

# Biodegradation of p-nitrophenol by aerobic granules in a sequencing batch reactor

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# **Biodegradation of *p*-Nitrophenol by Aerobic Granules in a Sequencing Batch Reactor**

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## ABSTRACT

*p*-Nitrophenol (PNP) is one of the most important industrial raw materials and can be found as a pollutant in industrial wastewater streams and groundwater resources. Since PNP contamination can pose a significant environmental and public health risk, the US EPA has listed PNP as a priority pollutant and recommended restricting its concentration in natural waters and industrial effluents. There is therefore a demand to effectively remediate industrial wastewaters and groundwaters that are contaminated with PNP. Biodegradation or bioremediation can be a viable technology for remediating PNP-contaminated waters, since substantial information is available that bacteria are capable of mineralizing PNP under aerobic conditions. However, PNP biodegradation bioreactors that have been reported so far are still limited and the PNP-degrading populations within bioreactors are still poorly understood.

This study aims to deploy a novel bioreactor system employing aerobic granulation in a sequencing batch reactor (SBR) for biodegradation or bioremediation of PNP-contaminated wastewaters. This study also seeks to identify and characterize the indigenous functionally important PNP-degrading populations in the aerobic granules for better understanding the underlying mechanisms of PNP biodegradation by aerobic granules in the granulation SBR.

In order to deploy aerobic granulation in SBR for biodegradation or bioremediation of PNP-contaminated wastewaters, aerobic granules were successfully developed at a PNP loading rate of 0.6 kg/m<sup>3</sup>·day in a SBR using activated sludge as inoculum. A key step for the cultivation of PNP-degrading aerobic granules was the conditioning of the activated sludge seed to enrich biomass with improved settleability and higher PNP degradation activity by implementing progressive decreases in settling time and stepwise increases in PNP concentration. The cultivated granules had a clearly defined shape and appearance, settled

significantly faster than activated sludge and were capable of nearly complete PNP removal. 16S rRNA gene fingerprint analysis showed a gradual temporal shift in microbial community succession as the granules developed from the activated sludge seed. Specific oxygen utilization rates (SOUR) at PNP concentration of 100 mg/l increased with the evolution of smaller granules to large granules and stabilized at 22 mg O<sub>2</sub>/g VSS·h, suggesting that the granulation process could enhance metabolic activity towards biodegradation of PNP.

After aerobic granulation SBR reached a steady state, mature PNP-degrading aerobic granules were characterized for their mechanical stability, specific PNP degradation kinetics, morphology, and broad phenolic substrate utilization. Firstly, PNP-degrading granules possessed an integrity coefficient greater than 99%. Extracellular polymer (ECP) content of PNP-degrading granules, in terms of extracellular polysaccharide (EPS) and extracellular protein (EP) was approximately 9.9% of granule biomass. Secondly, PNP-degrading granules were found having specific PNP degradation rates that increased with PNP concentration from 0 to 40 mg/l, peaked at 19 mg/g VSS·h, and declined with further increases in PNP concentration as substrate inhibition effects became significant. The kinetic analysis using Haldane equation showed that PNP-degrading granules had a maximum specific PNP degradation rate ( $V_{max}$ ) of 36 mg/g VSS·h, a half-saturation coefficient ( $K_s$ ) of 18 mg/l and an inhibition coefficient ( $K_i$ ) of 90 mg/l. Finally, PNP-degrading aerobic granules were also found to have a diverse morphotypes and can degrade broad phenolic substrates.

The microbial community study employing culture-dependent methods such as plate counting and isolation were performed to characterize the competent PNP-degrading bacterial population residing in the mature PNP-degrading aerobic granules. The microbial community study showed that the competent PNP-degrading bacteria accounted for 49% of total culturable heterotrophic bacteria. The physiological studies on the competent PNP-degrading bacteria showed that they possessed different PNP biodegradation activities, in term of specific PNP degradation rates and specific growth rates. Based on the analysis of 16S rRNA

gene sequencing, the competent PNP-degrading bacteria were found to belong to the different taxonomy affiliations of the genus *Burkholderia*, *Acidovorax* and *Paracoccus* respectively.

From the competent PNP-degrading bacteria isolated from PNP-degrading aerobic granules, a strict aerobic bacterium, designated as strain PNP-01 was identified as one of the functionally important PNP-degrading populations. The quantification analysis based on plate counting method indicated that strain PNP-01 constituted 2.5% of mature PNP-degrading granules. The Denaturing gradient gel electrophoresis (DGGE) analysis on the 16S rRNA gene fingerprints demonstrated that strain PNP-01 was persistently present in the bacterial community during the formation of PNP-degrading aerobic granules. The kinetic analysis using Michaelis-Menten equation estimated that strain PNP-01 had a specific maximum PNP degradation rate ( $V_{max}$ ) of 290 mg of PNP/g of DW·h and a half-saturation constant ( $K_m$ ) of 5.3 mg/l. In addition, physiological characterization of strain PNP-01 revealed that the cell density of strain PNP-01 affected the efficiency of PNP biodegradation by strain PNP-01 and strain PNP-01 was not able to form self-flocculation. These findings implied that the efficient PNP biodegradation of strain PNP-01 relied on its immobilized growth within granules in the SBR system. Finally, the phenotypic and phylogenetic characterization identified strain PNP-01 as the *Burkholderia* sp.

Besides *Burkholderia* sp. strain PNP-01, consortium PNP-04 was also identified as one of the functionally important PNP-degrading populations within PNP-degrading aerobic granules. Consortium PNP-04 was characterized as a highly stable microbial aggregate that can efficiently degrade PNP. Three bacterial members, designated as strain AG, AC and AY, were isolated from the consortium PNP-04 with specially designed isolation strategies, namely single colony repeat transfer method, micromanipulation method, as well as motility agar selection method, respectively. Phylogenetic analysis on the basis of 16S rRNA gene sequencing revealed that strain AG, AC and AY belonged to genus of *Brevibacterium*, *Micrococcus* and *Ochrobactrum*, respectively. Specific PNP

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biodegradation assay showed that consortium PNP-04 was able to completely degrade 100 mg/l PNP within 3.5 hours. The three isolates, strain AG, AC and AY, from consortium PNP-04, were found being not able to degrade PNP in monocultures but able to degrade PNP in a mixed culture by equally mixing three strains. The mixed culture of three isolates took more than 30 hours to completely degrade 100 mg/l PNP.

Based on the studies on two functionally important PNP-degrading populations, *Burkholderia* sp. strain PNP-01 and consortium PNP-04, the underlying mechanisms of PNP biodegradation by aerobic granules were revealed as either by the direct function of a single strain or by the concert function of a consortium.

In summary, this research developed and studied PNP-degrading aerobic granules to demonstrate that it is possible to deploy aerobic granulation SBR for efficient PNP biodegradation and to further elucidate the benefits of using aerobic granulation technology to target treatment of toxic and challenging organic compounds.

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## LIST OF ACRONYMS AND SYMBOLS

<b>ABR</b>	Anaerobic baffled reactor
<b>AMBR</b>	Anaerobic migrating blanket reactor
<b>APHA</b>	American Public Health Association
<b>ARDRA</b>	Amplified rDNA restriction analysis
<b>ATSDR</b>	Agency for Toxic Substances and Disease Registry
<b>BOD</b>	Biochemical oxygen demand (mg/l)
<b>CFU</b>	Colony forming unit
<b>COD</b>	Chemical oxygen demand (mg/l)
<b>CSTR</b>	Completely stirred tank reactor
<b>2,4-DCP</b>	2,4-dichlorophenol
<b>2,6-DCP</b>	2,6-dichlorophenol
<b>DGGE</b>	Denaturing gradient gel electrophoresis
<b>DNTP</b>	Deoxynucleotide triphosphates
<b>DW</b>	Dry weight
<b>EC<sub>50</sub></b>	The concentration of a compound where 50% of its maximal effect is observed (mg/l)
<b>ECP</b>	Extracellular polymer
<b>EP</b>	Extracellular protein
<b>EPA</b>	Environmental protection agency
<b>EPS</b>	Extracellular polysaccharides
<b>HSDB</b>	Hazardous Substances Data Bank
<b>HQ</b>	Hydroquinone
<b>HRT</b>	Hydraulic retention time (h)
<b>IC<sub>50</sub></b>	The concentration of a target compound causing a 50% reduction in microbial activity (mg/l)
<b>ICT</b>	Immobilized cell technology
<b>K<sub>s</sub></b>	Half-saturation constant (mg PNP/l)
<b>K<sub>i</sub></b>	Inhibition constant (mg PNP/l)
<b>K<sub>m</sub></b>	Half-saturation constant (mg PNP/l)
<b>LC<sub>50</sub></b>	Lethal dose for 50% of exposed population (mg/kg)
<b>MLSS</b>	Mixed liquor suspended solid (mg/l)
<b>μ<sub>max</sub></b>	The maximum specific growth rate (1/h)
<b>OLR</b>	Organic loading rate

*LIST OF ACRONYMS AND SYMBOLS*

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<b>PNP</b>	<i>p</i> -Nitrophenol
<b>PAP</b>	<i>p</i> -Aminophenol
<b>PNC</b>	<i>p</i> -Nitrocatechol
<b>PI</b>	Propidium iodide
<b>QS</b>	Quorum sensing
<b>SOUR</b>	Specific oxygen utilization rate (mg DO/g SS·h)
<b>SVI</b>	Sludge volume index (mg/l)
<b>SEM</b>	Scanning electron microscope
<b>SBR</b>	Sequencing batch reactor
<b>TOC</b>	Total organic carbon (mg/l)
<b>UASB</b>	Upflow anaerobic sludge blanket reactor
<b>UPGMA</b>	Unweighted Pair Group Method using Arithmetic Averages
$V_{max}$	The maximum specific substrate degradation rates (mg/g VSS·h)
<b>VSS</b>	Volatile suspended solid (mg/l)

# CHAPTER I

## INTRODUCTION

### 1.1 BACKGROUND

Nitroaromatic compounds, such as nitrophenol, nitrobenzene, nitrotoluene and nitrobenzoates, are of considerable industrial importance as they are the main raw materials in the manufacture of dyes, pharmaceuticals, pesticides, and explosives (ATSDR, 1992). *p*-Nitrophenol (PNP) is one of the most important nitroaromatic compounds, both in terms of quantities used and potential environmental impact (Podeh et al., 1995).

The annual demand for PNP in the US from 1989 to 1994 ranged from 22 to 25.5 million pounds (HSDB, 1999). PNP is used mainly for the manufacture of drugs (e.g. acetaminophen) and pesticides (e.g. methyl and ethyl parathions), and is also used in leather treatment, dyestuff production and for military purposes (Spain and Gibson, 1991).

Because of its frequent and widespread use, PNP can be found as a pollutant in industrial wastewater streams associated with its formulation, distribution and application. PNP can also be released into the subsurface and contaminate groundwater resources as a result of hydrolysis of pesticides and herbicides (Labana et al., 2005). Other origins, such as vehicular exhausts and photochemical reactions in the atmosphere, may also contribute a small amount of PNP contamination in the environment (Haderlein and Schwarzenbach, 1995).

If released into the environment, PNP contamination can pose a significant environmental and public health risk, owing to its acute toxicity and its mutagenic potential. Animal studies have demonstrated that PNP can cause blood disorders.

Acute exposure of PNP may lead to methemoglobin formation, liver and kidney damage, anemia, skin and eye irritation, and systemic poisoning (ATSDR, 1992; HSDB, 1999). In addition, PNP contamination of rivers and groundwater resources may cause deleterious effects to ecological systems (Zieris et al., 1988). For these reasons, the US EPA has listed PNP as a priority pollutant and recommended restricting its concentration in natural waters to below 10 ng/l (EPA, 1976). Regulations have also been established to restrict PNP levels in industrial effluents. For example, the average monthly concentrations of PNP should not exceed 162 µg /l (EPA, 1988a; EPA, 1988b).

There is therefore an obvious interest to effectively remediate PNP contaminated industrial wastewaters and groundwaters. Among the treatment methods, adsorption is the most frequently used technique for PNP removal (Vincent and Guibal, 2004). However, it suffers from the drawbacks that toxic slurries generated from this treatment remain technically or economically difficult to eliminate. This is because adsorption is just physically transferring the pollutants from waters to solids without any detoxification.

On the other hand, biological treatment is a viable alternative which can be environmentally and economically friendly. PNP is known to be mineralized by microorganisms under aerobic conditions (Crawford, 1995). Many studies on PNP degradation have been reported, particularly on aspects relating to degradation pathways in pure bacterial cultures (Spain and Gibson, 1991; Jain et al., 1994; Kadiyala and Spain, 1998; Chauhan et al., 2000b; Kitagawa et al., 2004).

However, studies on aerobic PNP degradation with bioreactor systems are relatively limited (Ray et al., 1999; Xing et al., 1999; Bhatti et al., 2002). This situation can be attributed to two issues that always challenge the microbial degradation of PNP with bioreactor systems. One is that PNP is an uncoupling agent for oxidative phosphorylation which will cause inhibitory effects to microbial activity and growth (Low et al., 2000). Another is that the presence of the nitro group, a strong electron-withdrawing group, makes PNP less susceptible to

electrophilic oxygenation (Rieger and Knackmuss, 1995), which sometimes causes PNP to appear recalcitrant to aerobic biodegradation ( $BOD_5/COD=0$ , (Yoon et al., 2001)).

Lessons from several bioreactor studies reported to date suggested that immobilization of biomass using attached biofilms can be a valid strategy to overcome the effects of PNP's toxicity and recalcitrance toward microbial degradation in the bioreactor (Heitkamp et al., 1990; Ray et al., 1999; Xing et al., 1999; Bhatti et al., 2002). Compared with suspended systems, such as activated sludge systems, immobilized systems were able to retain higher amounts of PNP-degrading bacteria, hence achieving higher PNP degradation activity and higher tolerance to PNP toxicity (Ray et al., 1999; Xing et al., 1999). However, these conventional cell immobilization technologies require carriers to support cell adhesion and growth, which may cause higher investment and raise operation costs. In addition, carriers in bioreactors may also cause clogging problems, increasing sludge handling and disposing costs.

Aerobic granulation is a recent innovation in biological wastewater treatment that can overcome the disadvantages associated with the use of carrier materials in conventional cell immobilization technologies. Aerobic granules are self-immobilized microbial aggregates that are cultivated in sequencing batch reactors (SBRs) without reliance on artificial surfaces for biofilm attachment, hence rendering carrier material and bulky settling devices unnecessary (Beun et al., 1999; Jiang et al., 2002; Moy et al., 2002).

The basis for the formation of aerobic granules in the SBR is a repetitive selection for sludge particles such that denser components are retained in the system while lighter and dispersed particles are washed out (Tay et al., 2005c). The aggregation of microorganisms into compact aerobic granules confers many benefits such as protection against predation, resistance to chemical toxicity, and development of syntrophic relationships of different species which may facilitate

horizontal gene transfer, interspecies substrate exchange and removal of metabolic products (Moy et al., 2002; Jiang et al., 2004b).

Because of these merits, aerobic granules had been previously shown to be suitable for enhancing COD removal in wastewater treatment (Beun et al., 1999; Moy et al., 2002) and for degrading phenol at levels that were known to cause the breakdown of conventional activated sludge processes (Jiang et al., 2002). In the case of PNP, the presence of the nitro group makes it more inhibitory and refractory to microbial degradation than its unsubstituted analog, phenol. The successful cultivation of aerobic phenol-degrading granules raised the question whether it is possible to cultivate aerobic PNP-degrading granules and what the performance of the granules is compared with those other reported biological systems.

In addition, there is interest in understanding the indigenous functionally important PNP-degrading microbial populations within aerobic PNP-degrading granules and their metabolic and physiological activities. Information on the functionally important PNP-degrading microbial populations will contribute valuable knowledge in understanding the underlying mechanisms involved in the PNP biodegradation with the aerobic granules and to develop optimal control and management strategies for PNP-degrading aerobic granulation systems. While the functionally important PNP-degrading bacteria and their metabolic and physiological activities have been reasonably well described for various ecosystems, such as soil and lake water (Jain et al., 1994; Kadiyala and Spain, 1998; Chauhan et al., 2000; Kitagawa et al., 2004), a gap in the understanding of the functionally important PNP-degrading bacteria residing in PNP-degrading aerobic granules still exists. This is because the selection pressures and the unique growth conditions of the aerobic granulation system may result in the microbial populations possessing different metabolic and physiological traits from those reported in other ecosystems.

## 1.2 OBJECTIVES AND SCOPE

The main objective of this study is to deploy aerobic granulation for effectively removing high strength PNP pollutant and to study the indigenous functionally important PNP-degrading microbial populations in the aerobic granules for understanding the underlying mechanisms of PNP biodegradation by the granules. In order to achieve this objective, research efforts will be directed in the following aspects:

- To develop strategies to cultivate the aerobic PNP-degrading granules, and to investigate the evolution of microbial metabolic efficiency towards biodegradation of PNP during aerobic granulation;
- To characterize the PNP-degrading granules on their physico-chemical properties, and to study the specific rate and extent that granules degrade PNP as sole substrate;
- To isolate and characterize the competent PNP-degrading bacterial population residing in the PNP-degrading aerobic granules, and to investigate the physiological characteristics and ecological importance of functionally important PNP-degrading bacterial populations from the aerobic PNP-degrading granulation system; and finally
- To understand the different PNP degradation mechanisms involved in the aerobic granules.

The results from this research would provide a sound understanding of aerobic granulation as an effective immobilization strategy in biodegradation of highly toxic and recalcitrant compounds in industrial wastewater. In addition, the development of aerobic PNP-degrading granulation process would lead to a novel biotechnology which could be potentially applied to eliminate PNP in industrial effluents and contaminated groundwaters. In addition, the knowledge on the physiological characteristics of functionally important PNP-degrading bacteria will be useful in developing optimal control and management strategies for PNP-degrading aerobic granulation systems. Finally, the isolated PNP-degrading

bacterial strain can be used for bioaugmentation to enhance the performance of bioreactors.

### **1.3 ORGANIZATION OF THE DISSERTATION**

This dissertation contains six chapters. Chapter I gives a brief background of this study and lists the objectives and scope of the study. Chapter II is a comprehensive literature review which discusses PNP contamination in the environment, the current status of the biodegradation of PNP wastewater, and the advances of aerobic granulation. Chapter III describes the cultivation of aerobic granules for PNP biodegradation. The physico-chemical properties and kinetic behavior of PNP biodegradation were also investigated for mature PNP-degrading aerobic granules. Chapter IV details the isolation and characterization of competent PNP-degrading bacterial populations residing in the steady-state PNP-degrading aerobic granules. The ecological importance and physiological characteristics of functionally important PNP-degrading bacteria, *Burkholderia* sp. strain PNP-01 were investigated in detail. Chapter V investigates the physiological characteristics of a stable aggregated microbial consortium PNP-04 isolated from PNP-degrading aerobic granules. The members within consortium PNP-04 were isolated with specially designed isolation strategies. The PNP biodegradation with mono- or mixed- culture of the members from consortium PNP-04 was also investigated. Chapter VI concludes the important findings from this study and outlines the recommendations for future work.

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## CHAPTER II

# LITERATURE REVIEW

### 2.1 BACKGROUND ON *P*-NITROPHENOL (PNP)

#### 2.1.1 PNP and Its Environmental Concerns

Nitroaromatic compounds, such as nitrophenol, nitrobenzene, nitrotoluene, and nitrobenzoates, are a group of aromatic compounds bearing one or more nitro group as substituent(s). They are among the most important and versatile industrial organic compounds and are widely used in the manufacture of dyes, pharmaceuticals, pesticides, and explosives. PNP is one of the isomeric forms of mononitrophenols and is one of the most important nitroaromatic compounds in terms of quantities used and potential environmental impacts (Podeh *et al.*, 1995).

- PNP as an Important Industrial Organic Compound

PNP is of considerable industrial importance and has been listed as a high production volume chemical by the United States Environmental Protection Agency (EPA, 1990). The annual production of PNP is approximately 20 million kg in the US in 1987 (Donlon *et al.*, 1996). In subsequent years, from 1989 to 1994, the annual demand for PNP was still above 9 million kilogram in the US, albeit the consumption of PNP was gradually reduced (HSDB, 1999) (Figure 2.1).

As showed in Figure 2.2, the chemical structure of PNP, a phenolic structure bearing one nitro-group at *para* position, determines that PNP is susceptible to the chemical reactions, such as substitution and reduction on its functional groups and aromatic ring (ATSDR, 1992).

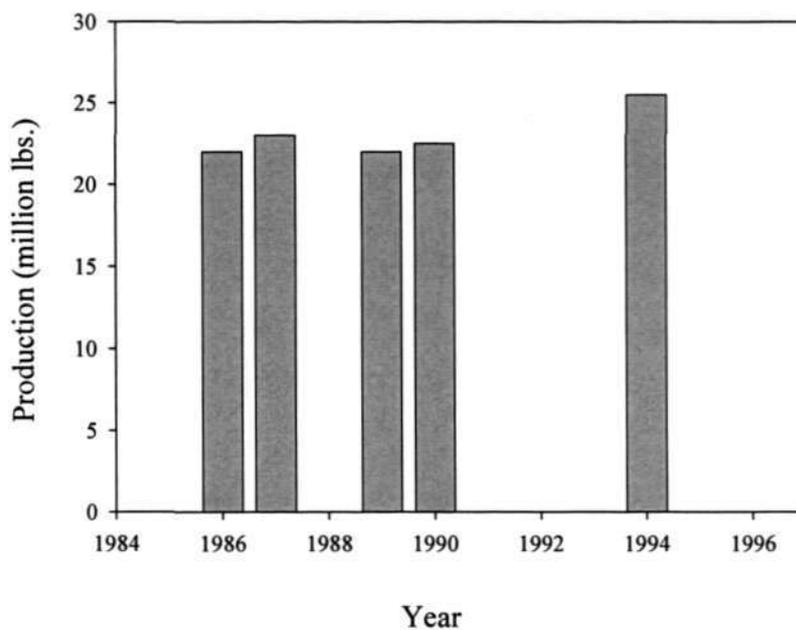


Figure 2.1 Annual demand of PNP in the US (HSDB, 1999).

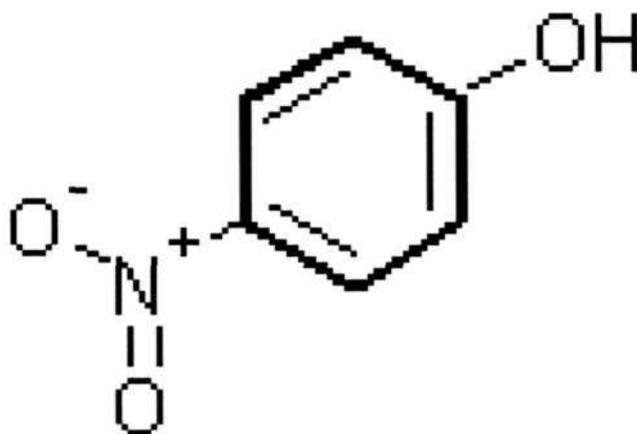


Figure 2.2 Chemical structure of PNP (UM-BBD, 2005).

Figure 2.3 shows several utilization patterns of PNP in the US in 1971, 1984 and 1987. In general, the utilization of PNP can be classified into two categories. In the first category, PNP is used directly as insecticide or fungicide, leather treatment material, and laboratory reagents (ATSDR, 1992). This category of PNP utilization only accounts for a small proportion of total PNP consumption. In the second category, PNP is used as an intermediate for synthesizing other nitrogen containing organic chemicals, such as organic phosphate pesticides (parathion and methyl parathion), non-aspirin pain reliever drug (acetaminophen), dyestuff and military products. This category of PNP utilization as an intermediate accounts for the major part of total PNP consumption.

- PNP Contaminations and Their Environmental Concerns

Due to the frequent and wide use of PNP and its derivatives, PNP may enter the environment through various anthropological origins (ATSDR, 1992). For example, the formulation, production and utilization of PNP in industries may result in its release into the environment through various waste streams. The application of parathion and methyl parathion may also release PNP into the subsurface and contaminated soil and groundwaters due to the microbial hydrolysis of the pesticides (Munnecke, 1976; Stevens *et al.*, 1991). In addition, PNP may be emitted in vehicular exhaust from both gasoline and diesel engines to contaminate the air. PNP is also a product of photochemical reactions of nitrobenzene or aromatic hydrocarbons in air (Haderlein and Schwarzenbach, 1995). Table 2.1 lists some typical concentration of PNP contaminants that have been identified in various compartments of the environment.

When PNP enters into the environment, the fate and partitioning of PNP contaminants are of great concern. Yoshida *et al.* (1983) assessed the distribution of PNP in different environments using a nonsteady-state equilibrium model which predicted about 94% PNP partitioning into aquatic environments and the remainder into air, soil, sediment and biota.

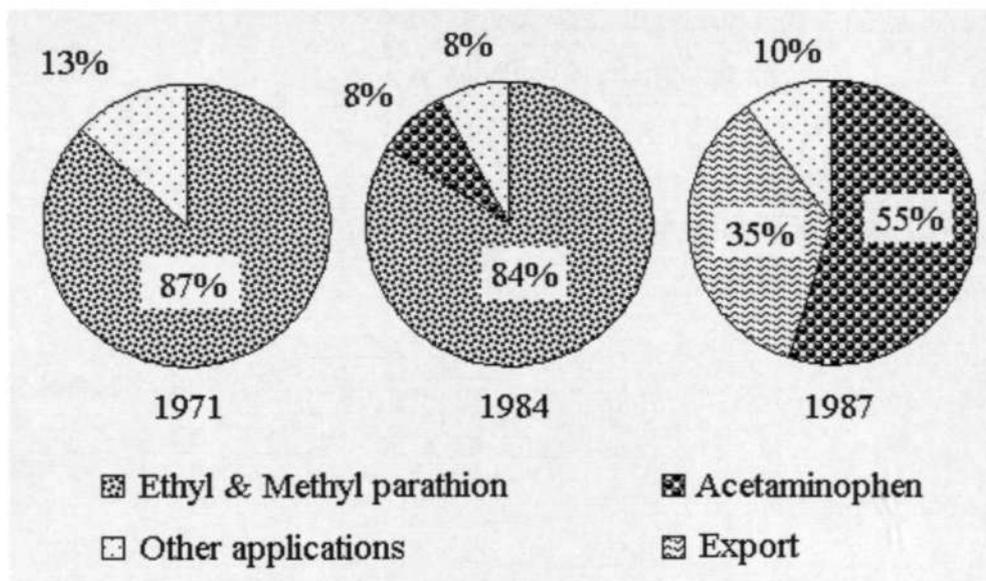


Figure 2.3 The usage pattern of PNP (HSDB, 1999).

Table 2.1 Occurrence and typical concentrations of PNP in various environmental systems.

Compartment	Concentration	Description (Place)
Water	0.3-6.5 µg/l	Groundwater (Ville Mercier, Quebec, Canada)
	5.8-84 µg/l	Groundwater beneath a former munitions plant (Saxony, Germany)
	200 µg/l	Groundwater (Biscayne Aquifer, FL)
	Identified not quantified	Drinking water (Taiwan)
	Identified not quantified	Surface water, (Taiwan)
	10 µg/l	River, (Potomac Virginia)
	Identified, not quantified	Rain (Yokohama, Japan)
	0.49-17.1 µg/l	Rain and snow (Hannover, Germany)
	1.11-16.27 µg/l	Rain (Vosges mountains, France)
Air	0.1-1.0 µg/l	Exhausts, (vehicles without catalytic converters)
	Trace amounts to 2.5 ppb	Exhausts, (gasoline and diesel engines)
	5.1-42 ppm	Urban area, (Yokohama, Japan)
Sediments	2.6-70,400 µg/kg	Soil, (Ville Mercier, Quebec, Canada)
Food	0.061 ppm	Lettuce sprayed with parathion (0.5 lb/acre)

Source: Hazardous Substances Data Bank [HSDB] (1999).

Based on the assessment from the physical and chemical characteristics of PNP (Table 2.2), the natural attenuation through the physico-chemical processes, such as adsorption to suspended solid and sediment, volatilization, and hydrolytic degradation are not likely to contribute to the removal of aquatic PNP contaminations (HSDB, 1999). PNP is usually considered to be persistent in the environment, albeit photolysis (Gorontzy *et al.*, 1994) and microbial degradation (HSDB, 1999) can be mainly attributed to the PNP natural attenuation. This is because photolysis is a slow process and half-lives of PNP in water exposed to sunlight were 5.7, 6.7, and 13.7 days at pH 5, 7, and 9, respectively. In addition, the microbial degradation of PNP always involves a long lag period ranging from several days to weeks before the onset of biodegradation (ATSDR, 1992).

Persistent PNP contamination in the environment poses a significant environmental and public health risk, owing to the acute toxicity and high mutagenic potential of PNP. Toxicological studies reported that the oral LD<sub>50</sub> (lethal dose for 50% of exposed population) of PNP for a male rat is 250 mg/kg which is much lower than the value, 530 mg/kg of phenol, PNP's unsubstituted analog, suggesting an acute toxicity of PNP to the animals (HSDB, 1999). Animal studies suggest that PNP may cause a blood disorder (ATSDR, 1992), and acute exposure of PNP may lead to methemoglobin formation, liver and kidney damage, anemia, skin and eye irritation, and systemic poisoning. In addition, when PNP enters the rivers and groundwater resources, it can be highly inhibitory to the aquatic living organisms (Madhavi *et al.*, 1995) and can cause deleterious effects to the ecological systems (Zieris *et al.*, 1988).

Due to its persistence in the environment and its acute toxicity to the environment and public health, the US EPA has listed PNP as a priority pollutant and recommended a restriction on its concentration in natural waters to less than 10 ng/l (EPA, 1976). Regulations have been established to restrict PNP levels in industrial effluents (EPA, 1980; EPA, 1988a). For example, average monthly concentrations of PNP should not exceed 162 µg/l (EPA, 1988b).

**Table 2.2** Summary of physical and chemical characteristics of PNP.

Physical and chemical properties of PNP		Reference
Synonyms	4-Nitrophenol, 4-Hydroxynitrobenzene, mononitrophenols, Niphen	
Formula	C <sub>6</sub> H <sub>6</sub> NO <sub>3</sub>	
Molecular weight	139.11	
Physical state	Colorless to light yellow crystalline solid	
Melting point	113-114°C	
Boiling point	297°C	
Density	1.270 g/cc at 20°C	
pKa	7.15 at 25°C	
Odor	Slight odor	
Odor threshold		
Water	2.5 mg/l	UM-BBD, 2005;
Air	2.3 mg/m <sup>3</sup>	ATSDR, 1992;
Solubility		HSDB, 1999
Distilled water	16,000 mg/l at 25°C	
Sea water	10,795 mg/l at 20°C	
Organic solvents	Soluble in toluene, ethanol, chloroform, ethyl ether, and alkali hydroxides	
Partition coefficients:		
Log K <sub>ow</sub>	1.91	
Log K <sub>oc</sub>	2.18-2.42	
Vapor pressure	0.005 mm Hg at 20°C	
Henry constant	1.3×10 <sup>-8</sup> atm/m <sup>3</sup> ·mol at 20°C	
Oral LD <sub>50</sub>		
Rat	250 mg/kg	
Mouse	380 mg/kg	

LD<sub>50</sub>: Lethal dose for 50% of exposed population;

K<sub>oc</sub>: Organic carbon coefficient;

K<sub>ow</sub>: n-Octanol/water partition coefficient.

pKa: Dissociation constant

▪ Current Decontamination Technologies for Industrial Wastewater or Groundwater Contaminated with PNP

There is an obvious interest to effectively remediate industrial wastewater and groundwater that are contaminated with PNP. Among the treatment methods, adsorption to the activated carbon (Nevskaia *et al.*, 2004) or polymeric adsorbents (Li *et al.*, 2002) is still the most frequently used method for decontaminating the PNP pollutants in industrial wastewater and groundwater. However, this method does not really degrade the PNP pollutants but just physically transfer the pollutants from waters to solids. The toxic slurries generated from the adsorption method remain technically or economically difficult to eliminate.

Chemical oxidation (Vincent and Guibal, 2004) is also used for remediating the PNP contaminated waters. However, this method suffers from the generation of secondary contaminated waste streams due to the incomplete degradation of PNP pollutant. Recently developed advanced oxidation processes (AOPs) including ozonation, sensitized sunlight (Xiong and Xu, 2005), photo catalysis and photo Fenton (Einschlag *et al.*, 2002) as well as electro-Fenton method (Oturán *et al.*, 2000) are able to overcome the drawbacks from incomplete degradation by conventional chemical oxidation. It should be pointed out that these processes need to add a large amount of reaction reagents for completely degrading PNP which makes the complete mineralization by the AOPs an uneconomical goal for treatment.

Due to the disposal problems of undesirable wastes and the high cost of physical and chemical decontamination technologies, biodegradation and bioremediation represent important alternative approaches for environmental remediation of PNP contaminated waters (Crawford, 1995; Spain, 1995). In the following sections, the biodegradation of PNP will be reviewed in detail.

### 2.1.2 Microbial Degradation of PNP with Pure Bacterial Cultures

Microbial degradation of PNP may occur by two mechanisms currently known (Marvin-Sikkema and de Bont, 1994):

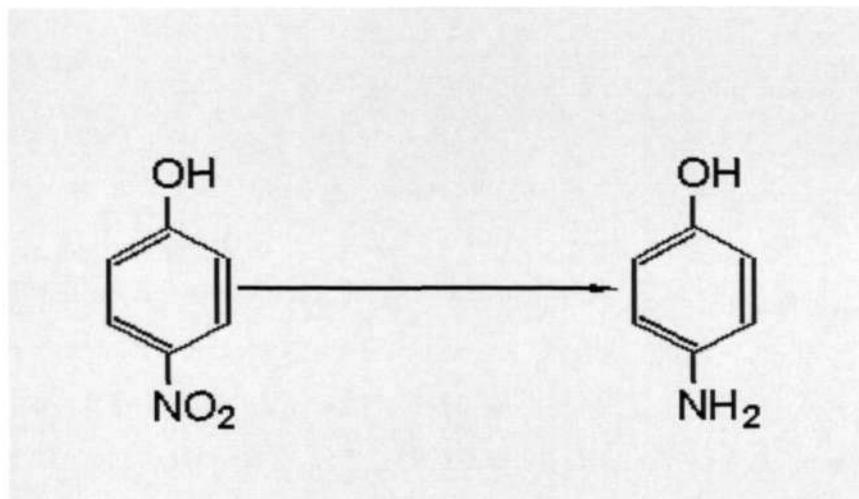
1. Biotransformation of PNP to *p*-aminophenol (PAP) by reductive reaction under anaerobic conditions; or
2. Mineralization of PNP to CO<sub>2</sub> and H<sub>2</sub>O by microbial oxidation under aerobic conditions.

In the first reaction described above, PNP acts as an electron acceptor. But in the second reaction, PNP serves as a carbon and energy source for microorganisms.

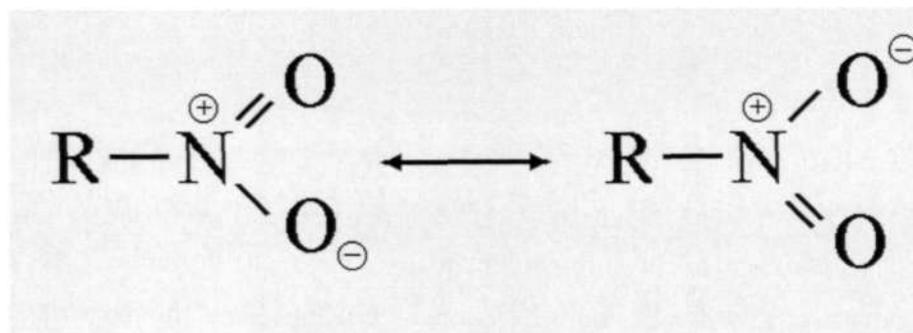
#### ▪ Biotransformation of PNP by Reductive Reaction under Anaerobic Conditions

There have been many publications on the reductive transformation of PNP to PAP by strictly anaerobic bacteria (Figure 2.4) (Marvin-Sikkema and de Bont, 1994), but PAP is usually not subjected to degradation by anaerobic microorganisms. In this section, this transformation will be described.

The fact that many anaerobic bacteria are able to catalyze the reductive reaction of PNP is due to the chemical properties of the nitro group (Spain, 1995). The nitro group consists of two different elements that are both highly electronegative and therefore compete for the available electrons. The nitro group is usually described as a resonance hybrid between the structures shown in Figure 2.5 (Preuß and Rieger, 1995). The electronegativity of the oxygen is higher than that of the nitrogen atom, resulting in a polarization of the N-O-bond. The partially positive charge on the nitrogen atom, combined with its high electronegativity, makes the nitro group easily reducible to amino group.



**Figure 2.4** The pathway of biotransformation of PNP to PAP by reductive reaction under anaerobic conditions.



**Figure 2.5** Canonical forms of the nitro group. The electro negativity of the oxygen is higher than that of the nitrogen atom, resulting in a polarization of the N-O bond. The polarization of the partially positive charge on the nitrogen atom, combined with its high electro negativity makes the nitro group easily reducible (Preuß and Rieger (1995)).

However, further reduction of amino group or hydroxyl group on the aromatic ring is usually not so easy as the reduction of the nitro group. The difficulty of this reduction can be attributed to the fact that the rate of nitro reduction by anaerobic bacteria is influenced by electron-withdrawing power of the functional group and the position of the other substitute groups on the aromatic ring (McCormick *et al.*, 1976). The rank of substituents corresponding to their electron-withdrawing power is:  $-\text{NH}_2 < -\text{OH} < -\text{H} < -\text{CH}_3 < -\text{COOH} < -\text{NO}_2$ . Once the strong electron-withdrawing nitro group ( $-\text{NO}_2$ ) is converted to amino group ( $-\text{NH}_2$ ), a substituent with a strong electron-offering power, the electron density of aromatic ring is increased. As a result, the further reduction of the compound molecule is hindered (Preuß and Rieger, 1995). The reduction of nitro group occurs more easily at *ortho* position rather than *meta* or *para* position (Gorontzy *et al.*, 1993).

Table 2.3 shows the variety of strictly anaerobic bacteria that can carry out nitro reduction. Nitroreductases from these bacteria seem to have a wide spectrum of substrates (Marvin-Sikkema and de Bont, 1994). PNP can be used as an electron acceptor under anaerobic conditions by these bacteria. Therefore, PNP is transformed to PAP, which is usually not further converted under anaerobic conditions.

- Mineralization of PNP by Microbial Oxidation under Aerobic Conditions

The presence of the nitro group makes PNP more difficult to biodegrade aerobically than its aromatic analogs (Spain, 1995). This recalcitrance is due to the strong electron-withdrawing character of the nitro group, which causes the electron deficiency of the aromatic ring. As a result, PNP is less susceptible to electrophilic oxygenation (Rieger and Knackmuss, 1995). Nevertheless, the degradation and mineralization of PNP by microbial oxidation under aerobic conditions have been demonstrated with various pure bacterial cultures as listed in Table 2.4.

**Table 2.3** Strictly anaerobic bacteria that have been reported to mediate nitro reduction of PNP.

<b>Bacteria</b>	<b>Reference</b>
<i>Clostridium pasteurianum</i>	Gorontzy <i>et al.</i> , 1993
<i>Clostridium</i> sp. strain W1	
<i>Desulfovibrio desulfuricans</i>	
<i>Desulfovibrio gigas</i>	
<i>Desulfovibrio</i> sp. strain AS	
<i>Desulfovibrio</i> sp. strain GB	
<i>Desulfococcus multivorans</i>	
<i>Desulfotomaculum orientis</i>	
<i>Desulfotomaculum</i> sp. GROL	
<i>Haloanaerobium praevalens</i> DSM 2228	
<i>Methanobacterium formicicum</i>	
<i>Methanobacterium thermoautotrophicum</i>	
<i>Methanoculleus oldenburgensis</i>	
<i>Methanogenium tationis</i>	
<i>Methanosarcina barkeri</i>	
<i>Methanosarcina frisia</i>	
<i>Methanosarcina</i> sp. strain KS2002	
<i>Methanospirillum hungatei</i>	
<i>Pseudomonas</i> sp. strain CBS3	Schackmann and Müller, 1991
<i>Rhodobacter capsulatus</i> E1F1	Blasco and Castillo, 1992
<i>Sporohalobacter marismortui</i> ATCC 35420	Oren <i>et al.</i> , 1991

**Table 2.4** Bacteria reported that can degrade or mineralize PNP through oxidation.

Bacteria	Reference
<i>Arthrobacter aurescens</i> TW17	Hanne <i>et al.</i> , 1993
<i>Arthrobacter protophormiae</i> RKJ100	Jain <i>et al.</i> , 1994; Chauhan <i>et al.</i> , 2000a
<i>Bacillus sphaericus</i> JS905	Kadiyala <i>et al.</i> , 1998
<i>Burholderia cepacia</i> RKJ200	Bhushan <i>et al.</i> , 2000
<i>Flavobacterium</i> sp.	Raymond and Alexander, 1971
<i>Moraxella</i> sp.	Spain and Gibson, 1991
<i>Nocardioides nitrophenolicus</i> sp. nov.	Yoon <i>et al.</i> , 1999
<i>Nocardia</i> sp. strain TW2	Hanne <i>et al.</i> , 1993
<i>Pseudomonas putida</i>	Kulkarni and Chaudhari, 2005
<i>Pseudomonas putida</i> PNP1	Löser <i>et al.</i> , 1998
<i>Ralstonia</i> sp. SJ98	Bhushan <i>et al.</i> , 2000
<i>Rhodococcus wratislaviensis</i> J3	Navratilova <i>et al.</i> , 2005
<i>Sphingomonas</i> sp. UG30	Leung <i>et al.</i> , 1999

**Biodegradation Pathway**

Two alternative pathways have been proposed for PNP mineralization under aerobic condition (Crawford, 1995) (Fig 2.6).

In the first catabolic pathway proposed with a *Moraxella* sp, hydroquinone is produced from PNP via a monooxygenase attack at the para position with concomitant  $\text{NO}_2^-$  release. The hydroquinone is oxidized subsequently by a novel ring cleavage dioxygenase to yield  $\gamma$ -hydroxymuconic semialdehyde which is further enzymatically converted to maleylacetate and to  $\beta$ -keto adipic acid (Spain and Gibson, 1991). Consequently, 2 mol of molecular oxygen is required for the conversion of PNP to  $\beta$ -keto adipic acid.

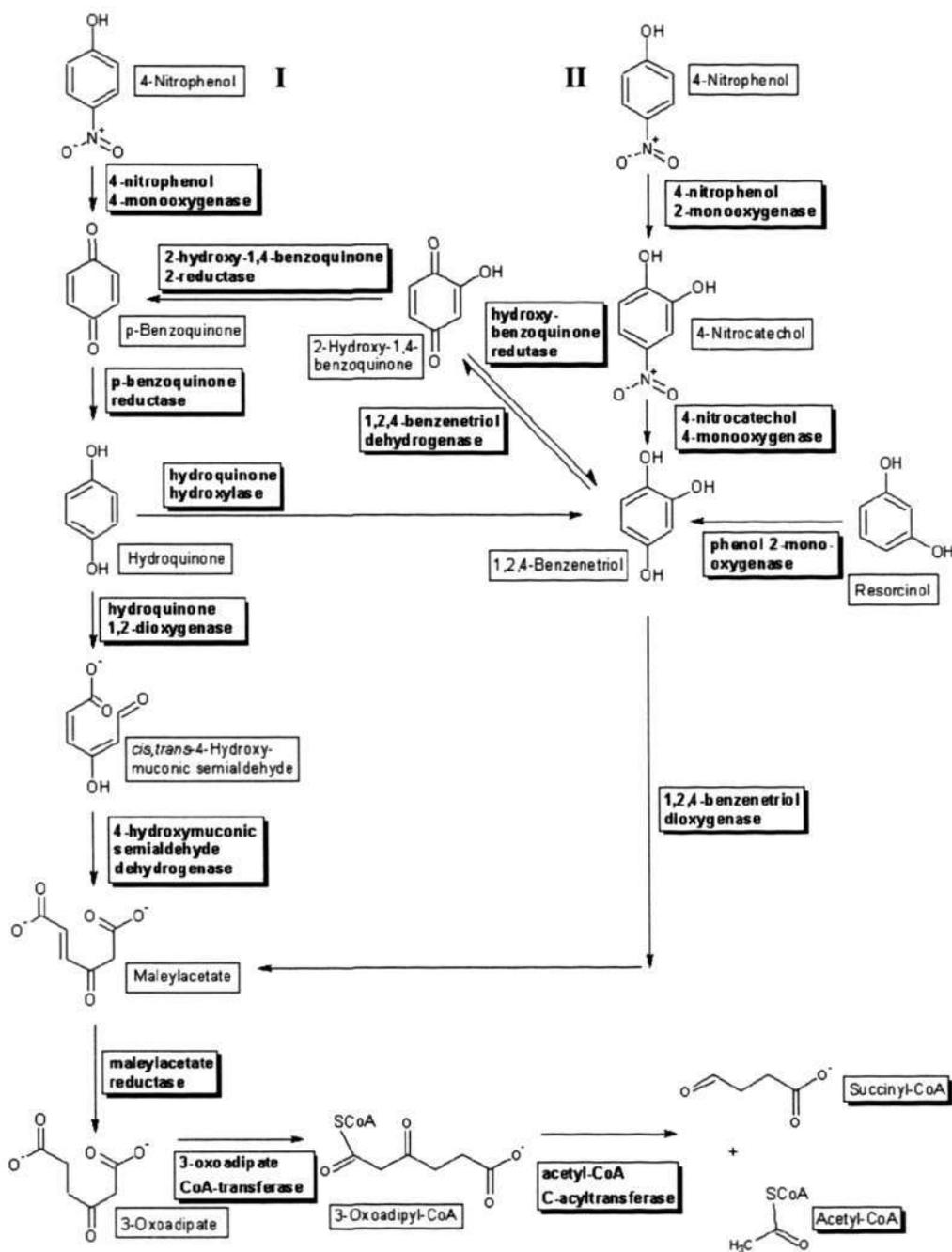


Figure 2.6 The pathway of the aerobic catabolism of PNP by microorganisms (UM-BBD, 2005).

In the second catabolic pathway proposed with an *Arthrobacter* sp., 4-nitrocatechol or 4-nitroresorcinol is produced from PNP via a monooxygenation reaction at the *ortho* or *meta* position followed by a second monooxygenase attack in the *para* position to yield 1, 2, 4-benzenetriol (BT) with concomitant release of nitrite. BT is further oxidized by a ring cleavage dioxygenase to yield maleylacetate which is further converted enzymatically to  $\beta$ -keto adipate (Jain *et al.*, 1994). In the second pathway, 3 mol of O<sub>2</sub> is required for transformation of each mol of PNP to  $\beta$ -keto adipate (Kadiyala *et al.*, 1998).

Both pathways converge at the level of  $\beta$ -keto adipate (Crawford, 1995) and further enter into TCA cycle in which PNP is finally degraded into H<sub>2</sub>O and CO<sub>2</sub>.

#### **Biokinetic Parameters**

Successful application of PNP degrading bacteria to remove the contaminants from the environment depends on biokinetic parameters, such as the maximum specific removal rate of PNP,  $V_{\max}$  (mg PNP/mg cells·t) or the maximum specific growth rate on PNP  $\mu_{\max}$  (1/t); the half saturation coefficient,  $K_s$  (mg PNP/l); the self inhibition coefficient,  $K_i$  (mg PNP/l); and the specific decay coefficient,  $b$  (1/t). However, only a few studies are available on the kinetics of PNP degradation or PNP-degrading bacteria growth.

Kadiyala *et al.* (1998) reported the kinetic studies of PNP oxidation by *Bacillus sphaericus* JS905, using a respirometric assay. The oxygen uptake curve at low PNP concentration (1.04 and 2.08 mg PNP/l) could be described very well by using Monod kinetics. *B. sphaericus* JS905 displayed very high substrate affinity ( $K_s$ ) values of 0.003 and 0.023 mg PNP/l, as well as low maximum specific growth rate ( $\mu_{\max}$ ) values of 0.021 and 0.019 /h at 30 and 37°C, respectively. However, a significant substrate inhibition was evident at a PNP concentration of 4.16 mg/l. Therefore, the Andrews (or Haldane) kinetic expression replaced Monod kinetics to describe the oxygen uptake curves. The measured specific growth rates ( $\mu_{\max}$ ) were 0.055 and 0.043 /h at 30 and 37°C, respectively.

In addition, linear regressions were performed on the portions of the oxygen uptake curves before PNP was added to estimate the endogenous decay coefficient  $b$  (1/t). The values for the endogenous decay coefficient (0.015 and 0.020 /h at 30 and 37°C, respectively) were unexpectedly of the same size as the specific growth rate. This result indicated that the net growth of *B. sphaericus* JS905 was very small with PNP as sole substrate. Therefore, the advantage of the high affinity PNP removal by this strain probably depends on its growth on other cosubstrates. The O<sub>2</sub>/PNP stoichiometry indicates the consumption of 96 g (3 mol) of molecular oxygen for the oxidation of 139 g (1 mol) of PNP. This result implies that *B. sphaericus* JS905 degraded PNP through the benzenetriol pathway.

Recently, three bacterial species, *Ralstonia* sp. SJ98, *Arthrobacter protophormiae* RKJ100, and *Burkholderia cepacia* RKJ200, have been examined for their efficiency and kinetics behavior toward PNP degradation (Bhushan *et al.*, 2000). In this study, the whole cell was used as an enzyme system for PNP degradation. Therefore, the Michaelis-Menten curves were adopted to describe the degradation. The apparent  $K_m$  values of PNP degradation by SJ98, RKJ100, and RHJ200 were 44.5, 38.9, and 32.0 mg, respectively. The maximum rates of PNP degradation ( $V_{max}$ ) were 1.6, 1.1, and 0.5 mg PNP degraded/min·mg dry biomass, respectively. In addition, the interpretation drawn from the Lineweaver-Burk plots indicated the effects of metabolic intermediates on the degradation. The plots showed that the PNP degradation by SJ98 was stimulated by 4-nitrocatechol and 1,2,4-benzenetriol. PNP degradation by RKJ200 was inhibited by benzoquinone and hydroquinone in a noncompetitive and competitive manner, respectively. PNP degradation by RHJ200 was uncompetitively inhibited by benzoquinone and hydroquinone.  $\beta$ -Ketoacid did not affect the rate of PNP degradation in any case.

The limited literature database on PNP degradation kinetics does not allow an extensive comparison of the measured kinetic parameters. Nevertheless, these studies did present some quantitative information which is crucial to predict or guide the application of pure cultures in actual bioremediation or treatment practice.

### 2.1.3 Biological Treatment of PNP-Containing Wastewater

Due to the frequent use of PNP as an important intermediate in the production of pesticides, dyes, and pharmaceuticals, PNP can be found as a pollutant in industrial wastewater streams associated with its formulation, distribution and application. PNP can also be released into the subsurface and contaminate groundwater resources as a result of hydrolysis of pesticides and herbicides. The commercial and effective treatment technology, such as biodegradation and bioremediation, should therefore be implemented to eliminate the remaining PNP in industrial wastewater and groundwater. However, the toxicity of PNP to microorganisms, together with its recalcitrant nature, is the biggest challenge for effective biological treatment. The feasibility of treating PNP containing wastewater with biological systems under anaerobic, combined anaerobic/aerobic, as well as aerobic processes has been investigated (Table 2.5).

- PNP Biodegradation with Anaerobic Processes

Under anaerobic conditions, PNP is easily reduced to PAP by microorganisms. The reduced product, PAP, was believed to be less toxic by several orders of magnitude than its parent compound PNP (Donlon *et al.*, 1996). It is based on this detoxification mechanism that the treatment of PNP containing wastewater was conducted with anaerobic processes.

High rate anaerobic wastewater treatment processes, such as upflow anaerobic sludge blanket (UASB) reactor have proven to be capable of treating various xenobiotic containing wastewaters with a high degree of efficiency and stability (Fang and Zhou, 1999). The feasibility of using UASB to treat highly toxic nitrophenolic compounds has been investigated under methanogenic (Donlon *et al.*, 1996; Karim and Gupta, 2001) and denitrifying conditions (Karim and Gupta, 2003).

**Table 2.5** Summary of biological systems treating PNP-containing wastewater.

Biomass	Reactor	Operational mode	HRT (h)	PNP loading (mg/l-d)	PNP removal efficiency (%)	Intermediate	Reference
<b>Anaerobic process</b>							
1	Granule sludge	UASB	Continuous	8	790	99	PAP Donlon <i>et al.</i> , 1996
2	Granular sludge	UASB	Continuous	12-30	1.6-60	99	PAP Karim and Gupta, 2001
3	Granular sludge	UASB	Continuous	24	30	90	PAP Karim and Gupta, 2003
4	Granular sludge	ABR	Continuous	249	0.97-67.9	79-99	PAP & phenol Kuscu and Sponza, 2005
<b>Combined anaerobic and aerobic process</b>							
1	Attached biofilm	SBR	Sequential batch	47-57	25	100	Nil Melgoza <i>et al.</i> , 2000
2	Attached biofilm	SBR	Sequential batch	14	1.9-122	100	Nil Melgoza and Biotron, 2001
3	Granular sludge & Activated sludge	AMBR CSTR	Continuous	166-249	3.85	95	Nil Sponza and Kuscu, 2005
<b>Aerobic process</b>							
1	Attached biofilm	Aeration tank	Batch mode	25	Up to 96	100	Nil Xing <i>et al.</i> , 1999
2	Attached biofilm	Aeration tank	Continuous	11	1600	100	Nil Bhatti <i>et al.</i> , 2002
3	Activated sludge	SBR	Sequential batch	16	0-477	100	Nil Tomei <i>et al.</i> , 2003
4	Activated sludge	SBR	Sequential batch	16	477-597	100	Nil Tomei <i>et al.</i> , 2004
5	Activated sludge	SBR	Sequential batch	14.4-16	134-533	100	Nil Tomei and Annesini, 2005

The granular sludge UASB reactors were able to anaerobically transform PNP under methanogenic conditions at concentrations which were much higher than those that normally caused severe inhibition. Donlon *et al.* (1996) reported that VFA removal efficiency was greater than 99% even at volumetric loading of PNP up to 790 mg/l·d and the substrates, such as hydrogen, butyrate, propionate, and ethanol, being able to provide interspecies reducing equivalents, were found able to stimulate nitrophenol reduction (Donlon *et al.*, 1996). Karim and Gupta (Karim and Gupta, 2001) studied the effects of hydraulic retention time (HRT) on the PNP removal with UASB and showed that HRT actually did not effect the overall removal of PNP, but methanogenic inhibition was observed at lower HRTs.

Karim and Gupta (2003) studied the effect of COD/NO<sub>3</sub><sup>-</sup>-N ratio on the biotransformation and removal of nitrophenols including PNP in bench scale UASB reactors under denitrifying condition. The results in this study revealed that the removal of nitrophenols increased with lowering of COD/NO<sub>3</sub><sup>-</sup>-N ratio. The PAP was found as the major intermediate metabolite of PNP.

Besides UASB, the feasibility of treating PNP containing wastewater was also studied in other high rate anaerobic reactors like, anaerobic baffled reactor (ABR) with PNP loading rates from 0.97 to 67.9 mg/l·d (Kuşçu and Sponza, 2005). The results in this study showed that besides PAP, phenol was also the product from PNP anaerobic transformation in ABR.

The results of these studies indicate that while anaerobic processes can efficiently convert PNP to other less toxic phenolic compounds, they cannot totally mineralize PNP to H<sub>2</sub>O, and CO<sub>2</sub>. To meet the needs for pollutant control, the anaerobic process can serve as a pretreatment which should be followed by an aerobic treatment process.

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- PNP Biodegradation in Combined Anaerobic and Aerobic Bioreactors

Although anaerobic transformation of PNP to PAP is facile, anaerobic consortia usually do not degrade PAP. This is due to the electron offering properties of the amino group which makes PAP more easily subject to oxidation under aerobic conditions. Recently, combined anaerobic and aerobic processes were proposed to treat the PNP-containing wastewater (Melgoza *et al.*, 2000; Melgoza and Buitron, 2001; Sponza and Kusçu, 2005). These combined processes can be obtained through either time or space orientated strategy.

The time-oriented process uses a SBR to provide an anaerobic and aerobic alteration. Recently, such time oriented combined process was proposed to treat the PNP containing wastewater (Melgoza and Buitron, 2001). A pilot biofilter which was packed with volcanic stones to support activated sludge attachment was used in this study. An initial PNP concentration of 25 - 35 mg/l was used for adapting the attached sludge for 75 days (25 cycles), and then the PNP concentration was gradually increased and finally maintained at 60 mg/l until the end of the experiment. The PNP removal efficiency was 99% during the experiment and the cycle time for SBR operation was gradually reduced. The SBR cycle time was finally reduced to 11.5 h (8 h for the anaerobic phase and 3.5 h for the aerobic one) after a 230-day operation. By using such a process, the PNP was transformed to PAP in the anaerobic phase with efficiencies near to 100% and the PAP was nearly 100% mineralized in oxidative stage. The reaction rates with this process were 16 mg PNP/l·h (PNP uptake), 14 mg PAP/l·h (PAP formation) and 20 mg/l·h (PAP mineralization).

The space-oriented combined process uses two different reactors to carry out the anaerobic PNP transformation and aerobic PAP mineralization, respectively. For example, recently Sponza and Kusçu (2005) reported a process in which PNP anaerobic reduction was carried out in an anaerobic migrating blanket reactor (AMBR) and the PAP oxidation was carried out in a completely stirred tank reactor (CSTR). The results in this study found that the efficiencies around 96% and 97%

could be achieved for total PNP and COD removal, respectively, at PNP loading rate of 3.85 mg/l·d.

The combined anaerobic and aerobic process offers a good option for treatment of PNP-containing wastewater. However, from a commercial application perspective, some challenging issues prevent the orientation of this process in real industrial applications. First of all, the alternation of anaerobic/aerobic phases raises capital and operating costs. Secondly, the operation and control of such reactors becomes complicated, and a highly skilled and experienced operator is needed. Thirdly, this process needs a longer start-up period, compared to most aerobic processes.

- PNP Biodegradation with Aerobic Processes

Whilst many studies have shown that PNP is readily mineralized by aerobic bacteria, only a few studies focused on the engineering aspects of aerobic treatment of PNP-containing wastewater. In most of these studies, immobilized cell technology (ICT) was adopted to remove PNP contaminants in aquatic waste streams.

Two interesting studies were conducted with immobilized pure bacterial cells to compare the performances of PNP degradation between immobilized and suspended biomass. In the first study, Cho *et al.* (2000) investigated the effect of biomass immobilization on PNP-degrading activity as well as on tolerance to PNP toxicity. PNP degradation by immobilized and suspended *Nocardioides* sp. NSP.41 was compared under various PNP concentrations. The results showed that the volumetric degradation rate and tolerance for PNP toxicity improved significantly as a result of biomass immobilization. On the other hand, the specific degradation rate declined with immobilization. The reduction in specific degradation rate appeared to be attributed to internal diffusion limitation in immobilized biomass. In addition, this study also investigated the effect of the presence of an alternative

toxic compound, phenol on the PNP degrading activity of immobilized and suspended biomass. High volumetric PNP and phenol degradation rates could be achieved with immobilized biomass but not with suspended biomass.

Besides the PNP-degrading activity and tolerance to toxicity, the response of the system to the disturbances is often of great interest in the real practice of wastewater treatment. In the second study, Ray *et al.*, (1999) studied the performance of the reactor after a sudden change of cultivation conditions such as media composition and pollution load. In this study, the biodegradation of PNP-containing wastewater was carried out in a continuously working aerobic solid-bed reactor. The results showed that the microorganisms immobilized in the biofilm responded less sensitively to disturbances than the suspended one did. The microorganisms fixed in the solid-bed reactor adapted almost without delay to changed cultivation conditions. Only when the change in conditions which resulted in a pollution load that exceeded the maximum capacity of the reactor was there a delay in the establishment of the new equilibrium. On the contrary, suspended bacteria cultivated in shaking flasks always showed a long lag phase when the PNP concentration was increased (Löser *et al.*, 1998).

These two studies clearly demonstrated that immobilized cells can potentially be applied for efficiently degrading PNP pollutants in industrial wastewater. Furthermore, in another study with pure bacterial cells, Heitkamp *et al.*, (1990) proved the feasibility of using immobilized cell technology for the removal of high concentrations of PNP from synthetic aqueous waste streams. In this study, 91%-99% removal of PNP could be achieved by immobilized bacteria at influent PNP concentrations of 1,200 to 1,800 mg/l under flow rates of 10 and 12 ml/min, respectively. Possible reasons for the effectiveness of immobilized cell systems in PNP degradation are postulated as follows:

- ICT offers a system with a high biomass density which in turn will increase the volumetric degradation rate as well as the tolerance to PNP

toxicity of the system (Cho *et al.*, 2000);

- In ICT systems, the pollutant is carried into the microbial consortia by diffusion (Ray *et al.*, 1999). Therefore, there is a concentration gradient of toxic substrate within the immobilized biomass. In this way, ICT offers a good protection mechanism to the microbial consortia that is exposed to the highly toxic substrate.
- ICT offers a compact living regime for bacteria with different metabolic capabilities, which may facilitate interactions between different bacteria. Consequently, the effective removal of complex chemical pollutant can be more easily achieved (Xing *et al.*, 1999).

All the studies mentioned above were conducted using pure bacteria cultures. In a real industrial practice, microbial communities consisting of mixed cultures, such as activated sludge are considered more favorable since these communities generally contain diverse and versatile metabolic capabilities which are impossible for pure bacterial cultures (Allison *et al.*, 2000). Therefore, activated sludge retained on various materials was adopted.

Xing *et al.* (1999) examined the microbial degradation of PNP by activated sludge retained on porous carrier particles. In this study, artificial wastewater containing PNP was used as the sole carbon source. The adaptation of microbes retained on porous carrier particles to PNP was faster than that of suspended microbes by more than 20 d. PNP was degraded completely by attached microbes without significant accumulation of intermediate metabolites. The PNP degradation activity of the retained microbes was more than twice higher than that of the suspended microbes.

In another study, Bhatti *et al.* (2002) demonstrated the use of inexpensive and durable nonwovens as a biomass retainer for PNP degradation. Results of the continuous flow experiments demonstrated that using the nonwovens could attain consistent high-rate PNP degradation. The highest specific and volumetric PNP

loading rates were determined to be 165 mg PNP/g MLSS·d and 1600 mg PNP/l·d, respectively.

Sequencing batch reactor (SBR) is a viable alternative for the treatment of xenobiotic compounds that are difficult to treat by traditional processes (Wilderer and McSwain, 2004). The variable nature of the SBR systems allows the manipulation of the selective pressure on the microorganisms so that the activity of the community can be dynamically adjusted to meet changing effluent conditions. In the studies reported recently, the feasibility of using SBRs for the efficient biodegradation of PNP was reported (Tomei *et al.*, 2003; 2004; Tomei and Annesini, 2005). The results from these studies indicated that the PNP degradation could be achieved at a higher concentration through adjusting the flexible operation of the SBRs. Another big achievement in treating PNP in SBRs is that an anoxic period could be integrated in the operation cycle to remove the nitrite released from PNP oxidation degradation.

#### 2.1.4 Factors Influencing PNP Biodegradation

Many factors affect the performance of PNP biodegradation and the more important ones will be discussed in this section.

- Inoculum Size

The inoculum size (number of cells) used in PNP biodegradation may affect the lag time, the rate and extent of degradation. Since practicality dictates that if the inoculum size is small, then the organism may fail to survive in initial biotic and/or abiotic stress. In that event, the target chemical would not be degraded. It is widely accepted that the smallest inoculum has the longest lag time (Chudoba *et al.*, 1992; Comeau *et al.*, 1993; Nishino and Spain, 1993; Thouand *et al.*, 1996). Thouand *et al.* (1996) further worked out an equational expression of the lag time to the bacterial density for using adapted *Pseudomonas putida* degrading PNP, as  $y = 3.5 - 5.7(\log_{10}X_0)$  ( $y$ , lag time [h];  $X_0$ , initial number of cells per liter). However, the

addition of inoculum does not always stimulate the PNP degradation (Zaidi *et al.*, 1996). Other factors, such as bacteria origin, the viable bacteria number, the adaptation of the bacteria to the target compound and the presence of protozoans, will also influence the degradation.

- Concentration of PNP

The toxicity of PNP to microorganisms is related to PNP's concentration. A high concentration of PNP can inhibit microbial growth and activity. For this reason, the concentration of PNP, especially the concentration at which PNP produces a toxic effect to the microorganisms, is considered as an important parameter influencing PNP biodegradation. Table 2.6 summarized the PNP concentrations at which microbial growth or activity was inhibited for the different microbial species reported in the literature. For example, the  $IC_{50}$  ( $IC_{50}$  is defined as the concentration of a target compound causing a 50% reduction in microbial activity (Grady *et al.*, 1999).) of PNP toward growth of two cyanobacteria, *Nostoc muscorum* and *Nostoc linckia* has been reported as 41 and 32 mg/l, respectively (Madhavi *et al.*, 1995). The  $EC_{50}$  ( $EC_{50}$  represents the concentration of a compound where 50% of its maximal effect is observed.) of PNP toward activated sludge microorganisms has been reported as 64 mg/l (Volskay and Grady, 1990). Kinetic parameters such as  $K_i$ , inhibitory coefficient of Haldane equation has also been used to quantify the concentration of PNP that caused inhibition effect in the bioreactors. For example, Tomei *et al.* (2003; 2004) found that the  $K_i$  values of activated sludge from SBR treating PNP containing wastewater were ranged from 12.0 to 30.7 mg/l. The highest PNP concentrations in bioreactors reported thus far were 600-700 mg/l which were found significantly toxic and completely suppressed microbial growth (Ray *et al.*, 1999; Bhatti *et al.*, 2002). Limited information in the literature as well as various measures used to indicate the PNP concentration does not allow a meaningful comparison of the PNP concentration among different microbial species. Nevertheless, the information presented herein did provide some quantitative guide that is crucial to PNP biodegradation and bioremediation practice.

**Table 2.6** The concentrations at which PNP produced toxic effect to microorganisms reported in the literature.

Microbial Species	Measures	PNP concentration (mg/l)	Reference
Pure bacterial culture <i>Nostoc muscorum</i> <i>Nostoc linckia</i>	IC <sub>50</sub>	41 32	Madhavi <i>et al.</i> , 1995
Activated sludge microorganisms	EC <sub>50</sub>	64	Volskay and Grady, 1990
Activated sludge	K <sub>i</sub>	12.0-30.7	Tomei <i>et al.</i> 2003; 2004
Attached biofilm	Completely suppressed microbial growth	600-700	Ray <i>et al.</i> , 1999; Bhatti <i>et al.</i> , 2002

- Dissolved Oxygen (DO) Concentration

The presence of molecular oxygen is important in aerobic PNP degradation. Equation 2.1 shows the stoichiometry of aerobic PNP mineralization calculated using half reactions. Theoretically, 1.55g O<sub>2</sub>/g PNP (6.5 mol O<sub>2</sub>/mol PNP) is needed to mineralize PNP. PNP degrading pathway studies have revealed that 64-96g (2-3 mol) oxygen is needed to degrade 139g (1 mol) PNP to *p*-nitrocatechol (PNC) (Kadiyala *et al.*, 1998). Löser *et al.* (1998) further worked out that the oxygen uptake coefficient Y (O<sub>2</sub>/PNP) is about 1.005g/g by *Pseudomonas putida* PNP1. Ray *et al.* (1999) found that the oxygen limitation in the biofilm was the main reason for incomplete PNP degradation when the reactor was subjected to high loading. Increasing the aeration rate will definitely increase the maximum degradation capacity of the reactor at the conditions when oxygen is limited.



- Second Carbon Source

Secondary compounds can influence PNP degradation. For example, Swindoll *et al.* (1988) found that alternative carbon sources, such as glucose and amino acids, decreased the rate of PNP mineralization by subsurface microbial communities (Herman and Costerton, 1993). On the contrary, it has been found that the addition of glucose (20 mg/l) to pure cultures of *Pseudomonas* sp strain K enhanced PNP (1 mg/l) degradation (Schmidt *et al.*, 1987) and the addition of amino acids (400 mg/l) to lake water samples also enhanced PNP degradation (Zaidi and Mehta, 1995). Interestingly enough, differences in the preferential utilization of PNP or alternative substrate by *Corynebacterium* Z-4 were detected depending on whether this strain was incubated in industrial wastewater or lake water sample.

- Nitrogen Source

Nitrite release is frequently observed during PNP biodegradation. Bacteria are able to use this released nitrite as a nitrogen source. Many studies based on pure culture experiments have shown that bacteria are able to use PNP as a sole nitrogen source (Spain and Gibson, 1991; Herman and Costerton, 1993; Ray *et al.*, 1999; Bhushan *et al.*, 2000). However, other nitrogen sources are usually preferred. This is because nitrite (or nitrate) needs to be assimilatively reduced to ammonium before it can be incorporated into cellular amino acids (Madigan and Martinko, 2006). The reduction of nitrite to ammonium requires energy, which is often limiting PNP degrading bacteria. Ammonium was preferred by *Pseudomonas putida* PNP1 as a nitrogen source (Löser *et al.*, 1998; Ray *et al.*, 1999). In addition, the presence of other nitrogen sources may significantly shorten the time for the degradation, even though it will not increase the efficiency of PNP degradation. In a starvation-survival study of PNP degrading bacteria, an *Actinomycetes* sp. showed a shorter time to reach the plateau of PNP mineralization in the presence of yeast extract (Herman and Costerton, 1993).

Until now there is no information on the utilization of PNP as nitrogen source by mixed culture. Therefore, no comparison is available to indicate the degradation performance with or without the presence of other nitrogen source in the bioreactor. However, in the PNP degradation bioreactors, ammonium was usually added as an easy-using nitrogen source for bacteria uptake (Xing *et al.*, 1999; Bhatti *et al.*, 2002).

## **2.2 BACKGROUND ON AEROBIC GRANULATION**

### **2.2.1 The Advances of Aerobic Granulation in Wastewater Treatment**

Although biological wastewater treatment practice has become more developed in the last century, the design of most treatment processes was based on meeting simple water quality criteria like biochemical oxygen demand (BOD) and chemical oxygen demand (COD). With the setting of more comprehensive water quality criteria, demand for more complex performance has required the modification of existing treatment processes and proliferation of novel treatment technology (Hallas and Heitkamp, 1995).

Aerobic granulation in SBRs is a novel biological wastewater treatment technology which depends on the physiological capabilities of aerobic granules to break down the undesired pollutants in wastewater (Morgenroth *et al.*, 1997; Beun *et al.*, 1999). Aerobic granules are self-immobilized microbial aggregates that are cultivated in sequencing batch reactors (SBRs) without reliance on artificial surfaces for biofilm attachment, hence rendering carrier material and bulky settling devices unnecessary (Tay *et al.*, 2005c).

The aerobic granulation was reported in early 1990's (Mishima and Nakamura, 1991; Shin *et al.*, 1992). The aerobic granulation reported therein employed either pure bacterial culture or pure oxygen in a continuously operated upflow sludge blanket reactor. In 1997, Morgenroth and his colleagues first

reported the successful cultivation of aerobic granules in a SBR using activated sludge as seeding materials (Morgenroth *et al.*, 1997). Since then, many studies have been conducted to investigate both basic and applied aspects of the novel technology, granulation in SBRs. In this section, previous studies on factors affecting aerobic granule cultivation, characteristics of aerobic granules, microbial communities within aerobic granules and exploitation of aerobic granules in biodegradation of toxic and recalcitrant chemicals.

- Primary Factors Influencing Aerobic Granule Cultivation

The concept of granules can be traced back to the 1970's and since then granules have been successfully cultivated in upflow anaerobic sludge blanket (UASB) bioreactors for the biological treatment of wastewaters. For many years, granule cultivation was commonly hypothesized to be dependent on specific syntrophic bacterial interactions in the methanogenic systems. However, with the observation of granule formation by acidifying, nitrifying, denitrifying, and aerobic heterotrophic bacteria, granule cultivation now is believed not to be restricted to the microbial populations in the methanogenic systems (Beun *et al.*, 1999).

Many research efforts have been carried out to cultivate granules in aerobic condition and to understand the granulation processes. The factors affecting the cultivation of aerobic granules were studied extensively in these pioneering studies. Based on the results from these studies, the factors affecting the aerobic granule cultivation can be divided into two categories; primary and secondary factors.

Beun *et al.* (1999) has proposed that sludge particles can be concomitantly present in bioreactors either in dense and compact forms as granules or in light and dispersed forms as suspended cells and flocs. Dense and compact sludge particles usually grow slower than light and dispersed sludge particles, because of a diffusion limitation in highly aggregated biomass. Dense and compact sludge particles therefore are usually competed-out by light and dispersed particles and cannot become the dominant forms of biomass in conventional bioreactors. However, if

light and dispersed sludge particles can be removed from bioreactors, the dense and compact sludge particles could hence accumulate and develop, and finally become the dominant form of biomass in bioreactors.

The basis for the aerobic granule cultivation is a repetitive selection in a SBR for sludge particles in such a way that denser and compact components are retained in the system while lighter and dispersed particles are washed out (Tay *et al.*, 2005c). The factors that have determinative effects on this selection process are therefore defined as primary factors which include reactor configuration and operation, settling time, cycling time, and hydrodynamic shear force.

### **Reactor Configuration and Operation**

The reactors used for aerobic granule cultivation were usually configured as column type reactors with relatively high H/D (height over diameter) ratio.

Column type reactors rather than rectangle tank reactors are usually used for cultivation of aerobic granules. The reasons can be attributed to the fact that unified upflow trajectory, hydrodynamic shear force and collisions generated in column-type reactor can encourage the microbial aggregation into granules. In contrast, the dispersed flow pattern generated in the rectangle tank will disperse the microbial aggregates and jeopardize the microbial aggregation into granules. Under such circumstance, only the flocs with irregular shape and size are present in the rectangle tank reactor (Liu and Tay, 2004).

A higher reactor favors the selection of granules from suspended cells and flocs than the shorter reactor with the same reactor diameter. This is because the higher reactor can provide sufficient settling length that allows the separation of granules and suspended cells and flocs based on their differences in settling velocity (Beun *et al.*, 1999). A higher reactor can also provide a smaller footprint, which is especially favorable to where territory is limited and precious.

The reactors used for granule cultivation were usually operated with sequencing batch mode in a regime of fill, react, settle, and withdraw. Such cycle

manipulation in sequencing batch reactor can easily create strong selective pressures through adjusting the reacting and settling time (Hallas and Heitkamp, 1995). Therefore, SBR is specifically tailored to the operational need for granulation.

### **Settling Time**

Settling time or the time allowed for sludge to settle in SBR cycle, is the main parameter for granules cultivation (Beun *et al.*, 2000; McSwain *et al.*, 2004; Qin *et al.*, 2004a; b). This is because the settling time acts as a major selection pressure exerted to granulation reactor. A short settling time favorably selects the dense granules with high settling velocity from the light floc with low settling velocity (Beun *et al.*, 1999). As a result, the granules could be kept in the reactor while the floc will be washed out from the reactors. Qin *et al.* (2004a) reported that aerobic granules could be successfully cultivated and become dominant only if the SBR was operated at a settling time of less than 5 minutes. While mixtures of aerobic granules and suspended sludge were observed in the SBRs which ran at a settling time of 20, 15 and 10 minutes, a similar observation was also reported in the study by McSwain *et al.* (2004) where only the reactor with 2 minutes settling time formed a completely granular sludge, but not the reactor with a 10-minute settling time. The short settling time results in the granular sludge having a higher extracellular protein (EP) content and a less diverse but more stable microbial community.

### **Cycle Time**

The cycle time affects granulation through two aspects: frequency of solid discharge and starvation period.

Cycle time determines the frequency of solid discharge through effluent withdrawal, when exchange ratio (exchange ratio in SBR is defined as the volume of effluent discharged over the working volume of the SBR) is fixed. A study on nitrifying bacteria granulation in SBR (Tay, 2002b) has shown that there is an optimal range of cycle time for granule cultivation. It was reported that no nitrifying granulation was observed in the SBR operated either at the longest cycle

time of 24 hours or shortest cycle time of 3 hours. Excellent nitrifying granules were developed in the reactor operated at cycle times of 6 or 12 hours.

Similarly, such optimal range also exists in other granulation systems, albeit the optimal range in other granulation system may not be necessarily the same as that in nitrifying bacterial granulation system. The underlying reasons could be that if the cycle time is greater than the optimal range, the growth of suspended flocs will overwhelm the granulation. On the other hand, if the SBR is running at an extremely short cycle time, the sludge loss due to hydraulic washout from the system could not be compensated by the growth of bacteria, thus results in a granulation failure. However, within the optimal range, a short cycle time should suppress the growth of suspended solids because of frequent washout of the suspended solids, and thus superior granules can be formed.

Cycle time also determines the length of starvation period. Starvation period may play an important role in granulation. Previous studies on the response of bacteria to starvation have demonstrated that, under starvation conditions, bacteria become more hydrophobic which, in turn, facilitated microbial adhesion and aggregation (Kjelleberg and Hermansson, 1984). In addition, cells in starved colonies were found to form connecting fibrils which, in turn, strengthened cell-to-cell interaction and communication (Varon and Choder, 2000). Consequently, starvation-induced changes in the characteristics of the cell surface seem to favor the formation of strong microbial aggregates. In the case of aerobic granulation, Tay *et al.* (2001b) proposed that the periodical starvation in the sequencing batch reactor (SBR) would play a role in triggering changes in the cell surface and metabolism and lead to stronger microbial aggregates. Under such circumstances, microbial aggregates would become increasingly dense with the cycling operation of SBR. Therefore, the microbial structure becomes more and more compact as the result of periodical starvation in SBR systems.

#### **Hydrodynamic Shear Force**

High hydrodynamic shear force favors the formation of granulation and the stability of granules (Tay *et al.*, 2001a). Tay *et al.* (2001a) pointed out that

hydrodynamic shear force in granulation reactors is from hydrodynamic turbulence caused by upflow aeration. Therefore, a superficial upflow air velocity can represent the measure of the hydrodynamic shear force. The results in their study showed that compact and regular aerobic granules were formed in the reactor with a superficial upflow air velocity higher than 1.2 cm/s. It was found that the shear force has a positive effect on the production of polysaccharide, biomass activity, and hydrophobicity of cell surface and specific gravity of granules, which was believed to have positive effects on the granules stability.

- Secondary Factors Influencing Aerobic Granule Cultivation

The secondary factors usually do not exert a strong selection pressure on granulation process, but will influence the characteristics of granules.

#### Characteristics of Seed Sludge

Previous studies have shown that aerobic granules can be cultivated with various seed sludge, including normal municipal activated sludge (Beun *et al.*, 1999), anaerobic sludge (Hu *et al.*, 2005a) or even bulky activated sludge (Peng *et al.*, 1999). This implies that the characteristics of seed sludge have little effects on granule cultivation. However, the characteristics of seed sludge such as the metabolic activity to the substrate, the activity to form aggregates, the hydrophobicity of sludge surface as well as the settleability of sludge will definitely influence the cultivation time for granules. Therefore, research efforts were conducted to select the better seed sludge to speed up the granulation process.

Acclimation of seed to the substrates being used in the reactor could shorten the granulation process. Jiang *et al.* (2002) reported that a two-month acclimation of seed to 50 – 500 mg/l phenol resulted in a very fast granule formation in the reactor (9 days from reactor start-up). Likewise, Tay *et al.* (2002c) reported the formation of nitrifying granules around 10 days after a 4-week pre-cultivation with ammonium.

The bioaugmentation of seed sludge with aggregation-accelerating pure cultures was able to enhance and shorten the granulation process. Ivanov *et al.* (2005b) recently reported a much faster granule cultivation (around 8 days) with a bioaugmented seed sludge with two pure bacterial cultures (*Klebsiella pneumoniae* strain B and *Pseudomonas veronii* strain F) with high self-aggregation and coaggregation ability. The bioaugmentation of seed sludge with enrichment cultures possessing high cell surface hydrophobicity was also able to speed up the granule cultivation (Ivanov *et al.*, 2005a).

Good settleability of sludge can speed up the granule cultivation process as well. Recently, Tay *et al.* (2005a) reported that acetate-fed granules with good settleability can replace activated sludge to be used as the seed sludge for cultivating phenol-degrading granules. The stable phenol-degrading granules were cultivated with acetate-fed granules as a starter culture within 1 week after initiating the reactor, which was 2 months faster than cultivation with activated sludge as a starter culture (Jiang *et al.*, 2002).

### **Characteristics of Wastewater**

**Substrate composition:** It seems that aerobic granules can be cultivated with a wide spectrum of feeding compounds. Granulation with organic substrates, such as molasses (Morgenroth *et al.*, 1997), glucose (Moy *et al.*, 2002), acetate (Peng *et al.*, 1999; Tay *et al.*, 2001a), and ethanol (Beun *et al.*, 1999) has been extensively reported. Recently, the granulation with ammonium and inorganic carbon (Tay *et al.*, 2002b) as well as with high concentrations of toxic phenol (Jiang *et al.*, 2002) and recalcitrant *tert*-butyl alcohol (TBA) (Tay *et al.*, 2005c) as sole source of growth and energy has also been reported.

**Substrate strength:** Beun *et al.* (1999) studied the formation of granules with COD loading ranging from 2.5 to 7.5 kg COD/m<sup>3</sup>·d. The results revealed that the COD loading did not seem to have a direct effect on granulation within the range tested. However, different COD loading influenced the final appearance of granules.

**Substrate N/C ratio:** Aerobic granules can form in a wide range of substrate N/C ratio (mass/mass) from 5/100 to 30/100 (Liu *et al.*, 2003). The formation of aerobic granules seemed insensitive to the substrate N/C ratio in the range studied. However, different N/C ratio would affect the granule formation in many aspects, such as elemental composition, microbial distribution and characteristics of aerobic granules. It was found that the substrate N/C ratio was closely related with the cellular N/C ratio, and a high N/C ratio would induce superior activity of nitrifying bacteria. In addition, a high N/C ratio was found to stimulate high cell surface hydrophobicity which resulted in a more compact and stable microbial structure of aerobic granules.

#### **Cell Surface Hydrophobicity**

Cell surface hydrophobicity is an important factor involved in microbial aggregation. Wilschut and Hoekstra (1984) proposed that the strong repulsive hydration interaction was the main force keeping the cells apart, and when bacterial surfaces were strongly hydrophobic, irreversible adhesion would occur. Tay *et al.* (2002a; 2002b) proposed that from the view point of thermodynamics theory, an increase of the cell hydrophobicity would cause a corresponding decrease in the excess Gibbs energy of the surface, which promoted cell-to-cell interaction and further served as a driving force for bacteria to self-aggregate out of the liquid phase. Therefore, a high cell surface hydrophobicity would result in a dense and stable granule structure.

#### **Extracellular Polymeric Substances (ECPs)**

ECPs consist of extracellular polysaccharides (EPSs), extracellular protein (EPs) and any polymeric substances from biological origins and generally are a major component of sludge flocs, biofilm and granules (McSwain *et al.*, 2005). ECPs can mediate both cohesion and adhesion of cells, and play an important role in maintaining the structural integrity of granular matrix (Flemming *et al.*, 2000). Tay *et al.* (2001b) proposed that the polysaccharides in ECPs can assist cell-to-cell attachment and induce the formation of initial microbial aggregation. McSwain *et al.* (2005) found that granules contained a large amount of protein in the ECPs' content, and furthermore this large amount of protein was found mainly distributed

in the core area of aerobic granules, which implies that granule formation and stabilization are dependent on this noncellular protein core (McSwain *et al.*, 2005).

### **Calcium Cation**

Divalent metal ions such as  $\text{Ca}^{2+}$  are known as playing an important role in microbial aggregation of biomass.  $\text{Ca}^{2+}$  may function by bridging negatively charged sites on extracellular biopolymers, or acting as a constituent of the extracellular polymeric matrix (Morgan *et al.*, 1990).

It has been reported that  $\text{Ca}^{2+}$  addition stimulates the formation of anaerobic granules and acidogenic biofilm (Huang and Pinder, 1995). Recently, the effect of  $\text{Ca}^{2+}$  on aerobic granulation was studied by Jiang *et al.* (2003) who found that augmentation with 100 mg/l  $\text{Ca}^{2+}$  significantly decreased the time to cultivate aerobic granules. In addition, the granules with  $\text{Ca}^{2+}$  augmentation exhibit better physical characteristics, and demonstrated better settleability and higher strength than granules with no  $\text{Ca}^{2+}$  augmentation.

- Mechanisms on Granulation Process

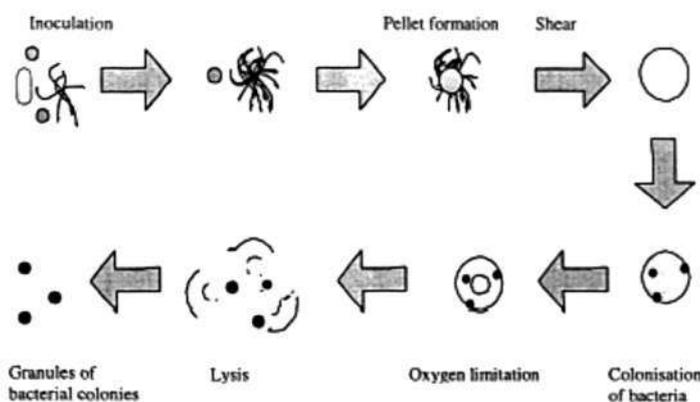
### **Earlier Hypothesis for Granulation Process**

Beun *et al.* (1999) used microscopic observations to formulate a hypothesis for the granulation process (Figure 2.7). After inoculation, filamentous fungal pellets dominated the reactor. These pellets functioned as an immobilization matrix in which bacteria could grow into colonies. After some time, the fungal pellets fell apart due to lysis in the inner part of the pellets. The bacterial colonies could then remain in the reactor because they were large enough to settle sufficiently fast. These colonies further grew into granules.

### **A Four-Step Model for Granulation Process**

Liu and Tay (2002) proposed a 4-step model for aerobic granulation. First step was physical movement to initiate bacterium-to-bacterium contact. Second step was stabilization of the multi-cell contacts resulting from the initial attractive forces. Third step was maturation of cell aggregation. Forth step was shaping of the steady state three-dimensional structure of microbial aggregate. Factors

involved in each step of granulation were proposed in the study (Table 2.7). Among these factors, cell surface hydrophobicity was believed play an important role in the initiation of aerobic granulation (Liu *et al.*, 2003) and ECPs was important to maintain the structural integrity in a community of immobilized cells (Tay *et al.*, 2001C).



**Figure 2.7** Proposed mechanism of aerobic granulation in a SBR reactor (Beun *et al.*, 1999).

### **Process of Granulation**

Tay *et al.* (2001b) based on a microscopic observation, reported that aerobic granulation was a gradual process involving the progression from seed sludge to compact aggregates, further to granular sludge and finally to mature granules. Seed sludge was observed having a loose and irregular structure, dominated by filamentous bacteria. After a week operation, compact aggregates appeared in granulation reactors. After two-week operation, the granular sludge with clear boundary and round shape was formed in reactors. After operation for 3 weeks, aerobic granules matured in reactors. At this stage, granules had a very regular round-shaped outer surface.

**Table 2.7** Factors involved in four-step model for granulation process (Liu and Tay, 2002).

Step	Factors
1	hydrodynamics, diffusion mass transfer, gravity, thermodynamic effects, and cell mobility
2	Physical attractive forces, such as Van der Waals forces, opposite charge attraction, thermodynamically driven reduction of the surface free energy, surface tension, hydrophobicity, and filamentous bacteria that can bridge individual cells; Chemical attractive forces, and Biochemical forces, such as cell surface dehydration, cell membrane fusion, signaling, and collective action in bacterial community
3	Production of extracellular polymer, growth of cellular clusters, metabolic change, environment-induced genetic effects that facilitate the cell-cell interaction and result in a highly organized microbial structure.
4	Hydrodynamic shear forces.

Su and Yu (2005) based on the macro-aspect observation and analysis, proposed a four-phase granulation process which included acclimating, shaping, developing, and maturing. This four-phase process could be divided by three turning points, granules initiated, shaped and matured. The first phase was the acclimating period that was from sludge inoculation to the first turning point. During this phase, sludge flocs in seed sludge aggregates and formed denser and irregular aggregates. Small granules appeared in this phase and the settling velocity and specific gravity of the sludge did not change significantly. The second phase was the shaping period which was defined as the process of change from dense and irregular shaped aggregates to regular granules. Size and the settling velocity of the sludge increased slowly, but specific gravity increased significantly. The third

phase was the developing phase that was the conversion process from the shaped granules to matured ones. Granules in this period grew drastically with a high specific growth rate by diameter of 0.12 /d. The final phase was the matured phase that was defined as the period after the granules became matured when the specific growth rate by diameter declined and the settling velocity and specific gravity were kept as a relatively stable value.

- Characteristics of Aerobic Granules

The conventional activated sludge process may encounter problems arising from poor biomass quality. Treatment efficiency may deteriorate as a result of dispersed growth, pinpoint flocs, rising sludge, non-filamentous bulking or filamentous bulking/foaming (Bitton, 1994). Compared with conventional activated sludge, aerobic granules possess several quality characteristics. Table 2.8 lists the characteristics of aerobic granules summarized from a recently published review article on aerobic granules (Liu and Tay, 2004). First of all, granules have regular spherical shape with defined outer layer and can be visible as separate entities in the mixed liquor during reaction and settling operation phase in SBRs. Secondly, granules have denser, stronger and compact microbial structure, which determines the outstanding settleability and strength of granules. Thirdly, granules have high biomass retention which was reported ranging from 6 to 12 g/l which is significantly more than 0.5 to 2 g/l in activated sludge systems. Fourthly, granules can tolerance high organic loading rates and are resilient to high chemical toxicity. Finally, granules can store up to several weeks without loss of integrity and metabolic potential. Considering these quality characteristics of granules, aerobic granule system can provide a higher volumetric conversion capacity and render the external settling devices small in size. With these advantages, aerobic granule system can have enormous potentials for widespread application and could revolutionize the wastewater treatment industry.

**Table 2.8** Comparison of characteristics of aerobic granules to activated sludges.

Characteristics	Aerobic granules	Activated sludges	Reference
Morphology	Spherical shape with defined outside layer	Amorphous shape and outer layer	Liu and Tay, 2004
	Separate entities in the mixed liquor		Liu and Tay, 2004
Size	0.2-5 mm	< 0.2 mm	Liu and Tay, 2004
Compactness & settleability	SVI < 50 ml/g	SVI $\approx$ 80-200 ml/g	Liu and Tay, 2004
Settling velocity	30-70 m/h	8-10 m/h	Liu and Tay, 2004
Specific gravity	1.004-1.065	Slightly >1.000	Liu and Tay, 2004
Physical strength	> 95%	NA	Liu and Tay, 2004
Biomass retention	6 – 12 g/l	0.5 – 2 g/l	Liu and Tay, 2004
Biomass production	3-fold less than activated sludge	NA	Liu <i>et al.</i> , 2005b
Storage stability	> 7 weeks	NA	Liu and Tay, 2004
Chemical toxicity resilience	Can degrade phenol at levels (1 - 2.5 kg phenol/m <sup>3</sup> ·d) that were known to cause the breakdown of activated sludge system		Tay <i>et al.</i> , 2005c
External settler	Smaller in size	Bulky in size	De Bruin <i>et al.</i> , 2004

NA: not applicable

#### ▪ Questions to Be Addressed in Future Studies on Aerobic Granulation

Until now, aerobic granulation has been received many research interests. Knowledge and understanding on the aerobic granulation imply the prospect for deploying aerobic granulation as a better option than conventional activated sludge

systems with higher treatment efficiency and smaller footprint in wastewater treatment practice (de Bruin *et al.*, 2004). However, many questions and potential drawbacks still need to be addressed before the widespread application of this technology in water treatment industry.

Firstly, many lab-scale experiments have shown that aerobic granulation in a SBR requires hydraulic selection pressures such as short settling and discharge time in reactor operation (Qin *et al.*, 2004a; b). However, providing such hydraulic selection pressures for aerobic granulation may be challenging in a pilot-scale or full-scale SBR, since high energy consumption for pump-equipment operation is necessary to ensure discharging a huge amount of treated effluent within a short period. The concern on energy consumed for maintaining such hydraulic selection pressures needs to be properly addressed.

Secondly, aeration has been identified as a key operation for cultivation and maintenance of aerobic granules in SBRs (Beun *et al.*, 1999; Liu and Tay, 2002). Aeration is believed executing three important functions for aerobic granulation in SBRs, such as providing a pneumatic agitation for the content in the bioreactors, providing oxygen demanded for oxidation of organic carbon and providing hydrodynamic shear force to shape the form of granules (Liu and Tay, 2004). High aeration rates were usually employed in granulation SBR systems. However, high aeration rate may lead to high energy consumption which can increase the operation cost for granulation system.

Thirdly, previous studies have shown that aerobic granulation process can be deployed to handle challenging requirement in wastewater treatment practice, such as reclaiming industrial wastewater containing recalcitrant or toxic organic compounds (Jiang *et al.*, 2002; Zhuang *et al.*, 2005), and treating wastewater containing both organic carbon and nutrient (Yang, *et al.*, 2003). However, a start-up period of several weeks or longer time was usually compulsory to launch the granulation system from seeding activated sludge. Such long start-up period may pose a problem in deploying aerobic granulation for pilot-scale or full-scale

industrial application. Up to date, not much is known about how start-up time are controlled in aerobic granulation system, although the presence of divalent cations has been shown to significantly reduce the time needed to cultivate aerobic granules (Jiang, *et al.*, 2003).

Finally, aerobic granulation has almost been successfully cultivated in sequential mode bioreactors. Although granulation in SBRs may offer many advantages, a continuous process in real application is more favorable for treating a large amount of wastewater as being able to avoid bulky storage tank. There have been a few studies reported the aerobic granulation in continuous mode aerobic upflow reactors (Mishima and Nakamura, 1991; Shin *et al.*, 1992; Tsuneda *et al.*, 2003). However, special conditions such as pure bacterial culture as seeding sludge, pure oxygen, or long cultivation period (over 200 days) were employed to cultivate aerobic granules, which seems not applicable for pilot-scale or full scale industrial application.

It needs to point it out that more and more research efforts have been directed to the questions and concerns addressed above. For example, Jiang *et al.* (2006) reported that addition of coaggregation bacteria could enhance granulation process and lessen the requirement for short settling time and withdraw time. Liu and Tay (2006) reported that reduction of aeration rate in famine period of a SBR cycle could lessen the energy consumption in granulation SBRs. Several recent studies reported that start-up time for granulation system could be shortened from several weeks to a couple of days by inoculating granules as seeding sludge (Tay *et al.*, 2005a) or introducing of auto-aggregation bacteria (Ivanov, *et al.*, 2005b). Up to date, cultivation of aerobic granules in a continuous mode bioreactor still remains a challenging topic in the area of granulation research (Liu and Tay, 2004).

### **2.2.2 Microbial Community Studies on Aerobic Granules**

In the last decade, the microbial communities in sludge flocs have been reasonably well described, and the knowledge gained from these studies has

assisted in many ways to improve the system design and performance (Wagner *et al.*, 1994; Watanabe *et al.*, 1999). Aerobic granules can be regarded as a special form of biofilm but without carriers for biofilm attachment (Liu and Tay, 2004; Wilderer and McSwain, 2004; Tay *et al.*, 2005c). Microbial communities in biofilms have been known to be highly distinct from the suspended biomass, even within a single reactor system, because of the difference in the growth environment for biofilm communities and planktonic communities (Davey and O'Toole, 2000; Briones and Raskin, 2003). An in-depth knowledge of the microbial communities residing in aerobic granules is therefore of great benefit in enhancing the design and performance of granulation bioreactors. To date, studies on the microbial communities in aerobic granules have provided interesting insights into this novel technology, albeit such studies are still limited.

The studies on microbial communities can be subcategorized into three areas; microbial diversity, function, and microstructure of aerobic granules.

- Microbial Diversity and Function of Aerobic Granules

#### **Microbial Diversity**

During the last twenty years, the applications of molecular tools in wastewater microbiology have revolutionized our understanding of the microbial communities of these systems (Amann *et al.*, 1995; Head *et al.*, 1998). In addition, cultivation-based methods such as isolation are not supplanted because they could provide better understanding of physiology and the function of microbial communities (Briones and Raskin, 2003). Both molecular tools as well as cultivation-based methods were used for investigating the microbial communities within aerobic granules.

Extraction of DNA from the granules and then performing a DGGE analysis of amplified 16S rRNA gene fragment was frequently used for studying the composition and diversities of microbial communities in aerobic granules (Jiang *et al.*, 2004; McSwain *et al.*, 2004; Zhuang *et al.*, 2005; Tay *et al.*, 2005c). The findings in these studies showed that bacterial populations in aerobic granules

usually have lower diversity than the sludge flocs. In addition, the composition of bacterial populations was very stable during a 27-day reactor operation in stabilized TBA(turt butyl alcohol)-degrading aerobic granules (Tay *et al.*, 2005c). Such low diversity and high stability of microbial communities in aerobic granules can be attributed to the selection pressures exerted by the SBR operation or the specificity required for substrate degradation, which would favor the enrichment of certain bacterial species to the detriment of others.

Semi-quantified method using PCR, cloning and sequence analysis of partial 16S rRNA gene of dominant bacteria was also carried out to study the changes in bacterial diversity and composition of microbial communities in young, mature and old glucose-fed granules (Yi *et al.*, 2003). The composition and relative abundance of bacterial populations were compared among the granules at different living stage. The young granules were found containing higher bacterial diversity than mature granules. The strictly anaerobic bacteria were found in large size old granules where oxygen transfer limitation could occur in the inner layer of large size aerobic granules (Tay *et al.*, 2002).

In the traditional culture-base method, isolation was also used for detecting the microbial communities in aerobic granules. Jiang *et al.* (2004) reported a collection of ten pure bacterial cultures which represented the major functionally important bacterial populations from phenol-degrading aerobic granules (Jiang *et al.*, 2004).

#### **Complementary Functional Roles of Bacterial Populations in Aerobic Granules**

Gaining an understanding of the functions of microbial communities is important because population diversity alone does not drive ecosystem stability. This can be aided by the isolation and characterization of functionally important bacterial population.

Recently, Jiang *et al.* (2004) studied the phenol degradation and aggregation abilities of dominant bacterial strains isolated from phenol degrading aerobic granules and a trade-off in the two functions was found to exist in the bacterial

isolates. Among ten bacterial isolates, none of the isolates had both a high growth rate or high phenol degradation rate and high autoaggregation activity. Based on the finding on this trade-off, a simple functional model of the microbial community within the aerobic granules was proposed. Phenol degradation and structure stabilization are two basic functions in the aerobic granules. The functional microorganisms within the aerobic granules generally fall into two groups. One group of microorganisms is mainly responsible for phenol degradation while the other group is mainly responsible for maintaining granule structure. At the same time, there may be some functional overlaps as some microorganisms may demonstrate modest levels of both functions. All these microbial groups cooperate with each other to ensure the formation and stability of aerobic granules exposed to high phenol concentrations in the bulk milieu. Within the granules, the bacteria will distribute and organize themselves to best meet the needs for each other and the community.

This kind of model has important implications for system management and can provide a basis for a more knowledge-driven approach to improve the design of granule-based systems for practical application.

- Structure of Aerobic Granules

**Three-Level Structure**

Peng *et al.*, (1999) reported the three-level structure of aerobic granules. The granules were cultivated in a SBR in which a synthetic wastewater containing sodium acetate was fed as a carbon and energy source. Microscopic examination showed that the granules were organized in three levels:

- The lowest level was the individual particles, including living cells, lysed decaying cells, non-biodegradable cell debris, and influent solids and of the order of 0.5-5  $\mu\text{m}$ .
- The secondary level was microcolonies, which consisted of aggregates of individual particles that were encapsulated in a clearly defined polymer matrix. It was believed that within this microcolony matrix, the

particles were held in constant positions stabilized relative to one another. These microcolonies were of the order of 5-50  $\mu\text{m}$ .

- The highest level of structure was the granule which was made up of numerous individual particles and microcolonies enmeshed in exopolymers. The size of granules in this study was about 0.3-0.5 mm.

### **Layered Structure**

A three-layer structure was found in glucose-fed granules using fluorescence in situ hybridization (FISH) combined with scanning confocal laser microscopy (SCLM) (Tay *et al.*, 2002a).

- The aerobic layer in granules was detected by the presence of strictly aerobic ammonium-oxidizing bacteria *Nitrosomonas* spp. This layer was situated at a depth of 70-100  $\mu\text{m}$  from the edge of granules.
- An anaerobic layer was detected by the presence of obligately anaerobic bacteria *Bacteroides* spp. This layer was situated at a depth of 800-900  $\mu\text{m}$  from the surface of granules.
- A dead cell layer was detected at a depth of 800-1000  $\mu\text{m}$  from the surface of the granules.

Channels and pores were detected by penetrating 0.1  $\mu\text{m}$  fluorescent microsphere standards into granules. The results showed that the most channels in aerobic granules can extend to 300-500 $\mu\text{m}$  depth. A dense polysaccharide layer was found at a depth of 400 $\mu\text{m}$  from the surface. This may imply that it is the accumulation of polysaccharide at this depth that causes the channel blocking in granules.

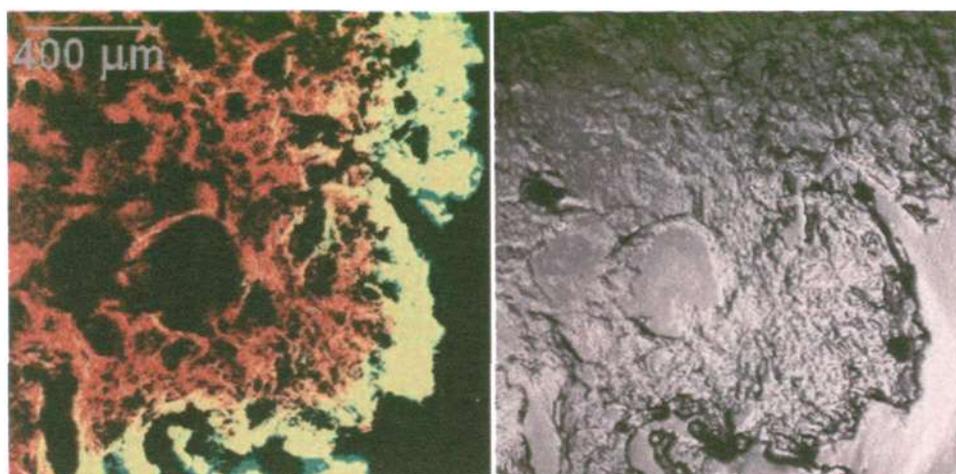
The layered structure was proposed for development due to its diffusion limitation. Since the oxygen and substrate in wastewater can only diffuse for a certain distance, strictly aerobic microbes can stay at the outer layer of the granules. The presence of channels and pores can overcome a part of diffusion limitation. However, when these channels and pores are blocked by polysaccharides, a strictly

anaerobic microbial layer can be formed. When the channels and pores are completely clogged, the dead cells dominate the center core.

**Other Studies Related to the Structure of Granules**

***Distribution of Viable Cells in Granules of Different Sizes:*** The viability of microflora residing in the aerobic granules was analyzed using in situ membrane permeable and non-permeable DNA stains Syto 9 and Propidium iodide (PI), respectively (Toh *et al.*, 2003). In this study, viability distributions in different size-categorized granules were assessed. The preliminary findings revealed that the distribution of biomass in the granules of all sizes followed a common pattern. The biomass was densest in the outer zone; and, beneath this zone, the biomass was greatly reduced, with clusters of biomass scattering evenly across the inner region. In relation to the distribution of live and dead cells (staining by Syto 9 and PI respectively), it was found that biomass in the peripheral zone was stained by both Syto 9 and PI, whereas biomass in the core region of the granules was only stained by PI (Figure 2.8). In addition, there were numerous patches in the granules that were not stained by either PI or Syto 9. Under the light transmission channel (Figure 2.8), the non-stained locations could be identified as void spaces or non-biomass materials.

The distribution of both live and dead cells was evaluated by drawing a line from the periphery towards the core across the section. Figure 2.9 presents the distribution of live and dead cells in various size-ranged granules. The distribution patterns clearly demonstrated that live biomass converged only at a certain depth below the surface of the granules, regardless of their increasing diameter; and this proved that there was mass transport limitation in the growing granules.

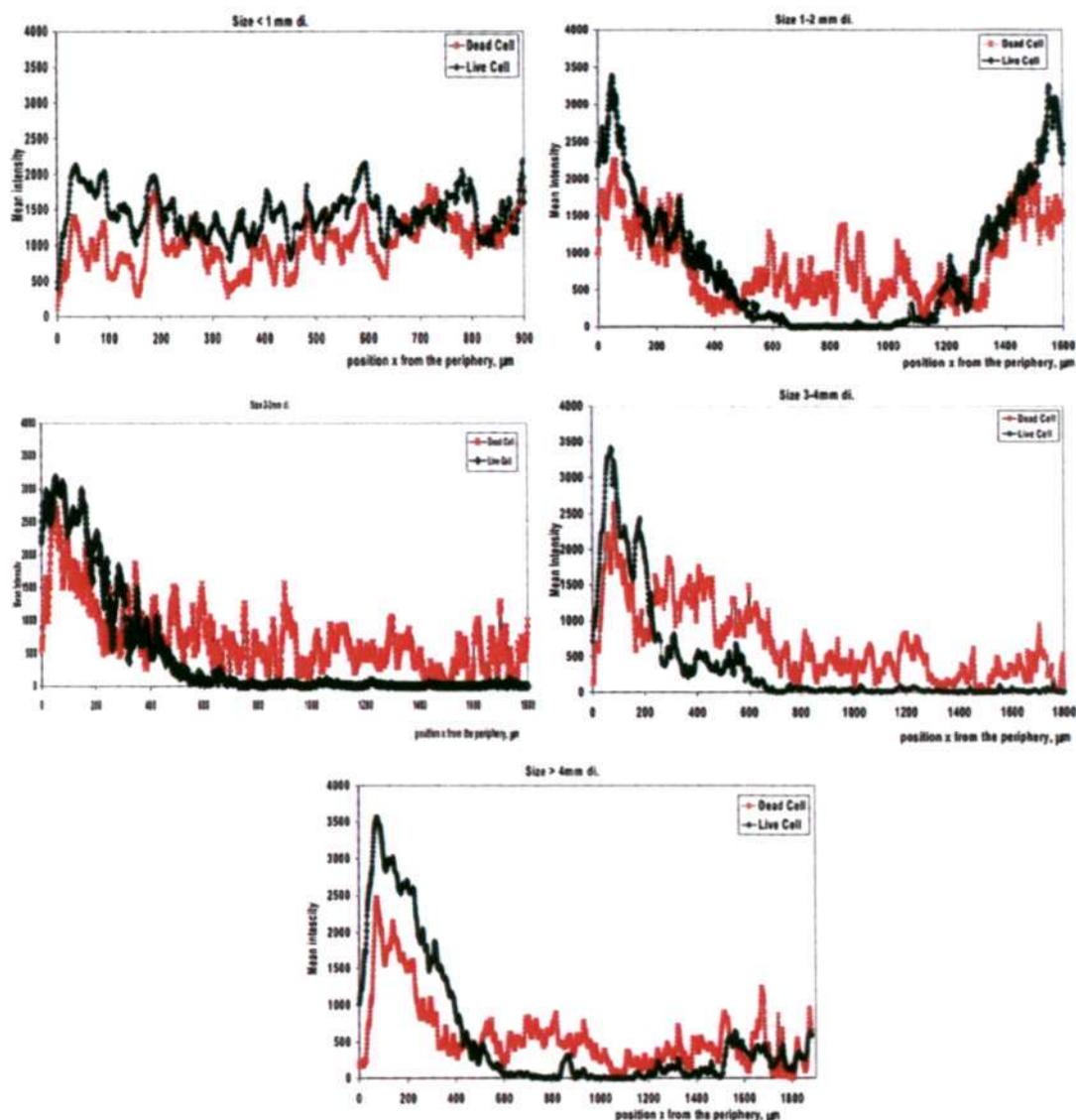


**Figure 2.8** Confocal laser scanning micrographs of a cross-section through an aerobic granule. The live biomass stained with Syto 9 produced a green fluorescence, whereas dead biomass stained with PI produced a red fluorescence. The light transmission micrographs show the unstained portions as non-biomass or voids (Toh *et al.*, 2003).

***Distribution of Extracellular Polymeric Substances (EPS) in Aerobic Granules:*** Three fluorescent staining dyes were used to visualize the distribution of EPS in aerobic granules (McSwain *et al.*, 2005). Fluorescein-isothiocyanate (FITC) (0.01%), Concanavalin A (ConA) lectin conjugated with Texas Red, and Syto 63 were used to stain proteins, sugar residues and cells respectively. The results indicated that the center of the granules was mostly comprised of noncellular proteins and the outer edge of granules was mostly comprised of cells and polysaccharides.

### **2.2.3 Exploitation of Aerobic Granules in Biodegradation of Toxic and Recalcitrant Organic Chemicals**

The efficiency of biological wastewater treatment depends, firstly, upon the selection and growth of metabolically capable microorganisms and secondly, upon



**Figure 2.9** Distribution of live and dead cells within a cross-section of different size-categorized granules from a SBR. Y-axis: Average mean intensity, as brightness of fluorescence per pixel. X-axis: Position of cell within granule, as distance from surface of granules. di: Diameter of granule (Toh *et al.*, 2003).

the efficient separation of those organisms from the treated effluent (McSwain et al., 2005). The efficiency of biological wastewater treatment can be improved by using aerobic granulation, since aerobic granulation technology can offer higher volumetric conversion rate and effective separation of biomass from treated effluent. Therefore, the footprint of granulation system will be reduced significant. Other advantages from such a process include no need for a carrier, which therefore reduces the cost of capital and disposal of biomass and tolerance for chemical toxicity etc. Granulation coupling with the characterized flexible operation of SBR ensures that granulation technology could have a broad application in wastewater treatment practice. Currently, several directions for deploying this technology in wastewater treatment practice have been reported, such as treating high-strength organic wastewater, simultaneous organic and nitrogen removal, phosphorous removal, biosorption of heavy metal by aerobic granules, as well as treating industrial wastewater. In this section, exploitation of aerobic granulation technology in biodegradation of toxic and recalcitrant organic chemicals is reviewed (Table 2.9).

Since the industrial wastewater usually contains specific chemical contamination, biological industrial wastewater treatment technology therefore requires that the biomass possess specific chemical degradation capability which is different from the request that only demand for total COD removal in most municipal wastewater treatment practices.

- Exploitation of Aerobic Granules in Biodegradation of Toxic Organic Chemical, Phenol

It is reported that aerobic granules can be used to treat phenol-containing wastewater (Jiang *et al.*, 2002).

#### **Cultivation of Granules with Phenol as Sole Carbon Source**

Granules can be cultivated using phenol as sole carbon and energy source, and the characteristics of phenol-degrading granules were comparable to the

granules formed with non-toxic substrates. The mean size of granules was about 0.52 mm, and SVI is 40 ml/g. The phenol removal efficiency was more than 99%. The activity of granules could be described by SOUR, which is about 110 mg DO/g VSS·h.

**Table 2.9** Summary of granulation systems that were deployed for biodegradation of toxic and recalcitrant organic chemicals.

	1	2
Substrate	Phenol	TBA
Reactor configuration		
Diameter (cm)	5	10
Working volume (l)	2	2
Operational condition		
Target compound loading rate (mg/l·d)	1500	100 to 600
HRT (h)	8	6-48
Influent filling time (min)	2	5
Settling time (min)	5-30	20-30
Effluent withdraw time (min)	3	5
Airflow rate (l/min)	3.5	0.4-05
Characteristics of granules at steady state		
Size (mm)	0.35-0.6mm	0.32
SVI (mg/l)	40	57
SOUR (mg DO/g VSS·h)	110	NA
Degradation rate (mg target compound/VSS·h)	NA	8.7
Kinetics		
$V_{max}$ (mg target compound/g VSS·h)	233 ± 17	18.2 ± 1.2
$K_s$	481 ± 30	47.4 ± 7.3
$K_i$	212 ± 34	1861 ± 297
Reference	Jiang <i>et al.</i> , 2002	Zhuang <i>et al.</i> , 2005

### **Biokinetic Parameters**

Biokinetic parameters are very crucial for real application of aerobic granules to remove phenol and toxic chemicals in industrial wastewater. In this

study, phenol-degrading granules were examined for their efficiency and kinetics behavior toward phenol degradation. A non-growth kinetic study was conducted in a batch study. The resulting data was properly described by the Haldane equation and the calculated kinetic parameters are  $V_{max}=5.6$  g phenol/g VSS·d,  $K_s=481$  mg/l and  $K_i=213$  mg/l.

### **Aerobic Granulation, a Good Immobilized Biomass System**

Microbial degradation of phenol is often hindered by toxicity effects exerted at high concentrations. The aggregation of microbial cells into compact granules likely served as an effective protection against the high phenol concentrations used in the study. Such protection of cells from inhibitory chemicals generally exists in all environments in which forms of immobilization or cell adhesion are present. Some underlying mechanisms of such protection were summarized. Firstly, the concentration gradient that normally develops because of diffusion resistance can protect the microorganisms by diluting the toxic chemical below some threshold value to avoid inhibition and allow continued microbial activity and substrate utilization. In addition to inhibition of diffusion, other possible explanations that have been proposed for the elevated phenol tolerance include binding of phenol by cells on biofilm exteriors that protected internal bacteria which continued to multiply without any inhibition, and modification in lipid composition of cell membranes to compensate for the phenol-induced increase in membrane fluidity. Although the underlying mechanism for phenol tolerance in the granules is not yet understood, it is clear from this study that the granules can tolerate high phenol concentrations and achieve good settling and biodegradation abilities.

- Exploitation of Aerobic Granules in Biodegradation of Recalcitrant Organic Chemical, *tert*-Butyl Alcohol (TBA)

Recently, Tay *et al.* (2005c) reported that aerobic granules can be cultivated in SBR for TBA biodegradation.

Aerobic granules were developed in a SBR using the strategy involving step increases in TBA loading rate achieved through either increasing TBA

concentrations in the influent (Tay *et al.*, 2005c) or reducing the cycle time of SBR operation (Zhuang *et al.*, 2005). Aerobic granules were first observed about 125-180 days after reactor startup and eventually became the dominant form of biomass in the reactor. The adapted granules were capable of nearly complete TBA removal.

The results in this study showed that aerobic granules as dense bacterial aggregates can facilitate the horizontal gene transfer (HGT) among bacterial populations, which is a strategy used by microbial communities to spread existing catabolic pathways and adapt to the presence of xenobiotics in their environment.

In summary, these studies indicated that it is feasible to exploit aerobic granules as an immobilized biomass system to treat the industrial wastewater containing the either toxic or recalcitrant organic compounds.

### 2.3 SUMMARY

In view of the environmental impacts, industrial wastewater and groundwater contaminated with PNP should be effectively cleaned before being discharged into the environment. Biological treatment systems may have promising applications for the removal of PNP pollutants. Aerobic biological treatment processes have several advantages in treating PNP contaminated wastewater, such as fast and complete PNP mineralization, as well as straightforward operation. Although many studies have been conducted to investigate the PNP degradation with pure and mixed bacterial cultures in shaking flask, the studies on the aerobic PNP treatment processes are still limited. Moreover, there is little up-to-date research effort on the investigation of microbial communities involved in the PNP treating processes.

Previous studies on PNP treatment processes have showed that microbial degradation of PNP containing wastewater is often hindered by the recalcitrant nature and inhibitory effects of PNP at high concentration (>100 mg/l). To

overcome these two problems, an immobilized biomass system should be adopted. Aerobic granulation technology which was developed recently has a number of advantages over conventional aerobic treatment processes, and this technology has been successfully applied to treat high strength either toxic or recalcitrant compound-containing wastewater. Aerobic granulation technology therefore has the potential to treat wastewater containing PNP which is both toxic and difficult to microbial degradation. In addition, there is an important research interest to reveal the functionally important PNP-degrading bacterial populations within this novel PNP-degrading system.

It is believed that the development of PNP-degrading granules would lead to a novel biotechnology which could be extensively applied to eliminate PNP in industrial effluents and contaminated groundwaters. The understanding and knowledge gained from this study will be useful in understanding how aerobic granules can be used to effectively treat PNP and other challenging organic compounds. In addition, the understanding on the functionally important PNP-degrading bacteria by this novel process will expand our knowledge on system management and can provide a basis for a more knowledge-driven approach to improve the design of granule-based systems for practical application.

# CHAPTER III

## CULTIVATION AND CHARACTERIZATION OF AEROBIC GRANULES FOR PNP BIODEGRADATION

### 3.1 INTRODUCTION

Biological wastewater treatment systems can degrade environmentally hazardous contaminants to environmentally benign substances, such as microbial biomass, CO<sub>2</sub> and H<sub>2</sub>O. Activated sludge processes are frequently used in aerobic biological wastewater treatment systems in which microorganisms with required degradation ability are able to aggregate into sludge flocs and settle quickly in the treatment system (McKinney, 2004). However, the stability of sludge flocs may be adversely affected when treating the contaminant with a high chemical toxicity (Di Gioia *et al.*, 2004). The treatment efficiency will deteriorate as a result of deflocculation and washout of the active microorganisms from the treatment systems (Bitton, 1994; Watanabe *et al.*, 1999).

In contrast to sludge flocs, aerobic granules have shown high stability when treating the contaminant with high chemical toxicity. Jiang *et al.* (2004b) reported that aerobic granules can tolerance and degrade phenol at concentrations that were known to cause the process failure of activated sludge systems. This therefore implies the potential of deploying the aerobic granule system to treat the environmental contaminant with high chemical toxicity. However, up-to-date research studies in this area are still limited. In this study, the aerobic granule system was deployed to treat PNP, a nitroaromatic compound with high chemical toxicity (ATSDR, 1992).

The main objective of this study was to investigate the feasibility of cultivating aerobic granules for PNP biodegradation. While several past studies have reported the successful cultivation of aerobic granules using target contaminant (e.g. phenol and *tert*-butyl alcohol) as sole carbon and energy source (Jiang *et al.*, 2002; Tay *et al.*, 2005c), it still could be challenging to cultivate granules using PNP as the sole carbon and energy source. This is because the presence of the nitro group makes PNP more toxic and difficult for microbial degradation than its unsubstituted analog (Rieger and Knackmuss, 1995). A major problem resulting from PNP's toxicity and difficulty for microbial degradation would be the failure to maintain the active PNP-degrading biomass within the SBR before successful granule cultivation.

This situation is similar to the problems encountered when immobilizing biomass onto carriers for PNP biodegradation. A continuous decrease in MLSS concentration was reported when PNP was added as the sole carbon and energy source for 2-3 months to an activated sludge system (Bhatti *et al.*, 2002). It is reported that adding glucose as co-substrate was able to solve the problems and maintain enough active biomass for immobilization onto the carriers for PNP biodegradation (Bhatti *et al.*, 2002). A similar strategy, adding glucose as a benign cosubstrate to the reactor, can also be adopted for cultivating the aerobic granules for PNP biodegradation. In addition, because activated sludge might not be suitable for direct inoculation into a reactor with a high input of PNP, conditioning strategy for activated sludge seed was developed to enrich biomass that possessed both improved settleability as well as higher PNP degradation activity.

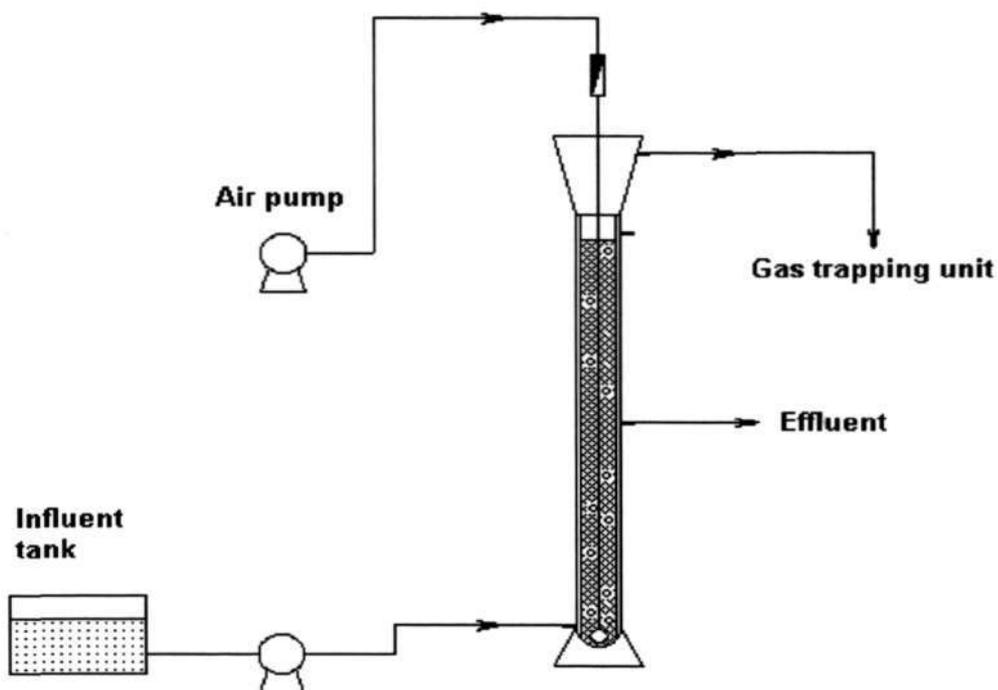
## 3.2 MATERIALS AND METHODS

### 3.2.1 Experimental Set-Up

A 2 l column-type acrylic-glass SBR (120 cm height; 5 cm diameter) (Figure 3.1) used for the study was housed in a temperature-controlled room at 25°C and operated sequentially in 4-hour cycles, with 2 min of influent filling, 213-229 min of aeration, 4-20 min of settling and 5 min of effluent withdrawal. Fine air bubbles for aeration were supplied through a dispenser at the reactor bottom at a superficial gas velocity of 2.5 cm/s. Effluent was discharged at a volumetric exchange ratio of 50%, giving a hydraulic retention time (HRT) of 8 hours.

The SBR was first inoculated with activated sludge from a full-scale wastewater reclamation plant in Singapore. The operation scheme of SBR for seed sludge acclimation as well as granule cultivation is summarized in Table 3.1. In the preliminary study, the PNP-degrading biomass was acclimated using PNP as sole carbon and energy source. However, the significant decrease of biomass concentration rendered the SBR deficient starting biomass for granule cultivation. Glucose was therefore added as a co-substrate during biomass acclimation and granule cultivation.

The seed sludge was conditioned over a 23-day acclimation period to allow the biomass to adapt to PNP and to the short settling times required for a successful granulation. During this acclimation period, the reactor was fed with a synthetic wastewater containing 500 mg/l of glucose and PNP. An initial PNP concentration of 50 mg/l was applied, and this was step-wise increased to a final PNP concentration of 200 mg/l towards the end of the acclimation period. In addition, the settling time in each 4-hour cycle was reduced progressively from 20 min initially to 5 min towards the end of the acclimation period.



**Figure 3.1** Schematic diagram of the sequencing batch reactor (SBR) used for cultivating PNP-degrading aerobic granules.

**Table 3.1** Operation scheme of seed sludge acclimation as well as granule cultivation.

	<b>Seed sludge acclimation</b>	<b>Granule cultivation</b>
Operation time	23 days	97 days
Influent PNP concentration	Influent PNP concentration was stepwise increased from 50-200 mg/l.	Influent PNP concentration was fixed at 200 mg/l until the end of experiment.
Settling time	Settling time was gradually decreased from 20 to 5 min.	Settling time was 5 min for day 0-17 and 4 min for day 18-97.

During the granule cultivation phase, the SBR was fed with a synthetic wastewater containing 500 mg/l glucose and 200 mg/l PNP. The settling time in each 4-hour cycle was set at 5 min for the first 17 days, and then shortened to 4 min from day 18 until the end of the experiment.

The synthetic wastewater consisted of a buffered mineral salt medium (pH  $7.1 \pm 0.3$ ) (Table 3.2) with the following compositions:  $\text{Na}_2\text{HPO}_4$ , 650 mg/l;  $\text{KH}_2\text{PO}_4$ , 200 mg/l;  $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 30 mg/l;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 25 mg/l; and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 20 mg/l. Micronutrients were supplemented to the medium at 1 ml/l (Moy *et al.*, 2002). The wastewater was supplemented with 500 mg/l of glucose and between 50 and 200 mg/l of PNP. Ammonium chloride ( $\text{NH}_4\text{Cl}$ ) was used as nitrogen source, and the COD (chemical oxygen demand): $\text{NH}_4^+$ -N ratio was maintained at 20:1. Wastewater containing 200 mg/l of PNP had a corresponding COD concentration of  $0.83 \text{ kg COD/m}^3$  and an organic loading rate (OLR) of  $2.5 \text{ kg COD/m}^3 \cdot \text{d}$ . The PNP loading rate was  $0.6 \text{ kg/m}^3 \cdot \text{d}$  and OLR attributed to PNP alone was  $0.9 \text{ kg COD/m}^3 \cdot \text{d}$ .

### 3.2.2 DNA Extraction, PCR and Denaturing Gradient Gel Electrophoresis (DGGE)

Genomic DNA of PNP-degrading biomass was extracted based on a protocol described by Kowalchuk *et al.* (1997). Approximately 200-300 mg (wet weight) of biomass was harvested in duplicate during the aeration stage of the SBR cycle and used immediately for DNA extraction. This involved beadbeating followed by extraction with saturated phenol (pH 8.0), phenol-chloroform (1:1) and chloroform:isoamyl alcohol (24:1). The extracted DNA was precipitated overnight with a sodium acetate-ethanol mix at  $-20^\circ\text{C}$  and dissolved in sterile deionized water. Extracted DNA samples were stored in a  $-20^\circ\text{C}$  freeze before use.

PCR primers P2 and P3 (containing 40 bp of GC clamp) were used to amplify the variable V3 region of the bacterial 16S rRNA gene (corresponding to positions 341 to 534 in the *Escherichia coli* sequence) (Muyzer *et al.*, 1993).

Touchdown PCR was performed (Watanabe *et al.*, 1998) with a Mastercycler Gradient thermal cycler (Eppendorf AG) using a 100  $\mu$ l (total volume) mixture

**Table 3.2** Synthetic wastewater composition for reactor operation.

	<b>Substrates</b>	<b>Seed sludge acclimation</b>	<b>Granule cultivation</b>
Carbon source	PNP	50 ~ 200 mg/l	200 mg/l
	Glucose		500 mg/l
Nitrogen source	NH <sub>4</sub> Cl		110 ~ 160 mg/l
Basal mineral salt	Na <sub>2</sub> HPO <sub>4</sub>		650 mg/l
	KH <sub>2</sub> PO <sub>4</sub>		200 mg/l
	CaCl <sub>2</sub> ·H <sub>2</sub> O		30 mg/l
	MgSO <sub>4</sub> ·7H <sub>2</sub> O		25 mg/l
	FeSO <sub>4</sub> ·7H <sub>2</sub> O		20 mg/l
	Micronutrient		1 ml/l
Composition of micronutrient	H <sub>3</sub> BO <sub>3</sub>		50 mg/l
	ZnCl <sub>2</sub>		50 mg/l
	CuCl <sub>2</sub>		30 mg/l
	MnSO <sub>4</sub>		50 mg/l
	(NH <sub>4</sub> )Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O		50 mg/l
	CoCl <sub>2</sub> ·6H <sub>2</sub> O		50 mg/l
	NiCl <sub>2</sub>		50 mg/l

containing Taq DNA polymerase (Promega Co., USA), 1× thermophilic DNA Taq polymerase Buffer B, MgCl<sub>2</sub>, deoxynucleotide triphosphates (dNTPs), primers and DNA extract as described previously (Tay *et al.*, 2005c). Successful PCR was confirmed by 2.0% agarose gel electrophoresis.

The PCR-amplified fragments were separated by DGGE using a DCode universal mutation detection system (Bio-Rad Laboratories) as described previously (Muyzer *et al.*, 1993). A 25 ml 30% to 70% urea-formamide denaturant gradient gel [10% (w/v) acrylamide solution (40% acrylamide and bis acrylamide, 37.5:1 stock solution) in TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM Na<sub>2</sub>-EDTA, pH 8.0)] was covered by a 4 ml acrylamide stacking gel (10%) without denaturant. 40 µl of PCR amplicons from DNA of biomass samples were loaded with 8 µl loading dye in each well. The gel was placed in a TAE buffer and ran at 40 V for 30 min and then at 85 V for 15 h at 60°C. After electrophoresis, the gel was stained with ethidium bromide for 30 min, and photographed with an EDAS 290 gel imaging system (Kodak). GelCompar II (version 1.5) software (Applied Maths) was used for DGGE band pattern analysis. Binary coefficient Dice was applied to calculate the similarity of band patterns. Unweighted Pair Group Method using Arithmetic Averages (UPGMA) was used to construct the DGGE dendrograms.

### **3.2.3 Broad Phenolic Substrate Utilization of Steady-State PNP-Degrading Aerobic Granules**

When granule formation reached a steady state, the ability of granules to degrade phenolic or nitroaromatic compounds was evaluated in 100-ml serum bottles which contained 50 mg/l of one of the following chemicals: phenol, hydroquinone (HQ), *p*-nitrocatechol (PNC), *p*-aminophenol (PAP), 2,4-dichlorophenol (2,4-DCP) or 2,6-dichlorophenol (2,6-DCP). The bottles were incubated overnight on a rotary shaker and substrate removal was confirmed

through TOC analysis at the beginning and end of incubation. The killed granules were used as negative control.

#### 3.2.4 Specific PNP Biodegradation Rates of Steady-State PNP-degrading Aerobic Granules

Specific PNP degradation rates for granules were calculated from PNP degradation curves obtained from experiments carried out in a small reaction volume of 100 ml at a range of PNP concentrations up to 300 mg/l. Triplicate batch tests were performed. A kinetic analysis of the degradation data was performed based on Haldane's equation for an inhibitory substrate,  $V = V_{\max} \cdot S / [K_s + S + (S^2 / K_i)]$ , where  $V$  and  $V_{\max}$  are the specific and the maximum specific substrate degradation rates (mg PNP/g VSS·h) respectively, and  $S$ ,  $K_s$  and  $K_i$  are the substrate concentration, half-saturation constant and inhibition constant (mg PNP/l), respectively (Tomei *et al.*, 2003).

#### 3.2.5 Abiotic Test

Abiotic PNP removal tests were conducted to prove that the PNP degradation in the reactor was mainly due to the microbial activity, rather than the abiotic effects such as volatilization, photo oxidation, adsorption and chemical reduction.

In Test 1, PNP was added to autoclaved basal mineral salt medium (as listed in Table 3.2) at a final concentration of approximately 100 mg/l. In Test 2, 50 ml sludge samples (MLSS approximately 2000 mg/l) were taken from the reactor and were autoclave-killed. The killed sludge was washed, centrifuged and resuspended in 50 ml sterile fresh mineral salt media with 100 mg/l PNP. In the two tests above, DI water with PNP of 100 mg/l was used as a negative control.

In Test 3, sterile fresh mineral salt medium, without biomass was added to three reactor columns. Column one was aerated and shielded with aluminum foil.

Column two was aerated, but not shielded with aluminum foil. Column three was not aerated, but shielded with the foil. The time courses of the PNP concentration in different columns were measured.

### 3.2.6 Other Analytical Methods

During the granule cultivation phase, pH, biomass concentration (mixed liquor suspended solids [MLSS] and volatile suspended solids [VSS]), sludge volume index (SVI), and specific gravity were periodically analyzed in accordance with the standard methods (APHA, 1998). The mean biomass size was measured with a laser particle size analysis system (Malvern Mastersizer 2600).

For determinations of specific PNP-associated oxygen utilization rate (SOUR), a certain amount of biomass sample taken from the reactor was transferred into a pre-cleaned biochemical oxygen demand (BOD) bottle. The standard BOD bottle was fully filled with the pre-aerated mineral salt medium with 100 mg/l PNP, and the oxygen sensing probe (YSI5000, USA) with stirring mechanism was immediately inserted into the BOD bottle. DO levels were measured at time intervals of 10 seconds over a 15 minute period or until DO became limiting, whichever occurred earlier. The oxygen consumption rate in mg/l·s was determined from the slope of the line of best fit to a slop of DO against time. The SOUR in term of mg DO/g VSS·h was calculated with oxygen consumption rate over VSS. The biomass concentration of 0.5 g VSS/l was employed for the measurement.

TOC (total organic carbon) concentration was measured using a TOC analyzer (TOC-500, Shimadsu). Ammonium, nitrite and nitrate concentration were measured using an IC analyzer (LC-2010, Shimadsu). To measure PNP concentration, samples were passed through a 0.22 µm syringe filter (Millipore) and adjusted to pH 10±0.5 with 0.1 N NaOH as described by Ray *et al.* (1999). The concentrations of PNP were spectrophotometrically determined using the absorbance values at 400 nm with a UV/vis spectrophotometer (UV/VIS spectrometer, Lambda Bio 20, Perkin Elmer). The concentrations of TOC,

ammonium, nitrite and nitrate, as well as PNP were quantified using standards of known concentrations. The detect limitations for TOC, PNP, as well as ammonia, nitrite and nitrate were 0.2 mg/l, 0.025 mg/l, and 2.5 mg/l, respectively.

Granule strength was expressed as an integrity coefficient (%) which is defined as a ratio of residual VSS to total VSS after a 5 min of shaking on a rotary shaker at 200 rpm (Ghangrekar *et al.*, 1996). Granule extracellular polymers (ECPs) were extracted with a formaldehyde–NaOH extraction method (Liu and Fang, 2002). The extraction efficiency was optimized so that ECPs could be effectively extracted without serious cell lysis (which can be detected by abrasive release of DNA into the extraction supernatant). A phenol-sulfuric acid method was used to quantify polysaccharides, with glucose as the standard and Lowery assay was used to quantify proteins, with bovine serum albumin (BSA) as the standard (Daniels *et al.*, 1994). The detect limitations for polysaccharides and protein were both 5 mg/l.

For morphological observations with scanning electron microscopy (SEM), granules were fixed for 1-4 hours in 2% (v/v) glutaraldehyde and then washed 3 times for 20 min with 0.10 M sodium cacodylate buffer. Granule samples were dehydrated in a graded *t*-butyl alcohol series (50, 70, 85, 95, 100% v/v). The dehydrated granule samples were then filtered through a 0.2 µm polycarbonate filter (Millipore, USA) and dried using a freeze dryer (Bal-Tec CPD 030). The dried samples were sputter-coated with gold at 20 mA in a high vacuum ( $2.8 \times 10^{-6}$  Torr) and low temperature (-170°C) cryo-chamber for 60 seconds, and viewed with a scanning electron microscope (Stereoscan 420, Leica, Cambridge Instruments) at 20 KV as described previously (Jiang *et al.*, 2002).

### 3.3 RESULTS

#### 3.3.1 Abiotic Test

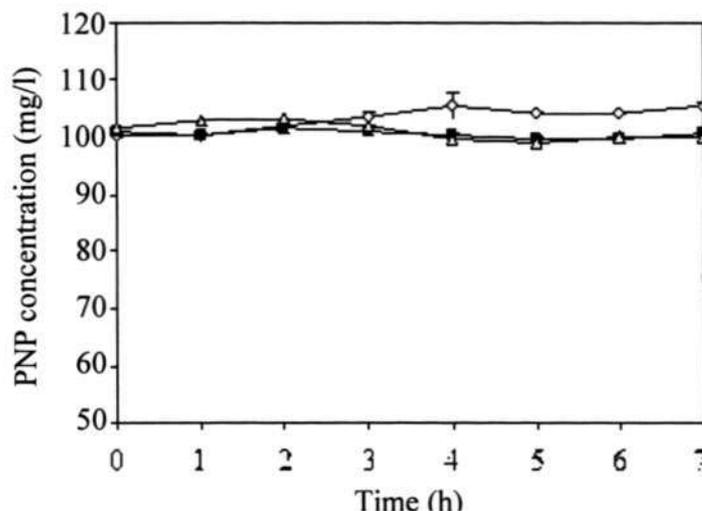
The purposes and results of the first two abiotic tests are summarized in Table 3.3. Test 1, which was designed to study the effect of chemical reduction on PNP removal, indicated that the mineral salt medium used in the feed of reactor could not cause any PNP removal. Test 2 was meant to study the effect of cellular adsorption on PNP removal indicated that only a small amount of PNP (<10%) was adsorbed by inactive biomass.

**Table 3.3** Abiotic removal of PNP.

Test name	Purposes	PNP concentration (mg/l)
	Negative control	96 (1) <sup>a</sup>
Test 1	Chemical reduction	96 (2) <sup>a</sup>
Test 2	Cellular adsorption	87 (7) <sup>a</sup>

<sup>a</sup> The value in the parenthesis is the standard deviation from triplicate experiment.

The results of Test 3 (Figure 3.2) indicated that both evaporation and photo oxidation did not cause any significant loss of PNP within the test period. The slight increase in PNP concentration in the aerated column may be due to the loss of water during aeration.



**Figure 3.2** Time course of PNP concentration in aerated reactor column (◇), in aerated and shielded reactor column (■), and in non-aerated and shielded reactor column (△) in abiotic Test 3.

### 3.3.2 Cultivation of Aerobic PNP-Degrading Granules

Activated sludge from a wastewater reclamation plant was first conditioned over a 23-day period to allow the biomass to adapt to PNP and to the short settling times required for a successful granulation. The initial seed sludge had a mean biomass size of 0.14 mm, an SVI value of 134 ml/g and a specific gravity of 1.0002 (Table 3.4). SOUR was not detectable during incubation with 100 mg PNP/l, indicating that the unacclimated sludge was initially unable to utilize PNP. The acclimated sludge showed remarked improvements in settleability and PNP degradation activity. The mean biomass size fell to 0.07 mm, the SVI value dropped to 32 ml/g, the specific gravity increased to 1.0011, and SOUR activity was detected at 13 mg O<sub>2</sub>/g VSS·h.

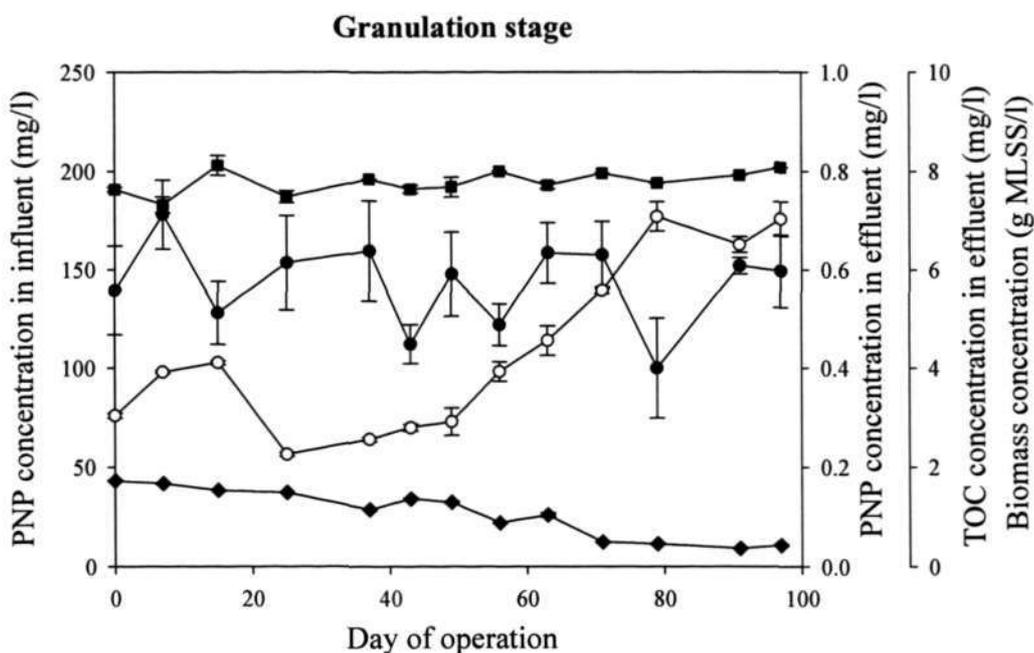
**Table 3.4** Characteristics of seed sludge, acclimated sludge and granules.

Characteristics	Seed sludge	Acclimated sludge	Granules
Mean biomass size (mm)	0.14±0.01	0.07±0.01	0.39±0.02
Sludge volume index (SVI, ml/g)	134±7	32±4	12±3
Specific gravity	1.0002±0.0003	1.0011±0.0004	1.0035±0.0002
SOUR (mg O <sub>2</sub> /g VSS·h)	Not detectable	13±0	22±1

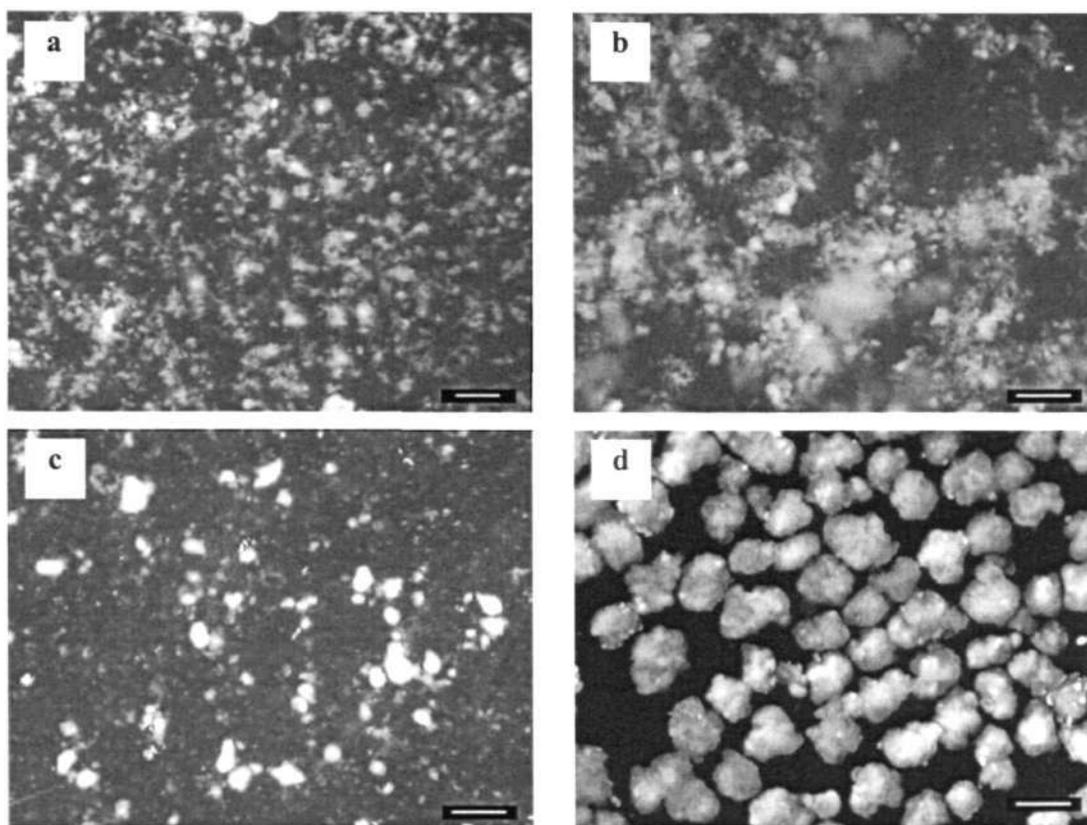
The acclimated sludge was used as the starting seed for the cultivation of aerobic PNP-degrading granules. This starting seed had a biomass concentration of 3040 mg MLSS/l and demonstrated good PNP degradation activity (Figure 3.3). Compared to a PNP concentration of 200 mg/l in the SBR influent, PNP concentrations in the effluent were less than 0.2 mg/l during the first several weeks of the granule cultivation phase, and further declined to below 0.05 mg/l beyond day 71. Compared to a TOC concentration of 300 mg/l in the influent, TOC concentrations in the effluent ranged from 4.0 to 7.1 mg/l, and were probably attributed to the soluble organic matter from biomass lyses.

Aerobic granules were first observed in the SBR as small spherical particles dispersed within amorphous sludge flocs on day 7 (Figure 3.4) of the granule cultivation phase. A distinct separation of granules from the sludge flocs was observed on day 25. The granules eventually grew to become the dominant form of biomass in the reactor, as evidenced by the gradual increase in mean biomass size and decrease in SVI beyond day 25 (Figure 3.5). Except for a brief duration of several days when the system adjusted to the reduction in settling time from 5

minutes to 4 minutes implemented on day 18, the biomass concentration in the reactor generally showed an upward trend to plateau at 7080 mg MLSS/l from day 79. Stable granules were obtained in the SBR when conditions stabilized from day 79 to the end of the reactor operation. The mean biomass size, SVI and specific gravity eventually stabilized at 0.39 mm, 12 ml/g and 1.0035, respectively (Table 3.4).



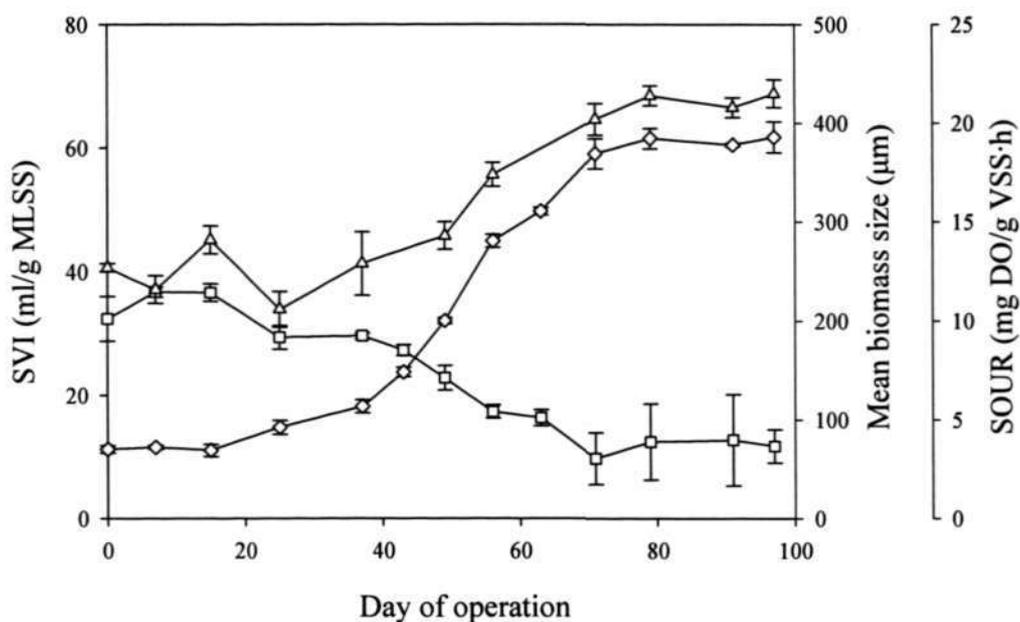
**Figure 3.3** Profiles of PNP concentration in influent (■) and effluent (◆), TOC concentration in effluent (●) and in-reactor biomass concentration (○) during granulation.



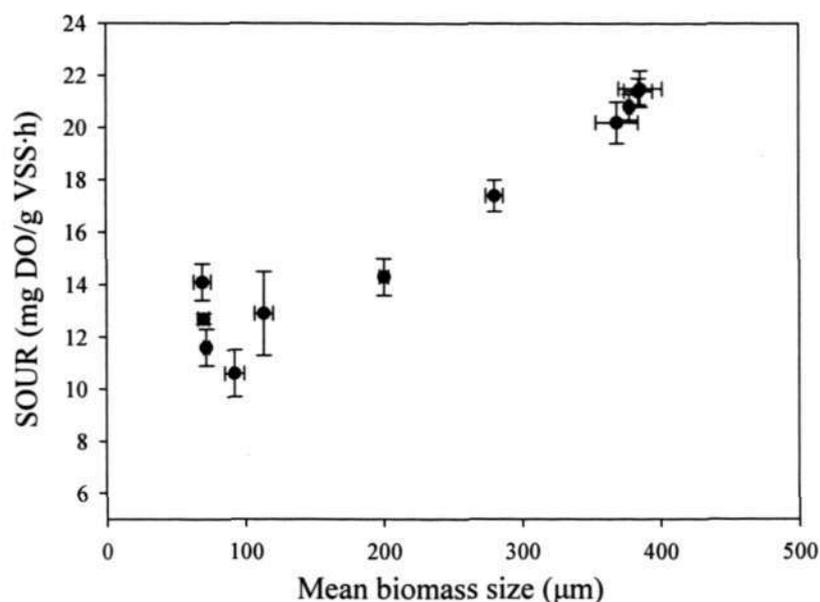
**Figure 3.4** Stereomicroscope images of flocs and granules. (a) Sludge flocs were the only form of biomass on day 0; (b) Small granules were first observed among sludge flocs on day 7; (c) Small granules became the dominant form of biomass on day 25; (d) Mature granules were the only form of biomass on day 90. bar=200  $\mu\text{m}$ .

SOUR was measured for reactor biomass in batch incubations with a PNP concentration of 100 mg/l to monitor changes in metabolic activity. The acclimated sludge on day 0 exhibited metabolic activity towards PNP, with an SOUR of 13 mg  $\text{O}_2/\text{VSS}\cdot\text{h}$  (Figure 3.5). From days 7 to 25, the SOUR values fluctuated from 11 to 14 mg  $\text{O}_2/\text{g VSS}\cdot\text{h}$ . These fluctuations were associated with events such as the onset of granulation on day 7, the reduction in settling time on day 18,

and the brief decline in biomass concentration from days 18 to 25. After day 25, the SOUR values showed an upward trend, and increased to finally stabilize at 22 mg O<sub>2</sub>/g VSS·h from day 79 to the end of the reactor operation. The steady-state SOUR values achieved at the end of the experiment were approximately two-fold higher than the initial values. A plot of SOUR against mean biomass size showed that larger aerobic granules had higher SOUR activity (Figure 3.6).



**Figure 3.5** Profiles of mean biomass size ( $\diamond$ ), sludge volume index (SVI,  $\square$ ), and specific oxygen utilization rate at an initial PNP concentration of 100 mg/l (SOUR,  $\triangle$ ).



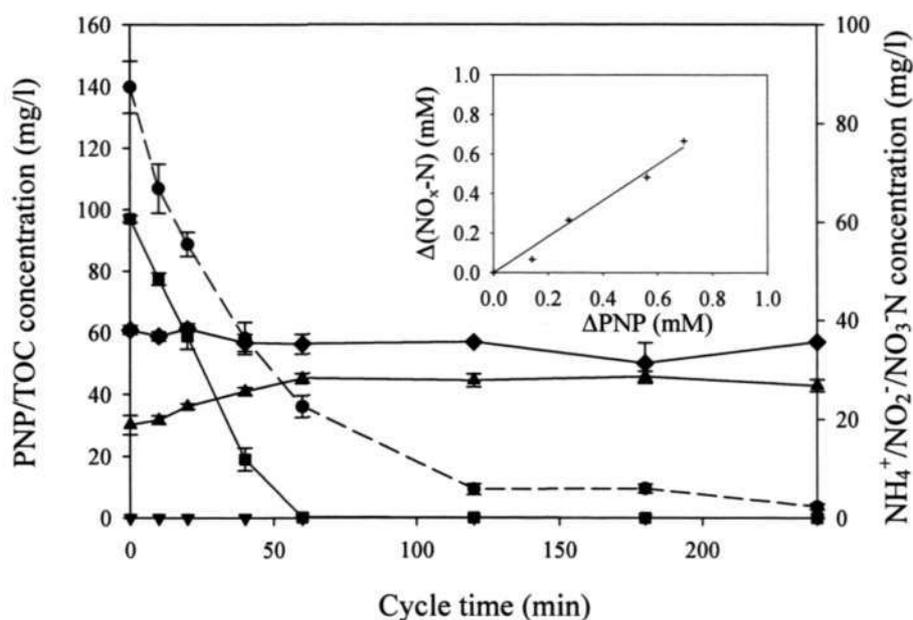
**Figure 3.6** Relationship between SOUR and mean biomass size.

### 3.3.3 PNP Degradation and TOC Removal in One SBR Cycle

The removal of PNP and TOC and the release of nitrite and nitrate during one SBR cycle were examined on day 85 after reactor conditions have stabilized (Figure 3.7). PNP and TOC removal commenced immediately after the influent feeding. Since the influent contained both glucose and PNP, the immediate removal of PNP and TOC indicated that glucose was not preferentially degraded at the expense of PNP when the benign substrate was supplied together with PNP. The start-of-cycle PNP concentration of 100 mg/l was rapidly degraded within one hour while the removal of the start-of-cycle TOC concentration of 140 mg/l took much longer. The PNP removal rate was greater than 99% and the TOC removal rate was 95%.

Ammonium concentration was measured during a SBR cycle. Slightly decrease in ammonium concentration (approximately 2.5 mg/l  $\text{NH}_4^+\text{-N}$ ) was most

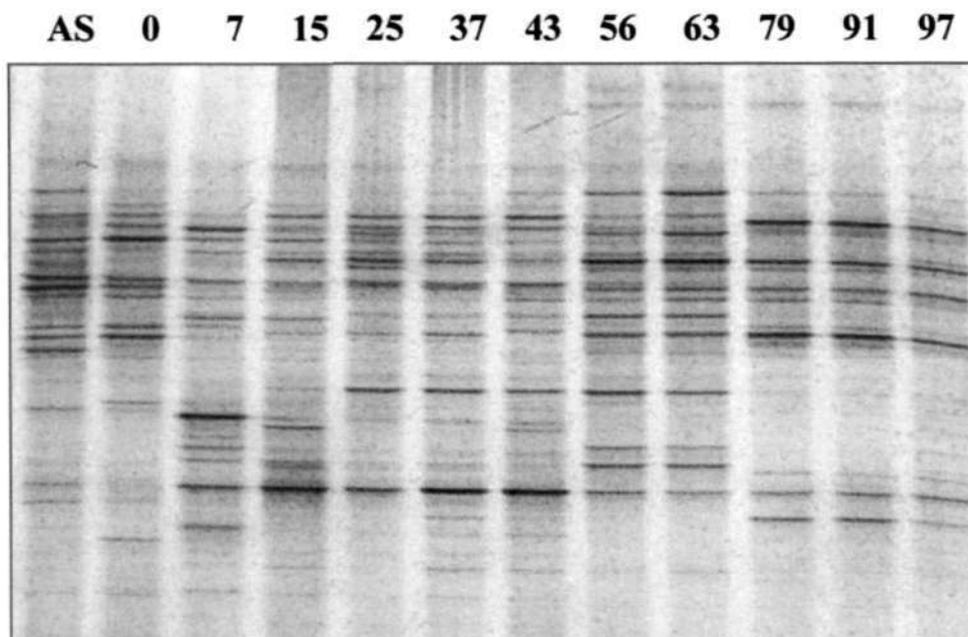
likely due to the assimilation of the reactor biomass. Although the stoichiometric release of nitrite has previously been observed during the aerobic biodegradation of PNP in biofilm reactors (Ray *et al.*, 1999; Bhatti *et al.*, 2002), no nitrite release was detected during the SBR cycle investigated in the current study. However, increases in nitrate concentration from 19 to 27 mg  $\text{NO}_3^-$ -N/l were measured that were stoichiometrically related to the amounts of PNP degraded (Figure 3.7). The estimated stoichiometric coefficient ( $E$  is defined as a molar ratio of N released from PNP [ $\text{C}_6\text{H}_5\text{NO}_3$ ]) of N released from PNP biodegradation with granules was  $0.91 \pm 0.04$ .



**Figure 3.7** Profiles of PNP (■), TOC (●),  $\text{NO}_2^-$ -N (▼),  $\text{NO}_3^-$ -N (▲), and  $\text{NH}_4^+$ -N (◆) concentrations within a representative 4-h SBR cycle. Inset, plot of  $\text{NO}_x$ -N released ( $\text{NO}_x^-$ -N =  $\text{NO}_3^-$ -N +  $\text{NO}_2^-$ -N) against PNP degraded (⊕) at various times in the first hour of the SBR cycle. Values were calculated from the data in Figure 3.6. The stoichiometric coefficient ( $E$ ) of N released from PNP biodegradation with granules was obtained through linear regression.  $E = 0.91 \pm 0.04$ .

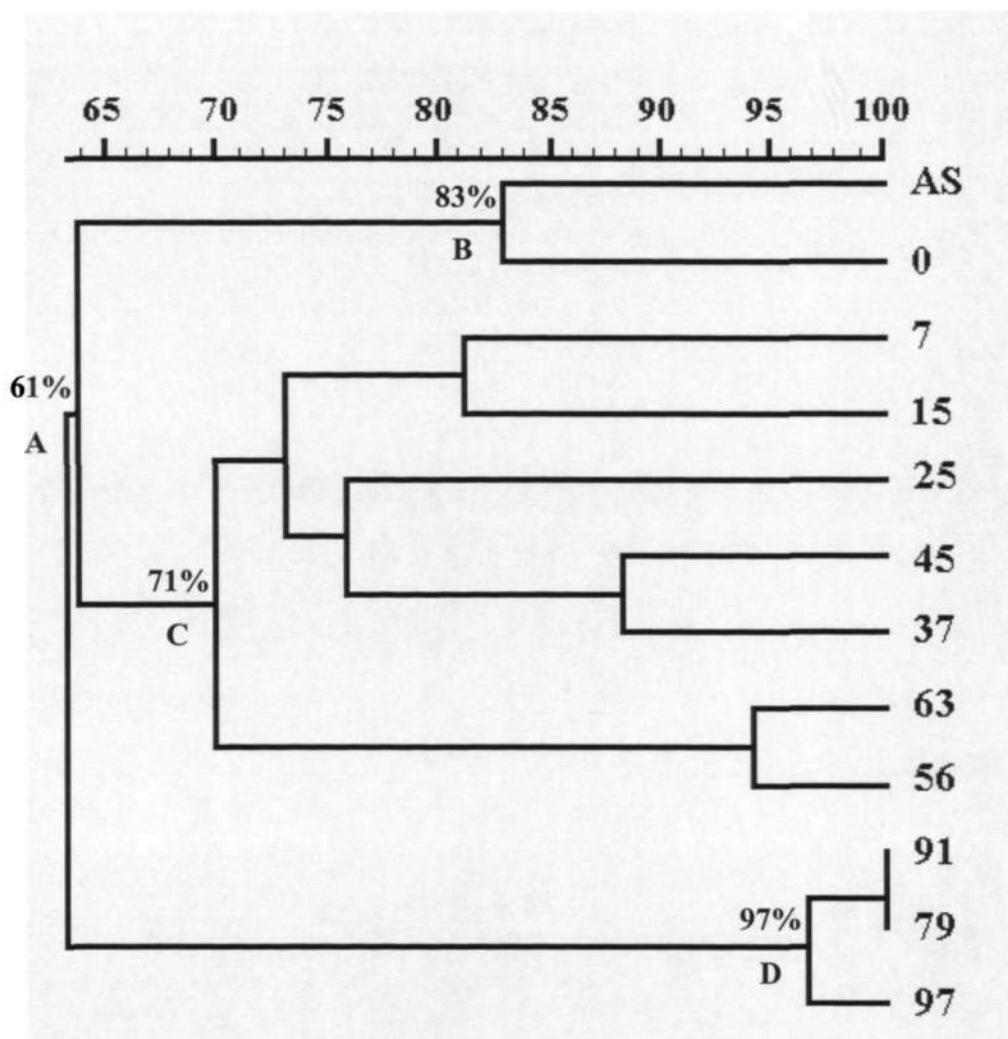
### 3.3.4 DGGE and Microbial Community Succession

DGGE was employed to generate genetic fingerprints to provide information on the composition and diversity of microbial communities in the reactor biomass. Figure 3.8 shows DGGE fingerprints of amplified 16S rRNA gene fragments from the reactor biomass sampled over a 97-day period. Fingerprint similarity was analyzed by applying the unweighted pair group method using arithmetic averages (UPGMA) algorithms. A gradual succession in microbial community was observed, and DGGE fingerprints from similar sampling periods were highly similar to one another. The UPGMA analysis clustered the 12 DGGE



**Figure 3.8** Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA gene fragments from PNP-degrading biomass. AS refers to activated sludge. The number above each lane refers to the day the biomass was sampled.

fingerprints into three groups, with inter-group similarity of 61% (A in Figure 3.9) and intra-group similarity of 71% to 97% (B, C and D in Figure 3.9). Group I



**Figure 3.9** UPGMA analysis of DGGE fingerprints of 16S rRNA gene fragments from PNP-degrading biomass. AS refers to the activated sludge seed. The number beside each branch in the dendrogram refers to the day the biomass was sampled. The percentage values and corresponding capital alphabets refer to the inter-group and intra-group similarities. A refers to the inter-group and B, C and D refer to the intra-groups.

contained DGGE fingerprints from seed sludge (AS) and acclimated sludge (day 0) (similarity  $\approx 83\%$ ) and was associated with reactor start-up. Group II contained DGGE fingerprints from days 7 to 63 and was associated with the development of granules (similarity  $\geq 71\%$ ). Group III contained DGGE fingerprints from days 79 to 97 and was associated with stable granules (similarity  $\geq 97\%$ ).

### **3.3.5 Morphological Characteristics of Steady-State PNP-Degrading Aerobic Granules**

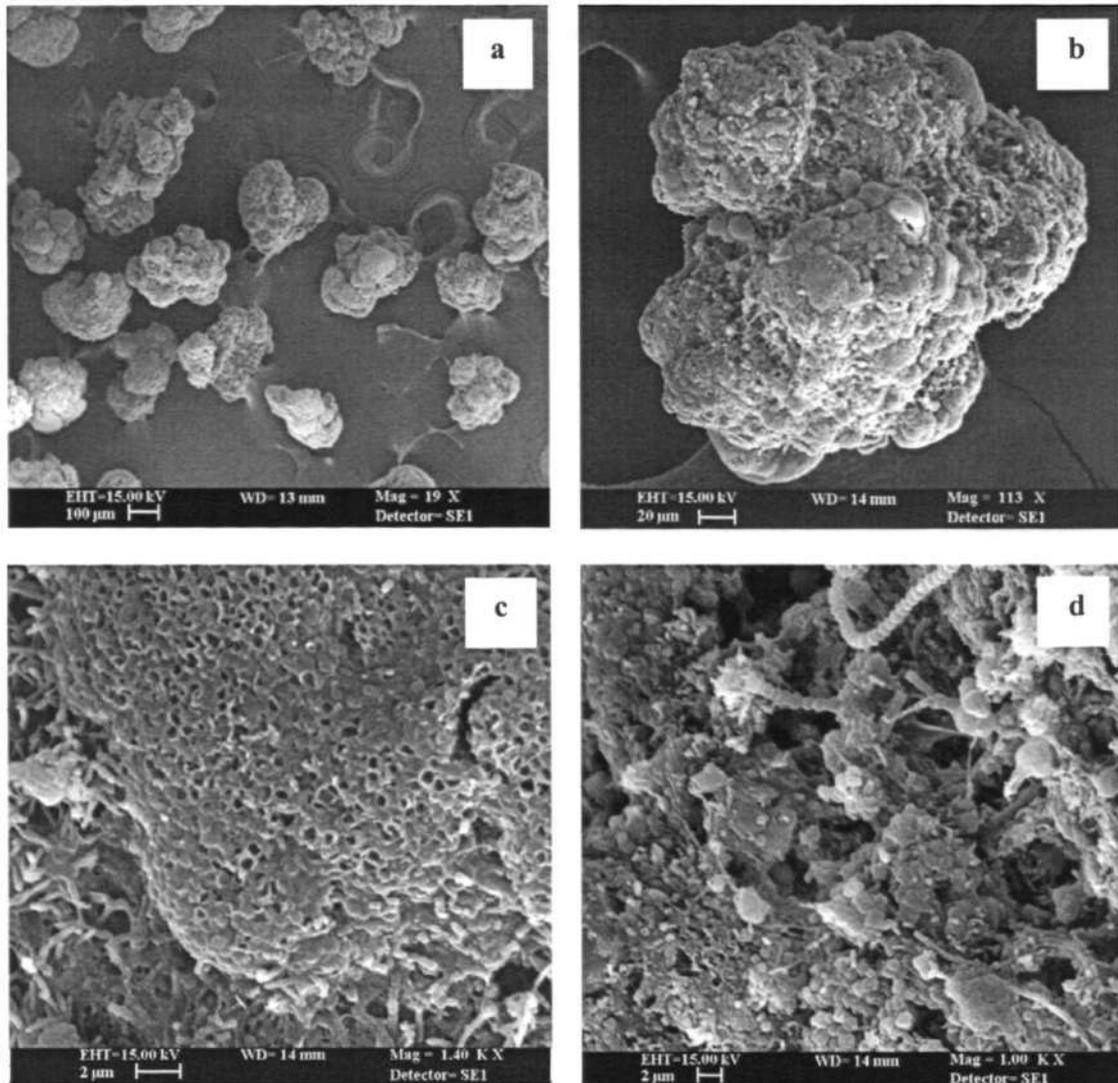
SEM images showed that the PNP-degrading granules had a compact structure with many cauliflower-like clusters (Figure 3.10a and 3.10b). The outer surface of these clusters typically consisted of short rod-shaped bacteria embedded in an extracellular polymeric matrix (Figure 3.10c). Many slender rod-shaped bacteria could be observed below this matrix. In contrast, the zones between adjacent clusters contained a large diversity of microbial morphotypes, including bacterial rods and cocci, and protozoa (Figure 3.10d).

### **3.3.6 Physico-chemical Properties of Steady-State PNP-Degrading Aerobic Granules Related to the Mechanical Stability**

The PNP-degrading granules had an integrity coefficient greater than 99%, indicating a high mechanical strength. ECP content (in terms of EPS and EP) in mature granules was  $99 \pm 12$  (mg/g VSS) and the ratio of EPS/EP was near 1 as listed in Table 3.5.

### **3.3.7 PNP Degradation Kinetics with Steady-State PNP-degrading Aerobic Granules**

The ability of granules to degrade PNP was evaluated by monitoring PNP removal at different PNP concentrations in batch culture. Granules used in kinetic



**Figure 3.10** Scanning electron micrographs of aerobic PNP-degrading granules. (a) View of PNP-degrading aerobic granules, bar = 100 μm; (b) View of an entire granule, bar = 20 μm; (c) View of a cauliflower-like cluster, bar = 2 μm; and (d) View of an inter-cluster region, bar = 2 μm.

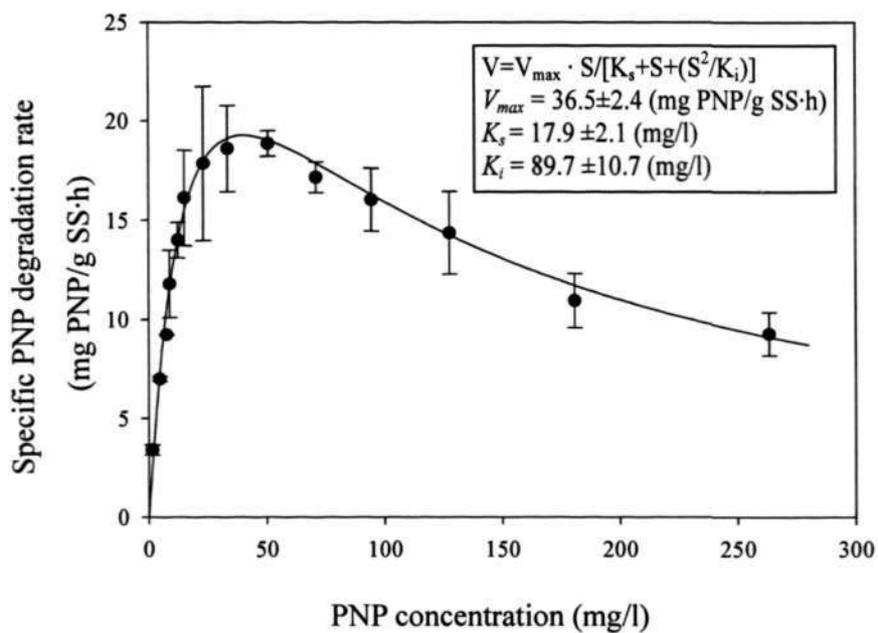
**Table 3.5** The properties of granules related to the mechanical stability.

Properties	Values
Integrity coefficient (%)	>99
ECP content (mg/g VSS)	
EPS (mg/g VSS)	48±9
EP (mg/g VSS)	50±2
EPS/EP Ratio	0.96±0.23

study were sampled on day 95. The specific PNP degradation rate increased with PNP concentration from 0 to 40 mg PNP/l, peaked at 19 mg PNP/g VSS·h, and declined with further increases in PNP concentration as substrate inhibition effects became significant (Figure 3.11). The Haldane equation was used to fit the degradation data, with calculated kinetic parameters of  $V_{max} = 36$  mg PNP/g SS·h,  $K_s = 18$  mg/l and  $K_i = 90$  mg/l, and a correlation coefficient ( $R^2$ ) of 0.97.

### 3.3.8 Broad Phenolic Substrate Utilization with Steady-State PNP-degrading Aerobic Granules

Direct detection of the intermediates of PNP degradation from the bulk solution in the reactor was not successful, suggesting that the degradation process was very rapid. Therefore, the possible PNP degradation pathways employed by the granules were assessed indirectly by examining the ability of granules to utilize potential intermediates. Under aerobic conditions, the major intermediates would include hydroquinone (HQ) and *p*-nitrocatechol (PNC) (Crawford, 1995), while *p*-aminophenol (PAP) would be an intermediate encountered under anaerobic conditions (Marvin-Sikkema and de Bont, 1994). The PNP-degrading granules could degrade both HQ and PNC, but not PAP. The PNP-degrading granules also possessed broad phenolic substrate utilization, as they could degrade phenolic compounds such as phenol, 2,4-dichlorophenol (2,4-DCP) and 2,6-dichlorophenol (2,6-DCP).



**Figure 3.11** Specific PNP degradation rates of aerobic PNP-degrading granules at different PNP concentrations. Error bars represent the standard deviation from triplicate batch experiments (experimental results, ●; Haldane fit, —,  $R^2=0.97$ ).

## 3.4 DISCUSSION

PNP is an inhibitory and challenging organic compound for microbial biodegradation. This study demonstrates that it is feasible to cultivate aerobic granules in a SBR for the biodegradation of PNP. The strategy of adding glucose was adopted in this study to develop stable aerobic granules efficient at PNP degradation.

### 3.4.1 Seed Sludge Acclimation for Cultivation Aerobic PNP-Degrading Granules

A key step in the development of aerobic PNP-degrading granules was the acclimation strategy adopted to condition the activated sludge seed in the preparation for granule formation.

A successful result should be dependent on the enrichment of appropriate microbial strains, and their subsequent survival and activity within the chosen environment. It is noted that many previous cases of selective enrichment typically involve the exclusive selection of strains that express the required degradation ability. However, such enrichments may not necessarily be typical or representative of the communities that will survive and thrive in the target habitat. Instead, a knowledge-based approach should be taken on the basis of understanding the kinds of microbial communities present in the habitat of interest. A proper enrichment strategy should favor not only degradation ability but also the other traits that are required for strains to be competitive and effective in the target environment.

The acclimation strategy adopted in this study is based on a priori knowledge that successful granule formation depends on the exertion of the appropriate combination of microbial and hydraulic selection pressures to facilitate the cultivation of microbial aggregates that can actively degrade the target contaminant, and effectively separate of this biomass from the wastewater liquor (Tay *et al.*, 2005c). The activated sludge seed was therefore conditioned during the

acclimation phase by implementing stepwise increases in PNP concentration and stepwise decreases in settling time to enrich the biomass that possessed both the ability to degrade PNP and the ability to settle rapidly (Table 3.4). The subsequent results on biomass concentration (Figure 3.3) showed that such acclimated sludge can effectively be retained within the SBR for granules formation.

### 3.4.2 Granulation Enhances Biomass Metabolic Activity towards PNP

As showed in Figure 3.5, the metabolic activity of biomass towards PNP, expressed as SOUR, increased during the granulation and stabilized at 22 mg O<sub>2</sub>/g VSS-h for matured granules. This value was approximately two-fold higher than the values from the acclimated sludge flocs. A plot of SOUR against mean biomass size showed that larger aerobic granules had higher SOUR activity (Figure 3.6). This finding contrasted with observations of biofilms growing at the expense of benign substrates, where an increase in biomass size was usually associated with a decrease in specific biomass activity because of mass-transfer limitation (Jiang *et al.*, 2002). However, the finding in this study was consistent with observations of biofilms cultivated on toxic substrates. For example, sludge biomass immobilized on carriers possessed higher specific PNP degradation rates than suspended sludge flocs when acclimated to increased PNP concentrations (Xing *et al.*, 1999). Similar observations were also reported for granulation systems where higher specific phenol degradation activities were attained after microbial cells aggregated into compact aerobic granules (Jiang *et al.*, 2002).

Many mechanisms could be involved in the enhancement of metabolic efficiency in the granules. Firstly, PNP metabolic activity could be enhanced by the retention of PNP-degrading microorganisms in the granules. Granulation could significantly increase the retention of these microorganisms by providing a protected microniche that facilitates the growth and proliferation of PNP-degrading microorganisms and prevents their washout from the system (Jiang *et al.*, 2002; Jiang *et al.*, 2004b). Secondly, this enhancement could be the result of syntrophic interactions between the community members within the granules. Thirdly, the

prolonged acclimation of biomass on PNP may also contribute the enhancement. Finally, metabolic enhancement could result from the increase of specific degradation activity through the exchange of genetic material among the bacteria within the compact living environment in granules. The increased PNP-degrading activity associated with increasing granule size could be the result of the specific or synergistic effects of the mechanisms mentioned above.

### **3.4.3 Transfer of Nitrite to Nitrate with Aerobic Granules**

The nitrite released from PNP biodegradation is an important environmental issue that should be addressed when applying aerobic biological treatment to degrade PNP-containing wastewater. This is due to the fact that nitrite itself is an environment toxin. The nitrite released from PNP degradation in this study was converted to nitrate. Although the underlying mechanism of this conversion was not clear, this unexpected finding can be attributed to the richness of sludge systems which contains a high diversity of metabolic and physiological capabilities not found in single-species bacteria system (Allison *et al.*, 2000).

### **3.4.4 Microbial Community Succession Revealed by DGGE**

Although there have been a few studies reported the microbial community in mature aerobic granules (Tsuneda *et al.*, 2003; Jiang *et al.*, 2004; Zhuang *et al.*, 2005), the study investigated the microbial community shift during granulation is still missing. In this study, the DGGE band patterns for samples from reactor biomass in the process of granule cultivation revealed a gradual succession in microbial community (Figure 3.8). The cluster analysis grouped all the DGGE patterns into three groups associated with the three different stages for granule formation: reactor start-up stage, granules formation stage and granule stabilization stage (Figure 3.9).

The inter-group similarity of 61% among three groups indicated that the microbial community of reactor biomass did not changed dramatically albeit the

reactor biomass went through three different stages for granulation. The dynamic behavior in the bacterial community structure in laboratory-scale activated-sludge reactors has been widely reported before. The bacterial communities could vary significantly over time even when the reactors were operated under stable conditions (Kaewpipat and Grady, 2002). Saikaly *et al.* (2005) have pointed out that the dynamic changes in the bacterial community structure in laboratory-scale activated-sludge reactors could be attributed to several biotic and abiotic factors such as resource competition, predation, and new selective pressure imposed on domestic sludge.

The lower variability in the microbial community observed in this study was probably due to three reasons. Firstly, the seed sludge used in this study was acclimated sludge that had already adapted to the operation conditions in the SBR such as the presence of PNP and the short settling time. Secondly, compared to the biomass in non-granulation SBRs, the biomass in granulation SBRs possesses compact and density structure. This rendered the bacterial community in granules less susceptible to the challenges such as predation and hydraulic washout and able to dwell stably in the SBR. Finally, the granulation SBRs were operated with high selection pressure, such as short settling time and fast withdraw, as a result the exogenous bacterial populations were not easy to be retained in the granulation system. The lower variability in the microbial community of reactor biomass in this study could be the result of specific or synergistic effects of the reasons mentioned above.

Among the three intra-group similarities of DGGE patterns from the cluster analysis, group II associated with the granule formation showed a relatively lower value ( $\approx 71\%$ ) than group I ( $\approx 83\%$ ) or group III (97%). This indicates that there were considerable changes in the population structure in the microbial community during the granule formation. These changes in the population structure might be related to the enrichment of PNP degradation population, as well as to the microbial attachment and deattachment process during granule formation.

### 3.4.5 ECP Content of PNP-Degrading Aerobic Granules

ECPs are of major importance for the mechanical strength of granules since ECPs are well-known for their important roles in development and structural integrity of microbial aggregates such as flocs, biofilms and granules (Tay *et al.*, 2005b). They also serve as a protective function and are known to form a shield against the adverse influences of the external environment by acting as a diffusion limitation barrier to delay or prevent toxicants from reaching the microorganisms (Wingender *et al.*, 1999). In this study, the ECP content of PNP-degrading granules from a steady-state reactor operation was approximately 9.9% of granule biomass. This compares favorably with the ECP contents ranging from 6.2% to 23% in other PNP-degrading immobilized cell system (Bhatti *et al.*, 2002) or granulation systems (Table 3.6).

The roles of EPS are believed to have mainly structural functions in forming and stabilizing the matrix of microbial aggregates, such as sludges, biofilms and granules (Flemming *et al.*, 2000). The roles of EP, however, are considered mostly related to their enzymatic activities which are believed to function in the extracellular degradation of macromolecules to low molecular weight products which can be transported into the cells and are available for microbial metabolism (Frolund *et al.*, 1995). The ratio of EPS/EP was nearly 1 in the PNP-degrading granules, which compares favorably with the ratio of 1.7 reported in other PNP-degrading immobilized cell system (Bhatti *et al.*, 2002), but differs significantly from the EPS/EP ratio of 2.5-15 (Tay *et al.*, 2001; Tay *et al.*, 2002b) and 0.13-0.15 (McSwain *et al.*, 2005) in other reported granule systems (Table 3.6).

**Table 3.6** ECPs composition of biofilm and granule samples reported in other studies.

Extraction method	Sample type	EP			Total ECPs* (% biomass)	EPS/EP Ratio	Reference
		EP	EPS	(mg/g of VSS)			
NaOH extraction	Granules	210±28	26 ± 9	23.6	0.13	McSwain <i>et al.</i> , 2005	
Dowex extraction	Granules	73±5	11 ±2	8.4	0.15	McSwain <i>et al.</i> , 2005	
Heat	Granules	NA	NA	NA	15	Tay <i>et al.</i> , 2001c	
Cold saline extraction	Granules	NA	NA	NA	2.5-3.5	Tay <i>et al.</i> , 2002b	
Heat	Biofilm	39.3	22.6	6.2	1.7	Bhatti <i>et al.</i> , 2002	
NaOH extraction	Granules	48±9	50±2	9.9	0.96±0.23	This study	

\*Total ECPs were calculated in terms of the sum of EPS and EP.

### 3.4.6 PNP Degradation Kinetics in Aerobic Granules

Stable PNP-degrading granules were harvested from the SBR on day 95 for batch tests to assess PNP degradation kinetics (Figure 3.11). The non-growth kinetic model (enzyme kinetics) was employed in the test in which entire granules were treated as enzyme systems for PNP degradation. The Haldane equation has been widely used to describe the degradation kinetics of many phenolic compounds (Cooper *et al.*, 1990) and was used to calculate the kinetic parameters in this study. The granules had a maximum specific PNP removal rate ( $V_{\max}$ ) of 36 mg PNP/g VSS·h.  $V_{\max}$  in other studies involving SBRs for PNP removal ranged from 85.4 to 194 mg PNP/g VSS·h (Table 3.7). These rates were associated with sludge flocs and were 2.3 to 5.3 times higher than the  $V_{\max}$  reported for the aerobic granules in this study.

The smaller rate in this study could be attributed to two main reasons. Firstly, the compact structure of aerobic granules meant that a smaller specific surface area was presented to the substrate than in the case of the sludge flocs. Moreover, the compact granular structure created a diffusion barrier such that microorganisms in the granule interior would encounter a lower concentration of PNP than existed in the bulk milieu.

Secondly, the ratio of PNP to total COD used in this study was 0.36, which was lower than the ratios used elsewhere (Tomei *et al.*, 2004). Tomei *et al.* (2003) found that  $V_{\max}$  correlated positively with the PNP/COD<sub>total</sub> ratio. The PNP/COD<sub>total</sub> ratio reflects the portion of specific PNP-degrading population in the total heterotrophic microbial community (Magbanua *et al.*, 1998). A ratio of 0.36 in this study pointed to the existence of a large group of bacteria within the granules that did not contribute directly to PNP degradation. Therefore, normalizing the rate of PNP removal to the total biomass concentration resulted in a lower specific PNP degradation rate. However, the ability to retain high amounts of biomass allowed aerobic granules to paradoxically achieve higher volumetric PNP removal rates than reported for other non-granular systems. The maximum volumetric PNP removal,  $R_{\max}$  rate for the SBR

in this study was 256 mg PNP/l·h. This was significantly higher than the range of 94 to 188 mg PNP/l·h reported in other studies (Tomei *et al.*, 2003; 2004).

Kinetic studies of granules with PNP as sole substrate yielded a half-saturation coefficient  $K_s$  of 18 mg/l and a substrate inhibition coefficient  $K_i$  of 90 mg/l.  $K_s$  was similar to values reported elsewhere (Tomei *et al.*, 2003; 2004), but  $K_i$  was significantly higher than the range of 12 to 30.7 mg PNP/l reported previously (Tomei *et al.*, 2003; 2004) (Table 3.7). The higher  $K_i$  indicated that the aerobic granules were more resilient than other forms of biomass towards PNP toxicity. One explanation, which has been elucidated elsewhere, was that the compact granular structure served to shelter the microorganisms against the chemical toxicity that might exist in the surrounding milieu (Jiang *et al.*, 2002). Compared to other biofilm systems, aerobic granules are less susceptible to the effects of substrate inhibition and can potentially be deployed to degrade higher concentrations of PNP.

**Table 3.7** Parameters for PNP degradation kinetics.

Study	Biomass type	Biomass concentration in the reactor(s)	$K_s$	$K_i$	$V_{max}$	$R_{max}^*$
		mg VSS/l	mg PNP/l	mg PNP/g VSS·h	mg PNP/l·h	
1 <sup>a</sup>	Sludge flocs	656-1066	20	12	85.4-187	94.6-188
2 <sup>b</sup>	Sludge flocs	502-850	17.6	30.7	138-194	108-160
This study	Granules	Approximately 7000	18	90	36	256

<sup>a</sup> Study 1: (Tomei *et al.*, 2003)

<sup>b</sup> Study 2: (Tomei *et al.*, 2004)

\*  $R_{max}$  is maximum volumetric PNP removal rate and is calculated by  $V_{max} \times X$  (mean biomass concentration)

### 3.5 SUMMARY

This study demonstrates the successful cultivation of aerobic granules in a SBR for PNP biodegradation. Operating strategies, such as the addition of glucose as well as incremental adjustments in settling time and PNP concentrations, were adopted to develop aerobic PNP-degrading granules with excellent settling characteristics and nearly complete PNP removal. The aerobic granules were cultivated at a PNP loading rate of  $0.6 \text{ kg/m}^3\cdot\text{d}$ , with glucose supplement to boost the growth of biomass. The granules had a clearly defined shape and appearance, settled significantly faster than activated sludge and were capable of nearly complete PNP removal. DGGE analysis of 16S rRNA gene fragments showed a gradual temporal shift in microbial community succession as the granules developed from the activated sludge seed. Specific oxygen utilization rates at  $100 \text{ mg/l}$  PNP were found increasing with the evolution of smaller granules to large granules and stabilizing at  $22 \text{ mg O}_2/\text{g VSS}\cdot\text{h}$ , suggesting that the granulation process can enhance metabolic efficiency towards biodegradation of PNP.

When granule formation reached stable stage, PNP degrading granules were characterized for their mechanical stability, specific PNP degradation kinetics, morphology, and broad phenolic substrate utilization. Firstly, PNP-degrading granules were found possessing an integrity coefficient greater than 99%. ECP content of PNP-degrading granules, in terms of EPS and EP, was approximately 9.9% of granule biomass. Secondly, PNP-degrading granules were found having specific PNP degradation rates that increased with PNP concentration from 0 to  $40 \text{ mg/l}$ , peaked at  $19 \text{ mg/g VSS}\cdot\text{h}$ , and declined with further increases in PNP concentration as substrate inhibition effects became significant. The kinetic analysis using Haldane equation estimated that PNP-degrading granules had a maximum specific PNP degradation rate ( $V_{max}$ ) of  $36 \text{ mg/g VSS}\cdot\text{h}$ , a half-saturation coefficient ( $K_s$ ) of  $18 \text{ mg/l}$  and an inhibition coefficient ( $K_i$ ) of  $90 \text{ mg/l}$ . PNP-degrading aerobic granules were also found having a diverse morphotypes and can degrade broad phenolic substrates.

This study broadens the benefits of using the SBR to target the biodegradation of toxic and recalcitrant organic compounds. From a practical perspective, refinements

that leverage on the operational flexibility of the SBR, such as introduction of anoxic-aerobic phases within an SBR cycle, can be employed to remove nitrate and improve the treatment process.

# **CHAPTER IV**

## **ISOLATION AND PHYSIOLOGICAL CHARACTERIZATION OF PNP-DEGRADING BACTERIA RESIDING IN AEROBIC GRANULES**

### **4.1 INTRODUCTION**

There is a general interest in understanding the indigenous functional microbial populations involved in a bioremediation or biodegradation process. Identification and characterization of the key microorganisms which play important roles in the pollutant biodegradation will contribute valuable knowledge on the development and optimization of bioremediation and biodegradation strategies (Abed et al., 2002).

Culture-independent 16S rRNA approaches have been used to detect the key organisms in pollutant degradation process (Leys et al., 2005). An analysis of a population shift accompanied by a change in the degradation function of a microbial community can yield information useful for identifying functionally dominant populations (Morgan et al., 2002; Li and Moe, 2004; Roling et al., 2004). However, the physiological characteristics and ecological roles of the functionally dominant populations cannot be obtained by employing the culture-independent 16S rRNA approaches alone (Heuer et al., 1999).

In fact, physiological characteristics, such as pollutant degradation rate and growth rate of microorganisms will significantly affect the biodegradation process. Therefore, a detailed analysis on the physiological characteristics based on the culture-dependent methods, such as isolation, are certainly necessary for

understanding the physiological and ecological roles of functionally important populations in biodegradation process (Watanabe *et al.*, 1998; Ellis *et al.*, 2003).

Although many PNP-degrading bacteria have been isolated and characterized from various ecosystems, such as soil and lake water (Jain *et al.*, 1994; Kadiyala and Spain, 1998; Chauhan *et al.*, 2000; Kitagawa *et al.*, 2004), currently there is little information on the indigenous PNP-degrading bacteria isolated from engineered ecosystems such as bioreactors. Studies on PNP biodegradation with bioreactors have mainly focused on process development and operation (Xing *et al.*, 1999; Bhatti *et al.*, 2002; Tomei *et al.*, 2004; Tomei and Annesini, 2005), with little research studies concerning the microbial community within the PNP biodegradation bioreactors.

A recent study on aerobic granulation SBR demonstrated that the aerobic granules can be cultivated for PNP biodegradation (see Chapter III). Compared with the conventional non-granulation SBR system, the aerobic granulation SBR offers several advantages such as higher protection effects from PNP toxicity, higher PNP metabolic activity and higher volumetric conversion capacity. These advantages are the consequent results from metabolic and physiological activities of indigenous microbial populations chosen by the selection pressures supplied for granulation. Therefore, it is important to know the roles of the dominant microbial populations in the PNP biodegradation and the physiological characteristics of these microbes affecting PNP biodegradation. The information on the PNP-degrading bacterial population not only helps in understanding the PNP degradation processes with granules but is also important in the development of optimal control and management strategies for the bioreactor (Abed *et al.*, 2002).

The main objective of this study therefore is to isolate and characterize PNP-degrading bacterial populations residing in the aerobic granules. The numerically abundant and functionally important populations within the PNP-degrading aerobic granules will also be identified and characterized for its ecological importance in the aerobic PNP-degrading granules as well as its physiologic characteristics. The

ecological importance of the strain PNP-01 was investigated through the quantifying of isolated strain PNP-01 from the mature PNP-degrading aerobic granules, detecting the temporal changes of relative fluorescence of strain PNP-01-like cells during the granules' formation in 16S rRNA fingerprint analysis, and analyzing the specific PNP biodegradation ability of strain PNP-01. The physiologic characteristics of strain PNP-01, such as its cell density effects on PNP biodegradation and its capability of self-flocculation were investigated through batch studies. In addition, the phenotypic and phylogenetic characteristics of strain PNP-01 were also investigated.

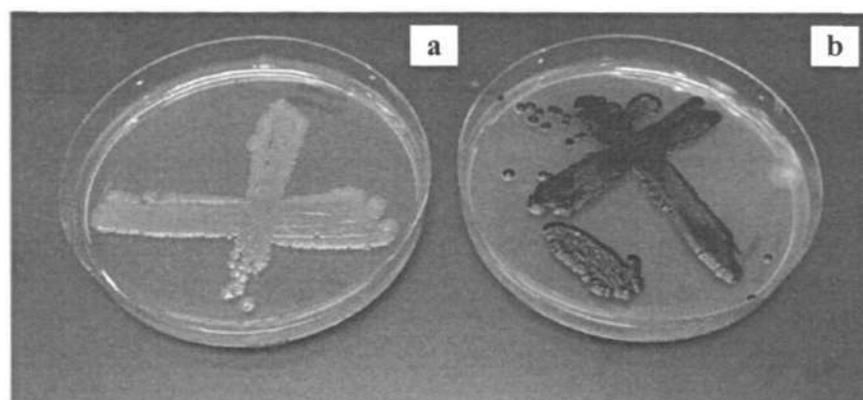
The findings in this study will enhance knowledge on the functionally important PNP-degrading bacteria in aerobic granulation bioreactor, which will provide an insight into the mechanisms involved in the PNP biodegradation with this novel aerobic granulation bioreactor. In addition, the knowledge on the physiological characteristics of strain PNP-01 will be useful in developing optimal control and management strategies for PNP-degrading aerobic granulation bioreactor. Finally, the isolated strain PNP-01 can be used for bioaugmentation to enhance the performance of the bioreactor.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Bacterial Strains, Consortium and Media

Strain PNP-01, PNP-02, PNP-03 and consortium PNP-04 were isolated using direct plating of serially diluted dispersed mature PNP-degrading granules.

Bacteria strain *Chromobacterium violaceum* CV026 obtained from the national collection of type cultures (NCTC, U. K.) under the number NCTC 13278 was used as the reporter strain to detect the quorum sensing (QS) signal molecules (N-acyl-homoserine lactones, AHLs) produced by strain PNP-01. The reporter strain can produce violet pigment when exposed to AHLs supplied in the culture medium as shown in Figure 4.1. Bacto<sup>®</sup> tryptic soy agar and LB agar were used as the growth medium for *Chromobacterium violaceum* CV026.



**Figure 4.1** Reporter strain *C. violaceum* CV026 on Tryptic Soy Agar: (a) without AHLs supplied; and (b) supplied with 2.0  $\mu\text{g}$  AHLs.

Bacto<sup>®</sup> plate count agar was used to enumerate total heterotrophic bacteria in the granules. PNP-MS agar used for enumeration, isolation and growth of PNP-degrading bacteria was prepared with autoclave-sterilized mineral salt agar (mineral salt medium [MS] with 15% Bacto<sup>®</sup> agar was autoclaved at 121°C for 15 min) and filter-sterilized PNP solution.

Bacto<sup>®</sup> R2A agar, Bacto<sup>®</sup> tryptic soy agar (TSA), 1/5-strength of Bacto<sup>®</sup> tryptic soy broth (6 g/l, containing 3.4 g Bacto<sup>®</sup> Tryptone, 0.6 g Bacto<sup>®</sup> Soytone, 0.5 g Bacto<sup>®</sup> Dextrose, 1 g sodium chloride and 0.5 g dipotassium phosphate) and PNP-MS medium were used as the growth medium for bacterial strains and consortium. PNP mineral salt medium (PNP-MS) was prepared as described in Chapter III, containing Na<sub>2</sub>HPO<sub>4</sub>, 650 mg/l; KH<sub>2</sub>PO<sub>4</sub>, 200 mg/l; NH<sub>4</sub>Cl, 160 mg/l; CaCl<sub>2</sub>·H<sub>2</sub>O, 30 mg/l; MgSO<sub>4</sub>·7H<sub>2</sub>O, 25 mg/l, FeSO<sub>4</sub>·7H<sub>2</sub>O, 20 mg/l, micronutrient, 1 ml/l and PNP, 100mg/l.

#### 4.2.2 Reactor Operation

PNP-degrading aerobic granules were cultivated in a SBR from conditioned activated sludge as described in Chapter III. The biomass was periodically sampled for ribosomal-based experiments during granule formation. When granule formation reached a steady state, the mature granules were sampled for cultivation experiments.

#### 4.2.3 Bacterial Enumeration and PNP-Degrading Bacteria Isolation

Bacterial counts for total heterotrophic bacteria and PNP-degrading bacteria were determined with Bacto<sup>®</sup> plate count agar and PNP-MS agar, respectively.

Mature granule samples taken from reactor were washed three times with 0.85% saline and directly used for enumeration and isolation. Portions of granules samples were added to 5 ml 0.85% saline and aseptically ground for 20 min with fine porcelain mortar and pestle in an ice-water bath. Microscopy inspection

coupling with acridine orange staining was implemented to ensure the ground efficiency at which the granules could be properly dispersed into single cells or at least small microbial clusters.

2 ml dispersed granule samples were taken and serially diluted from  $10^0$  to  $10^7$  with 0.85% saline. 4-fold serial dilutions of cell suspension ( $10^4$  to  $10^7$ ) were spread in triplicate on either Bacto<sup>®</sup> plate count agar or PNP-MS agar. All operations were performed in a biohazard flow cabinet (ESCO, Singapore). Plates were sealed with parafilm, inverted and incubated in a 25°C incubator (Sanyo, Japan) and monitored over 2 weeks. Visible colonies were enumerated after 1 week of incubation.

Colonies formed on PNP-MS agar were then picked up and restreaked either onto fresh PNP-MS agar or Bacto<sup>®</sup> R2A plates. Axenic cultures were obtained after several such transfers. Purity of the cultures was confirmed by a microscopic examination.

#### **4.2.4 Morphological and Phenotypic Characterizations of PNP-Degrading Bacteria**

Cells were observed with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM) (Stereoscan 420, Leica, Cambridge Instrumentns). Cells were fixed, dehydrated, dried and viewed for SEM with the method described by Zhuang et al. (2002). Gram-stain test, oxidase activity and catalase activity were performed as described by Smibert and Krieg (1994). A non-staining Gram-stain method was also performed to validate the Gram-stain results (Maszenan, 2000). Motility test was performed with Bacto<sup>®</sup> Motility Medium S according to the manufacture's instructions (Difco Laboratories, Division of Becton Dickinson and Company, Sparks, Maryland, USA). *Escherichia coli* ATCC<sup>®</sup> 25922 was used as a quality control in the tests.

Enzyme profiles and carbon substrate utilization characteristics of PNP-01 were determined using the API ZYM and API 20E systems according to the manufacturer's instructions (bioMérieux, France). The suspension of cells (which was prepared according to the manufacturer's instructions) was inoculated into each minitube in the API ZYM and API 20E strips and the strips were incubated at 37°C according to the manufacturer's instructions. Sterilized mineral oil was added when necessary. After incubation, reagents were added to each minitube according to the manufacturer's instructions. The strips were then compared to a reading chart supplied by the manufacturer and all reactions were scored accordingly.

#### **4.2.5 Direct Lysis PCR Amplification and ARDRA Fingerprints of PNP-degrading bacteria**

Modified whole cell direct lysis PCR amplification method (Maszenan, 2000) was used to amplify the 16S rRNA gene of all isolates. Cells were cultured either on R2A or Bacto<sup>®</sup> TSA plates at 25°C for 4 days. A single colony was aseptically collected with a sterile pipette tip and smeared onto the bottom of a PCR tube. Cell lysis was performed at 98°C for 30 minutes in a thermal cycler (Mastercycler, Eppendorf, Germany) with 90 µl of mixture containing 10 µl of 10× thermophilic DNA Taq polymerase Buffer B (Promega Co., USA), 8 µl 25 mM MgCl<sub>2</sub>, and 72 µl sterilized MilliQ water. The nearly full-length 16S rRNA gene was amplified by PCR with forward primer Eubac27F and reverse primer Universal 1492R1 (Table 4.1). Thermal cycling was carried out as follows: 30 cycles consisting of 1.5 min at 94°C, 1.5 min at 62°C, and 2 min at 72°C. The last step of the last cycle continued for 10 min, followed by cooling at 4°C. The PCR products were purified with a Qiagen PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. Agarose gel electrophoresis of the harvested products was used to confirm purity and concentration of the amplified DNA.

Amplified rDNA restriction analysis (ARDRA) of the isolates was performed to identify the identical strains. Aliquots (approximately 0.5µg) of 16S rRNA gene were digested with restriction endonuclease *CfoI* (Promega, USA) and

separated by gel electrophoresis in 3% metaphor agarose gels (FMC, Rockland, Maine) to generate restriction fragment length polymorphism (RFLP) patterns. The strains with the same RFLP patterns were considered the identical strains.

**Table 4.1** Primers used in this study for PCR and sequencing.

Name	Sequence (5' – 3')	Specificity	Reference
27F	AGAGTTTGATCMTGGCTCAG	Most eubacteria	Lane, 1991
357F	CTCCTACGGGAGGCAGCAG	Most eubacteria	Lane, 1991
530F	GTGCCAGCMGCCGCGG	Most eubacteria, eukaryotes, archaeobacteria	Lane, 1991
800F	GTCCACGCCGTAAACGATG	Eubacteria	Lane, 1991
1100F	GTCCCGCAACGAGCGCAAC	Eubacteria	Lane, 1991
1392F	CTTGTACACACCGCCCGT	Most eubacteria	Lane, 1991
338R	GCTGCCTCCCGTAGGAGT	Most eubacteria	Lane, 1991
519R	GWATTACCGCGGCKGCTG	Most eubacteria, eukaryotes, archaeobacteria	Lane, 1991
800R	CATCGTTTACGGCGTGGAC	Most eubacteria	Lane, 1991
1100R	TTGCGCTCGTTGCGGGACT	Most eubacteria	Lane, 1991
1392R	ACGGGCGGTGTGTACAAG	Most eubacteria, eukaryotes, archaeobacteria	Lane, 1991
1492R1	TACGGYTACCTTGTTACGACTT	Most eubacteria, archaeobacteria	Tay et al., 1998

\*M = C : A    W = A : T    Y = C : T    K = G : T

#### 4.2.6 16S rRNA Gene Sequencing and Phylogenetic Analyses of PNP-Degrading Bacteria

The nucleotide sequences of 16S rRNA gene from the isolates were determined using the dideoxy chain termination chemistry and the ABI model 310A sequencer (Applied Biosystems, Perkin-Elmer). The ABI PRISM<sup>®</sup> BigDye<sup>™</sup> Terminator Cycle Sequencing ready-reaction kit (version 3.0) (Applied Biosystems) was used as specified by the manufacturer. Both strands of 16S rRNA gene

sequences were sequenced by using the primers listed in Table 4.1. The partial sequences were compiled and aligned using BioEdit software (Hall, 1999) (available from <http://www.mbio.ncsu.edu/RnaseP/info/programs/BIOEDIT>) and analyzed with BLAST search program (Altschul et al., 1990) and other algorithms as described previously (Zhuang et al., 2002).

#### **4.2.7 Screening PNP Degradation of PNP-Degrading Isolates**

The isolates were screened for their abilities to grow on PNP as well as to degrade PNP on the agar plates. Axenic cultures were streaked onto fresh PNP-MS agar plates in duplicate and incubated in a 25°C incubator (Sanyo, Japan). Plates were observed daily. For the ability to grow on PNP, the plate was counted as positive if visible colonies were detected. For the ability to degrade PNP, the plate was counted as positive if the yellow color induced by PNP disappeared on the plate.

#### **4.2.8 Specific Growth Rate of PNP-Degrading Isolates**

Cells used in the experiment were exponential phase cultures grown with PNP as sole carbon and energy source. The PNP-MS medium was used with addition of 1% (v/v) filter-sterilized vitamin solution (Vitamin solution [100 ml] was prepared as described by Su et al. (2001), containing biotin, 0.02 g; folic acid, 0.02 g; pyridoxine hydrochloride, 0.1 g; riboflavin, 0.05 g; thiamin hydrochloride, 0.05 g; nicotinic acid, 0.05 g; pantothenic acid, 0.05 g; cyanocobalamine, 0.01 g; p-aminobenzoic acid, 0.05 g and thioctic acid, 0.05 g.). Experiments were performed in triplicate in 250 ml shaking flasks. The medium solution volume in each bottle was 50 ml with the initial PNP concentration of 100 mg/l. The individual strain was then introduced to liquid solution with optical density (OD) of 0.08-0.1, and incubated at 25°C with active shaking (180 rpm). PNP concentration was assayed at regular intervals of incubation. Cell growth was monitored by OD measurement at 600 nm using a spectrophotometer (Beckman DU-650 uv-vis Spectrophotometer). The specific growth rates were calculated from the cell growth measurement during

the initial period. The initial period was defined as the time duration in which there was less than 5% of substrate degraded. Negative controls were performed for each isolate under the same experimental conditions except no carbon source being supplied to the culture medium.

#### 4.2.9 Chemical and Cell Concentration Analyses

PNP concentrations were measured as described by Ray et al. (1999) in Chapter III. Dry weight (DW) of strain PNP-01 cells was determined as described by Onysko (2000). In brief, cell suspension was filtrated through a 0.2  $\mu\text{m}$ -pore size cellulose acetate membrane filter (Advance MFS Inc, CA) and dried to a constant weight for 24 hours at 80°C. Cell numbers were determined by the CFU count with Bacto<sup>®</sup> R2A agar plates. Cell growth was monitored by OD measurement at 600 nm using a spectrophotometer (DR/2400 Portable Spectrophotometer, Hach, Loveland, CL, U.S.A.). The linear relationship between the cell numbers (CFU) and OD<sub>600</sub> as well as DW and OD<sub>600</sub> for strain PNP-01 were established through linear regression (Table 4.2).

**Table 4.2** The linear relationship between CFU and OD<sub>600</sub> as well as DW and OD<sub>600</sub> for strain PNP-01.

<b>Equation: <math>Y = a \cdot X</math></b>				
<b>Function</b>	<b>Variables</b>		<b>a Coefficient <math>\pm</math> Std Error</b>	<b>R<sup>2</sup></b>
	<b>Y</b>	<b>X</b>		
CFU vs OD <sub>600</sub>	CFU	OD <sub>600</sub>	2.3 $\pm$ 0.1 $\times 10^8$ (cells/ml)	0.99
DW vs OD <sub>600</sub>	DW		0.21 $\pm$ 0.01 (mg/ml)	0.99

#### 4.2.10 Induction of PNP Degradation of Strain PNP-01

For a constant experimental result in a batch study, the PNP-degrading ability of strain PNP-01 was induced before every batch study according to the method describe by Kadiyala et al. (1998) with modification. Cells used for induction were grown overnight in 200 ml cultures of 1/5-strength of Bacto<sup>®</sup> tryptic soy broth. The cells were harvested by centrifugation, washed three times and suspended in PNP-MS broth to a final OD<sub>600</sub> of 1 and incubated at 25°C with shaking at 180 rpm. When the yellow color of PNP disappeared, another dosage of PNP was added into the culture flasks at a final concentration of 100 mg PNP/l. This step was repeated five to six times until the yellow color of PNP disappeared at a rate of approximately 50 mg of PNP/OD<sub>600</sub>·h, suggesting that a sufficient induction was achieved.

#### 4.2.11 Cell Density Effects and Specific PNP Degradation Rate

Since maximum in-cycle PNP concentration was 100 mg/l in the granulation SBR, specific PNP degradation rate for strain PNP-01 was assessed at the PNP concentration up to 100 mg/l.

The effect of cell density on PNP degradation was studied with initial different concentrations of well-induced cells in the reaction mixture containing 100 mg/l PNP. The samples were withdrawn from the reaction mixture and the PNP concentration was determined at every 10 minute-interval for up to 280 minutes.

Strain PNP-01 was characterized for its specific PNP degradation rate using PNP as the sole substrate. Cells used in determination of specific growth rate were well-induced stationary-phase cultures. An initial cell concentration of around  $1.6 \times 10^8$  CFU/ml was employed to avoid the cell density effects on PNP biodegradation. Experiments were performed with triplicate sets of cultures in 100 ml PNP-MS medium at 25°C with shaking. Kinetic parameters,  $K_m$  and  $V_{max}$  were estimated by fitting the initial rate of PNP degradation under different PNP

concentrations up to 100 mg/l to the Michaelis-Menten equation ( $V = V_{max} \cdot S / [K_m + S]$ ), where  $V$  and  $V_{max}$  are the specific and the maximum specific substrate degradation rates [mg of PNP/mg of cell DW·h], respectively, and  $S$ , and  $K_m$  are the substrate concentration, half-saturation constant [mg PNP/l], respectively) using a nonlinear analysis.

#### 4.2.12 Self-Flocculation Assay of Strain PNP-01

Self-flocculation of strain PNP-01 was assayed with the method described by Malik et al. (2003). Cell suspension for the assay was prepared from a liquid culture of PNP-01 in PNP-MS broth incubated at 25°C with shaking (180 rpm). Cells were harvested in the late exponential growth phase by centrifugation at 3000 rpm for 20 minutes, washed twice and suspended in the MS broth with the OD<sub>600</sub> of the cell suspension at the range of 0.4 to 0.5.

The cell suspension (200 ml) was allowed to stand still for an hour in a cylinder. Duplicates of 3-ml aliquot of cell suspension were periodically sampled from the supernatant of cell suspension and measured the OD<sub>600</sub> as described by Malik et al. (2003).

#### 4.2.13 Relative Fluorescence Intensity Analysis of DGGE Fingerprints

The relative fluorescence intensity analysis of DGGE fingerprints was performed with the methods described by Casamayor et al. (2002). High-resolution digital DGGE images were analyzed using 1D Image Analysis software (Kodak, USA). Lanes were manually framed, and bands in each lane were automatically searched. A pixel intensity profile was automatically generated with the software. The relative fluorescence intensity (expressed as percentage of the net intensity [the sum of the background subtracted pixel values in the band rectangle] of a particular band relative to the total of all bands in the lane) of each band was calculated with the software and the error among replicates was < 4%.

#### 4.2.14 Recovery and Purification of DNA Template from DGGE bands

The recovery and purification of DNA templates from DGGE bands were performed according to Tay et al. (2005c) with modification. Selected DGGE bands were aseptically excised and crushed. DNA was eluted by incubation of the crushed band overnight in 20  $\mu$ l of sterile MilliQ water at 30°C with gentle shaking. After centrifugation at 5000 g for a minute, 1- $\mu$ l aliquot of the elutant was used as the template for PCR amplification with the original primer set of P2 and P3 as described in Chapter III.

DGGE analysis on the PCR amplicons was performed to check the migratory position and purity of bands. The correct migratory position of PCR amplicons was confirmed using PCR amplicons of PNP-01 as a DGGE marker. The purity of the bands was confirmed when no band besides the band of interest was present in each lane on DGGE gel. For those impure bands, additional rounds of band excision, PCR amplification and DGGE analysis were performed until the band was pure.

The confirmed PCR amplicons (which only contained one DGGE band) were subsequently purified with the Qiagen PCR purification kit (Qiagen, Germany) and quantified in an agarose gel.

#### 4.2.15 DNA Sequencing

Approximately 20 to 40 ng purified PCR amplicons were used for the sequencing reaction with the ABI PRISM<sup>®</sup> BigDye<sup>™</sup> Terminator Cycle Sequencing ready-reaction kit (version 3.0), using primer P2 (Watanabe et al., 1998). The 16S rRNA gene fragments were sequenced with the ABI model 310A sequencer (Applied Biosystems, Perkin-Elmer). The sequences obtained were manually aligned using BioEdit software (Hall, 1999) and the similarities of sequences were analyzed with the BioEdit software and PHYLIP package of

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programs (version 3.5c, available from the website <http://evolution.genetics.washington.edu/phylip.html>).

#### 4.2.16 AHL Extraction and Bioassays

The AHL extraction and bioassays were performed as described by Swift (1997). Spent supernatants (500 ml) from over night (12-16 hours) cultures of strain PNP-01 with an initial cell density greater than  $1.6 \times 10^8$  CFU/ml in PNP-MS were extracted three times with dichloromethane (700:300 of supernatant over dichloromethane). The dried extract was reconstituted with methanol and water and filter-sterilized. The AHL extraction was spotted onto the agar plates which contained reporter strain *C. violaceum* CV026. Positive assays were judged as induction of the purple pigment violacein in the *C. violaceum* CV026 at the spotted place.

AHL production by strain PNP-01 was also detected by cross-streaking against reporter strains. Positive assays were judged as induction of the purple pigment violacein in the *C. violaceum* CV026 reporter as described by McClean (1997).

## 4.3 RESULTS

### 4.3.1 Competent Biomass Fraction

Since the granules were cultivated with both PNP and glucose, the presence of PNP-degrading bacteria was evaluated. Counts of colony-forming units (CFUs) revealed that the granules contained  $8.2 \pm 2.2 \times 10^7$  CFU/mg MLSS of heterotrophic bacteria, and  $4.0 \pm 1.4 \times 10^7$  CFU/mg MLSS of PNP-degrading bacteria. PNP-degrading bacteria accounted for 49% of total heterotrophic CFUs.

### 4.3.2 Isolation and Characterization of PNP-Degrading Bacteria

All colonies from the PNP-degrading aerobic granules obtained by a direct isolation technique on PNP-MS agar plates were initially screened based on colony morphology, color, and size and then were subjected to ARDRA analysis to identify identical stains. A final set of 4 strains designated PNP-01 to PNP-04 were obtained from ARDRA analysis of all colonies isolated (Table 4.3). Each strain in the final set had a unique ARDRA pattern.

Only colonies associated with strain PNP-01 appeared on plates with dilution factors higher than  $10^7$ , indicating that PNP-01 is the highest number of active culturable PNP-degrading bacterial population in aerobic granules compared to the other three isolates. Colonies associated with strain PNP-04 was found accounting for 23.5% in total PNP-degrading competent fraction, suggesting that PNP-04 was the second most abundant PNP-degrading population in granules. PNP-02 was the third abundant bacteria in the granules. Strain PNP-03 was estimated to be present in the PNP-degrading aerobic granules at a cell density of  $1.4 \times 10^5$  CFU/mg granule MLSS, suggesting strain PNP-03 only accounted for a small proportion of bacterial community in granules (Table 4.3). The phenotypic characteristics are summarized in Table 4.4. Colonies formed on agar plates of strain PNP-02 was highly mucoid. Liquid cultures of strain PNP-03 and PNP-04

**Table 4.3** The summarization of final set of 4 PNP-degrading strains.

Strain name	Cell counts (CFU/mg MLSS)	Colony morphology			Self aggregation	Phenotypic characteristics			Microscopy morphology	
		Size	Color	Slime production		Motility	Oxidase	Catalase	Shape	Gram-stain
PNP-01	$2.7 \pm 0.8 \times 10^7$	2-3 mm	White	-	-	+	-	-	Rods	Gram -
PNP-02	$3.7 \pm 1.7 \times 10^6$	3-4 mm	White	++	-	+	+	+	Cocci	Gram -
PNP-03	$1.4 \pm 0.1 \times 10^5$	1-2 mm	Gray white	-	+	+	+	+	Rods	Gram +
PNP-04	$9.4 \pm 0.9 \times 10^6$	4-5 mm	Yellowish	-	++	-	+	+	Rods & cocci	Gram +&-

could form aggregates. Strains PNP-03 and PNP-04 were found positive in the motility test. Strain PNP-01, PNP-02 and PNP-03 were found positive in the oxidase test and Strains PNP-02, PNP-03 and PNP-04 were found positive in the catalase test.

Microscopy coupling with gram-staining was used to visualize the morphology of each strain. Strains PNP-01 and PNP-03 were bacterial rods, while strain PNP-02 was cocci. Strain PNP-04 was not composed of a single bacterial species but a bacterial consortium containing both Gram negative and Gram positive bacteria. However, conventional streaking technique was not able to separate the different bacterial species from each other in PNP-04. The isolation and characterization of PNP-04 will be described in detail in Chapter V.

#### **4.3.3 Microbial Diversity among the Isolated Bacteria from PNP-Degrading Granules**

The 16S rDNA sequence analyses indicated that two PNP-degrading strains, PNP-01 and PNP-03 which fall in the  $\beta$ -Proteobacteria group, are highly similar (>98%) with their nearest neighbors in genus *Burkholderia* and *Acidovorax*, respectively (Table 4.4). PNP-01 is related to *Burkholderia* sp. FDS-1 (AY550913), a fenitrothion-mineralizing bacterium strain (Zhang et al., 2005) and PNP-03 is related to *Acidovorax* sp. PD-10 (AB195159), a culturable denitrifier isolated from a solid-phase-denitrification process (Horiba et al., 2005). Strain PNP-02 belongs to the  $\alpha$ -Proteobacteria group, genus *Paracoccus* and shows 98.4% identity with *Paracoccus yeei* (AY528674) (Funke et al., 2004).

#### **4.3.4 Physiological Analyses of Isolates from Aerobic Granules**

Screening experiments on the growth of three strains on PNP as sole carbon and energy source showed that the strain PNP-01 could form colonies on PNP-MS plates after 1 to 2 days of incubation, while strain PNP-02 and PNP-03 could form

pin-point colonies after 4 to 5 days of incubation (Table 4.5). The different time needed for three strains to form colonies indicated that strain PNP-01 grew much faster than the other two strains.

**Table 4.4** The nucleotide sequence analyses of the 16S rRNA gene of three PNP-degrading bacterial strains.

Strain	Taxonomy affiliation	Closest relative in GeneBank	Identity (%)	Number of bases used in establishing identity
PNP-01	$\beta$ -Proteobacteria	<i>Burkholderia</i> sp. FDS-1 (AY550913)	98.9	1497
PNP-02	$\alpha$ -Proteobacteria	<i>Paracoccus yeei</i> (AY528674)	98.4	1423
PNP-03	$\beta$ -Proteobacteria	<i>Acidovorax</i> sp. PD-10 (AB195159)	99.7	1428

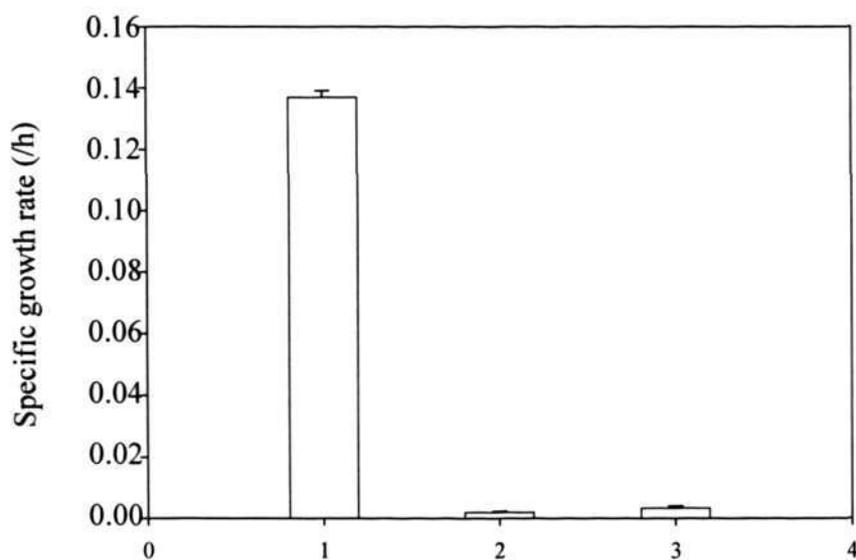
**Table 4.5** Screening experiments of isolated bacteria for their capability to grow and to degrade PNP on PNP-MS agar plates.

Strain	Growth	Color removal
PNP-01	+	+
PNP-02	+	-
PNP-03	+	-

Screening experiments on degradation of PNP with three strains showed similar trends (Table 4.5). Strain PNP-01 could transform the yellow color induced by PNP into colorless on agar plate in 1 to 2 days after the appearance of colonies. However, the other two strains, PNP-02 and PNP-03, were not able to transform the

yellow color into colorless within a 2-week incubation, indicating a very slow degradation of PNP. That strains PNP-02 and PNP-03 were proven not being able to form colonies on MS agar plates without any carbon source, indicates that they were not oligotrophic bacteria.

Specific growth rates of three strains were measured in batch experiments. Strain PNP-01 was found having the highest specific growth rate, 0.14 /h among the three strains isolated from PNP-degrading granules (Figure 4.2). Strain PNP-02 and PNP-03 were found growing slowly using PNP as the sole carbon and energy source.



**Figure 4.2** The specific growth rate of three isolated strain. 1, PNP-01; 2, PNP-02; and 3, PNP-03.

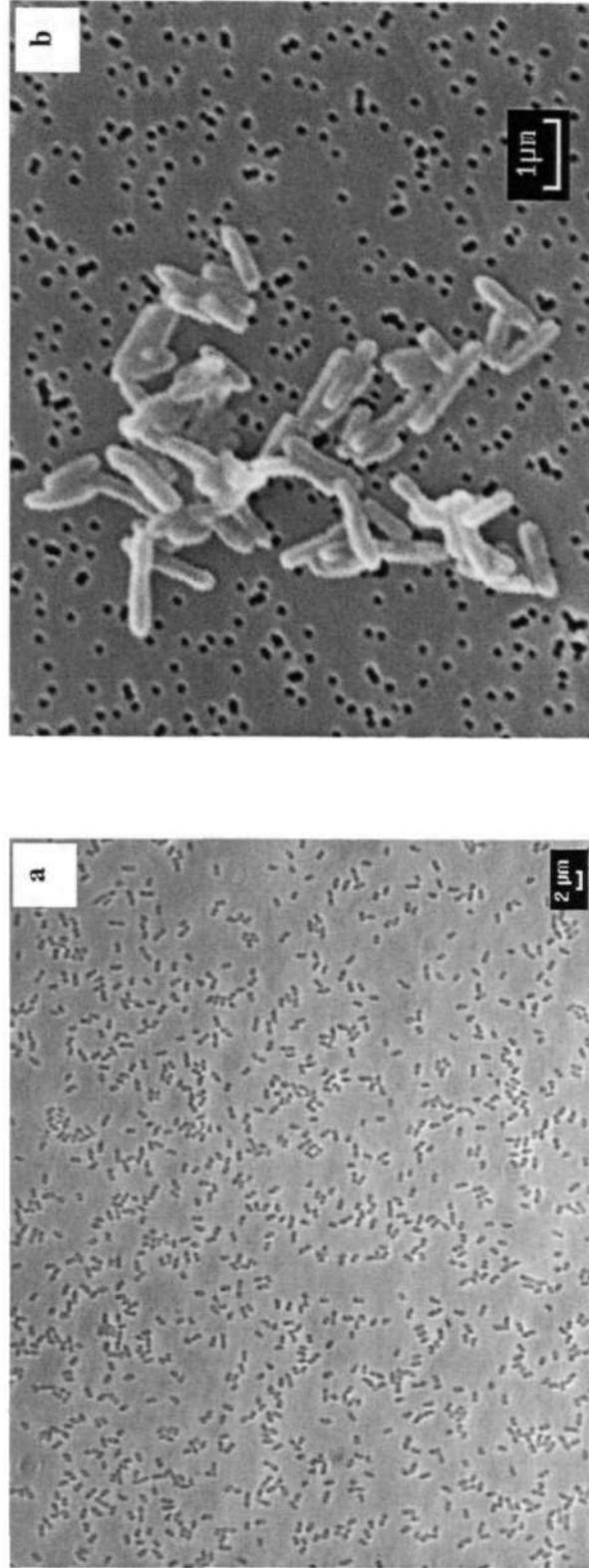
#### 4.3.5 Isolation and Quantification of PNP-Degrading Strain PN-01

Strain PNP-01 was obtained from a 2 to 3 mm diameter white colony formed on PNP-MS agar plates spread with 100  $\mu$ l of a  $10^6$ -fold diluted dispersed granule samples. Based on its appearance on the  $10^6$ -fold dilution plate, strain PNP-01 was estimated to be present in the PNP-degrading aerobic granules at a concentration of  $2.7 \pm 0.8 \times 10^7$  cells/mg of granule MLSS. This cell density is equivalent to 0.025 g DW of cells /g MLSS of granules or 2.5% of granules (g DW/g MLSS), based on the calculated relationships of cell number (CFU count), biomass concentration and the OD<sub>600</sub> for a batch culture of strain PNP-01 grown on PNP. Since culturable bacteria usually account for 0.1% to 15% of total microbial population in the environmental sample (Amann *et al.*, 1995), the fact that strain PNP-01 represented 2.5% of granules (on a dry weight basis) suggested that it constituted a significant fraction of the total bacteria residing in the PNP-degrading granules.

#### 4.3.6 Phenotypic and Phylogenetic Characterization of PNP-Degrading Strain PN-01

Strain PNP-01 was a rod-shaped, gram negative, non-spore forming aerobic bacterium. Cells were between 0.7 to 1.4  $\mu$ m in length and 0.2 to 0.4  $\mu$ m in diameter when grown 18-24 hours in 1/5 strength Bacto<sup>®</sup> tryptic soy broth at 25°C (Figure 4.3a). Cells were nonmotile, and flagella were not observed using scanning electron microscopy (SEM) (Figure 4.3b).

The following biochemical characteristics tested by API 20E and API ZYM were positive for strain PNP-01: Voges-Proskauer test, arginine dihydrolase, ornithine decarboxylase, alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, N-acetyl- $\beta$ -glucosaminidase, citrate utilization, indole production, nitrate reduction, glucose, rhamnose, and arabinose.



**Figure.4.3** Micrographs of strain PNP-01. (a) Phase contrast microscopic image (bar = 2 μm); and (b) scanning electron micrograph (bar = 1 μm) of strain PNP-01.

The following biochemical characteristics tested by API 20E and API ZYM were negative for strain PNP-01: lipase, trypsin,  $\alpha$ -chymotrypsin, esterase, esterase lipase, cystine arylamidase,  $\alpha$ -glucosidase,  $\beta$ -galactosidase,  $\alpha$ -mannosidase,  $\alpha$ -fucosidase, lysine decarboxylase, gelatinase, H<sub>2</sub>S production, urease, tryptophane deaminase, melibiose, mannitol, inositol, sorbitol, sucrose, and amygdalin.

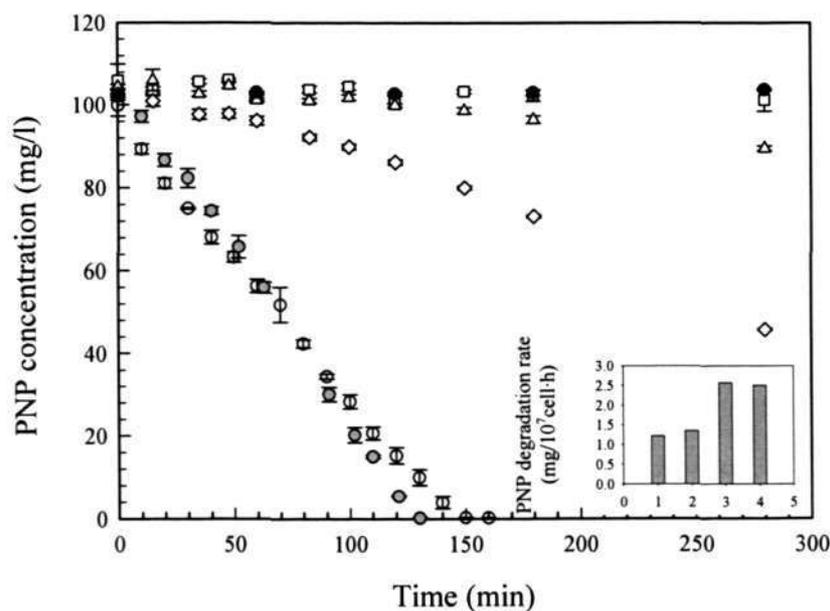
The nucleotide sequence of the 16S rRNA gene of strain PNP-01 (1,497 bp) was 98.9% and 97.3% identical to that of *Burkholderia* sp. FDS-1 (AY550913) and *Burkholderia* sp. strain AK-5 (AB103080), respectively (Takenaka et al., 2003; Zhang et al., 2005). According to its 16S rRNA gene sequence and phylogenetic analysis, strain PNP-01 was therefore identified as belonging to the genus of *Burkholderia*.

#### 4.3.7 Density-Dependent PNP Degradation with Strain PNP-01

PNP biodegradation assay with different initial cell density of strain PNP-01 showed that cell density had significant influence on the PNP biodegradation efficiency. Several densities of PNP-01 cells, ranged from  $1.6 \times 10^7$  CFU/ml to  $2.0 \times 10^8$  CFU/ml, were inoculated into the degradation assay setup, but efficient PNP degradation could only occur at cell densities of  $1.0 \times 10^8$  CFU/ml and higher (Figure 4.4).

The length of the lag phase before the onset of PNP biodegradation was found inversely associated to the initial density of PNP-01 cells (Figure 4.4). At higher cell densities, such as  $1.6 \times 10^8$  CFU/ml and  $2.0 \times 10^8$  CFU/ml, PNP biodegradation was immediately started after cell inoculated into the degradation assay. In contrast, an hour lag phase was observed before the PNP biodegradation started at the cell density of  $1.0 \times 10^8$  CFU/ml, and a 2-hour lag phase was found at the cell density of  $3.2 \times 10^7$  CFU/ml. At lower cell density of  $1.6 \times 10^7$  CFU/ml, the lag phase was more than 4 hours.

PNP biodegradation rate was found higher in a higher cell density (Inset of Figure 4.4). For example, at cell density of  $1.6 \times 10^8$  CFU/ml and  $2.0 \times 10^8$  CFU/ml, average PNP biodegradation rates were  $2.6 \text{ mg PNP}/10^7 \text{ CFU}\cdot\text{h}$  and  $2.5 \text{ mg PNP}/10^7 \text{ CFU}\cdot\text{h}$ , respectively. However, at cell density of  $3.2 \times 10^7$  CFU/ml and  $1.0 \times 10^8$  CFU/ml, average PNP biodegradation rates were  $1.2 \text{ mg PNP}/10^7 \text{ CFU}\cdot\text{h}$  and  $1.3 \text{ mg PNP}/10^7 \text{ CFU}\cdot\text{h}$ , respectively.

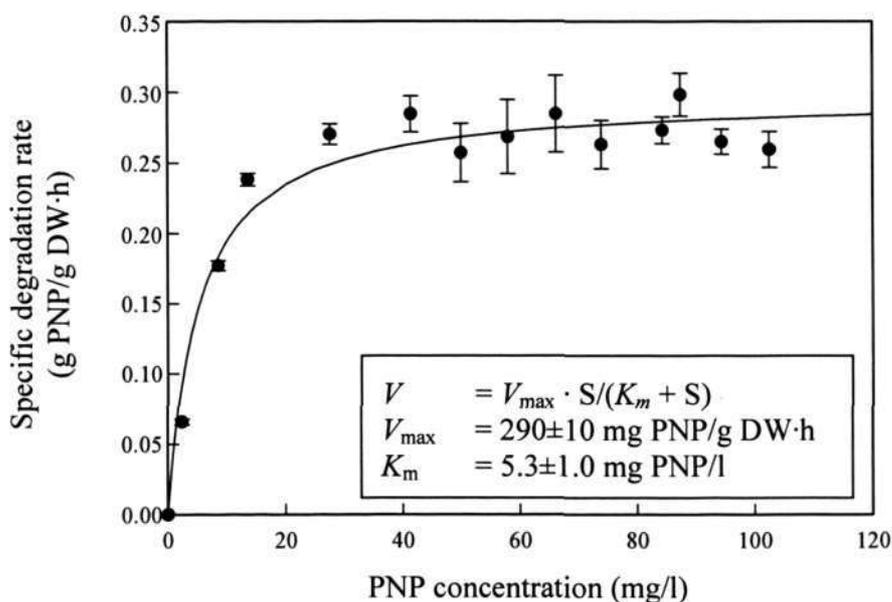


**Figure 4.4** PNP degradation by strain PNP-01 with different initial density of cells (●,  $2.0 \times 10^8$  CFU/ml; ○,  $1.6 \times 10^8$  CFU/ml; ◇,  $1.0 \times 10^8$  CFU/ml; △,  $3.2 \times 10^7$  CFU/ml, □,  $1.6 \times 10^7$  CFU/ml, and ●, negative control). Inset, average PNP degradation rate at different cell density. 1,  $3.2 \times 10^7$  CFU/ml; 2,  $1.0 \times 10^8$  CFU/ml; 3,  $1.6 \times 10^8$  CFU/ml and; 4,  $2.0 \times 10^8$  CFU/ml.

### 4.3.8 Kinetic Analysis of PNP Biodegradation with Strain PNP-01

Well-induced stationary-phase cells of strain PNP-01 were harvested for batch tests to assess PNP degradation kinetics (Figure 4.5). In order to circumvent the effect of cell density, an initial cell concentration of approximately  $1.6 \times 10^8$  CFU/ml was used for the kinetic analysis.

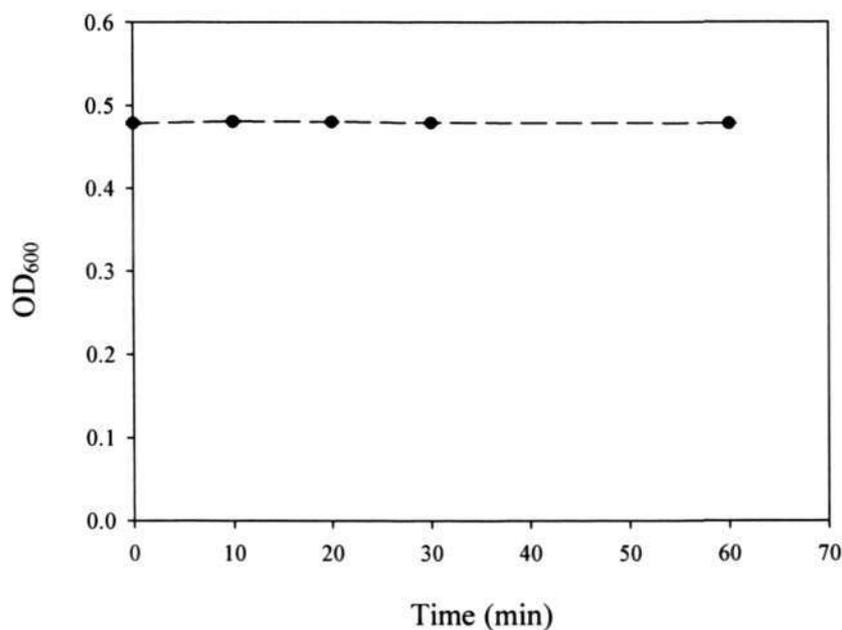
The non-growth kinetic model (enzyme kinetics) was employed in the test in which entire cells were treated as enzyme systems for PNP degradation. The Michaelis–Menten equation was used to calculate the kinetic parameters since the substrate inhibition was not apparent in the range of PNP concentrations used for the kinetic study. As shown in Figure 4.5, the strain PNP-01 had a maximum specific PNP degradation rate ( $V_{max}$ ) of 290 mg of PNP/g of DW·h and a half-saturation constant ( $K_m$ ) of  $5.3 \pm 1.0$  mg of PNP/l.



**Figure 4.5** Kinetics for PNP biodegradation of strain PNP-01. Error bars represent the standard deviation from triplicate batch experiments (●, experimental results; —, fit.  $R^2 = 0.97$ ).

#### 4.3.9 Self-Flocculation Assay of Strain PNP-01

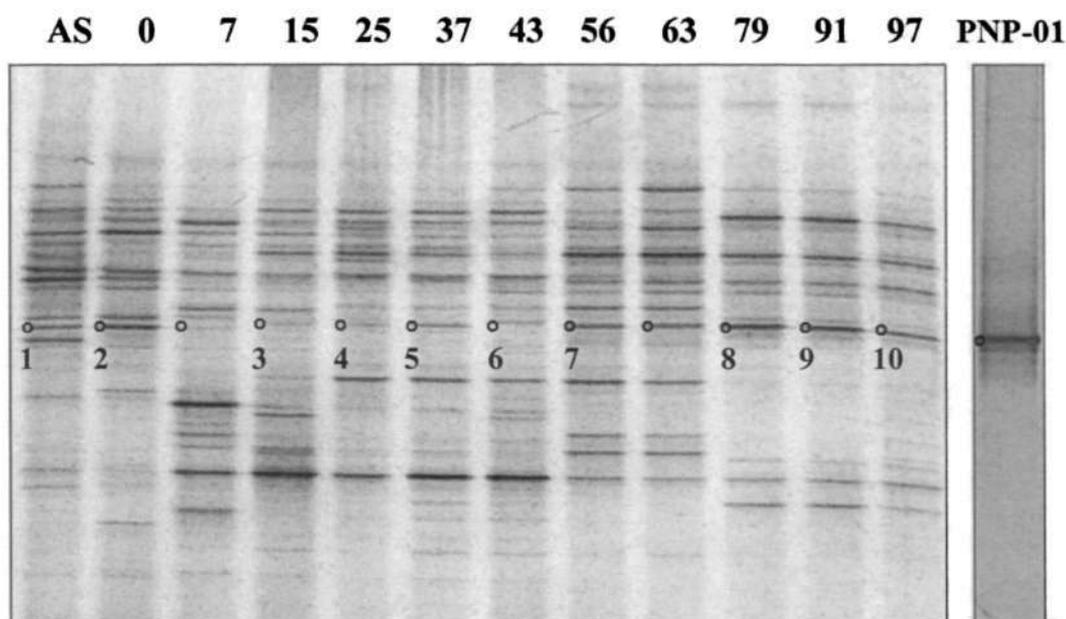
When grown in PNP-MS and 1/5 strength Bacto<sup>®</sup> Tryptic Soy Broth, strain PNP-01 was found to prefer a suspension growth rather than self-aggregation or attachment onto the inner surface of the shake flasks. A settling assay was therefore performed to exam the aggregation and settling capability of this strain. The extent of self-aggregation and attachment on surface of strain PNP-01 was measured using a 1-hour settling assay. The results showed that OD<sub>600</sub> of late exponential phase cell suspensions growth in PNP-MS liquid medium did not change significantly after a 1-hour stand-still assay in the cylinder (Figure 4.6). The results confirmed that strain PNP-01 was not an aggregate or biofilm-forming bacterium.



**Figure 4.6** Settling assay of strain PNP-01 (●, Absorbance at OD<sub>600</sub>).

#### 4.3.10 Detection the Presence of *Burkholderia* sp. Strain PNP-01-related bacteria During Granulation with DGGE

PCR-DGGE analysis on the microbial communities in the reactor biomass showed that there was a band present in every lane comigrating with the band of strain PNP-01 (bands marked with a small round circle in Figure 4.7). To confirm that the comigrating bands corresponded to the same DNA sequence of strain PNP-01, the bands of interest were excised from DGGE gel and sequenced (bands marked with numbers in Figure 4.7).



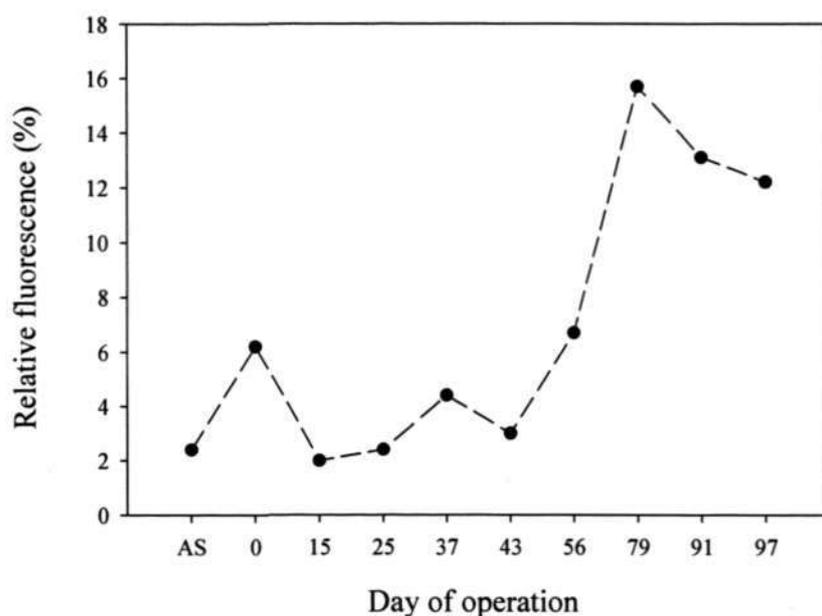
**Figure 4.7** Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA genes from sludge or granule samples during granulation. AS stands for activated sludge. Numbers stand for the day of operation. PNP-01 stands for strain PNP-01. Small circles correspond to the bands found to comigrate with strain PNP-01, and numbers are given to the bands excised and sequenced. The sequenced bands showed a similarity of >98% to strain PNP-01, based on 160 basepairs of 16S rRNA gene fragments for the similarity calculation.

The sequences recovered from the bands (marked with numbers in Figure 4.7) were aligned and compared with the corresponding sequences of strain PNP-01. The similarity of sequences between excised bands and strain PNP-01 was calculated based on 160 basepairs of 16S rRNA gene sequences. It was found that there were high similarities between the sequences recovered from DGGE bands and the sequences of PNP-01 (Table 4.6). Most sequences were identical to the sequences of PNP-01, and two sequences from day 15 and day 43 had a similarity of 98.8% (2/160) and 99.1% (1/160) to strain PNP-01 respectively. Therefore, the excised bands were confirmed being related with strain PNP-01.

**Table 4.6** The similarities between excised bands and the corresponding position of strain PNP-01 based on 160 basepairs of 16S rRNA gene sequences.

Band No.	Sample	Similarity to corresponding position of strain PNP-01 (%)
1	AS	100
2	Day 0	100
3	Day 15	98.8 (2/160)
4	Day 25	100
5	Day 37	100
6	Day 43	99.3 (1/160)
7	Day 56	100
8	Day 79	100
9	Day 91	100
10	Day 97	100

It was found that the band related to strain PNP-01 was present in every sample on the DGGE profile, from the seed sludge to the mature granules. Temporal changes in the relative fluorescence of strain PNP-01 associated bacteria were monitored through analyzing the changes in the intensity of the DGGE bands during the granule formation (Figure 4.8).



**Figure 4.8** Relative fluorescence of strain PNP-01-related bacteria during granule formation based on the estimation of band intensity in PCR-DGGE analysis.

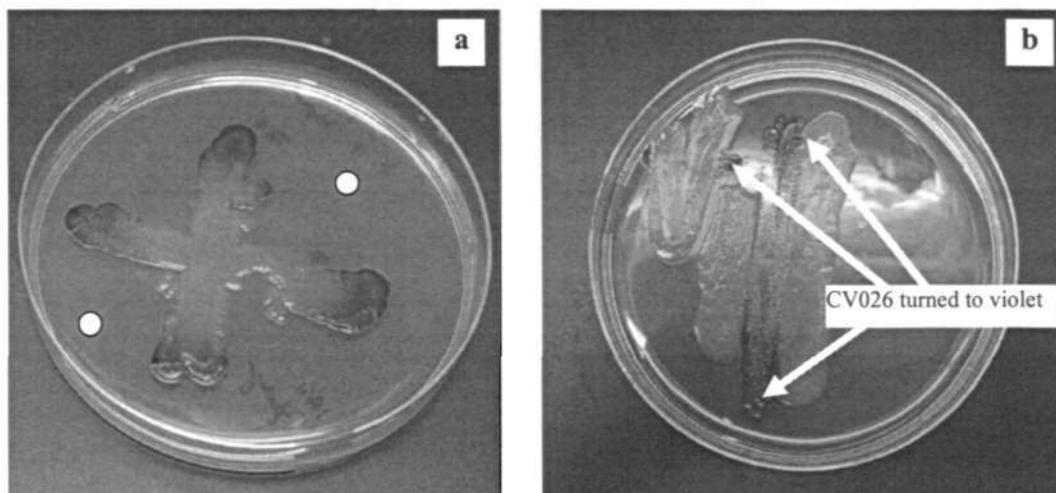
As showed in Figure 4.8, strain PNP-01-related bacteria were present in the activated sludge with a relative fluorescence of 2%, after acclimation, the relative fluorescence of PNP-01-related bacteria increased to 6%. During the process of granulation, the relative fluorescence of PNP-01-related bacteria fluctuated initially and subsequently gradually increased to >12%. The constant presence of PNP-01-related bacteria during the process of granulation indicated that they were a group

of bacteria that were well-adapted to the PNP-degrading granulation SBR system and they may play an important role in the PNP-degrading granulation SBR system. The observation of PNP-01 being present in both seed sludge and in granules indicated that PNP-01 was not only an important population in water treatment plant, but also a good survivor and competitor in the granulation SBR system.

#### 4.3.11 AHLs Production by Strain PNP-01

In this study, cell density was found related to the efficient PNP biodegradation by strain PNP-01. QS is a mechanism by which bacteria communicate and obtain information on cell density in their environment (Keller and Surette, 2006). N-acyl-homoserine lactones (AHLs) are widely employed by gram-negative bacteria as diffusible QS signal molecules (Whitehead et al., 2001).

In order to investigate whether strain PNP-01 possesses QS, the produce of QS signal molecules, AHLs by strain PNP-01 was investigated. The supernatant from PNP-01 cultures in PNP-MS medium assayed with *C. violaceum* CV026 gave a positive result from the spotted plates (Figure 4.9, a), indicating that diffusible QS sensing molecules, AHLs were present in the PNP biodegradation. Cross-streaking experiments with PNP-01 against the *C. violaceum* CV026 (Figure 4.9, b) also confirmed that PNP-01 produced diffusible AHLs.



**Figure 4.9** Strain PNP-01 was tested for QS signal molecules synthesis using the reporter strain *C. violaceum* CV026 as an indicator. (a) Extracted bacterial culture medium from high cell number in PNP-MS was spotted on the TSA agar (white circles), and reporter strain *C. violaceum* CV026 turned violet because QS molecules diffused from spotted point; (b) T-streak method was performed on TSA agar plate.

## 4.4 DISCUSSION

This study describes the PNP-degrading bacterial populations that obtained through direct isolation. Strain PNP-01 was identified to play an important role in PNP-degrading aerobic granules. In addition, this study reveals that PNP-01 possessed the physiological characteristics, such as its cell density effects on PNP biodegradation and its incapability of self-flocculation, implying that the efficient PNP biodegradation of strain PNP-01 relied on the immobilized growth within granules in the SBR system.

### 4.4.1 The Competent Biomass Fraction in PNP-Degrading Aerobic Granules

The biodegradation of a target xenobiotic with sludge biomass is supposed to be performed by the competent biomass which possesses the specific capability to metabolize the target xenobiotic (Magbanua et al., 1998). The competent biomass fraction can be estimated using either the culture-dependent enumeration or the COD estimation (which is defined as the ratio of the COD of target compound to the total COD [ $\text{COD}(\text{target compound})/\text{COD}_{\text{total}}$ ]) (Hu et al., 2005b).

In this study, the competent biomass fraction in PNP-degrading aerobic granules estimated using the colony counts showed that the competent fraction of PNP-degrading population accounted for 49% to the total culturable bacteria population. This fraction estimated by the cell enumeration compares higher than the COD estimation, which is around 36%. The similar discrepancy between the estimation by cell enumeration and by COD estimation was also reported in other xenobiotic-degrading sludge and granule systems (Magbanua et al., 1998; Hu et al., 2005b).

One possible reason for the discrepancy between the estimation by cell enumeration and by COD estimation in this study can be attributed to the fact that the bacterial abundance within the reactor may not only have been the result of their capability to metabolize the available nutrients but also the result of their capability

to tolerate a toxic environment in the reactor. PNP is highly toxic and sludge microorganisms usually are not able to tolerate the toxicity of PNP. It has been reported that the  $EC_{50}$  of sludge microorganisms to PNP is 64 mg/l (Volskay and Grady, 1990). The in-cycle-concentration of 100 mg/l of PNP may exert a important selection pressure to the microorganisms in the reactor so that only the microorganisms who were either able to metabolize PNP or able to tolerate the toxicity of PNP could live well in the reactor. The high fraction of PNP-degrading culturable bacterial population may imply the prevalent roles of the microorganisms, such as to metabolic PNP and to protect other bacteria from PNP toxicity in a whole granule microbial community.

#### **4.4.2 Physiological and Phylogenetic Diversity of PNP-Degrading Bacterial Isolated from Aerobic Granules**

Although many PNP-degrading bacteria have been isolated from different ecosystems, the PNP-degrading bacteria residing in the engineered bioreactor treating PNP-containing wastewater have not been well studied. The physiological traits of the PNP-degrading bacteria within the bioreactor are still poorly understood. In this study, a collection of three PNP-degrading bacterial strains were obtained. The physiology and phylogenetic studies showed that these three strains represent diverse bacterial populations that can degrade the PNP in aerobic granules.

Data obtained in this study revealed that the PNP degradation behavior with the three strains can be categorized into two groups; the first group which included strain PNP-01 has a fast PNP biodegradation rate, while another group, which included strains PNP-02 and PNP-03, has a slow PNP biodegradation rate. This indicated that the bacteria residing in aerobic granules have different PNP-degrading activities. Previous studies have shown that PNP-degrading bacteria isolated from different places exhibit diverse PNP degradation activities (Bhushan et al., 2000). In addition, two PNP-degrading bacteria isolated from the same PNP-degrading bioreactor was also showed having different PNP-degrading activities

(Shinozaki et al., 2002). The difference, observed in PNP-degrading activities among the reported bacteria, was hypothesized to be attributed to the difference in the initial PNP oxidation enzymes whose activity were induced by different PNP concentrations.

The possibility for the coexistence of bacteria with different PNP degradation activities within the granulation SBR can be attributed to the fact that different PNP concentrations could be developed in the granulation SBR to allow the microorganisms with different PNP degradation activity to reside together in the same system. The sequential batch mode of operation can exert a dynamic-changed PNP concentration in a bulk milieu to the bacteria within the SBR. In addition, the granules have three dimensional structures which allowed the development of different microniches in which bacteria can expose to different PNP concentrations.

Phylogenetic analysis in previous studies on PNP-degrading bacteria has showed that the reported strains to degrade PNP include species from the groups of gram-positive bacteria high G+C, gram-positive bacteria low G+C,  $\alpha$ ,  $\beta$ ,  $\gamma$ -Proteobacteria and CFB (Table 4.7). The bacteria isolated in this study were mainly from  $\alpha$ - and  $\beta$ -Proteobacteria. The bacterial strain PNP-01 which can quickly degrade high concentration of PNP is related with the genus of *Burkholderia*. Strains belonging to genus of *Burkholderia* or related genus of *Ralstonia* have been reported for their capabilities for degrading high concentration of PNP (Bhushan et al., 2000). The two strains, PNP-02 and PNP-03, which can only slowly degrade PNP are related with the genus of *Paracoccus* and *Acidovorax*. Strains belonging to these two genera have not been reported for their capability in PNP biodegradation.

It needs to be pointed out that strain PNP-01, presented in granules in a high quantity, possessed high PNP specific growth and degradation rates, implies that strain PNP-01 played important roles in PNP biodegradation in the aerobic granules. The PNP degradation with strain PNP-01 therefore was studied in detail.

#### 4.4.3 Strain PNP-01 Played Important Roles in PNP-Degrading Aerobic Granules

Strain PNP-01 was isolated from the PNP-degrading aerobic granules by the direct isolation method. The direct isolation method has been successfully used for selecting the important functional bacteria which were surviving in different

**Table 4.7** PNP-degrading bacteria reported in the literature.

Taxonomy affiliation	Bacteria	Reference
High G+C Gram positive bacteria	<i>Arthrobacter aurescens</i> TW17	Hanne et al., 1993
	<i>Arthrobacter protophormiae</i> RKJ100	Jain et al., 1994
	<i>Nocardioides nitrophenolicus</i> sp. nov.	Yoon et al., 1999
	<i>Nocardia</i> sp. strain TW2	Hanne et al., 1993
	<i>Rhodococcus wratislaviensis</i> J3	Navratilova et al., 2005
Low G+C Gram positive bacteria	<i>Bacillus sphaericus</i> JS905	Kadiyala et al., 1998
$\alpha$ -Proteobacteria	<i>Sphingomonas</i> sp. UG30	Leung et al., 1999
$\beta$ -Proteobacteria	<i>Burkholderia cepacia</i> RKJ200	Bhushan et al., 2000
	<i>Ralstonia</i> sp. SJ98	Bhushan et al., 2000
$\gamma$ -Proteobacteria	<i>Moraxella</i> sp.	Spain and Gibson, 1991
	<i>Pseudomonas putida</i>	Kulkarni and Chaudhari, 2005
	<i>Pseudomonas putida</i> PNP1	Löser et al., 1998
CFB	<i>Flavobacterium</i> sp.	Raymond and Alexander, 1971

environments (Tay et al., 1998; Watanabe et al., 1998; Zhuang et al., 2002). The isolated strain PNP-01 constituted a relatively high fraction (approximately 33%) of the total culturable heterotrophic bacteria residing in the mature PNP-degrading

aerobic granules. In addition, DGGE analysis of 16S rRNA fingerprints also showed that strain PNP-01-related bacteria was important in aerobic granules. All these lines of evidence suggested that strain PNP-01 was a numerical abundant member of the microbial community residing in the mature PNP-degrading aerobic granules.

The kinetic analysis of PNP biodegradation by strain PNP-01 revealed that the strain PNP-01 had a specific maximum PNP removal rate ( $V_{max}$ ) of  $290 \pm 10$  mg of PNP/g of DW·h. This  $V_{max}$  was approximately 15 times higher than the highest specific PNP biodegradation rate of PNP-degrading granules (19 mg PNP/g VSS·h) from which the strain PNP-01 was isolated. If taken into account the fraction of strain PNP-01 in whole granule biomass (2.5%), the strain PNP-01 was estimated to consume 7.25 mg PNP/h, indicating that approximately 38% of PNP degraded by granules was contributed by strain PNP-01. It should be pointed out that this simple calculation may not accurately reflect the real situation of PNP biodegradation with PNP-01 within PNP-degrading aerobic granules. On one hand, the specific PNP biodegradation rate estimated with planktonic growth of cells in this study might not represent the PNP biodegradation rate of aggregated growth of PNP-01 cells within granules. On the other hand, the fraction of strain PNP-01 in whole granule biomass estimated by CFU counts might underestimate the amount of strain PNP-01 within the granules, as overlooking the viable but not culturable fraction of strain PNP-01 within the granules. Nevertheless, this simple calculation did indicate that PNP-01 played an important functional role for PNP-degradation within the aerobic granules.

Given the evidence of strain PNP-01 being numerical abundant and played an important functional role in PNP biodegradation, strain PNP-01 was identified as a functionally important PNP-degrading bacterial strain within the granules. The PNP-biodegradation by strain PNP-01 represented one of important mechanism of the microcosm within the aerobic granules.

#### 4.4.4 PNP Degradation Kinetics with Strain PNP-01

The Michaelis–Menten equation has been used to describe the kinetics of PNP biodegradation (Bhushan et al., 2000) and was used to calculate the kinetic parameters in this study (Figure 4.5). Strain PNP-01 was estimated as having a specific maximum PNP removal rate ( $V_{max}$ ) of  $290 \pm 10$  mg of PNP/g of DW·h and a half saturation coefficient ( $K_m$ ) of  $5.3 \pm 1.0$  mg of PNP/l.

Although earlier studies have reported the successful isolation of PNP-degrading bacteria from various environments, the studies on those PNP degradation kinetics are still limited. Bhushan et al. (2000) reported the kinetic behavior of three PNP-degrading bacteria isolated from soil samples, namely *Ralstonia* sp. SJ98, *Arthrobacter protophormiae* RKJ100, and *Burholderia cepacia* RKJ200 which had specific maximum PNP removal rate of 98, 65, and 32 mg of PNP/g of cell DW·h, respectively. Compared with these reported PNP-degrading bacteria, strain PNP-01 possessed a specific maximum PNP removal rate 3-13 times higher than them (Table 4.8). The substrate affinity of strain PNP-01 which is defined as  $V_{max}/K_m$  (Battersby, 1990) was also the highest among the reported PNP-degrading bacteria. This result implies that a more effective and cleaner elimination of PNP can be expected with strain PNP-01.

The distinguished differences in PNP-degrading activity of PNP-01 and other published isolated PNP-degrading bacteria can be attributed to the differences in the selection pressures that exerted to the microbial communities where the isolated PNP degraders resided. Compared with the batch enrichment processes in the laboratory, selection pressures in a SBR, such as the feast and famine living regime, highly dynamic changes in substrate concentration, as well as frequent and repeated exposure to a high concentration of PNP might be responsible for inducing such microorganisms as PNP-01 which possessed a high specific PNP degradation rate and a high substrate affinity (Spain, 1995).

**Table 4.8** Parameters for PNP biodegradation kinetics by pure bacterial isolates.

Strain name	$V_{max}$ (mg PNP/gcell·h)	$K_m$ (mg PNP/l)	$X_0$ (mg/ml)	$S_0$ (mgPNP/l)	Reference
<i>Ralstonia</i> sp. SJ98	98	44.5	1-5 mg/ml	13.9-139	(Bhushan et al., 2000)
<i>Arthrobacter protophormiae</i> RKJ100	65	38.9	-----For all three-----		
<i>Burkholderia cepacia</i> RKJ200	32	32.0			
<i>Burkholderia</i> sp. PNP-01	290	5.3	0.15 mg/ml	0-100	This study

#### 4.4.5 Cell-Density-Dependent PNP Degradation Behavior of Strain PNP-01

Although strain PNP-01 has notably good degradation ability, the investigation of PNP degradation with different initial cell concentrations showed that a high cell density was favorable to an efficient PNP degradation with *Burkholderia* sp. strain PNP-01. While at a low cell density of  $1.6 \times 10^7$  CFU/ml to  $1.0 \times 10^8$  CFU/ml, PNP biodegradation was slow or even delayed as a lag phase during the test.

The cell density related PNP biodegradation has been observed in previous studies (Nishino and Spain, 1993; Thouand et al., 1996). However, the underlying mechanism for such density-dependent PNP biodegradation is still not clear. Nishino and Spain (1993) found that the length of lag phase for PNP biodegradation with *Pseudomonas putida* was inversely proportional to the initial cell density. The authors hypothesized that the lag phase before significant PNP removal with *P. putida* was caused by delayed induction of initial degradative enzyme. The induction of the initial enzyme was dependent upon the cell-density-associated accumulation of hydroquinone or an unknown factor whose release was stimulated by hydroquinone. Unfortunately, this hypothesis has still not been proven.

The cell density related PNP biodegradation observed in this study might be explained by the effects of the changes in the  $S_0/X_0$  ratio to the bacterial growth and activity (Liu et al., 2005a). In addition, it might not rule-out the possibility that the strain PNP-01 had a density-dependent mechanism as sensing of environmental conditions, which might regulate the biodegradation behavior of strain PNP-01.

QS is a mechanism of cell-cell communication which has been found involved in many density-dependent cell physiologies (Fuqua and Winans, 1994; Bainton et al., 1992; Eberl et al., 1996; Davies et al., 1998; Aguilar et al., 2003). In this study, *Burkholderia* sp. strain PNP-01 was detected producing diffusive QS signal molecules, both through cross-streaking and supernatant assays with AHL

reporter strain *C. violaceum* CV026. However, the relation of AHL-mediated QS and PNP biodegradation with *Burkholderia* sp. strain PNP-01 is still not clear.

It should be pointed out that the regulation of biodegradation could be very complex and population density might be only one environmental parameter sensed by bacteria. Other environmental parameters, such as the presence of metabolic intermediates as proposed by Spain and Nishino (1993) and  $S_0/X_0$  effects by Liu *et al.* (2005a) may also modulate the regulation of biodegradation process. To understand the mechanism of such cell-density-dependent PNP biodegradation of strain PNP-01, a further detailed and careful study should be carried out before the hypothesis can be proposed.

#### **4.4.6 The Function of PNP Biodegradation by Strain PNP-01 is Dependent on the Immobilized Form of Growth of the strain within Granules**

The study of the physiological characteristics of strain PNP-01 implied that immobilized growth of the strain within aerobic granules could enhance the survival chance of strain PNP-01 in the bioreactor and could also benefit PNP removal efficiency of the SBR system

From the results in this study (Figure 4.4), a high density of PNP-01 cells was necessary to the efficient PNP utilization. Thus, to maintain a high cell density within the bioreactor would be crucial for competition capability and higher survival chance of this strain. In general, physiological characteristics, such as a fast growth rate and the ability of fast settling are favorable to maintain a high cell density within a bioreactor. However, strain PNP-01 had a doubling time of 7.2 hours (specific growth rate of 0.14 /h). This doubling time was slightly less than the 8-h HRT applied to the SBR in this study. In addition, the settling assay showed that PNP-01 preferred a planktonic growth form and was not able to settle fast. The both characteristics could jeopardize the sustention of PNP-01 into a high cell density under the selection pressures applied in the SBR of this study.

However, if growing as an immobilized form within aerobic granules, it could be easier for PNP-01 to maintain a high cell density and to utilize PNP efficiently. This is because cells living in granules might be embedded in a matrix of extracellular polymeric substances which could effectively prevent cells from being washed out by the hydrodynamic pressure in the SBR. In addition, granules could provide a constrained living compartment for strain PNP-01 so that the cell density would be significantly higher in the granule microniches compared to in bulk milieu. This could play a positive role in competitive PNP utilization.

It worth to point out that, theoretically, the fluorescence intensity relates to PCR amplicon's concentration and PCR amplicon's concentration also relates to the DNA template concentration to certain degree. From DGGE results that the relative fluorescent of PNP-01-related bacteria showed an upward trend in the SBR during granulation, we speculated that granulation could support the successful retention and growth of PNP-01-related bacteria in the SBR to a high cell density. This might be one of the possibilities that led to the enhanced PNP metabolic activities after granule formation observed previously (Chapter III). Nonetheless, PCR-related methods may suffer from the bias and drawbacks and are not suitable to quantify the increase of PNP-01 in granules. Other quantitative methods such as quantitative-PCR or probing shall be implemented in the future to testify this hypothesis.

## 4.5 SUMMARY

The microbial community study showed that the competent PNP-degrading bacteria accounted for a portion of 49% of total culturable heterotrophic bacteria. The physiological studies on the competent PNP-degrading bacteria showed that they possessed different PNP biodegradation activities, in term of specific PNP degradation rates and specific growth rates. Based on the analysis of 16S rRNA gene sequences, the competent PNP-degrading bacteria, designated as PNP-01, 02 and 03 were found belonging to the different taxonomy affiliations of the genus *Burkholderia*, *Acidovorax* and *Paracoccus* respectively.

Strain PNP-01 was found to play an important role in PNP-degrading aerobic granules. The quantification analysis indicated that strain PNP-01 constituted 2.5% (dry weight) of mature PNP-degrading granules. The DGGE analysis on the 16S rRNA gene fingerprints exhibited that strain PNP-01-related bacteria was persistently present in the bacterial community during the formation of PNP-degrading aerobic granules. Kinetic analysis on the PNP biodegradation data estimated that strain PNP-01 had a specific maximum PNP degradation rate ( $V_{max}$ ) of 290 mg/g DW·h and a half-saturation constant ( $K_m$ ) of 5.3 mg/l. In addition, physiological characterization of strain PNP-01 revealed that the cell density of strain PNP-01 affected the efficiency of PNP biodegradation by strain PNP-01 and strain PNP-01 was not able to form self-flocculation. These findings implied that the efficient PNP biodegradation of strain PNP-01 relied on its immobilized growth within granules in the SBR system. Finally, the phenotypic and phylogenetic characterization identified strain PNP-01 as the *Burkholderia* sp.

The findings in this study have expanded the knowledge on the functionally important PNP-degrading bacteria in aerobic granulation bioreactor, which can provide an insight into the mechanisms involved in the PNP biodegradation with this novel aerobic granulation bioreactor. In addition, the knowledge on the physiological characteristics of strain PNP-01 could be useful in developing optimal control and management strategies for PNP-degrading aerobic granulation

bioreactor. The isolated strain PNP-01 can be used for bioaugmentation to enhance the performance of the bioreactor.

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## CHAPTER V

# CHARACTERIZATION OF PNP-DEGRADING POPULATION; CONSORTIUM PNP-04 ISOLATED FROM PNP-DEGRADING AEROBIC GRANULES

### 5.1 INTRODUCTION

The biodegradation process depends on the activity of microorganisms that display the appropriate metabolic potential for degradation and mineralization of the target pollutant to harmless end products. Pollutant biodegradation process can be performed by a single microorganism which possesses the entire set of enzymes capable of transforming the pollutant in a metabolic route that leads to its eventual breakdown to harmless end products (Briones and Raskiny, 2003). However, in some cases, the degradation process cannot be performed efficiently or which cannot be performed at all by a pure culture but depends on the cooperative action of two or more bacterial species (Sorensen *et al.*, 2002).

Cell immobilization technology, such as formation of multispecies granules and biofilms, may facilitate such cooperative biodegradation processes, since microbial aggregation into granules or biofilms helps establish syntrophic relationships among the community members and provides a compartment for metabolic commensalisms among multispecies bacteria (Tolker-Nielsen and Molin, 2000).

In Chapter IV, it was discussed that a microbial consortium PNP-04 was isolated from PNP-degrading aerobic granules. Consortium PNP-04 was found to be able to use PNP as a sole carbon and energy source. However, detailed information is still missing for this PNP-degrading consortium. Therefore, the objective of this study

is to characterize this consortium PNP-04. The members in the consortium were unraveled by isolation and the specific PNP degradation was assessed for consortium PNP-04, the mono-cultures and the mixed culture of the isolates from the consortium. This study will help to understand the characteristics of such PNP-degrading populations as consortium PNP-04 within PNP-degrading aerobic granules and shed light on the understanding of mechanisms involved in the PNP biodegradation with the novel PNP-degrading aerobic granulation system.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Microbial Culture and Media**

PNP-MS medium was prepared as described in Chapter IV. When needed, PNP-MS medium was solidified with 15 g of Bacto agar per liter to prepare PNP-MS agar plates. 1/5 strength Bacto<sup>®</sup> Tryptic Soy Broth (TSB) was used for liquid culture. Bacto<sup>®</sup> R2A agar, LB agar, and Bacto<sup>®</sup> Tryptic Soy agar (TSA) were used for isolation and maintenance of bacteria from consortium PNP-04.

Bacto<sup>®</sup> Motility Test Medium (Bacto<sup>®</sup>Tryptose, 10 g; Sodium Chloride, 5 g; Bacto<sup>®</sup> Agar, 5 g; pH 7.2 ± 0.2 at 25°C) (Tittsler and Sandholzer, 1936) was prepared as pour plates and used for detecting motility of microorganisms and for separating and isolating motile bacteria in consortium PNP-04.

### **5.2.2 Isolation of Three Bacterial Strains from Bacterial Consortium PNP-04**

As described in Chapter IV, consortium PNP-04 was first observed to form a single colony on PNP-MS agar plates. The colony was picked and transferred to R2A agar plates followed conventional pure culture isolation procedure. After several such transfers, it was noticed that the single colony still contained several different morphotypes of bacteria as examined by gram-staining under microscopy. The colonies were then subjected to repetitive procedures of dispersion, serial dilution and plating onto R2A agar plates until a pure microcolony containing only a single morphotype of bacteria was obtained. For isolating other members from consortium PNP-04, several other isolation methods were employed.

Micromanipulation using the equipment and methods of Skerman (1968) was employed in the isolation of consortium PNP-04 as described by Maszenan (2000). This method employed a micromanipulator consisting of a glass microtool which can be used to transfer cells on an agar surface across the surface and away from other cells

by trapping cells in the water film around the microtool. In brief, 9 cm plastic petri dishes containing Bacto<sup>®</sup> R2A agar was inoculated by running a 50- $\mu$ l drop of liquid culture of PNP-04 down the center of the plate to let the free moisture be absorbed by the agar. The plate was then examined and the cells displaying different morphology and size were manipulated away from the central inoculum line to a position on the surface of the same plate, well separated from the inoculation line. Plates were then incubated for 24 hours until microcolony formation was apparent. The microcolony was then transferred to a fresh R2A agar plate. Purity was checked by gram-staining under microscopy at each stage of the isolation until a pure bacterial culture was obtained.

Since the microcolonies of consortium PNP-04 were found motile on the surface of agar plates, the motility agar was then used for isolating the motile member in the consortium. A single colony of consortium PNP-04 was stabbed on a motility test medium agar plate. Motility was evidenced by the presence of diffuse growth away from the line or spot of inoculation, while nonmotile bacteria only grew along the line of inoculation. The motile bacteria recovered from 3-4 mm away from the inoculation point were repetitively stabbed on motility test medium agar. After 8-10 times of such stab-recover procedure, the motile bacteria recovered from the motility test and medium agar were streaked onto R2A agar. The purity was checked by gram-staining under microscopy.

### **5.2.3 Morphological and Phenotypic Characterizations of Consortium PNP-04 and Three Isolates**

Consortium and isolates were observed with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM). For morphological observations, bacteria was fixed, dehydrated, and viewed with a scanning electron microscope (Stereoscan 420, Leica, Cambridge Instruments) at 20 KV as described previously (Zhuang *et al.*, 2002). Gram-stain was performed as described by Smibert and Krieg (1994). Motility test was performed with Bacto<sup>®</sup> Motility Medium S according to the manufacturer's instructions (Difco Laboratories,

Division of Becton Dickinson and Company, Sparks, Maryland, USA). *Escherichia coli* ATCC® 25922 was used as a quality control in the tests.

#### **5.2.4 DNA Extraction, PCR Amplification, 16S rRNA Gene Sequencing and Phylogenetic Analyses of Consortium PNP-04 and Three Isolates**

16S rRNA gene of consortium PNP-04 was extracted and amplified as previously described in Chapter III. 16S rRNA gene of isolates was amplified using the modified whole cell direct lysis PCR amplification methods as described in Chapter IV.

The nucleotide sequences of 16S rRNA gene from the isolates were sequenced using the dideoxy chain termination chemistry and the ABI model 310A sequencer (Applied Biosystems, Perkin-Elmer) as described in Chapter IV.

#### **5.2.5 DGGE Analysis of Consortium PNP-04 and Three isolates**

The DGGE analysis of consortium PNP-04 as well as three isolates was performed as described in Chapter III.

#### **5.2.6 PNP Biodegradation with Consortium PNP-04, as well as Mono- or Mixed cultures of Three Isolates**

The PNP biodegradation ability by consortium PNP-04 as well as by mono- or mixed culture of three isolates were examined in batch studies for identifying functional important isolates as well as for revealing the underlying reasons for these bacteria forming a stable and tight microbial consortium. Cultures were grown in 1/5 strength Bacto® TSB medium supplemented with PNP concentration of 40 mg/l. Before the PNP biodegradation assay, cells were centrifuge collected, washed twice with 85% saline at room temperature, and re-suspended in PNP-MS medium (containing 100 mg/l of PNP). The initial cell concentration in the assay with consortium PNP-04 and mono-culture of each isolate was adjusted to around

0.15 mg DW/ml. In the artificial mixed-culture, equal-amount cells (around 0.15 mg DW /ml) of each isolates were added into the biodegradation assay setup.

### **5.2.7 Other Analytical Methods**

PNP concentrations were measured photometrically as described in Chapter III (Ray *et al.*, 1999). The dry weight (DW) of the cells was determined as described in Chapter IV (Onysko *et al.*, 2000).

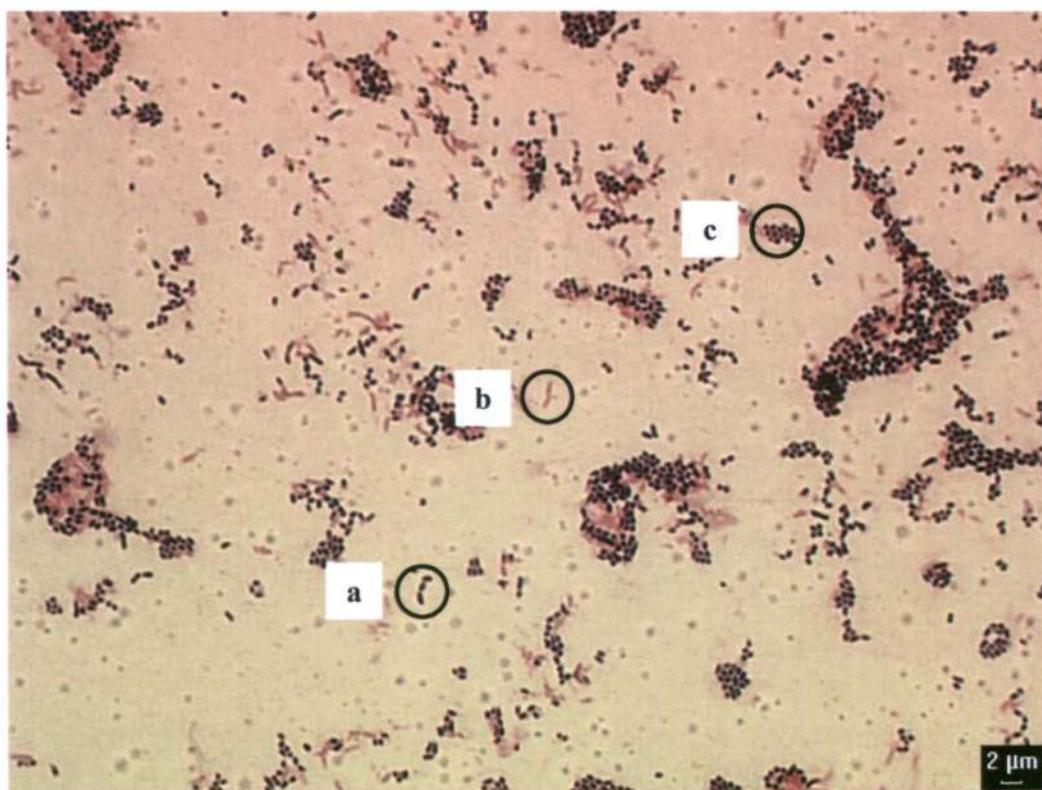
## 5.3 RESULTS

### 5.3.1 Phenotypic Characterization and DGGE Profile of PNP-Degrading Consortium PNP-04

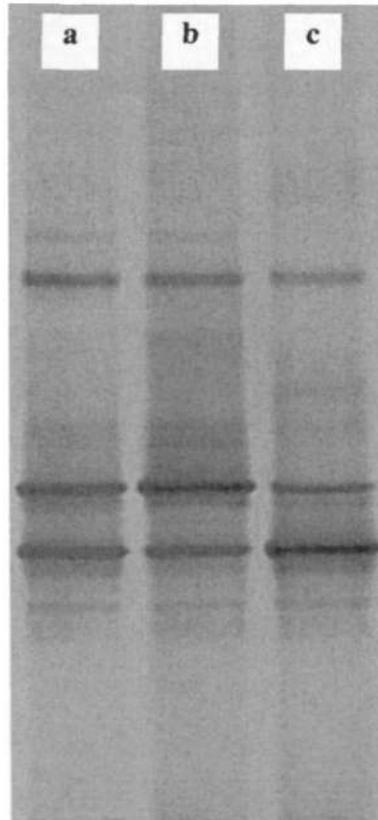
Consortium PNP-04 formed yellowish colonies with irregular edge on PNP-MS agar plates with 100  $\mu$ l of  $10^6$ -fold dilution of dispersed granule samples. On the basis of its appearance on the  $10^6$ -fold dilution plate, consortium PNP-04 was estimated to be present in the PNP-degrading aerobic granules at a concentration of  $9.4 \pm 0.9 \times 10^6$  CFU/mg of granule MLSS. This concentration is equivalent to 23.5% of total culturable PNP-degrading bacteria, suggesting PNP-04 constituted an important fraction of the PNP-degrading bacteria.

Consortium PNP-04 was initially regarded as a single species bacterial culture since it formed distinguished and consistent colony morphotype in the subculturing on either PNP-MS or Bacto<sup>®</sup> R2A agar plates. However, after gram staining and viewed under microscope, consortium PNP-04 was revealed to be constructed with at least three morphotypes of bacteria which belonged to gram-positive short rods, gram-positive cocci, and gram-negative rods (Figure 5.1).

To confirm the finding, a culture independent 16S rRNA gene based molecular method was employed to understand the microbial composition of consortium PNP-04. DGGE fingerprint microbial population analysis was performed to fast identify consortium PNP-04 compositions under different culture media. As showed in Figure 5.2, PNP-04 constantly showed a three-band pattern, when it was grown on R2A agar plate, PNP-MS medium or 1/5 strength Bacto<sup>®</sup> TSB medium liquid cultures.



**Figure 5.1** Gram-staining micrograph of consortium PNP-04 grown in liquid culture in PNP-MS broth, bar = 2μm. At least three morphotypes were found in the micrograph: a, gram-positive rod-shaped bacteria, b, gram-negative rod-shaped bacteria and c, gram-positive cocci.



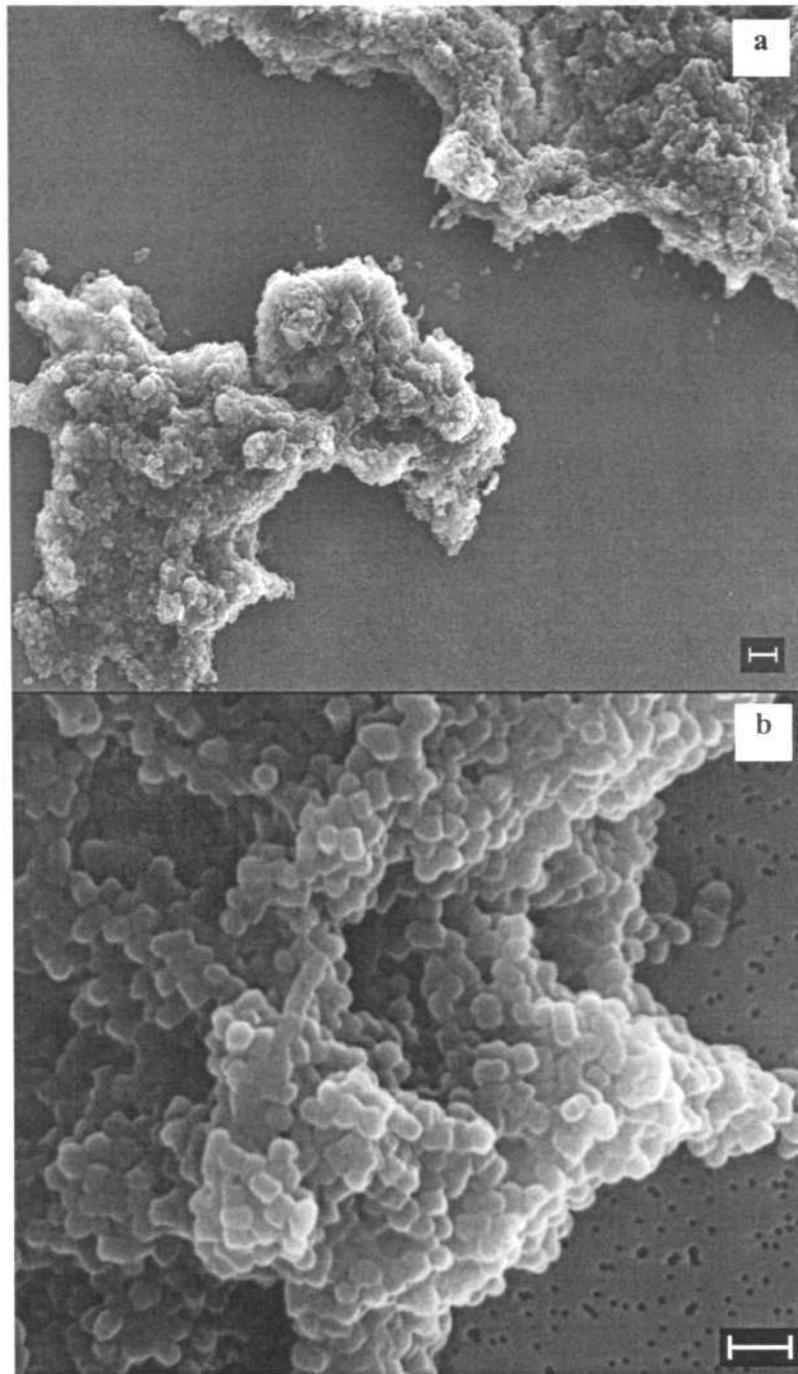
**Figure 5.2** DGGE fingerprint analysis of the important microbial populations of consortium PNP-04 grown in different culture media. a: in PNP-MS broth; b: in 1/5 strength Bacto<sup>®</sup> TSB medium; c: on Bacto<sup>®</sup> R2A agar plates. The results showed that consortium PNP-04 formed a very stable microbial community under different culture media.

### 5.3.2 Unraveling PNP-04 through Isolation

It is unusual that a multispecies microbial consortium could constantly form single colonies on various culture media agar plates. Attempts in isolating microbial members from the consortium PNP-04 were therefore carried out. Although classical serial-dilution method and repeat streaking method were intensively employed to try to unravel consortium PNP-04 on PNP-MS, R2A, LB and Bacto<sup>®</sup> TSA agar plates, only one pure culture was successfully isolated by these methods. SEM micrograph (Figure 5.3) showed that PNP-04 formed a tightly aggregated cluster in the PNP-MS liquid culture. This phenomenon suggested that this might be the reason that conventional streaking and dilution methods could not successfully disperse and isolate microbial members. Therefore, several other methods which can either better disperse the cluster or separate or purify a pure bacterial culture were employed to unravel consortium PNP-04.

A bacterial strain, designated as AG, was successfully isolated using the method of dispersion of the consortium PNP-04 through a repetitive procedure of vigorous agitation, serially dilution and spread onto Bacto<sup>®</sup> R2A agar plates. Another bacterial strain, designated as AC was obtained via micromanipulation and the third bacterial strain, designated as AY was obtained through repetitive inoculation and isolation in motility agar.

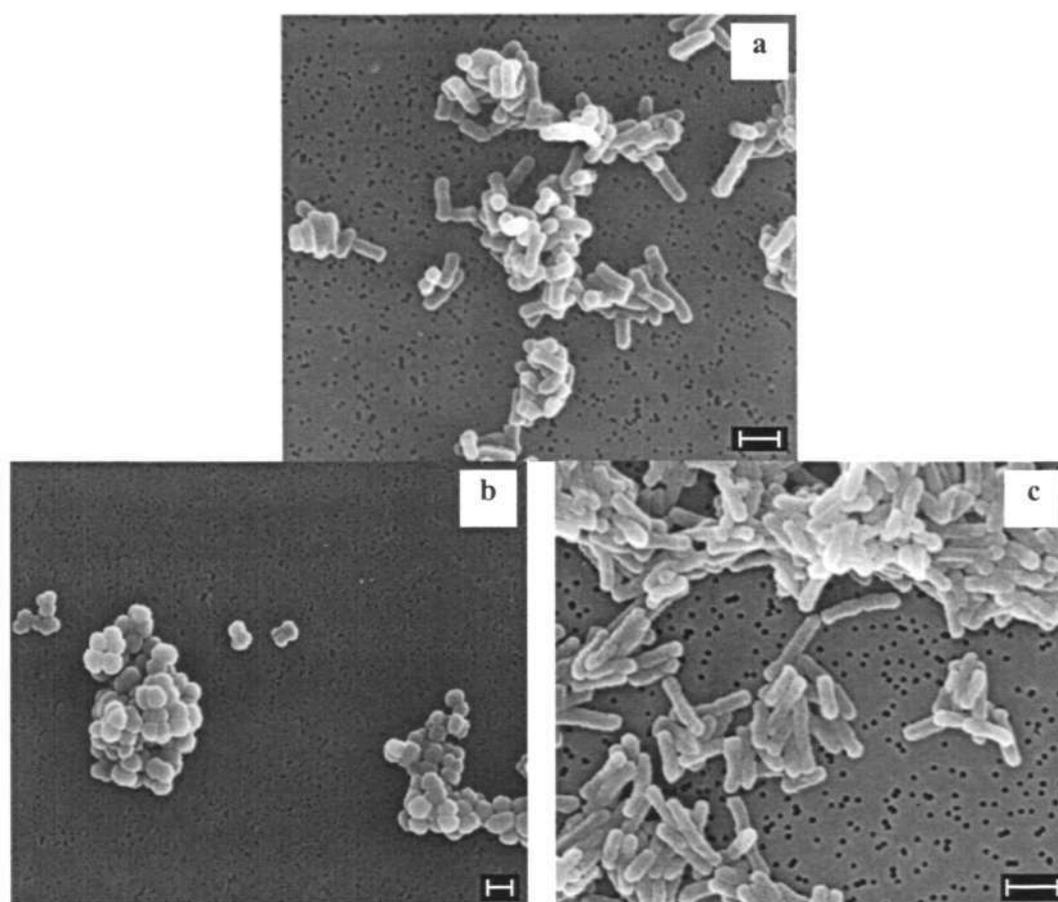
Table 5.1 summarizes the bacteria recovered from PNP-04 and their morphological characteristics. When grown on the Bacto<sup>®</sup> R2A agar, the colonies of strain AG were pale orange and convex. The colonies of AC were bright yellow and flat. The colonies of AY were pale yellow. Strain AG and AC were gram-positive bacteria, and strain AY was gram-negative bacteria. Cells of strain AG were rod-shaped bacteria with 0.5-1.0  $\mu\text{m}$  in length and 0.1-0.2  $\mu\text{m}$  in width (Figure 5.4a). Cells of strain AC were oval-shaped bacteria of nearly 1  $\mu\text{m}$  diameter (Figure 5.4b). Cells of strain AY were rod-shape bacteria with 1.0-2.0  $\mu\text{m}$  in length and 0.3-0.5  $\mu\text{m}$  in width (Figure 5.4c).



**Figure 5.3** SEM micrographs of the overnight cultures of PNP-04 grown in PNP-MS broth, a: overview, bar = 2  $\mu\text{m}$ ; b: zoom in view, bar = 1  $\mu\text{m}$ .

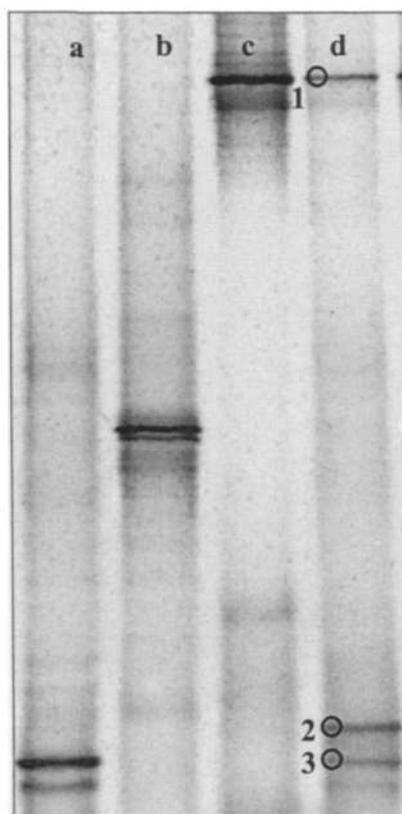
**Table 5.1** Isolated members in the consortium PNP-04.

Isolation method	Strain name	Colony morphology on R2A agar	Motility	Microscopy morphology
Serial dilution	AG	Pale orange, convex	-	Rods
Micromanipulation	AC	Bright yellow, dry, flat	-	Cocci
Motile agar	AY	Brown yellow, flat	++	Rods

**Figure 5.4** SEM micrographs of isolated bacteria from consortium PNP-04. a: strain AG; b: strain AC; c: strain AY, bar = 1 µm.

### 5.3.3 DGGE Fingerprint Analysis on Isolated Bacteria and Consortium PNP-04

The isolated bacteria and original consortium PNP-04 were compared with DGGE analysis of 16S rRNA gene fragments (Figure 5.5). Strain AG was found comigrating with the No. 3 band on PNP-04 profile, indicating that strain AG was important in the mixed culture. Strain AY was found comigrating with the DGGE band No. 1 on PNP-04 profile.



**Figure 5.5** DGGE analysis of 16S rRNA gene fragments from isolated bacteria and consortium PNP-04. a: strain AG; b: strain AC, c: strain AY, and d: consortium PNP-04. Small circles and numbers are given to the bands from consortium PNP-04.

There was no corresponding pure culture band comigrating with band No. 2 on the PNP-04 profile, suggesting that an important microbial population was probably not-yet-cultured from the consortium. In addition, pure culture AC has also no comigrating DGGE band from consortium PNP-04 profile.

### 5.3.4 Taxonomic Characterization of Three Isolates from Consortium PNP-04

The 16S rRNA gene was successfully amplified from three isolates and sequenced. The sequences were aligned to the most similar ones available from GenBank and used to determine the taxonomy affiliation of the isolates (Table 5.2).

Strain AG falls in the high G+C Gram positive bacteria (Actinobacteria) group, *Brevibacterium* genus and shows an identity of 95.0% with its nearest neighbor, *B. sp. BH* (AY577816), a bensulfuorn-methyl degrading bacterium. Strain AC falls in the high G+C Gram positive bacteria group, *Micrococcus* genus and shows an identity of 96.3% with its nearest neighbor *M. sp. TUT1210* (AB188213) (Narihiro *et al.*, 2004). Strain AY belongs to  $\alpha$ -proteobacteria group, genus *Ochrobactrum* and shows 99.7% identity with *O. sp. B2* (AY661464).

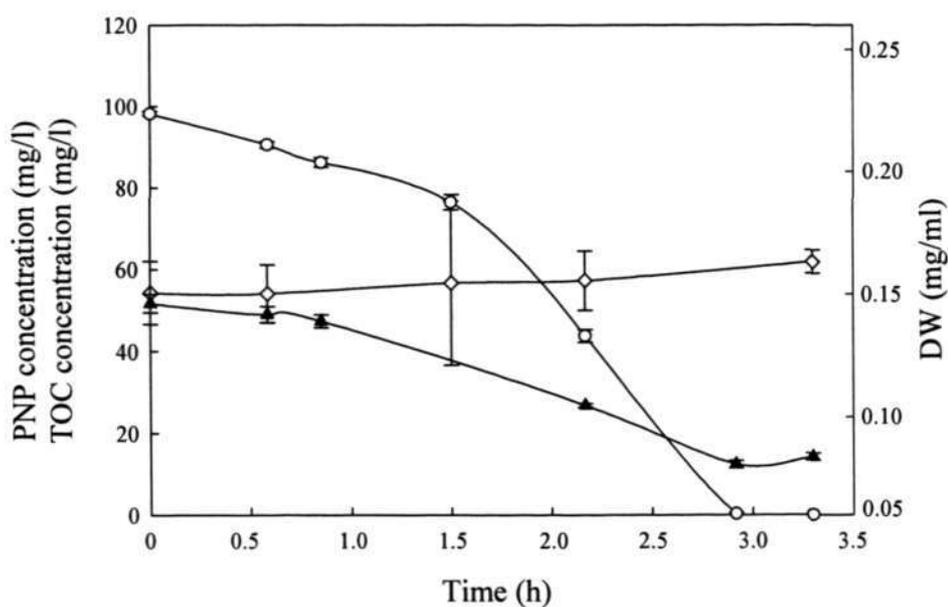
**Table 5.2** The 16S ribosomal DNA sequence analysis on 3 members in the consortium PNP-04.

Strain name	Taxonomy affiliation	Closest relative in GeneBank	Identity (%)	Number of bases*
AG	High G+C Gram positive bacteria	<i>Brevibacterium sp. BH</i> (AY577816)	95.0	1423
AC	High G+C Gram positive bacteria	<i>Micrococcus sp. TUT1210</i> (AB188213)	96.3	1457
AY	$\alpha$ - Proteobacteria	<i>Ochrobactrum sp. B2</i> (AY661464)	99.7	1438

\* Number of bases used in establishing identity.

### 5.3.5 Specific PNP Degradation as well as Growth Assay of Consortium PNP-04

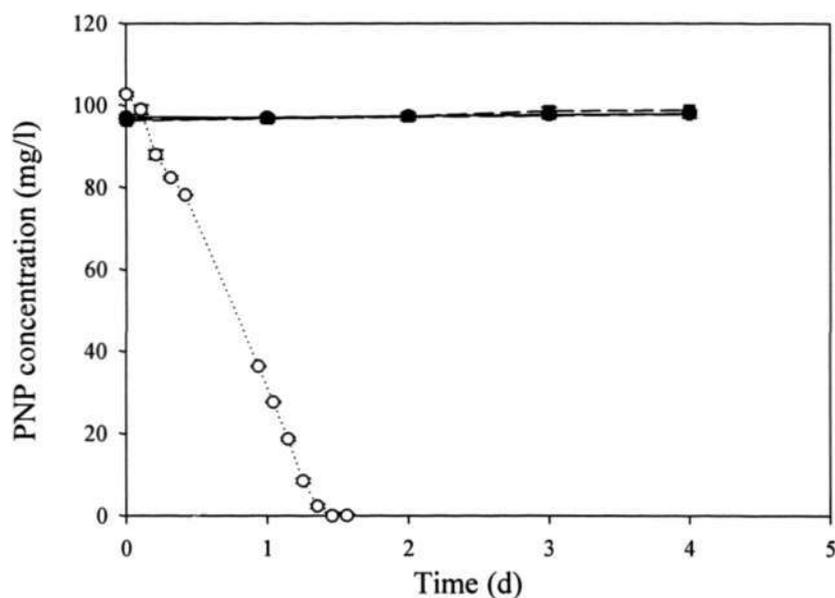
As showed in Figure 5.6, 100 mg of PNP/l can be degraded within 3.5 hours by PNP-04. TOC analysis indicated that 73.9% of TOC was removed by consortium PNP-04 and near 100 mg of PNP/l was completely removed by consortium PNP-04. Specific growth of PNP-04 was monitored during PNP biodegradation assay and revealed that 12.5 mg biomass/l yielded from the 37.5 mg TOC/l removed.



**Figure 5.6** Time course of PNP biodegradation by mixed-culture of PNP-04 in a batch incubation.  $\diamond$ , biomass concentration;  $\circ$ , PNP concentration; and  $\blacktriangle$ , TOC concentration.

### 5.3.6 Specific PNP Degradation with Mono-cultures and Mixed-Culture of Three Isolates from Consortium PNP-04

To determine the roles of the three isolates in biodegradation of PNP, biodegradation assays were performed in liquid PNP-MS medium with 100 mg/l PNP as the sole carbon and energy source. As showed in Figure 5.7, none of the three isolates could degrade PNP in mono-culture. In contrast, artificial mixed-culture can degrade PNP. This indicated that biodegradation of PNP by the members in consortium PNP-04 required the cooperative action by three isolates. However, it took more than 30 hours for mixed-culture to biodegrade 100 mg/l of PNP in the batch test.



**Figure 5.7** Time course of PNP biodegradation by mono-cultures of three isolates and by artificial mixed-culture of isolates. ○, artificial mixed-culture, ■, strain AG, ▲, strain AC; and ●, strain AY. The standard deviation is from triplicates of incubation.

## 5.4 DISCUSSION

This study characterizes consortium PNP-04 as a highly stable microbial aggregate that can efficiently degrade PNP. Three bacterial members were isolated from the consortium and were found being able to degrade PNP in a cooperative manner. In addition, consortium PNP-04 was identified as another functionally important PNP-degrading population within PNP-degrading aerobic granules, on the basis of its numerical abundance and its capability for efficient PNP removal.

### 5.4.1 Consortium PNP-04 as a Stable Multispecies Microbial Consortium and a Highly Associated Aggregate Isolated from Aerobic Granules

Although there has been many studies on the stable coexist multispecies microbial consortium exerting effective pollutant biodegradation function in diverse natural and engineered environments (Pelz *et al.*, 1999), the current study showed that consortium PNP-04 possessed several unique characteristics that were significantly different from those previously reported consortia.

A unique characteristic of consortium PNP-04 was that bacterial members in the consortium could stably coexist and consistently form a distinguished and single colony regardless of the growth on selective (PNP-MS) or non-selective media (R2A, TSA and LB). The medium supplied with a target organic compound as the sole carbon and energy source is usually employed as a selective medium to isolate the target compound degrading bacteria (Madigan *et al.*, 1999). Indeed, it is not surprising that a multispecies mixed-culture can form a distinguished and single colony on a selective agar plate supplied with the target compound as the sole carbon source. This is because the specific target compound, especially the toxic or recalcitrant compound, usually cannot be used as the sole carbon and energy source by every bacterial member in the multispecies mixed culture (Dejonghe *et al.*, 2003).

The bacterial members have to grow closely as a single colony on the selective agar plate so that they can survive on the basis of a substrate commensalism or symbiosis interspecies relationship. It has been found that such single colony would be quickly dispersed if the mixed culture was transferred to the non-selective culture medium containing a benign substance as carbon source (Carvalho *et al.*, 2002). This was because the benign substances could support the growth of most members in the mixed culture, so that the members in the mixed culture could grow independently and would not need the substrate commensalism or symbiosis interspecies relationship. As a result, the members would not form single colony anymore and separate from each other.

However, the situation for consortium PNP-04 was quite different from what mentioned above. The consistent single-colony-forming of consortium PNP-04 on different culturing media suggested that the members of consortium PNP-04 might not simply coexist on the basis of a substrate commensalism. Other mechanisms might also involve in the syntrophic relationship between the members in consortium PNP-04.

Another unique characteristic was that consortium PNP-04 formed tightly associated aggregates (Figure 5.3) which were not readily dispersed by conventional isolation methods. This finding indicated that the members of consortium PNP-04 preferred a closed contact, multispecies mixed living environment rather than a monoculture of a single species.

Therefore, it is not surprising that consortium PNP-04 could be isolated from aerobic granules. This is because aerobic granules, similar to biofilm, are cell self-immobilized microbial aggregates. Bacteria surviving in biofilms have been reported to be able to closely contact with each other as compared to planktonic growth style and can form multispecies microcolonies or microaggregates to enhance their survival in natural and engineered environments (such as to protect bacteria from being washed out and being predated by protozoa) (Sutherland, 2001). The fact that the members in consortium PNP-04 formed tight aggregates indicated that such multispecies aggregated growth form might be a better way for these members to compete for

substrate, tolerate the PNP toxicity and synthesize some unknown growth factors. The compact aerobic granules structure could prove to be a favorite condition for the members of the consortium PNP-04 adapted to the mixed growth formed after a long term competition and selection in the aerobic granules, so that the consortium like PNP-04 could form, maintain and grow within aerobic granules.

#### **5.4.2 Consortium PNP-04 as a Functionally Important PNP-Degrading Population in the Aerobic Granules**

As a stably coexisted and tightly aggregated multispecies microbial cluster, consortium PNP-04 was also identified as a functionally important PNP-degrading population surviving within the aerobic granules. The functional importance of consortium PNP-04 reflected on two aspects, numerical abundance and efficient PNP removal capability. It is worth to point out that even consortium PNP-04 formed single colonies on PNP-MS and other types of agar plates, the CFU count may not be able to accurately reflect the numerical abundance of the consortium in the PNP-degrading aerobic granules. However, the fact that consortium PNP-04 presented on the  $10^6$ -fold dilution agar plates, which was comparable with *Burkholderia* sp. strain PNP-01 (described in Chapter IV), can be an important line of evidence to indicate that consortium PNP-04 residing in PNP-degrading aerobic granules in a significant level.

In addition, the consortium PNP-04 could utilize PNP as a sole carbon and energy source. Batch studies showed that consortium PNP-04 had a mean PNP biodegradation rate of 180 mg PNP/g DW·h which is also comparable with the PNP degradation rate of *Burkholderia* sp. PNP-01 as 290 mg PNP/g DW·h (described in Chapter IV). The high PNP removal efficiency revealed the functional importance of the consortium PNP-04 in biodegradation of PNP within aerobic granules.

It is worth to mention that the PNP biodegradation by strain PNP-01 and by consortium PNP-04 represented two different important mechanisms of PNP biodegradation within aerobic granules. That is the PNP biodegradation can be

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performed by a single bacterial strain or by a multispecies consortium in a cooperative manner.

### 5.4.3 PNP Degradation by Consortium PNP-04 and Its Members

In order to characterize the consortium PNP-04, the consortium members were isolated. It was found that each individual isolate from consortium PNP-04 could not biodegrade PNP (Figure 5.7). However, the artificially reconstructed mixed culture, by equal-mixing three isolates, could biodegrade PNP, albeit the PNP degradation rate of this reconstructed mixed culture was several fold lower compared with the original consortium PNP-04.

The discrepancy in the PNP removal rate between original and artificial mixed cultures can be attributed to two reasons. Firstly, the composition of each member in the artificial mixed culture might not represent the composition ratio in the original consortium PNP-04. Therefore, the collaboration of the three members may not be performed at the optimized conditions. This hypothesis can be proven in future studies by analyzing the original abundance ratios of the consortium members.

Secondly, not all of the members involved in the consortium PNP-04 have been isolated. 16S rRNA gene fingerprints analysis of the isolates and consortium PNP-04 has provided a clue for this reason. The DGGE analysis revealed that at least one important member of the consortium, represented as the band 02 on DGGE analysis has not yet been isolated. This finding clearly indicated that the isolation procedure of the consortium PNP-04 has not yet completed and not revealed all the members from the consortium. Other isolation strategies shall therefore be designed for obtaining the yet-to-be cultured members in the consortium. In addition, other 16S rRNA gene-based molecular microbial population analysis methods, for example cloning library, should be used for a detailed analysis of the consortium. Information from both culture-based and culture-independent methods may help to understand the underlying reasons for the formation of consortium PNP-04.

## 5.5 SUMMARY

This study characterizes consortium PNP-04 as a highly stable microbial aggregate that can efficiently degrade PNP. Consortium PNP-04 was identified as another functionally important PNP-degrading population within PNP-degrading aerobic granules, on the basis of its numerical abundance and its capability for efficient PNP removal.

Three bacterial members, designated as strain AG, strain AC and strain AY, were isolated from the consortium PNP-04 with specially designed isolation strategies, namely single colony repeat transfer method, micromanipulation method, as well as motility agar selection method, respectively. Phylogenetic analysis on the basis of 16S rRNA gene sequencing revealed that strain AG, strain AC and strain AY belonged to genus of *Brevibacterium*, *Micrococcus* and *Ochrobactrum*, respectively.

Specific PNP biodegradation assay showed that consortium PNP-04 was able to completely degrade 100 mg/l PNP within 3.5 hours. The three isolates, strain AG, strain AC and strain AY, from consortium PNP-04 were found not to be able to degrade PNP in monocultures but able to degrade PNP in a mixed culture by equally mixing the three strains. However, the mixed cultures of the three isolates took more than 30 hours to completely degrade 100 mg/l PNP.

This study helps to understand the characteristics of the stably aggregated microbial consortium within PNP-degrading aerobic granules. The findings in this study will shed light on the understanding of mechanisms involved in the PNP biodegradation with the novel PNP-degrading aerobic granulation system.

## CHAPTER VI

# CONCLUSION AND RECOMMENDATIONS

### 6.1 CONCLUSION

This study demonstrates that it is feasible to deploy the aerobic granulation technology for remediating PNP contaminated wastewaters. Proper strategies were developed from this study for cultivation of PNP-degrading aerobic granules. The characteristics of PNP-degrading aerobic granules were investigated on their physico-chemical properties, and their kinetic behavior of PNP biodegradation. The competent PNP-degrading populations residing in the aerobic granules were isolated and characterized. The underlying mechanisms involved in the PNP biodegradation with PNP-degrading aerobic granules were investigated through the identification and characterization of two major functionally important PNP-degrading bacterial populations isolated from PNP-degrading aerobic granules, namely strain PNP-01 and consortium PNP-04 respectively.

#### 6.1.1 Cultivation of PNP-Degrading Aerobic Granules

Aerobic granules to treat PNP contaminated synthetic wastewater were successfully developed in a sequencing batch reactor (SBR) using activated sludge as inoculum. A key step for the cultivation of PNP-degrading aerobic granules was the conditioning of the activated sludge seed to enrich biomass with improved settleability and higher PNP degradation activity by implementing progressive decreases in settling time and stepwise increases in PNP concentration.

The aerobic granules were cultivated at a PNP loading rate of  $0.6 \text{ kg/m}^3\cdot\text{d}$ , with glucose supplement to boost the growth of biomass. The granules had a clearly defined shape and appearance, settled significantly faster than activated sludge and were

capable of nearly complete PNP removal. 16S rRNA gene fingerprint analysis showed a gradual temporal shift in microbial community succession as the granules developed from the activated sludge seed. Specific oxygen utilization rates (SOUR) at PNP concentration of 100 mg/l were found increasing with the evolution of smaller granules to large granules and stabilizing at 22 mg O<sub>2</sub>/g VSS·h, suggesting that the granulation process could enhance metabolic efficiency towards biodegradation of PNP.

After aerobic granulation SBR reached a steady state, mature PNP-degrading aerobic granules were characterized for their mechanical stability, specific PNP degradation kinetics, morphology, and broad phenolic substrate utilization. Firstly, PNP-degrading granules were found to possess an integrity coefficient greater than 99%. ECP content of PNP-degrading granules, in terms of EPS and EP, was approximately 9.9% of granule biomass. Secondly, PNP-degrading granules had specific PNP degradation rates that increased with PNP concentration from 0 to 40 mg/l, peaked at 19 mg/g VSS·h, and declined with further increases in PNP concentration as substrate inhibition effects became significant. The kinetic analysis using Haldane equation estimated that PNP-degrading granules had a maximum specific PNP degradation rate ( $V_{max}$ ) of 36 mg/g VSS·h, a half-saturation coefficient ( $K_s$ ) of 18 mg/l and an inhibition coefficient ( $K_i$ ) of 90 mg/l. Finally, PNP-degrading aerobic granules were also found to have a diverse morphotypes and can degrade broad phenolic substrates.

### **6.1.2 Underlying mechanisms involved in PNP biodegradation with PNP-degrading aerobic granules I – isolation and physiological characterization of PNP-degrading bacterial strains residing in PNP-degrading aerobic granules**

The microbial community study showed that the competent PNP-degrading bacteria accounted for a portion of 49% of total culturable heterotrophic bacteria. The physiological studies on the competent PNP-degrading bacteria showed that they possessed different PNP biodegradation activities, in term of specific PNP degradation rates and specific growth rates. Based on the analysis of 16S rRNA gene sequencing,

the competent PNP-degrading bacteria were found belonging to the different taxonomy affiliations of the genus *Burkholderia*, *Acidovorax* and *Paracoccus* respectively.

From the competent PNP-degrading bacteria isolated from the aerobic granules, a strict aerobic bacterium, designated as strain PNP-01 was identified as one of the functionally important PNP-degrading populations. The quantification analysis indicated that strain PNP-01 constituted 2.5% (dry weight) of mature PNP-degrading granules. The DGGE analysis on the 16S rRNA gene fingerprints demonstrated that strain PNP-01 bacteria was persistently present in the bacterial community during the formation of PNP-degrading aerobic granules. Kinetic analysis on the PNP biodegradation data estimated that strain PNP-01 had a specific maximum PNP degradation rate ( $V_{max}$ ) of 290 mg/g DW·h and a half-saturation constant ( $K_m$ ) of 5.3 mg/l. In addition, physiological characterization of strain PNP-01 revealed that the cell density of strain PNP-01 affected the efficiency of PNP biodegradation by strain PNP-01 and strain PNP-01 was not able to form self-flocculation. These findings imply that the efficient PNP biodegradation of strain PNP-01 relies on its immobilized growth within granules in the SBR system. Finally, the phenotypic and phylogenetic characterization identified strain PNP-01 as the *Burkholderia* sp.

### **6.1.3 Underlying mechanisms involved in PNP biodegradation with PNP-degrading aerobic granules II – physiological characterization of consortium PNP-04 and isolation of three members from the consortium**

Consortium PNP-04 was characterized as a highly stable microbial aggregate that can efficiently degrade PNP. Consortium PNP-04 was identified as another functionally important PNP-degrading population within PNP-degrading aerobic granules, on the basis of its numerical abundance and its capability for efficient PNP removal.

Three bacterial members, designated as strain AG, strain AC and strain AY, were isolated from the consortium PNP-04 with specially designed isolation strategies, namely single colony repeat transfer method, micromanipulation method, as well as

motility agar selection method, respectively. Phylogenetic analysis on the basis of 16S rRNA gene sequencing revealed that strain AG, strain AC and strain AY belonged to genus of *Brevibacterium*, *Micrococcus* and *Ochrobactrum*, respectively.

Specific PNP biodegradation assay showed that consortium PNP-04 was able to completely degrade 100 mg/l PNP within 3.5 hours. The three isolates, strain AG, strain AC and strain AY, from consortium PNP-04 were found not able to degrade PNP in monocultures but able to degrade PNP in a mixed culture by equally mixing three strains. However, the mixed cultures of three isolates needed to take more than 30 hours to completely degrade 100 mg/l PNP.

Based on the results from this and previous studies, the two mechanisms involved in PNP biodegradation by aerobic granules were figured out. The PNP biodegradation by aerobic granules was carried out either by single bacterial strains or by the concert effect of bacterial consortium.

In summary, comprehensive PNP biodegradation studies were conducted throughout this study to understand the microbial biodegradation of PNP with aerobic granules in SBR bioreactors. This research also serves to address the potential of biodegradation as treatment alternative in the removal of PNP from contaminated wastewater. PNP-degrading aerobic granules have been developed and studied to demonstrate that it is possible to use aerobic granules for efficient PNP biodegradation and broadens the benefits of using the SBR to target the treatment of toxic and recalcitrant organic compounds. The understanding of the characteristics of the PNP-degrading aerobic granules would confer the effective application and enhancement of the PNP biodegradation with aerobic granule process. The isolation and the analysis of physiological traits of functionally important PNP-degrading populations revealed information not only useful for understanding the underlying mechanisms of PNP biodegradation by PNP-degrading aerobic granules, but also helpful in the manipulation and optimization of the PNP-degrading aerobic granulation process. Special biodegradation mechanisms of aerobic granules can enhance the knowledge on the microbial interactions within a mixed culture biosystems and shed light on the

future application of the aerobic granulation system to degrade difficult compounds. The information on the physiological characteristics of functionally important populations related to the PNP biodegradation would provide an important guideline for the future application of the PNP-degrading population for the enhancement of the process performance.

## 6.2 RECOMMENDATIONS FOR FUTURE WORK

### 6.2.1 To analyze interactions among two major PNP-degrading populations, *Burkholderia* sp PNP-01 and consortium PNP-04

*Burkholderia* sp. PNP-01 and consortium PNP-04 represented two major PNP removal mechanisms within PNP-degrading aerobic granules. This means that PNP can be degraded either by a single species degrading bacteria or by a cooperative mixed culture. Both *Burkholderia* sp. PNP-01 and mixed culture PNP-04 showed some physiological characterizes associated with competitive and growth advantages. It is interesting to determine the abundance and in situ spatial distribution of these two PNP-degrading populations within aerobic granules in a long-term bioreactor operation for PNP biodegradation. Quantitative PCR with specific primers and fluorescence in situ hybridization (FISH) with specific probes should be employed to investigate the abundance and in situ spatial distribution of these two PNP-degrading populations. On the basis of the information obtained from this proposed future study, the interactions of these two PNP-degrading populations will be analyzed to facilitate the understanding and control of this novel PNP-degrading granulation biotechnology.

### 6.2.2 To improve the treatment capacity of the system through optimizing the operational parameters for PNP-degrading aerobic granulation systems

The PNP-degrading aerobic granulation SBR has shown being able to completely mineralize a start-of-cycle PNP concentration of 100 mg/l within 37.5% of a 4-hour cycle time (1.5 hour) at a PNP loading rate of 0.6 kg/m<sup>3</sup>·day. This indicated that the PNP-degrading aerobic granulation SBR still has room for an improved PNP treatment capacity. It is of applicable interest to develop an improved PNP treating capacity in the PNP-degrading aerobic granulation SBR. The operational strategies will be developed and the operational parameters will be optimized for the PNP-degrading aerobic granulation SBR to achieve an enhanced PNP treatment capacity.

### **6.2.3 To integrate an anoxic phase to remove the nitrite/nitrate generated from PNP biodegradation**

From the pollutant control perspective, the simultaneous removal of nitrite (or nitrate) released from PNP aerobic biodegradation is also desired. Therefore, it is interesting to know whether such PNP-degrading aerobic granules can be used for the simultaneous removal of carbon and nitrogen in PNP via the integration of a denitrification process. Proper strategies will be developed to integrate denitrification with PNP aerobic biodegradation in granulation SBR system. The major operational parameters, such as DO concentration, and COD/N ratio will be optimized for the process.

In addition, it is also interesting to know the impact of the integrated denitrification process on the PNP-degradation ability as well as on the characteristics of the granules. The effects of the integrated denitrification process on the changes of the characteristics of the PNP biodegradation aerobic granules and the microbial community will be evaluated.

This will lead to the development of a novel process for a successful and sustainable simultaneous removal of carbon and nitrogen in PNP. The knowledge obtained from this study will expand our understanding of this integrated process so that a better control of the process can be obtained.

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