.78\times10^7$	Rod	+
PA-02	10^7	$3.39\pm1.54\times10^{10}$	Rod	−
PA-03	10^5	$6.31\pm1.16\times10^7$	Rod	+
PA-04	10^6	$1.17\pm0.92\times10^8$	Cocci	+
PA-05	10^4	$2.43\pm1.84\times10^5$	Rod	−
PA-06	10^5	$4.47\pm2.82\times10^6$	Cocci	+
PA-07	10^5	$8.24\pm1.47\times10^6$	Cocci	+

[a] Negative results (-), positive results (+);

6.3.3 Phylogenetic Analysis of Isolates

To evaluate the polygenic diversity represented by the 7 isolates, the 16S rDNA gene was partially sequenced. The putative division and nearest relatives of these 7 sequencing types were invested by BLAST, and the results were summarized in Table 6-4. The isolates fell into 5 major lineages of the Bacteria domain: the α-, β-and γ-*Proteobacteria*, CFB group bacteria and the gram-positive bacteria with high G+C content. Two of the isolates (PA-03 and PA-05) were placed in the β subclass of *Proteobacteria*, the other two (PA-06 and PA-07) in the Gram-positive bacteria with high G+C group, and the remaining three (PA-02, PA-04 and PA-01) were assigned to the α, γ subclass of *Proteobacteria* and CFB group bacteria, respectively.

Table 6-4 Phylogenetic characterization of isolates.

Isolates	Closest relatives	Taxon affiliation	Identity value (%)	Number of bases used in
PA-01	*Flectobacillus sp.* GWF20A	CFB group bacteria	91.0	499
PA-02	*Sphingomonas capsulata* GIFU 11526	α-*proteobacteria*	99.2	489
PA-03	*Acidovorax sp.* BSB421	β -*proteobacteria*	92.5	468
PA-04	*Acinetobacter baumannii*	γ-*proteobacteria*	92.2	503
PA-05	*Acidovorax avenae* isolate CBA1	β-*proteobacteria*	95.1	767
PA-06	*Microbacterium resistens strain* AGP4-3	high GC Gram+	100	539
PA-07	*Microbacterium resistens strain* AGP4-3	high GC Gram+	100	651

Isolate PA-01 matched the sequence of *Flectobacillus sp.* with an identity of 91.0%. Isolate PA-02 was 99.2% identical with a type of strain of *Sphingomonas sp capsulata* GIFU 11526. Isolate PA-03 matched the sequence of *Acidovorax sp*. with a sequencing identity of 92.5%. PA-04 matched sequences of *Acinetobacter baumannii* with an identity of 92.2%. Isolate PA-05 had a 95.1% identity to the closest identified strain, with less than 95% identity, suggesting that they might represent novel or unknown bacterial species (Amann et al., 1995). However, this needs to be confirmed by further full sequencing results and other phenotype studies.

Table 6-5 shows the nucleotide identities of individual isolates to each other in the genebank database as compared by partial 16S rRNA gene sequences. Of all the

isolates, PA-02 and PA-04 had a close relationship, with an identity of 0.804, while PA-03 and PA-05 had the close relationship with an identity of 0.956. PA-01, PA-06, PA-07 had the closest relationship with PA-04. The identities were 0.741, 0.793 and 0.793, respectively. PA-06 and PA-07 were close to each other, with identities of 1. Although the identities between PA-06 and PA-07 were 1, they had different patterns based on rep-PCR analysis. Thus the two isolates were different species.

Table 6-5 16S rRNA identity matrix for isolates.

	PA02	**PA01**	**PA03**	**PA04**	**PA05**	**PA06**	**PA07**
PA02	1	0.727	0.78	0.804	0.78	0.77	0.77
PA01	---	1	0.73	0.741	0.723	0.707	0.707
PA03	---	---	1	0.802	0.956	0.777	0.777
PA04	---	---	---	1	0.789	0.793	0.793
PA05	---	---	---	---	1	0.768	0.768
PA06	---	---	---	---	---	1	1
PA07	---	---	---	---	---	---	1

6.3.4 Detection of Dominant Species by DGGE

In order to compare the DGGE profile of aerobic granules to the diversity assessment based on isolation, the individual isolates and aerobic granular samples were amplified with the same primer pair and separated via DGGE. A comparison was also made between the isolates and the aerobic granules. Of the 7 isolates, 6 distinct bands were identified on the gel shown in Fig. 6-4. The aerobic granules on day 0 and day 39 were called PA-degrading aerobic granules and DMP-degrading aerobic granules, respectively. The isolates and corresponding bands for the granules are listed in Table 6-6. The 6 isolates correspond to the six different DGGE bands from the granules (band G1, G2, G3, G4, G5 and G6).

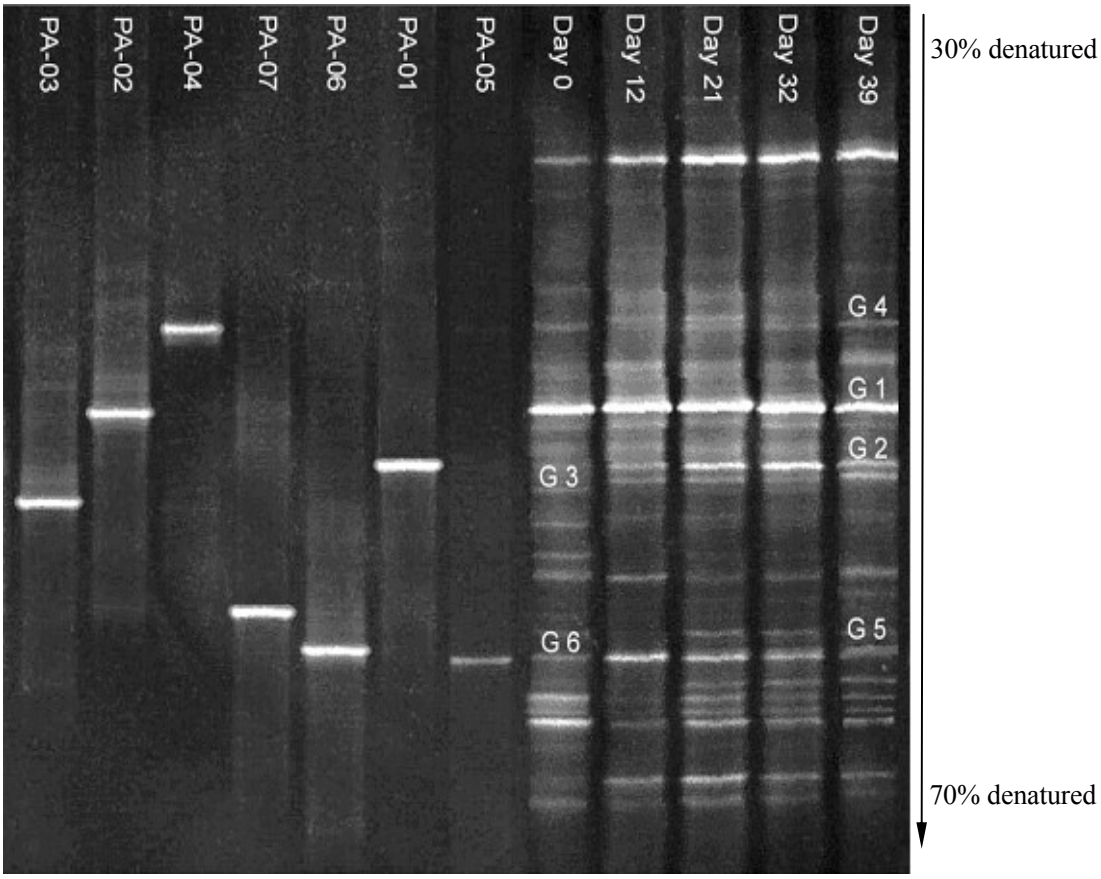


Fig. 6-4 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30%-70%) with DGGE profiles of the fragments of 16S rDNA after PCR amplification from matured aerobic granules and pure cultures. The designations of the gel patterns are indicated above the lanes.

Isolates PA-01, PA-02 and PA-04 correspond to DGGE band G2, G1 and G4, which were present in aerobic granules from day 0 to 39. G1 represented the dominant species both in PA and DMP-degrading aerobic granules. G4 represented minor species in both PA and DMP-degrading aerobic granules. G2 represents species which were minor in PA-degrading aerobic granules and gradually changed into dominance in DMP-degrading aerobic granules. PA-03 corresponds to band G3, which was a minor population in PA-degrading aerobic granules, and PA-05 corresponds to band G6, which was a minor population of PA-degrading aerobic

granules also. Isolate PA-06 corresponds to band G5 present in DMP-degrading aerobic granules. No band in granules matched with PA-07 (Table 6-6).

Table 6-6 Isolates and corresponding bands in granules.

Isolates	Band	PA granule[a]	DMP granule[a]
PA-01	G2	+	+
PA-02	G1	+	+
PA-03	G3	+	−
PA-04	G4	+	+
PA-05	G6	+	−
PA-06	G5	−	+
PA-07		−	−

[a] Corresponding (+), not corresponding (-);

6.3.5 Degradation Ability of Isolates from Aerobic Granules

Isolated bacteria were screened for growth on various phthalates and PAEs. As representatives, PA, DMP, DBP and di-(2-ethylhexyl) phthalate (DEHP) were evaluated for their potential to serve as growth substrates (Table 6-7). All of the isolates can grow on PA. PA-02 and PA-04 had obvious growth on PA. PA-03, PA-05, PA-07 did not growth when served with DMP, DBP and DEHP as substrates. The rest of them grew on DMP, DBP and DEHP.

Table 6-7 Isolates growth on PAEs.

Isolates	Compounds*			
	PA	**DMP**	**DBP**	**DEHP**
PA-01	+	+	+	+
PA-02	++	+	+	+
PA-03	+	−	−	−
PA-04	++	+	+	+
PA-05	+	−	−	−
PA-06	+	+	+	+
PA-07	+	−	−	−

* No growth (−), growth (+), obvious growth (++)

The specific PA degradation rates of all isolates were compared at an initial PA concentration of 350.0 mg l^{-1} (Fig. 6-5). PA-02 possessed the highest specific degradation rate. The next was PA-04. Isolate PA-05 had the lowest specific degradation rate. The specific growth rates of all isolates were also compared at an initial PA concentration of 350.0 mg l^{-1} (Fig. 6-6). PA-02 possessed the highest specific growth rate. Isolate PA-04 had the second highest growth rate. PA-06 had the lowest specific degradation rate among all the 7 isolates.

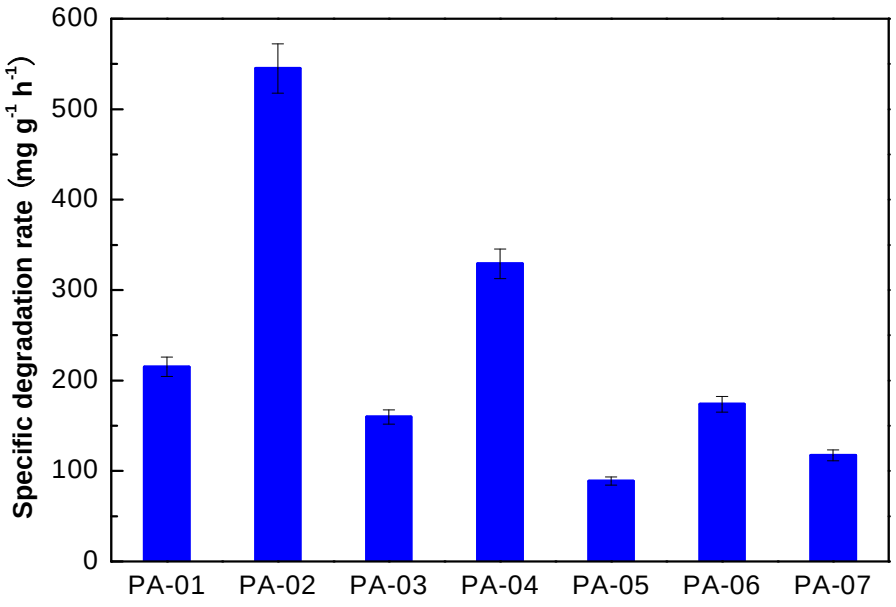


Fig. 6-5 Comparison of specific degradation rates at the PA concentration of 350.0 mg l^{-1} for all isolates.

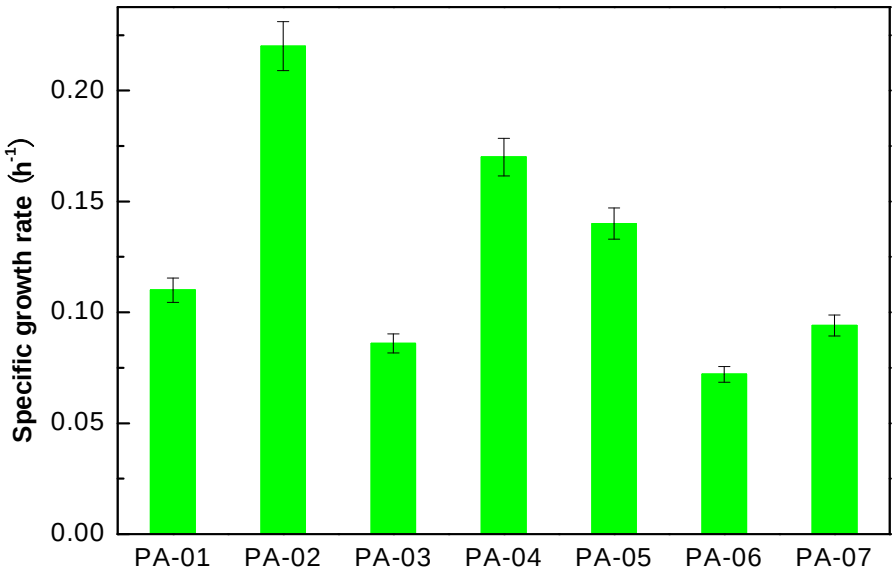


Fig. 6-6 Comparison of specific growth rates at the PA concentration of 350.0 mg l^{-1} for all isolates

6.3.6 Characteristics of Strain PA-02 Isolated from PA-degrading Aerobic Granules

The strain PA-02 showed high PA degradation ability and high growth rates and the strain with both PA and DMP degradation ability played an important role in the strategy of DMP degradation by PA-degrading aerobic granules. It was selected as representative to study the bacteria with PA and DMP degradation ability..

PA-02, a strain with both PA and DMP degradation activity was, isolated from PA-degrading aerobic granules by the direct isolation method, using MSM medium containing PA as the sole carbon source. This strain, which formed yellow colonies, was a gram-negative, non-sporulating, rod-shaped bacterium. Cells were between 0.3 and 1.0 μm in length and 0.2-0.4 μm in diameter. Its 16S rDNA sequence is closest to that of *Sphingomonas capsulate* GIFU 11526 (99.2% homology), suggesting that it phylogenetically belongs to the *Sphingomonas* species (Table. 6-8).

6.3.6.1 Flocculation Activities of PA-02

PA-02 possessed strong flocculation activity based on its visualized floc-like biomass. Auto-aggregation took place when PA-02 was cultivated in a shaker flask using PA medium or DMP, DBP or DEHP medium. Cell aggregates were compact with a mean size of 0.52 mm (Fig.6-7 d). The aggregates settled to the bottom of the reagent bottle after shaking was ceased for only 1 min, indicating a good settling ability for cell aggregates. Optical density of the supernatant after settling was very low, indicating most cells were retained in the aggregates. In the degradation experiment, a dispersing reagent, Tween-80, was used to get symmetrical biomass optical densities.

Table 6-8 16S rRNA identity matrix for strain PA-02 and related taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1	0.992	0.972	0.962	0.961	0.935	0.927	0.916	0.906	0.883	0.886	0.916	0.906	0.908	0.908	0.904	0.904	0.902	0.906	0.918
2	---	1	0.964	0.962	0.957	0.931	0.925	0.912	0.904	0.881	0.883	0.912	0.904	0.906	0.902	0.898	0.902	0.9	0.904	0.918
3	---	---	1	0.972	0.964	0.933	0.92	0.918	0.92	0.884	0.888	0.92	0.922	0.923	0.923	0.912	0.912	0.91	0.914	0.931
4	---	---	---	1	0.968	0.931	0.916	0.912	0.918	0.894	0.894	0.918	0.914	0.916	0.912	0.904	0.904	0.902	0.906	0.929
5	---	---	---	---	1	0.931	0.91	0.922	0.92	0.883	0.892	0.922	0.908	0.91	0.91	0.902	0.898	0.896	0.9	0.927
6	---	---	---	---	---	1	0.922	0.947	0.922	0.908	0.914	0.935	0.902	0.904	0.906	0.9	0.892	0.89	0.894	0.937
7	---	---	---	---	---	---	1	0.925	0.925	0.914	0.896	0.922	0.906	0.908	0.908	0.904	0.904	0.902	0.906	0.941
8	---	---	---	---	---	---	---	1	0.959	0.912	0.922	0.929	0.898	0.9	0.906	0.9	0.892	0.89	0.894	0.966
9	---	---	---	---	---	---	---	---	1	0.91	0.916	0.933	0.925	0.923	0.92	0.912	0.912	0.91	0.914	0.972
10	---	---	---	---	---	---	---	---	---	1	0.968	0.949	0.883	0.884	0.881	0.888	0.881	0.879	0.883	0.914
11	---	---	---	---	---	---	---	---	---	---	1	0.953	0.877	0.879	0.877	0.884	0.875	0.873	0.877	0.922
12	---	---	---	---	---	---	---	---	---	---	---	1	0.906	0.908	0.906	0.914	0.904	0.902	0.906	0.937
13	---	---	---	---	---	---	---	---	---	---	---	---	1	0.998	0.978	0.966	0.982	0.98	0.984	0.904
14	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.98	0.968	0.984	0.982	0.986	0.906
15	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.988	0.968	0.966	0.97	0.91
16	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.964	0.962	0.966	0.902
17	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.994	0.998	0.898
18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.996	0.896
19	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1	0.9
20	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	1

Note: 1. PA-02; 2. *Sphingomonas capsulata* GIFU 11526; 3. *Sphingomonas aromaticivorans* IFO 16084; 4. *Sphingomonas subterranea* IFO 16086; 5. *Sphingomonas stygia* IFO 16085; 6. *Sphingomonas subarctica str*. KF1 (T); 7. *Sphingomonas rosa* IFO 15208 (T); 8. *Sphingomonas macrogoltabidus str*. 203 IFO 15033 (T); 9. *Sphingomonas terrae str*. E-1-A IFO 15098 (T); 10. *Sphingomonas chlorophenolica* ATCC 33790 (T); 11. *Sphingomonas herbicidovorans str*. MBIC3166; 12. *Sphingomonas yanoikuyae* GIFU 9882 (T); 13. *Sphingomonas sanguinis* IFO 13937 (T); 14. *Sphingomonas parapaucimobilis* IFO 15100 (T); 15. *Sphingomonas paucimobilis* GIFU 2395 (T); 16. *Sphingomonas trueperi* LMG 2142 (T); 17. *Sphingomonas mali str*. Y-347 IFO 15500 (T); 18. *Sphingomonas pruni str*. Y-250 IFO 15498 (T); 19. *Sphingomonas asaccharolytica* IFO 10564; 20. *Sphingomonas adhaesiva* IFO 15099 (T)

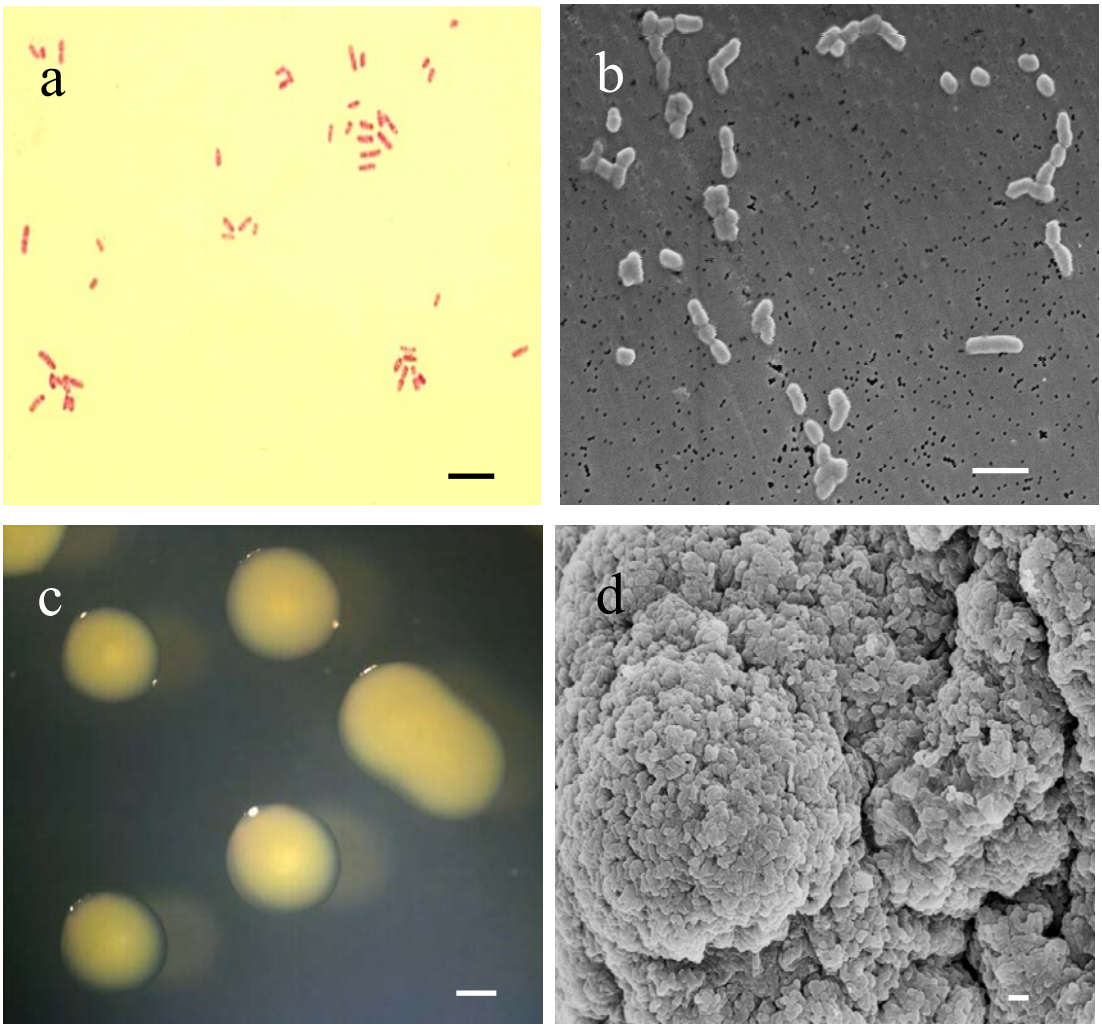


Fig. 6-7 Pictures of PA-02 (a) gram-staining photo (bar: 5 μm) (b) scanning electron micrograph (bar: 1 μm) (c) imagine analysis of PA-02 colony (bar: 1 mm) and (d) scanning electron micrograph of PA-02 aggregates (bar: 1 μm).

6.3.6.2 Biomass Growth, and PA and DMP Degradation

With a volumetric exchange ratio of 50%, PA concentrations in the reactors at time zero were approximately half of the sum of the influent and effluent PA concentration. Because the influent PA concentration was 692.0 mg l^{-1} and the effluent PA concentration in the reactor was less than 0.5 mg l^{-1}, the initial PA concentration in the reactor was about 350.0 mg l^{-1} (Chapter 4). Likewise, the initial DMP concentration in the reactor was about 290.0 mg l^{-1}. Thus the characteristics of strain PA-02 in the PA and DMP degradation process were investigated in shaking flasks using initial PA and

DMP concentrations of 350.0 and 290.0 mg l^{-1}, respectively.

Cell growth and substrate PA and DMP degradation profiles for strain PA-02 at initial PA and DMP concentrations of 350.0 mg l^{-1} and 290.0 mg l^{-1} are shown in Figs. 6-8 and 6-9. PA at an initial concentration of 350.0 mg l^{-1} was completely degraded in 7.5 h, accompanied by a biomass OD increase from 0.05 to 0.40. DMP at an initial concentration of 290.0 mg l^{-1} was degraded in 22 h. At the same time, the biomass OD increased from 0.23 to 0.34.

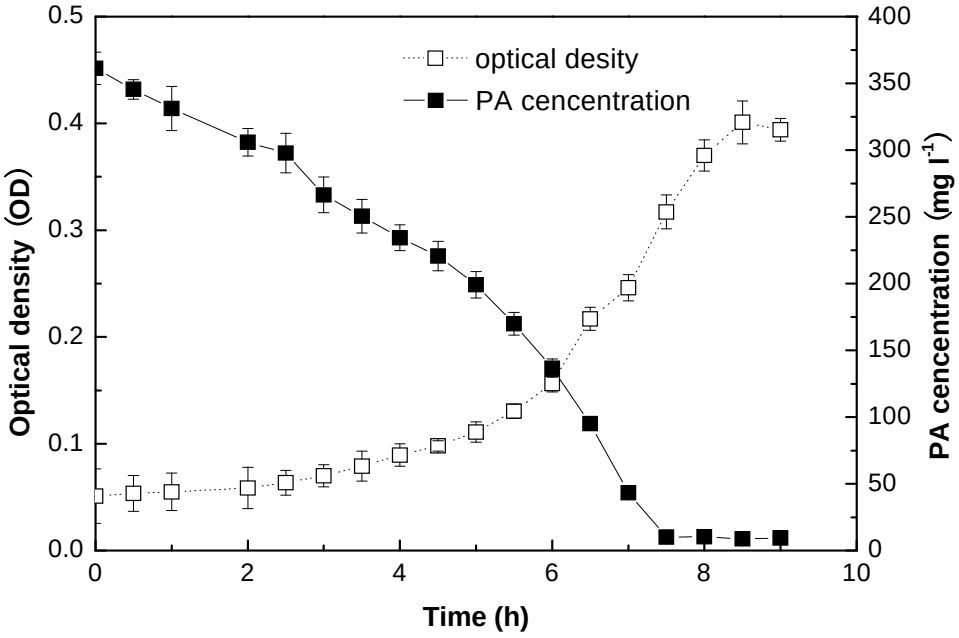


Fig. 6-8 PA degradation by strain PA-02 at the initial PA concentration of 350.0 mg l^{-1}.

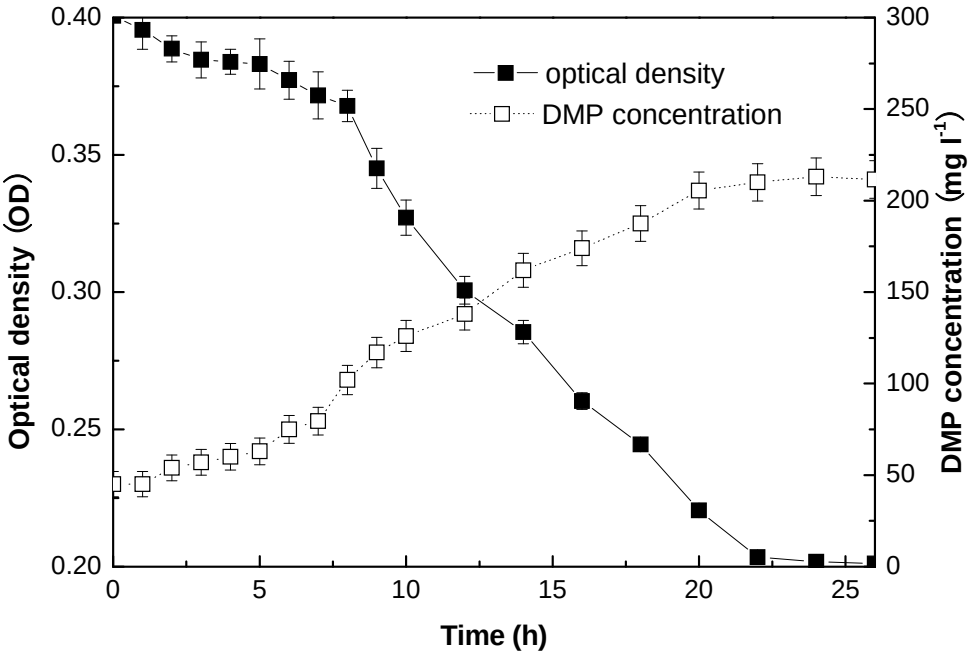


Fig. 6-9 PA degradation by strain PA-02 at the initial DMP concentration of 290.0 mg l^{-1}.

Based on the information of the initial biomass growth rate, PA and DMP concentrations as well as the biomass dry weight, the specific biomass growth (μ) and PA and DMP degradation rates (q) at the initial time were calculated with equations (6-1) to (6-4) and are listed in Table 6-9. The specific growth rate of PA-02 on PA was 5.3 times higher than that on DMP. The specific DMP degradation rate was 68% of the specific PA degradation rate. Y_{obs} on PA and DMP at the initial time were 0.388 and 0.192 g biomass g^{-1} substrate. The yield Y_{obs} on PA was almost 2 times that of the Y_{obs} on DMP.

Table 6-9 Specific growth rates, PA, DMP degradation rates and biomass yields of strain PA-02.

Substrate	Initial concentration (mg l^{-1})	Specific growth rate (μ) (h^{-1})	Specific degradation rate (q) (g g^{-1} biomass h^{-1})	Biomass yield [a] (μ/q) (g biomass g^{-1} substrate), Y_{obs}
PA	350.0 (±15)	0.220 (±0.010)	0.567 (±0.026)	0.388
DMP	290.0 (±10)	0.035 (±0.002)	0.182 (±0.009)	0.192

[a] Calculated for the initial time

6.3.6.3 Kinetics of PA and DMP Degradation by PA-02

The specific growth and degradation rates for strain PA-02 in the exponential growth phase were measured at various initial concentrations ranging from 100 to 1000 mg l^{-1} (Figs. 6-10 and 6-11). Stain PA-02 was capable of degrading PA and DMP at concentrations up to 1000.0 mg l^{-1}. The specific growth rates increased with the increase of PA concentration from 0 to 250.0 mg l^{-1} and declined with further increases in PA concentration as substrate inhibition effects became important. For substrate DMP, the specific growth rate increased with DMP concentration from 0 to 100.0 mg l^{-1}, and declined with further increases in DMP concentration. The specific PA degradation rate was higher than the specific DMP degradation rate, and the PA-02 specific growth rate using PA as substrate was higher than that using DMP as substrate (Tables 6-10 and 6-11).

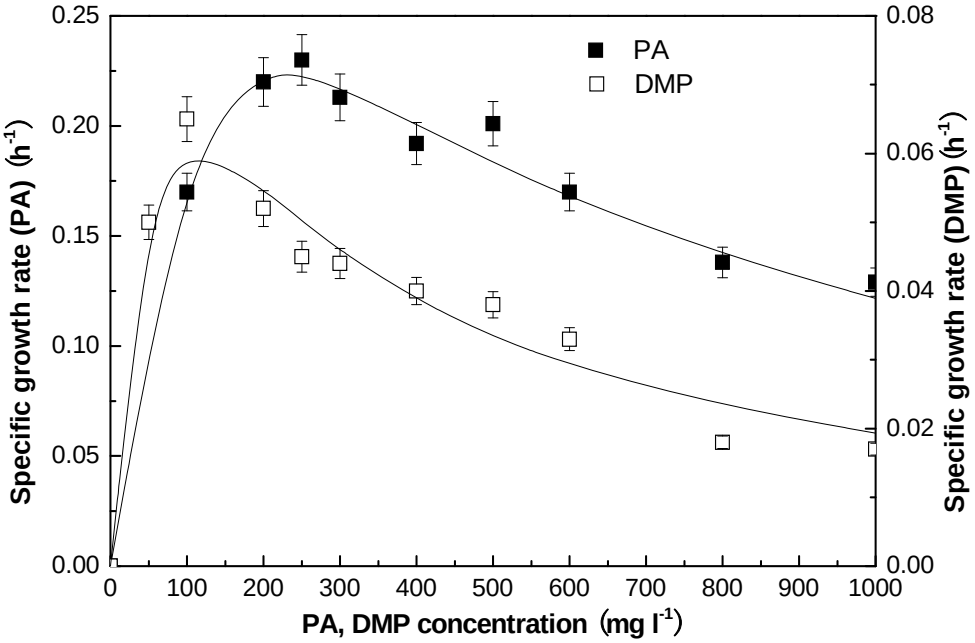


Fig. 6-10 Specific growth rate of strain PA-02 at different PA, DMP concentrations.

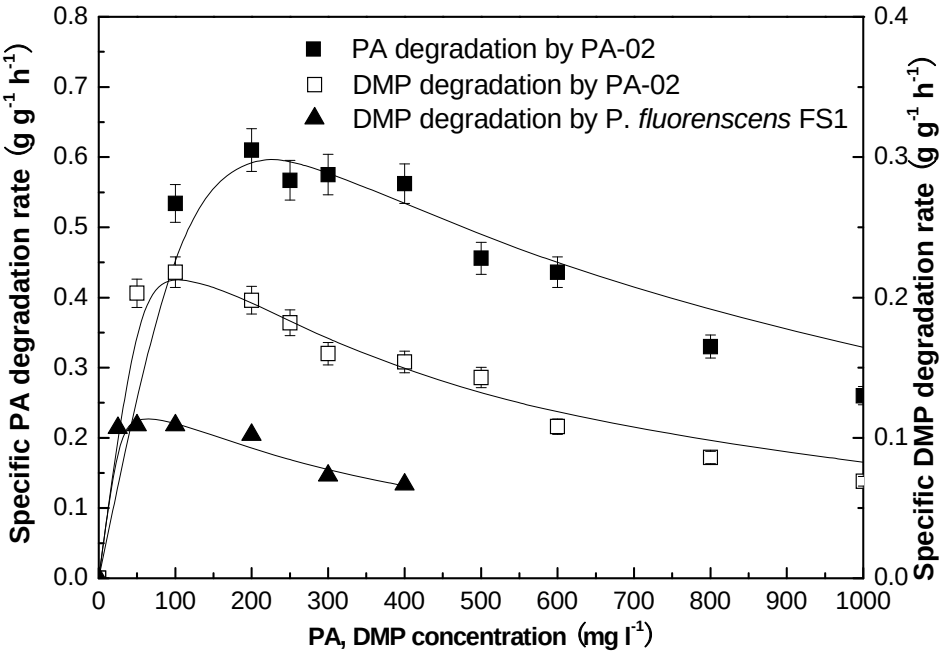


Fig. 6-11 PA, DMP specific degradation rate of strain PA-02 and P. *fluorenscens* FS1 at different PA, DMP concentrations (DMP degradation rate by strain P. *fluorenscens* FS1 calculated based on the original data of Zeng et al., (2004)).

Table 6-10 The growth parameters of strain PA-02.

Substrate	μ_{max} (h^{-1})	K_s (mg l^{-1})	K_i (mg l^{-1})	μ_{max}/K_s
PA	0.538 (±0.023)	148.1 (±7.2)	305.4 (±13.2)	0.0036
DMP	0.146 (±0.007)	73.2 (±3.5)	154.3 (±7.4)	0.0020

Table 6-11 The degradation parameters of strain PA-02 and stain *P. fluorescens* FS1.

Strain	Substrat	q_{max} (g g^{-1} h^{-1})	K_s (mg l^{-1})	K_i (mg l^{-1})	q_{max}/K_s
PA-02	PA	1.282 (±0.060)	115.0 (±5.9)	359.9 (±18.0)	0.0111
PA-02	DMP	0.352 (±0.018)	28.6 (±1.4)	309.7 (±15.3)	0.0123
P. fluorescens FS1*	DMP	0.116	14.1	253.9	0.0083

* kinetic parameters of DMP degradation by strain P. *fluorenscens* FS1 was calculated based on the original data of Zeng et al., (2004).

Because of inhibition effects, kinetic parameters were fitted using the Haldane equation to determine specific rates at different initial PA and DMP concentrations. The μ_{max}, K_s and K_i of PA-02 using PA as the substrate for growth kinetics were found to be 0.538 h^{-1}, 115.0 mg l^{-1} and 305.4 mg l^{-1}, respectively. When degrading DMP, PA-02 showed 3.7-fold lower μ_{max} (0.1462 h^{-1}), and 2.0-fold lower values of K_s (73.2 mg l^{-1}) and K_i (154.3 mg l^{-1}), respectively (Table 6-10). The ratio of q_{max} to K_s using PA as substrate was 0.0111, lower than that with DMP as the substrate. The q_{max}, K_s and K_i of strain PA-02 for PA degradation kinetics were calculated to be 1.282 h^{-1}, 115.0 mg l^{-1}, 359.9 mg l^{-1}, respectively (Table 6-11). The DMP degradation kinetic parameters of PA-02, with q_{max} of 0.352 h^{-1}, K_s of 28.6 mg l^{-1} and K_i of 309.7 mg l^{-1}, were all lower than those using PA as the substrate.

6.4 Discussion

6.4.1 Change of Microbial Community Structure in PA-degrading Aerobic Granules

Results from Chapters 4 and 5 indicated that PA-degrading aerobic granules can be used as bioseeds for the PAE treatment since the PA-degrading aerobic granules possessed compact structure, good settling ability, constitutive and induced enzyme system of PA-degrading aerobic granules. In this study, 16S rDNA DGGE for total community fingerprints were used to assess the diversities and change of microbial community structure in PA-degrading aerobic granules.

The microbial community diversity of conventional activated sludge, acclimated PA-degrading activated sludge and PA-degrading aerobic granules analyzed by DGGE using general bacterial primers indicated that more than 10 bands were detected in these microbial communities. Compared with conventional activated sludge fed with complex municipal wastewater, there were not many differences in the numbers of DGGE fragments for acclimated activated sludge and granule samples, even though they had been acclimated by a single substrate. The microbial population abundance was not affected by the change of substrate.

However, the positions of the DGGE bands for acclimated activated sludge were different from conventional activated sludge. The change of substrate resulted in population dynamics between conventional activated sludge and acclimated activated sludge. In contrast, more similarity existed between acclimated activated sludge and aerobic granules, as evidenced by the fact that several DGGE bands were in the same position (Fig. 6-1).

Due to the same substrate being fed to the acclimated activated sludge and aerobic granules, they had the same populations with the ability of PA degradation. The

difference between the two microbial communities lies in the aggregation process for aerobic granules' formation. Some bacteria with slow settling ability would be washed out by the selection pressure.

The microbial community in PA-degrading aerobic granules further changed when PA-degrading aerobic granules were fed with DMP as the sole carbon and energy source. In this process, some dominant bacteria appeared in aerobic granules throughout the whole process, although the substrate changed to DMP. This phenomenon indicated that these dominant populations possessed the ability to degrade both PA and DMP. During the operation, some minor species in the PA-degrading aerobic granules with DMP degradation ability became dominant due to adaptation of the microbial community to the change of substrate. Some new species with DMP degradation ability appeared after DMP was fed. The species without DMP degradation ability that had a slow settling ability were washed out of the system.

6.4.2 Community Structure of Aerobic Granules in DMP Degradation

In this study, 16S rDNA DGGE for typical community fingerprints at different operation times and isolates in PA-degrading aerobic granules were analyzed to assess the community structure and to investigate the functional diversity of the microbial community in PA-degrading aerobic granules.

There were four groups of isolates in the PA-degrading aerobic granules. The first group of the bacteria was represented by PA-02, which played an important role in the initial stage of DMP degradation. They were the dominant population in PA-degrading aerobic granules. This group of bacteria possessed both DMP and PA degrading ability. The second group, represented by PA-04, was a minor population in the PA-degrading aerobic granules. They were also capable of degrading PA and DMP at the same time. They were still a minor population when the reactor reached steady state after being fed with DMP. The third group, represented by PA-01, gradually

grew to be dominant population in the cultivation process. The forth group, such as PA-06, was induced after DMP was added as sole carbon source. They had the DMP degrading ability but without the PA degrading ability.

The first and the second group played an important role in the process of DMP degradation by PA-degrading aerobic granules. Because of their metabolisms, the granules could quickly adapt to the new substrate DMP, especially in the initial stage when the substrate changed from PA to DMP. As a result, no disturbance was posed on reactor operation, and it was easy for the reactor to reach steady state. With the appearance and increasing population of the third and the forth group bacteria, the DMP degradation ability of the system increased.

6.4.3 The Bacteria with PA and PAE Degradation Ability

6.4.3.1 Microbial identification and phylogenetic analysis

The bacterium with PA and DMP degrading abilities was successfully isolated from PA-degrading aerobic granules by direct isolation method. It was designated as strain PA-02. Phylogenetic analysis based on 519 unambiguous bases revealed that strain PA-02 (EF650644) was a member of the *Sphingomonas* genus. The genus *Sphingomonas* forms a phylogenetically-tight group of 33 reported species in the α-4 subclass of the Proteobacteria (Stackebrandt et al., 1988; Takeuchi, 1994; Yurkov et al., 1997; Boone et al., 2005). Fig. 6-8 illustrates the phylogenetic relationship of PA-02 with species of close similarity. Among them, *Sphingomonas* capsulate GIFU 11526 (99.2 % similarity) was the closest one.

Sphingomonas species are widespread in nature and can be obtained from various sources, such as water, soil, living organisms and human skin. Most of the *Sphingomonas* species are degraders of xenobiotic or endocrine disruptors. Many studies have reported the isolation of *Sphingomonas* species from environments

polluted by a variety of pollutants, such as polycyclic aromatic hydrocarbon (PAHs), bisphenol, di-methyl naphthalene, naphthalene, p-xylene, alkyphenol polythoxylate (APPEO) (Miyachi et al., 1993; Bramucci et al., 2002; Liu et al., 2004d; Leys et al., 2005; Nishio et al., 2005; Sasaki et al., 2005). Several earlier studies also reported the successful isolation of *Sphingomonas* species from a variety of PAE-contaminated environments. The strain *Sphingomonas* paucimobilis was isolated from activated sludge (Wang et al., 2003b; Chang et al., 2004) and soil (Vega et al., 2003) using DMP as the sole carbon source. The *Sphingomonas* strains have broad catabolic capabilities and, therefore, have high potential for bioremediation and waste treatment.

6.4.3.2 Morphology and physical characteristics of strain PA-02

Strain PA-02 was shown to be gram-negative, non-sporulating and aerobic rod-shaped bacterium. Cells were between 0.3 and 1.0 μm in length, 0.2-0.4 μm in diameter when grown in PA-MSM media at 30 °C. The PA-02 colony formed on PA-MSM agar plates was yellow, convex and circular, and had a smooth surface (Fig. 6-7 a-c). PA-02 possessed strong flocculation abilities as floc-like biomass could be observed. Auto-aggregation took place when PA-02 was cultivated in shaker using PA, DMP, DBP or DEHP medium. Cell aggregates were compact in a mean size of 0.52 mm (Fig. 6-7 d). The cell aggregates settled to the bottom of the reagent bottle after shaking was ceased for only 1 min, indicative of good settling ability. Optical density of the supernatant after settling was very low, indicating that most cells were retained in the aggregates.

PA-02 was the dominant species in aerobic granules, as PA-02 could be cultivated in plates with the highest dilution among all the isolates from aerobic granules (Table 6-3). PA-02 also showed a high capability to auto-aggregate. Similar scenario was previously reported. For example, the strain PG-08 isolated from phenol-degrading aerobic granules, and K. *pneumoniae* strain B, P. *veronii* strain F isolated from acetate aerobic granules could either form aggregates by themselves or coaggregate with

other microbial strains (Jiang et al., 2004b; Tay et al., 2004d; Ivanov et al., 2006). Such ability of these strains could play an important role in the fast formation and maintenance of the structure of aerobic granules.

6.4.3.3 Specific growth rates and degradation kinetics

The specific growth rates and degradation rates for strain PA-02 in the exponential growth phase were measured at various initial concentrations ranging from 100.0 to 1000.0 mg l^{-1} (Fig. 6-10 and 6-11). Stain PA-02 was capable of degrading PA and DMP at concentrations up to 1000.0 mg l^{-1}. Based on the data obtained, kinetic parameters were fitted using the Haldane equation (Wang et al., 1998). The results are shown in Tables 6-10 and 6-11.

Strain PA-02 was isolated from PA-degrading aerobic granules. The maximum specific growth rate (μ_{max}) of strain PA-02 was found to be 0.538 h^{-1} using PA as substrate. When the substrate was changed to DMP, the specific growth rate of PA-02 showed a 3.7-fold decrease to 0.146 h^{-1}. K_s and K_i also decreased indicating that DMP had a lower affinity and a higher inhibition effect on strain PA-02 compared to PA (Fig. 6-10, Table 6-10).

Strain PA-02 showed high PA and DMP degradation abilities. The highest degradation rates for PA and DMP were 1.3±0.10 g-PA g biomass^{-1} h^{-1} and 0.4±0.03 g-DMP g biomass^{-1} h^{-1} respectively. Even when the PA concentration was increased to 1000.0 mg l^{-1}, the specific PA degradation rate was about 0.25 g-PA g biomass^{-1} h^{-1}. The corresponding value for DMP was 0.067 g-DMP g biomass^{-1} h^{-1} at 1000 mg l^{-1}. Strain PA-02 exhibited 29%-33% of maximum degradation rate on PA and DMP even when the substrate concentration was increased to 1000.0 mg l^{-1}.

Strain PA-02 also showed higher DMP degradation ability when compared to strain P. *fluorescens* FS1 which was reported by Zeng et al. (2004). The parameters were

calculated based on the original data of Zeng et al (2004) and simulated using Haldane equation (Wang et al., 1998). The highest DMP degradation rate (q_{max}) by strain P. *fluorescens* FS1 was 0.1±0.01 g-DMP g biomass^{-1} h^{-1} at 60 mg l^{-1}, while q_{max} by strain PA-02 was 0.4±0.03 g-DMP g biomass^{-1} h^{-1} at 100 mg l^{-1}. Strain P. *fluorescens* FS1 possessed a lower K_i value than that of strain PA-02, suggesting that DMP had a higher inhibition on strain P. *fluorescens* FS1. Strain P. *fluorescens* FS1 showed a lower affinity with DMP with a lower K_s value compared to strain PA-02.

6.4.3.4 Degradation of dialkyl-phthalates by strain PA-02

Table 6-7 shows the 5-day incubation results that PA-02 was able to grow in solutions containing DMP (with initial concentration at 290 mg l^{-1}), DBP (100 mg l^{-1}), or DEHP (100 mg l^{-1}). The results indicated that PA-02 was able to degrade some other dialkyl-phthalic esters. Strain PA-02 was isolated directly from PA-degrading aerobic granules. However, it possessed PAE degradation ability without acclimation. It is possible that PA-02 could produce constitutive enzymes responsible for PAE degradation. Fan et al. (2001) reported the possibility that PAEs degradation would be enhanced by the addition of PA-degrading micro-organisms. Thus, strain PA-02 with both PA and PAE degradation ability was the potential candidate for enhancing PAE degradation.

6.5 Summary

The study on change of microbial community structure and function of microbial species in aerobic granules was carried out to better understand the strategy of enhancement of PAE degradation by bioaugmentation of PA-degrading aerobic granules.

The microbial community structure of PA-degrading aerobic granules changed in DMP degradation. Some dominant species in PA-degrading aerobic granules were

maintained as the major species throughout the DMP degradation process. The dominant species with both PA and DMP degrading capabilities contributed to the steady operation of the reactor, especially in the initial stage of DMP degradation. Some minor bacteria became dominant to adapt to the change of substrate. Some bacteria disappeared with the operation, and some new bacteria appeared gradually after DMP was fed.

Seven distinct isolates with DMP or PA degradation ability were isolated from PA-degrading aerobic granules. PA-02 and PA-04 had high PA degradation ability. DMP, DBP and DEHP also can be used as substrate for their growth. PA-01 and PA-06 also possessed the ability to degrade PA, DMP, DBP and DEHP. The other three isolates, PA-03, PA-05 and PA-07, were capable of PA degradation but without the ability for DMP, DBP and DEHP degradation.

Strain PA-02 was the representative dominant species in PA-degrading aerobic granules possessing both PA and DMP degradation ability. It was robust under high concentrations of PA and DMP degradation. Even when the PA concentration increased to 1000.0 mg l^{-1}, the specific PA degradation rate was about 0.250 g-PA g^{-1} biomass h^{-1}. The DMP degradation rate by stain PA-02 was 0.067 g-DMP g^{-1} biomass h^{-1}. PA-02 still possessed its 29%-33% of maximum degradation rate on PA and DMP degradation even when the substrate concentration increased to 1000.0 mg l^{-1}. Combined with its high growth rate and high flocculate ability, strain PA-02 could be potentially used for PAE degradation.

CHAPTER 7

THE INFLUENCE OF STORAGE ON THE MOPHOLOGY AND PHYSIOLOGY OF PHTHALIC ACID-DEGRADING AEROBIC GRANULES

7.1 Introduction

The DMP degradation by the system inoculated with fresh PA-degrading aerobic granules was studied in Chapter 4. However, freshly cultured granules are not always available and used as seeds. Stored anaerobic granules are widely used as seeds to shorten the start-up period of UASB (upflow anaerobic sludge blanket) reactors to treat specific contaminants (Syutsubo et al., 1997; Nollet et al., 2005). In addition, several studies on aerobic granules have also demonstrated that stored aerobic granules could recover their bioactivity within several days in bioreactors (Ng, 2002; Tay et al., 2002c; Zhu and Wilderer., 2003). Acetate, a highly biodegradable substrate, was used in all the studies. So far, it is not known whether aerobic granules cultivated on toxic and/or recalcitrant compounds could be stored and subsequently recover their biodegradability properties after using these granules as inoculants to restart a bioreactor.

PA-degrading aerobic granules were used in the current study to investigate the storage effects on these granules. The capability of these granules as inocula to restart an SBR was also studied. The study enhanced the current information on aerobic granules storage and reactivation. In addition, it showed a feasible way to rapidly start up an aerobic granulation system to treat specific toxic and/or recalcitrant compounds.

7.2 Material and Methods

7.2.1 Storage Condition

PA-degrading aerobic granules were collected using a 1 l beaker during the aeration stage of the SBR operation, settled for 5 min, and carefully removed the supernatant. The granules were then washed with tap water for three times to remove residual nutrient substrates, re-suspended in tap water, and transferred into a 1 l reagent bottle with a total liquor volume of 0.9 l. The final liquor contained 9.7 g l^{-1} dry weight granules. The bottle was then stored in a 4 ºC refrigerator for 8 weeks

7.2.2 Reviving of Stored PA-degrading Aerobic Granules

After 8 weeks of storage, color of the aerobic granules at lower 2/3 part slightly changed from yellow to brown-yellow. However, color of aerobic granules at upper 1/3 part turned to black. Hereafter, BCG refers to the black color granules, and YCG refers to the brown-yellow color granules. The two types of stored aerobic granules were manually separated and used for further studies.

To study the reviving capability of the stored aerobic granules, two identical column-type SBRs (5 cm diameter, 2.4 L working volume) were set up in the 25 ºC temperature-controlled room. The configuration and operations of reactors have been described in Chapter 3.

7.2.3 Analytical Methods

The analysis of SS, VSS, DO, TOC, SOUR, ECP and biomass size has been described in Chapter 3. Sulfur ion was analyzed by inductively coupled plasma emission spectrometry (ICP) (Perkin-Elmer, Plasma 2000, Perkin-Elmer

Corporation, Norwalk, USA). The observations of aerobic granular samples by SEM have been described in Chapter 3. Settling velocity was measured by recording the time taken for individual granules to fall a certain height in a measuring cylinder (Moy et al., 2002).

Similar to SOUR measurement, the granule samples for adenosine triphosphate (ATP) assay were collected at the end of a cycle. Bacterial ATP assay was performed using the ENLITEN ATP Assay System (Promega Corporation, Madison, WI, USA) and TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA) according to the manufactures' instructions. The Specific ATP concentration was normalized by VSS as follow:

$$\text{Specific ATP concentration} = \frac{mg\ ATP\ l^{-1}}{g\ VSS\ l^{-1}}$$

7.2.4 Bacterial Viability Measurement of Aerobic Granules

Cell viability is a direct indicator of live cells with bioactivity in biofilm. The traditional plate count techniques underestimate the cell viability of biomass due to the selectivity of media. The fluorescent DNA-binding stain method was used to measure cell viability of aerobic granules. Cell viability was analyzed using the Molecular Probes Live/Dead BacLight bacterial viability staining kit (Invitrogen Singapore Pte Ltd, Singapore) and a FP-6500 research fluorescence spectrometer (Jasco, Great Dunmow, Essex, UK) according to manufacture's instructions. Standard curve of live and dead cells was established using *Escherichia coli* suspension with different proportions of dead and live cells. Aerobic granules were washed with de-ionized water three times and disintegrated by bead-beater methods described previously (Tay et al., 2002b) before staining. The relative abundance of live bacteria was decided by the ratio of green fluorescence (live cells) to red fluorescence (dead cells) emissions.

7.3 Results

7.3.1 Morphology and Physiology of Aerobic Granular Sludge During Formation, Storage and Recovering Periods

The PA-degrading aerobic granules were formed in a sequencing batch reactor using acclimated activated sludge as the inocula. As the granules matured, the mean biomass size gradually increased to reach a steady value of around 1.02 mm and the SVI value was 34 ml g^{-1} SS (Table 7-1).

Table 7-1 Characteristics of PA-degrading aerobic granules at different stages.

Characteristics	Granules before storage	Granules after storage		Granules after reviving
		YCG	BCG	
Mean biomass size (mm)	1.02 (±0.06)	0.99 (±0.05)	0.92 (±0.04)	1.25 (±0.06)
SVI (ml g^{-1})	34 (±2)	36 (±0.3)	37 (±2)	34 (±2)
SOUR (mg DO g VSS^{-1} h^{-1})	59 (±4.1)	2 (±0.1)	3 (±0.1)	59 (±3.2)
Settling velocity (m h^{-1})	22 (±1.2)	21 (±1.0)	21 (±0.9)	23 (±1.0)
TOC removal efficiency (%)	totally removed	NA[a]	NA[a]	totally removed
Average ATP content (mg g VSS^{-1})	0.017 (±0.001)	ND[b]	ND[b]	0.016 (±0.001)

[a]NA: data not available

[b]ND: non-detectable

The PA-degrading aerobic granules were collected on day 180 after the reactor start-up and stored in a 1 L reagent bottle. In the first 3 weeks, no color change was observed upon the stored granules and the supernatant was clear and colorless. After 4 weeks, the supernatant gradually turned into dark color while the apparent color of aerobic granules turned to brown-yellow. It was also noticed that upper part granules were starting to turn to grayish-dark color at this moment. After two months of storage period, the upper 1/3 part of aerobic granules in storage bottle turned to grayish or dark black color, whereas the rest of granules were still brown-yellow color (Fig. 7-1).

Fig.7-1 Appearance of PA-degrading aerobic granules after 8-week storage.

The pH value of supernatant decreased from 7.5 to 6.1 after the 8 weeks storage. In addition, the sulfide concentration in the supernatant increased from non-detectable to 12.0 mg l^{-1} after the storage. The DO value of supernatant decreased from 2 mg l^{-1} before storage to non-detectable after storage (Table 7-2).

Table 7-2 The value of extracellular polymers (ECPs) contents of PA-degrading aerobic granules before storage, after storage, after reviving and the dissolved oxygen concentrations of granule storage liquor.

	ECPs in granular sludge (mg g VSS^{-1})		DO in supernatant (mg l^{-1})
Before storage	85 (±3)		2
After storage	YCG	BCG	non-detectable
	79 (±2)	59 (±3)	
54 cycles after restart	85 (±3)	81 (±3)	

To verify the difference between black color granules (BCG) and brown-yellow color granules (YCG), they were manually separated (Fig. 7-2) and returned into two identical SBRs (R1-YCG and R2-BCG), respectively. BCGs turned to brown-yellow color after 1 d operation. The stored aerobic granules remained integrity shape, compact and dense structure after the 8 weeks of storage (Fig. 7-3). The mean particle sizes shrunk about 3% for YCG and 10% for BCG (Table 7-1). The settling velocity and SVI of aerobic granules did not change by the storage. The extracellular polymers (ECPs) of YCGs had no change while ECPs of BCGs decreased from 85 mg l^{-1} to 59 mg l^{-1} (Table 7-2).

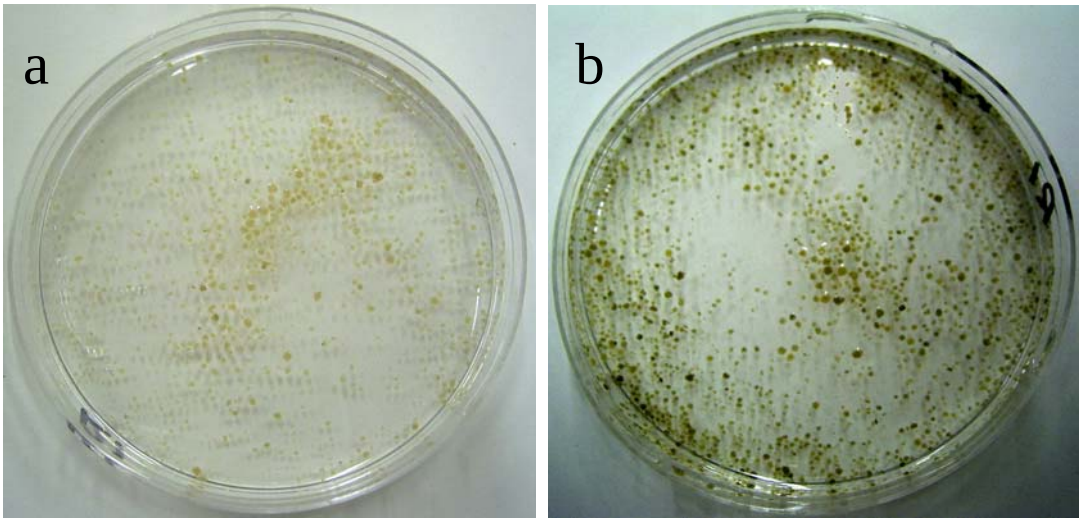


Fig. 7-2 Image of biomass at different status, (a) stored granules-YCG; (b) stored granules-BCG.

To find out the difference between the two types of stored granules, they were separated into two parts and put back in the reactor as seeds for a new system start-up. Black color granules (BCGs) turned yellowish-brown after one day aeration, and had the same appearance as the yellow color granules (YCGs). The SEM photos are shown in Fig. 7-3. The stored aerobic granules remained spherical, and compact and with a dense structure after 8 weeks of storage. The mean particle sizes decreased 2.7% for YCG and 10% for BCG. The settling velocity of the aerobic granules was not affected by the storage. And the SVI ranged between 30 to 40 ml g^{-1} during storage and recovery processes (Table 7-2).

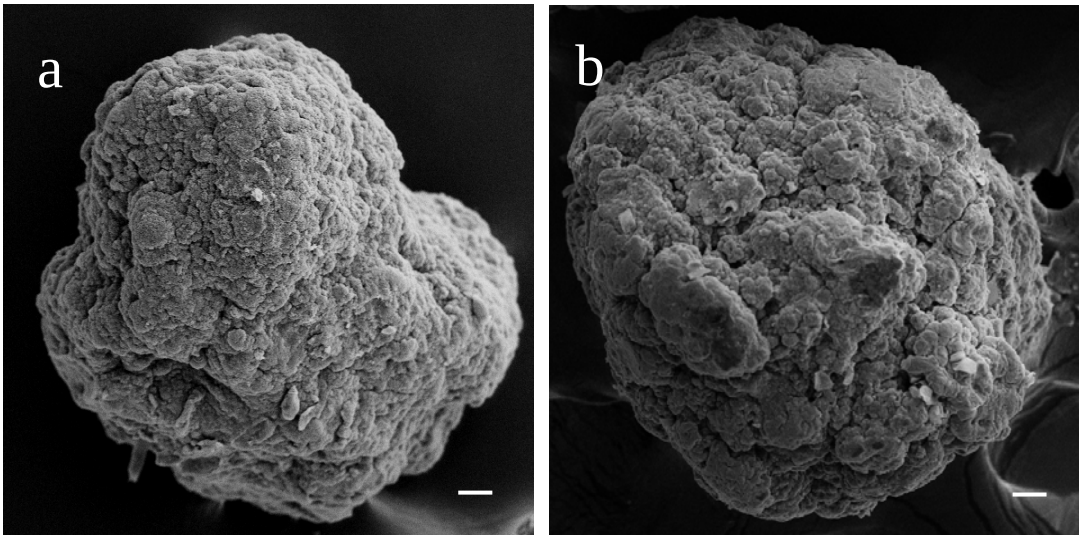


Fig. 7-3 Scanning electron micrographs of stored granules (a): R1-YCG (bar: 10 μm) and (b): R2-BCG (bar: 10 μm).

7.3.2 Stored Granules Reviving and Bioactivities Recovery

In this process, both reactors showed good performance and high function recovery capability in terms of TOC removal efficiency and SOUR (Fig. 7-4a & b). After one cycle of operation, TOC removal efficiency of R2-BCG recovered 88% while that of R1-YCG was 54%. After 24 cycles (4 d) operation, the TOC removal efficiency of the both reactors totally recovered to the same level as that of before storage

(Table 7-1, Fig. 7-4a).

The SOURs of the YCG and BCG were 2 mg DO g VSS^{-1} h^{-1} and 3 mg DO g VSS^{-1} h^{-1}, respectively. They were only 3-5% of the SOUR of granules before storage, which was 59 mg DO g VSS^{-1} h^{-1} (Table 7-1). From 6 to 24 cycles, SOUR of R1-YCG granules recovered from 12 to 47 mg DO g VSS^{-1} h^{-1}, while SOUR of R2-BCG granules increased from 8 to 52 mg DO g VSS^{-1} h^{-1}. Beyond that period, both of them increased and stabled at 59 mg DO g VSS^{-1} h^{-1}.

Figure 7-4 c presents the average specific ATP content during the recover process using storage aerobic granules as inocula. After storage, the ATP content of R1-YCG and R2-BCG granule was non-detectable. During the first 18 cycle operation, ATP content in R1-YCG and R2-BCG increased slowly. After 24 cycles, ATP content in R1-YCG increased to 0.014 mg ATP g VSS^{-1}, while in R2-BCG increased to 0.017 mg ATP g VSS^{-1}. The ATP content in both type of granules reached a steady level around 0.016 mg ATP g VSS^{-1} toward the end of the experiment.

7.3.3 Cell Viability in the Aerobic Granules

The change of live/dead cell ratios in aerobic granule during the period of this study was measured. The average value of live cell of freshly cultivated aerobic granules was approximately 80%. After storage, the live cells occupied 3% of total cells in R1-YCG granules, while the ratio was 15% in R2-BCG aerobic granules. After 54 cycles' operation, the live cells in R1-YCG aerobic granule increased to 52% of total cells and the ratio increased to 70% in R2-BCG granules.

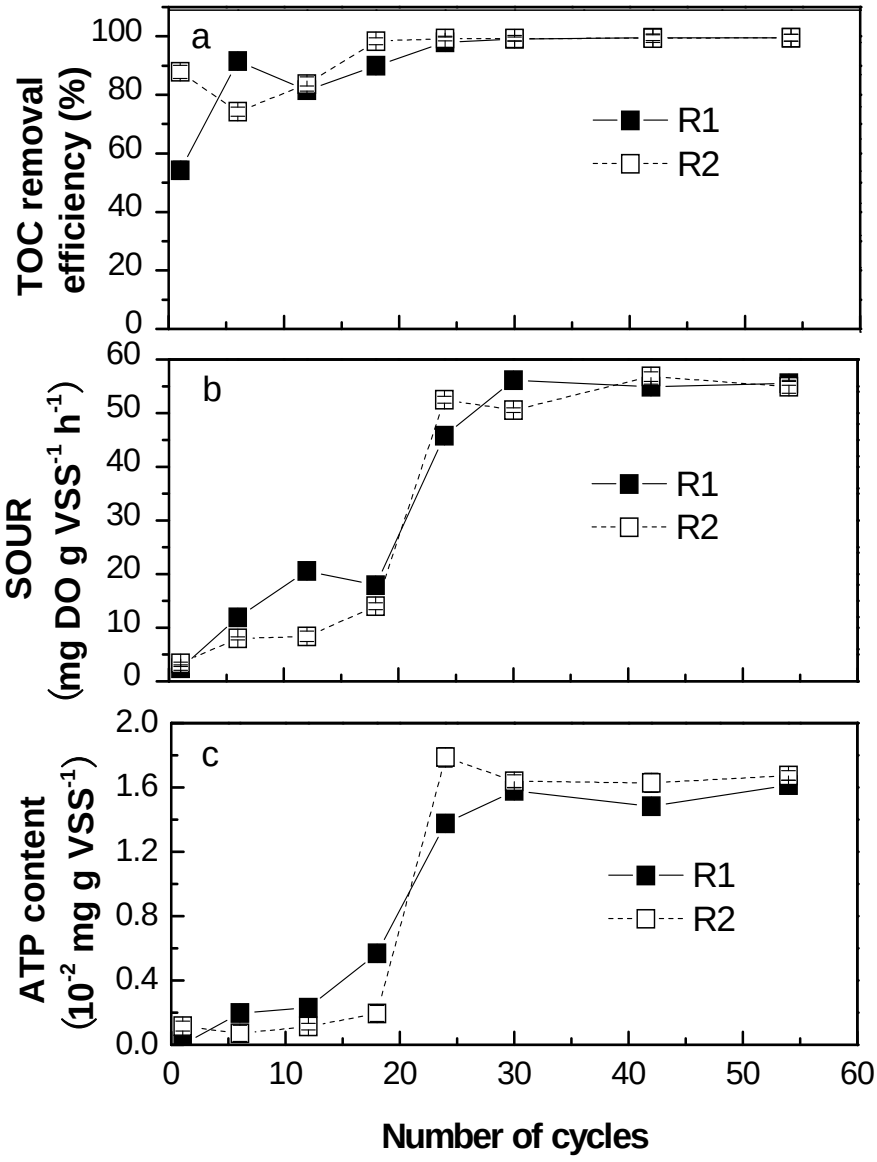


Fig. 7-4 Reactor performance during stored granules reviving process (a) profiles of TOC removal efficiency; (b) profiles of SOUR; (c) profiles of ATP content

7.4 Discussion

7.4.1 Storage and Reviving of PA-degrading Aerobic Granules

No disintegration of the stored PA-degrading granules was observed after the 8-week storage (Figs. 7-1, 7-2 & 7-3). Besides, no matter YCG and BCG, granular size, SVI and settling velocity were all maintained at good quality (Table 7-1). The granules cultivated by acetate/glucose (Tay et al., 2002c) or glucose/peptone (Zhu and Wilderer, 2003) showed similar results. Thus, it seems to be a common feature that the physicochemical characteristics of aerobic granules grown on different substrates may not be significantly affected by storage.

After the storage, the carbonaceous respiration activity (SOUR) of the PA-degrading granules decreased significantly from 59 mg DO g VSS^{-1} h^{-1} to 2 mg DO g VSS^{-1} h^{-1} (YCG) and 3 mg DO g VSS^{-1} h^{-1} (BCG) (declined about 95%) (Fig. 7-4b). Similar scenario was previously reported, for example, 60% activity decline of glucose-fed granules and 90% activity decline of the acetate-fed granules (Tay et al., 2002c). While the aerobic granules fed on glucose/peptone were found to lose 97% of bioactivity after a 7-week-storage in terms of oxygen consumption rate (Zhu and Wilderer, 2003). Bioactivity reduction of aerobic granules in storage process was affected by comprehensive factors, such as microbial dormancy, temperature, duration, environmental conditions, and structure properties of aggregates.

Nevertheless, the PA-degrading aerobic granules showed excellent recovering capability. In this study, TOC removal efficiency (Fig. 7-4a) as well as SOUR (Fig. 7-4b) of stored PA-degrading aerobic granules fully recovered after 24 cycles (4 d) and 42 cycles (7 d) in reviving process, respectively. The activity recovery behavior was comparable to the aerobic granules fed with benign substrate like glucose and peptone (Zhu and Wilderer, 2003). It worth to be pointed out that toxic or recalcitrant compounds degradation genes are frequently carried by plasmid, and these plasmids are easily eliminated during bacteria preservation under non-selective conditions. The fast recovery of bioactivity toward the complex

pollutant of aerobic granules probably indicated that, as a compact bacterial aggregate, aerobic granule maybe facilitate microbial interactions and enhance microbial genetic material exchanges (e.g. horizontal gene transfer). Although there is no direct evidence in this study could demonstrate the facilitating function, studies on biofilm area have shown that compact microbial aggregates could enhance horizontal gene transfer efficiency by magnitude. We are currently working on the topic of microbial genetic material exchanges within aerobic granules.

7.4.2 Morphology Changes during Granules Storage

In this study, the stored PA-degrading aerobic granules exhibited black and brown-yellow colors. The observation was different from the previously reported studies (Tay et al., 2002c; Zhu and Wilderer, 2003), in which the granules were either no color change (Zhu, 2004), or totally changed to grey/black color (Liu et al., 2004a). Liu et al. (2004a) discussed that anaerobic metabolism of granules was a reason to the color change. On the other hand, other researchers (Zhu and Wilderer, 2003; Zhu, 2004) revealed that sulfur generation by sulfur-reducing bacteria might cause the change of supernatant color.

For the current study, it seems that oxygen diffusion from the headspace of the reagent bottle into liquor phase caused the different color change behavior of aerobic granules stored at upper 1/3 part and lower 2/3 part. Generally, oxygen could slowly diffuse into liquor phase during the storage period. However, it will be consumed by the upper part of granules before it could finally transfer to the bottom. Thus, in the initial storage period, the upper part aerobic granules may still capable to access certain level of oxygen while the aerobic granules in the lower part stayed in almost absolute anaerobic conditions.

The oxygen is crucial in the current case because microaerophilic and/or facultative bacteria from upper 1/3 part of aerobic granules may still maintain certain level of their activities by using extracellular polymer substances as substrate (Wang et al.,

2006, 2007) and using oxygen as electron acceptor (Table 7-2). On the other hand, the lower 2/3 part granules went into dormant phase quickly because of no oxygen and other alternative electron acceptor from tap water. It can be approved from the ECPs level in which BCG was obviously lower compared with YCG (Table 7-2). In addition, the higher Live/Dead cells ratio of BCG compared with YCG also reinforced the postulation.

Since the metabolic activity and endogenous metabolism, cell lysis may be happened leading to sulfur elements release into supernatant. It was evidenced by the increase of sulfur concentration from zero to 12.0 mg l^{-1} in supernatant after storage. The facultative sulfur-reducing bacteria residing in aerobic granules may synthesize sulfur compounds using the released sulfur elements (Morgenroth et al., 2000). The precipitation of black color sulfide compounds on the granules leading to color changing of aerobic granules. On the other hand, as mentioned, the bottom part granules quickly went into dormant status because of no proper electron acceptors. As a result, the color of upper 1/3 part aerobic granules changed to be black while the granules in the lower 2/3 part remained brown-yellow color.

7.5 Summary

The results of this study provide valuable information of aerobic granules storage and bioactivity restoration. The storage had no influence on the structure of PA-degrading aerobic granules, and will not cause permanently lose of complex carbon source utilities. It implied the possibility that pre-cultured aerobic granules with special pollutants removal capability could be stored, transported and applied for fast start-up, rescue, or enhance aerobic biological wastewater treatment systems.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Aerobic granulation is a relatively new form of cell immobilization. Unlike activated sludge flocs, microbial granules have a well defined appearance and are still visible as separate entities after settling. Granulation facilitates the accumulation of high amounts of active biomass and the effective separation of this biomass from the wastewater liquor. The current study demonstrates that the degradation of phthalates can be enhanced by seeding with PA-degrading aerobic granules. The formation of PA-degrading aerobic granules, the metabolic responses of PA-degrading aerobic granules to loading of PAEs, and the microbial community of PA-degrading granules have been systematically investigated, by monitoring the reactor performances as well as employing traditional cultivation and molecular methods. The following conclusions can be drawn from this study.

- ✧ PA-degrading aerobic granules can be formed and matured in three weeks at an OLR of 3.0 kg COD m^{-3} d^{-1}. The PA aerobic granules possessed the high settling ability indicated by SVI of 41 ml g^{-1} and the high bioactivity with SOUR of 73 mg DO g VSS^{-1} h^{-1}. They also can sustain PA concentrations as high as 4000 mg l^{-1}. With these features, PA-degrading aerobic granules are proposed as bioseeds for phthalates degradation.

- ✧ The degradation of PA and PAEs by the systems inoculated with PA-degrading aerobic granules was investigated. The highest OLR achieved by PA-degrading aerobic granular system in PA degradation was 12.4 kg COD m^{-3} d^{-1}. In this

system, the biomass detached from aerobic granules retained well in the reactor and played an important role in achieving the high OLR.

✧ The system inoculated with PA-degrading aerobic granules could treat DMP wastewater without acclimation. The system reached steady state in 15 days at an OLR of 3.0 kg COD m^{-3} d^{-1}. The system showed higher tolerance to high OLR, possessed higher removal efficiency and took shorter time to reach steady state than the system inoculated with DMP acclimated activated sludge. Compared with the conventional DMP wastewater treatment, PA-degrading aerobic granular technology was a good choice for industrial DMP wastewater treatment.

✧ For a refractory compound DBP, the system inoculated with PA-degrading aerobic granules could handle the DBP as high as an OLR of 1.5 kg COD m^{-3} d^{-1} and the system reach steady state in 38 days. For the first time, the DBP was reported to be treated in such a high OLR with the DBP removal efficiency of 100% and the TOC removal efficiency of 92.7%.

✧ The system seeded with PA-degrading aerobic granules could treat DMP wastewater without acclimation because the PA-degrading aerobic granules were found to originally possess the constitutive enzymes catalyzing DMP and MMP hydrolysis and the dioxygenase with high enzyme activity. The activities of the esterase for DMP and MMP hydrolysis increased with the induction of DMP. At the same time, the enzyme activity of dioxygenase maintained a high value.

✧ The reaction rates in DMP degradation changed to be $k_3 > k_2 > k_1$ after the system was seeding with PA-degrading aerobic granules. Thus, the reaction

bottleneck step in traditional DMP degradation from MMP to PA was broken by seeding with PA-degrading aerobic granules. The DMP mineralization process was carried out smoothly.

✧ The microbial community structure of PA-degrading aerobic granules during DMP degradation was also investigated. Some dominant species can be kept in aerobic granules in the entire DMP degradation process. Seven distinct isolates were isolated from PA aerobic granules. Among them, strain PA-02 was the representative which possessed both PA and DMP degradation ability, and the auto-aggregation ability. They play an important role in the initial stage of DMP degradation and the steady operation of the system. Kinetic studies revealed that PA-02 was robust under high concentrations of DMP and PA.

✧ The results showed that the eight weeks of storage at 4 ºC refrigerator had no significant influence on the structure of PA-degrading aerobic granules. However, more than 94% of the PA-degrading aerobic granules bioactivity in terms of SOUR, was lost after storage. In recovery experiments, the system seeded with the storage PA-degrading aerobic granules could regain the full capability within 7 days.

✧ This study provided the information of phthalates wastewater treatment by the system inoculated with PA-degrading aerobic granules. Such information would be essential for the design of compact, high-rate aerobic granular systems for the treatment of industrial phthalates wastewater.

✧ Based on the information provided by this study. The industrial PA and PAEs wastewater treatment is proposed as follows: First, the PA-degrading aerobic granules are cultivated with PA at the OLR of 3 kg COD m^{-3} d^{-1} in three weeks.

8.2 Recommendation for Future Work

This study demonstrated the phthalates wastewater was successfully treated by the system inoculated with PA-degrading aerobic granules and provided the fundamental and deep insights into the underlying reasons that phthalate degradation by PA-degrading aerobic granules. Further study would be carried out in the following aspects.

- DEHP, a recalcitrant compound with a higher molecular weight and more complex structure, will be degraded by the system inoculated with PA-degrading aerobic granules.

- The amount of PA-degrading aerobic granules inoculated into a phthalates treatment system and the optimum operation conditions of phthalates degradation by the system will be studied to gain the high removal efficiency and economical cost.

- Interactions and relationships between population dynamics and the function of various types of enzymes will be studied. The population dynamics will also be investigated to find the way of improving the process stability and treatment capacity.

- The physiology and kinetic characteristics of the isolates from PA-degrading aerobic granules will be further investigated to peer into the microorganisms in PA-degrading aerobic granules

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