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Development of Aerobic Granules For Enhanced Biological Wastewater Treatment

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School of Civil & Environmental Engineering

A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Doctor of Philosophy

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3. Joo-Hwa Tay, Stephen Tiong-Lee Tay, Volodymyr Ivanov, Shun Pan, **He-Long Jiang**, Qi-Shan Liu (2003). "Biomass and porosity profiles in microbial granules used for aerobic wastewater treatment." Letters in Applied Microbiology 36: 297-301.
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SUMMARY

To investigate the ability of aerobic granules to efficiently degrade toxic and inhibitory substrates, a column-type SBR was operated with phenol as the sole carbon source at a rate of $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. Aerobic granules first appeared on day 9 of reactor operation and quickly grew to displace the seed flocs as the dominant form of biomass in the reactor. These granules were compact in appearance, had a mean size of 0.52 mm and a sludge volume index of $40 \text{ ml g}^{-1} \text{ SS}$. The compact structure of aerobic granules offers several advantages, namely easy separation of biomass from bulk liquid during settling phase, high biomass retention, and protection of cells against the effects of phenol toxicity. Compared to conventional activated sludge systems and SBR with flocculated sludge, phenol-degrading aerobic granules can be exploited to design compact, high-rate aerobic granulation systems for the treatment of industrial wastewaters containing high concentrations of phenol and other inhibitory chemicals.

Effect of phenol loading on aerobic granules was further studied. Compact granules were maintained at loadings up to $2.0 \text{ g phenol l}^{-1} \text{ day}^{-1}$, and structurally weakened granules with enhanced production of extracellular polymers and proteins and lower hydrophobicities were observed at the highest loading of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. The microbial community in the aerobic granules was capable of adaptive changes to tolerate the range of phenol loadings tested. At the highest loading applied, the anabolism and catabolism of microorganisms were regulated such that phenol degradation proceeded exclusively via the *meta* pathway, apparently to produce more energy for overstimulation of protein production against phenol toxicity.

16s rDNA DGGE and culture isolation methods were combined to assess the community structure and the functional diversity of microbial community in phenol-degrading aerobic granules cultivated at a loading of $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. The DGGE fingerprint indicated that about ten dominant populations were detected in aerobic granules. Ten pure cultures were isolated from phenol-degrading aerobic

granules, and characterized. Three isolates possessed identical 16s sequences to three dominant populations in granules. Both DGGE and isolation demonstrated that beta subclass of the *Proteobacteria* and high G+C Gram-positive bacteria had significant presence in phenol-degrading aerobic granules. The ten isolates showed different physiological traits, in terms of specific growth rates, kinetics, enzyme activities and flocculation activities.

Isolation, DGGE and hybridization with specific probes confirmed that isolate PG-01 was numerically abundant in the aerobic granules. Combined with its highest growth and phenol degradation rate among all isolates, isolate PG-01 could be regarded as one of the functionally dominant strains and may have contributed significantly to phenol degradation in the granules. Through analyzing its competition with the other fast-growth isolates, strain PG-01's dominance in granules may be due to the following factors: (1) high growth and phenol degradation rate; (2) use of *meta* degradation pathway; (3) high respiratory activities; (4) high energy production at the cost of low biomass yield,

Key Words:

Aerobic granules; Cell immobilization; Denaturing gradient gel electrophoresis (DGGE); Dominance; Fluorescence in situ hybridization (FISH); Function; Isolation; Kinetics; Loading; Metabolism; Microbial community; Phenol degradation.

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NOMENCLATURE

μ	specific growth rate, h^{-1}
Δs	total disappearance of phenol, mg l^{-1}
Δs_1	phenol for assimilation into biomass, mg l^{-1}
Δs_2	phenol transformed for energy production, mg l^{-1}
ΔX	net growth of biomass, mg l^{-1}
APHA	American Public Health Association
ARDRA	amplified ribosomal DNA restriction analysis
BOD	biochemical oxygen demand
C12O	catechol 1, 2-dioxygenase
C23O	catechol 2,3-dioxygenase
CFU	colony forming unit
CLSM	confocal laser scanning microscope
COD	chemical oxygen demand
DAPI	4',6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
dNTPs	deoxynucleotide triphosphates
DO	dissolved oxygen
DW	dry weight
ECPs	extracellular polymers
EPA	Environmental Protection Agency
F/M	feed/microorganism
FISH	fluorescence in situ hybridization
HRT	hydraulic residence time
k_d	endogenous metabolism or decay rate, h^{-1}
K_s	half-saturation constant, mg phenol l^{-1}
K_i	inhibition constant, mg phenol l^{-1}
NCBI	national centre for biotechnology information
OCT	optimum cutting temperature
OD	optical density
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PFA	paraformaldehyde
PGA	phosphoglycerate
PN	proteins
PS	polysaccharides
q	specific phenol degradation rate, mg phenol mg ⁻¹ biomass h ⁻¹
Q	specific oxygen utilization rates, mg DO g ⁻¹ DW h ⁻¹
Q_{max}	maximum specific oxygen utilization rates, mg DO g ⁻¹ DW h ⁻¹
RDP	ribosomal database project
s	substrate concentration, mg l ⁻¹
s₀	initial substrate concentration, mg l ⁻¹
SBR	sequencing batch reactor
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SOUR	specific oxygen utilization rate
SS	suspended solids
SVI	sludge volume index
TGGE	temperature gradient gel electrophoresis
TOC	total organic carbon
TOUR	total oxygen utilization rate
tRNAs	transfer RNAs
UASB	upflow anaerobic sludge blanket
VSS	volatile suspended solids
X	biomass concentration, mg l ⁻¹
X₀	Initial biomass concentration, mg l ⁻¹
Y	true yield of microorganism, mg biomass mg ⁻¹ substrate
Y_c	theoretical yield of cell mass on phenol, mg biomass mg ⁻¹ substrate
Y_E	yield of cell mass on phenol consumed for energy, mg biomass mg ⁻¹ substrate
Y_{obs}	observed biomass yield, mg biomass mg ⁻¹ substrate
Y_{obs/COD}	observed biomass yields based on COD removal, mg biomass mg ⁻¹ COD

CHAPTER ONE

INTRODUCTION

With a rapidly expanding world population, and a highly urbanized and industrialized society, the problems related to the management of wastewater have become of considerable importance. The growth of industry over the past century has been accompanied by an expansion in the production and use of a wide range of organic chemicals including synthetic or xenobiotic compounds. Release of many of these chemicals to the environment has resulted in detrimental consequences including immediate toxicity to exposed biological populations, accumulation and destruction of local sites, and transport leading to contamination of air, soil, groundwater and waterways at locations far from the original sites of release.

Phenol is one of the most widely used products in industry, and the demand for this product is constantly rising. All the industries that produce or use phenol may at some point be responsible for the release of this compound into environment. Phenols are present in effluents from industries such as petroleum and petrochemical, coal gasification, coke production, pharmaceutical, pesticide, fertilizer, dye manufacturing, pulp and paper (Loh et al., 2000; van Schie and Young, 2002). Phenol concentrations ranging from several up to 10, 000 mg l⁻¹ have been reported in industrial wastewater. Based on severe chronic toxicity, phenol has been classified as a high concern priority pollutant by the USA Environmental Protection Agency (USEPA, 1998). Phenol affects aquatic life, causing ecological imbalance. Phenol can be toxic to some aquatic species at concentrations in the low mg l⁻¹ range (Brown et al., 1967) and causes taste and odor problems in drinking water at far lower concentrations (Rittmann and McCarty 2001). Due to these concerns, a variety of quality guidelines have been

established for phenol levels in water. The removal of phenol from wastewater is essential before discharge into nature environments, and the need for technologies for environmental friendly treatment of phenol wastewater especially at high loadings is therefore important (Muyima et al., 1997).

Biological wastewater treatment is a cost-effective and environmentally sound approach as it takes advantage of naturally occurring biological processes that result in the decomposition of organic and inorganic pollutants into harmless products, such as CO₂ and water, to decrease the load of wastewater contaminants (Droste, 1997). This is accomplished by promoting in the bioreactor the environmental conditions required for the growth of a microbial culture capable of performing the biological reactions necessary for producing the design effluent quality. A wide range of microbes have the ability to utilize phenol as carbon and energy sources and can hence grow and thrive within phenol-contaminated environments. Compared to bulk removal of phenol by physical or chemical methods, biological degradation is generally preferred due to lower costs and the possibility of complete mineralization (Loh et al., 2000). However phenol-containing wastewater is difficult to biologically treat because of substrate inhibition, whereby microbial growth and concomitant biodegradation of phenol are hindered by the toxicity exerted by high concentrations of the substrate itself.

While phenol wastewaters are usually treated in continuous activated sludge processes, these systems are known to be sensitive to high phenol loading rates and to fluctuations in phenol loading (Watanabe et al., 1999; Kibret et al., 2000). The substrate inhibition difficulties associated with high-strength phenolic wastewaters can be overcome by strategies such as cell immobilization (Keweloh et al., 1989). Until now, most immobilization experiments used one pure culture or bacterial consortium consisting of two or several cultures, and were carried out

under sterile (monoseptic) conditions (Mordocco et al., 1999; Loh et al., 2000). This limited their application as nonsterile conditions are usually required for wastewater treatment. In addition, the demand for a natural or synthetic carrier that meets all these criteria is still relevant (Brányik et al., 2000). Furthermore, the carriers usually are expensive and they use up a significant amount of reactor space.

Recent research efforts had been dedicated to the development of aerobic granules in sequencing batch reactors (SBRs) (Beun et al., 1999; Peng et al., 1999; Tay et al., 2001a, b). As a relatively new form of cell immobilization, aerobic granules are self-immobilized bacterial aggregates from activated sludge. Compared to conventional activated sludge flocs, the advantages of aerobic granules are denser and more compact microbial structure, better settling ability and higher biomass retention. Aerobic granulation can create a more favorable microenvironment for microorganisms under unfavorable conditions. The dense structure of aerobic granules gives rise to a substrate concentration gradient because of diffusional resistance, which protects the microorganisms in aerobic granules by diluting the toxic chemical below some threshold value to avoid inhibition and allow continued microbial activity and substrate utilization (Villaverde and Fernández-Polanco, 1999; Rittmann et al., 2000). In addition, aggregation of activated sludge cells into aerobic granules helps establish syntrophic relationships, and facilitates horizontal gene transfer, interspecies substrate exchange and removal of metabolic product. Because of these characteristics, aerobic granules have been shown to achieve high organic loading rates with simple substrates such as glucose and acetate, and may lead to the design and operation of novel, compact and high-rate reactors (Moy et al., 2002). Effects of operational parameters and medium composition on aerobic granules have been widely studied (Tay et al., 2001b; Moy et al., 2002; Jiang et al., 2003; Liu et al., 2003b). However, the

microbial ecology aspects, e.g. the compositions of the microbial populations that are responsible for the development of granules, are not yet well understood.

During the early development of biological wastewater systems, scientists have tended to concentrate on the processes rather than on the microbial communities involved and community structure and function partially due to lack of adequate methods. The “black box” philosophy has dominated the understanding of these systems (Head et al., 1998). Now, it is widely accepted that the knowledge on the microbial ecology involved in these systems would assist in many ways in improving systems design and performance (Cloete, 1997). The advent and application of molecular tools in wastewater microbiology has revolutionized our view on the microbial communities of these systems during the last decade. Although the culture-based method has been considered to suffer from several shortcomings (Vallaeys et al., 1997), these methods such as isolation, however, cannot be totally supplanted. Isolates are certainly needed for a better understanding of their physiology, and will assist in obtaining new cultures that could mineralize recalcitrant compounds. The combination of both culture-independent and culture-dependent techniques can provide very useful and complementary information about the structures of microbial communities.

Molecular ecological approaches have detected diverse microbial populations in the natural environment, which make us realize that it would be almost impossible to analyze the behavior of all the individual species in a complex microbial community. Recent studies stressed the importance of gaining an understanding of the functions of microbial communities and revealed that population diversity alone does not drive ecosystem stability (Briones and Raskin, 2003). Recognizing the diversity and the links within each functional group of a system can lead to better ways to model diversity and function (Hulot et al., 2000). Thus, there has

been increasing interest in identifying dominant or functionally important microorganisms in different pollutant-degrading ecosystems (Watanabe et al., 1998a; Bruns et al., 2001; Tirola et al., 2002). Identification of the dominant organisms that play a key role in pollutant removal not only helps in understanding degradation processes in systems but is important in the development of optimal control and management strategies (Watanabe et al., 1998a; Abed et al., 2002; Manefield et al., 2002).

This research seeks to accomplish the following objectives:

1. To study the formation of aerobic granules using phenol as sole carbon substrate;
2. To investigate the effect of phenol loading on structure, activity, and metabolism of aerobic granules;
3. To survey the microbial community within phenol-degrading granules using conventional and advanced molecular methods;
4. To identify the functionally important populations in aerobic granules;
5. To analyze the interactions between microbial community structure and function in aerobic granules.

This work is expected to be useful in understanding the ability of aerobic granules to handle high-strength industrial wastewaters containing chemicals that are inhibitory to microbial growth and biodegradation. This study could also contribute to a better understanding of the microbial community of these aerobic granules and provide information useful for manipulation of the aerobic granulation process and optimization of microbial community.

Chapter 2 is a detailed literature review and discussion about phenol wastewater treatment methods, the phenomenon of aerobic granules, and the use of

culture-dependent and rRNA-based methods to study microbial communities. Chapter 3 describes the formation process and properties of aerobic granules using phenol as sole carbon source. Chapter 4 details the effect of phenol loadings on structure, activity, and metabolism of aerobic granules. Aerobic granules can make adaptive adjustments in metabolism against phenol toxicity at high phenol loading. So, the granule system can remain stable without compromising granule integrity and function even under a high loading of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. Chapter 5 describes the microbial community of phenol-degrading aerobic granules using both molecular methods and conventional isolation techniques. Ten pure cultures were isolated from phenol-degrading aerobic granules, and characterized. Based on the dominance of three isolates in the granules, a model for the microbial structure of granules was proposed. Chapter 6 analyzes the competition between dominant and non-dominant isolates in terms of kinetics and metabolism. Several physiological traits were identified that contributed to the dominance of individual microorganisms in the granules. Chapter 7 summarizes some important conclusions from the experimental results and outlines some recommendations for future work. Some of the results in Chapter Three and Four were developed into three papers published in the journals *Letters in Applied Microbiology*, *Journal of Environmental Engineering and Applied Microbiology* and *Biotechnology*, respectively. Part of the results in Chapter Five were developed into one papers in *Applied and Environmental Microbiology*.

CHAPTER TWO

LITERATURE REVIEW

2.1 ENVIRONMENTAL CONTAMINATION

The growth of industry over the past century has been accompanied by an expansion in the production and use of a wide range of organic chemicals including synthetic or xenobiotic compounds. Release of many of these chemicals to the environment has resulted in detrimental consequences including immediate toxicity to exposed biological populations, accumulation and destruction of local sites, and transport leading to contamination of air, soil, groundwater and waterways at locations far from the original sites of release. Many of these compounds continue to serve useful purposes in industry, while production of others has been discontinued due to the threat their use presents to the well being of biological populations and the natural environment. Regardless of whether these compounds occur within industrial effluents in currently operating processes, or within abandoned storage barrels in forgotten warehouses, once produced they present a serious potential source of environmental contamination. For reasons of both health and environmental concerns, methods of remediation and elimination of these chemicals is a vitally important area of study and development.

2.1.1 General aspects of phenol and phenol wastewater

Phenol is one of the most widely used products in many industries, and the demand for this product is constantly rising. The greatest commercial uses for phenol are in production of intermediates in manufacturing of nylon and resins, and bonding of laminated wood products. This compound is also used for explosive, paints, inks, perfumes, textiles, drugs, and as an antibacterial and antifungal agent. Phenol is produced by distillation of coal tar, and can also be made by synthetic process such as oxidation of methylethylbenzene, oxidation of toluene, fusion of sodium

benzenesulfonate with sodium hydroxide, or heating monochlorobenzene with sodium hydroxide under high pressure. Phenol ranks among the 40 most produced chemicals in the USA with an annual production of 4.77 billion pounds in 1998 and an expected production growth rate of 3% per year (van Schie and Young, 2002).

All the industries that produce or use phenol may at some point be responsible for the release of this compound into environment. Phenols are present in effluents from industries such as petroleum and petrochemical, coal gasification, coke production, pharmaceutical, pesticide, fertilizer, dye manufacturing, and pulp and paper (Loh et al., 2000; van Schie and Young, 2002). Phenol concentrations ranging from several to up to 10, 000 mg l⁻¹ have been reported in industrial wastewater, whereas domestic wastewater contains phenol with concentration between 0.1 to 1 mg l⁻¹.

Based on severe chronic toxicity, phenol has been classified as a high concern priority pollutant by the USA Environmental Protection Agency (EPA) (USEPA, 1998). Phenol affects aquatic life, causing ecological imbalance. Phenol can be toxic to some aquatic species at concentrations in the low mg l⁻¹ range (Brown et al., 1967) and causes taste and odor problems in drinking water at far lower concentrations (Rittmann and McCarty, 2001). In addition to inhalation, phenols can be absorbed through the skin and exposure, and highly irritate the skin, eyes and mucous membranes of humans (Allen and Allen, 1997). Due to these concerns, a variety of quality guidelines have been established for phenol levels in water. The USEPA criterion to protect freshwater aquatic life is 600 µg phenol l⁻¹ as a 24-hour average, never to exceed 3.4 mg phenol l⁻¹, and the drinking water limit is 1 µg phenol l⁻¹ (USEPA, 1979). Hence, the removal of phenol from wastewater is essential before discharge into nature environments.

2.2 PHYSICAL AND CHEMICAL TREATMENT OF PHENOL WASTEWATER

Traditional efforts to eliminate phenols have involved a variety of physical and chemical treatment methods including landfill, air stripping, adsorption, incineration, and oxidation (Nyer 1993; Bowlen and Kosson, 1995; Wu and Zhou, 2001; Esplugas et al., 2002; Feng and Li, 2003). The choice of treatment methods is influenced by a variety of issues including the nature of both contaminant and site of release, extent of contamination, potential for pollutant migration, the perceived level of threat to biological populations and the natural environment, cost efficiency, and commitment to remediation of the parties responsible for the pollutant release.

The implementation and effectiveness of these physical-chemical treatment methods, however, is continually limited by the degree and extent of contamination, monitoring requirements and the associated costs. The magnitude of contamination in both soil and especially waterway environments preclude the use of remediation methods such as adsorption or incineration (Nyer, 1993). Decreasing site availability relative to load, and concerns over run-off and re-release of contaminants to the environment has limited the use of landfill approaches. The high boiling points of phenol prevent the efficient use of any remediation techniques relying on volatilization. In fact, physical processes are especially limited since contaminants undergo transfer to a new medium (and larger volume) with little or no actual modification (degradation) to eliminate the hazardous chemicals (Omenn, 1988). One of issues with many chemical methods such as oxidation processes is related to possible formation of secondary toxic materials (e.g. cyanates, chlorinated phenols) that are even more troublesome, requiring further treatment (Bandhyopadhyay et al., 1998; Loh et al., 2000). Moreover, it should be pointed out that these physico-chemical treatment

processes have proven to be costly (Bandhyopadhyay et al., 1998; Wu and Zhou, 2001).

2.3 BIOLOGICAL TREATMENT OF PHENOL WASTEWATER

A wide range of microbes have the ability to utilize phenol as carbon and energy sources and can hence grow and thrive within phenol-contaminated environments. Compared to bulk removal of phenol by physical or chemical methods, biological degradation is generally preferred due to lower costs and the possibility of complete mineralization (Loh et al., 2000). However phenol-containing wastewater is difficult to biologically treat because of substrate inhibition, whereby microbial growth and concomitant biodegradation of phenol are hindered by the toxicity exerted by high concentrations of the substrate itself.

2.3.1 Phenol degradation and growth of pure cultures

Phenol can be degraded in aerobic or anaerobic condition by microorganisms. Aerobic degradation has been studied since the beginning of 1900s. A number of microorganisms are capable of aerobically degrading phenol at low concentrations, including *Pseudomonas* sp. (Hinteregger et al., 1992), *Rhodococcus* sp. (Bell et al., 1998), *Acinetobacter calcoaceticus* (Schirmer et al., 1997), *Alcaligenes eutrophus* (Leonard and Lindley, 1998), *Alcaligenes faecalis* (Bastos et al., 2000), *Bacillus stearothermophilus* (Gurujeyalakshmi and Oriel, 1989), and *Comamonas testosteroni* (Arai et al., 1998). The knowledge of phenol biodegradation in anaerobic conditions is less advanced than aerobic process. Several phenol-degrading anaerobes, such as phenol-degrading denitrifying *Azoarcus* sp. and *Magnetospirillum* sp. (van Schie and Young, 1998; Shinoda et al., 2000), and sulfate-reducing phenol-degrading organisms *Desulfotomaculum* strain Groll (Kuever et al., 1993) were more recently isolated.

2.3.1.1 Phenol degradation pathway and enzyme

Phenol and its derivatives are aerobically biodegraded by two main metabolic pathways, initiated either by *ortho* or *meta* cleavage (Müller and Babel, 1996; Leonard and Lindley, 1998). The general scheme of bacterial degradation of phenol under aerobic conditions is shown in Figure 2.1. The first step of phenol degradation in the *meta* pathway is the hydroxylation to catechol by phenol hydroxylase. In the next step, the aromatic ring is opened by catechol 2,3-dioxygenase (C23O) to yield 2-hydroxymuconic semialdehyde. Then an aldehyde dehydrogenase converts this compound to 4-oxalocrotonic acid. After decarboxylation, the resulting 2-oxo-pent-4-enoic acid, is converted to 4-hydroxy-2-oxovaleric acid by a hydratase. Then 4-hydroxy-2-oxovaleric acid is degraded into acetaldehyde and pyruvic acid. The first step in the *ortho* pathway of phenol degradation is also catalyzed by phenol hydroxylase to form a catechol. In the second step, catechol 1,2-dioxygenase (C12O) converts catechol into *cis*, *cis*-muconate. Then a lactonizing enzyme converts this compound to muconolactone. Next, in a two stage reaction a hydrolase acts on muconolactone to produce beta-ketoadipate. Finally, oxidative cleavage of beta-ketoadipate produces the common tricarboxylic cycle intermediates, succinate and acetyl-CoA.

Many bacteria frequently possess one or two of these pathways. In general most naturally occurring phenols are metabolized using the *meta* pathway. The enzymes phenol hydroxylase and C12O or C23O catalyze the first and second steps of phenol degradation, respectively. Two types of phenol hydroxylase including single-component and multi-component enzymes are known, and multicomponent enzymes are considered the major ones in the environment among them (Harayama et al., 1992). In recent years, a number of the genes for microbial phenol degradation had been determined (Schirmer et al, 1997; Futamata et al, 2001). The genes necessary for the expression of these enzymes can be either

Fig. 2.1 Phenol degradation by the (A) ortho- and (B) meta-pathway. (1) conversion of catechol (CAT) to 2-hydroxymuconic semialdehyde (2HMSA) by CAT 2, 3 dioxygenase; (2) conversion of 2HMSA to 4-oxalocrotonate enol (4OCE) by 2HMSA dehydrogenase; (3) formation of the keto form of 4OC (4OCK) by 4OC tautomerase; (4) formation of 2-oxy-pentanoate-4-enoate (2OP4E) from 4OCK by 4OCK decarboxylase or from 2HMSA hydrolase (7); (5) conversion of 2OP4E to 4-hydroxy-2-oxovaleric acid (4H2OV) by a hydrolase; (6) cleavage of 4H2OV to pyruvate (PYR) and acetaldehyde (AAH) by 4H2OV aldolase; (8) formation of *cis, cis* muconate (CCM) from CAT by CAT 1,2 dioxygenase; (9) conversion of CCM to muconolactone (ML) by CCM lactonizing enzyme; (10) formation of β -keto adipate enol lactone (β -KAEL) from ML by ML isomerase; (11) formation of β -keto adipate (β -KA) from β -KAEL hydrolase; (12) formation of β -keto adipyl CoA (β -KA-CoA) from β -KA and succinyl CoA (SUCC-CoA) by β -KA:SUCC-CoA transferase, with the concomitant formation of succinate (SUCC); (13) cleavage of β -KA-CoA to form SUCC-CoA and acetyl CoA (ACA) by β -KA-CoA thiolase (Caldwell, 1995).

chromosome- or plasmid-encoded. For example, multi-component phenol hydroxylase together with genes encoding the *meta* cleavage are organized in operons located on the chromosome of *Pseudomonas* sp. (Ng et al., 1995) or the TOL plasmid (Nordlund et al., 1990).

In all aerobic degradation pathways the highly reactive molecular oxygen is used for the initial attack(s) on the aromatic nucleus as well as for the final de-aromatising ring splitting step. A similarly reactive co-substrate is not available in anaerobic pathways. Therefore, anaerobic aromatic degradation has to rely on completely different mechanisms for channeling diverse compounds into degradation pathways and dearomatising reactions. In contrast to aerobic pathways, the reactions involved in anaerobic aromatic metabolism are largely reductive modifications of the substrate. In particular, the actual dearomatizing reaction in the different anaerobic pathways proceeds by reduction of the aromatic ring to nonaromatic cyclohexane-derivatives. Pure cultures of anaerobic bacteria can completely oxidize phenol to carbon dioxide using nitrate, ferric iron or sulphate as terminal electron acceptor. At least two phenol degradation pathways have been suggested to occur under methanogenic conditions (Karisson et al., 2000). The most studied anaerobic pathway is phenol transformation via the “benzoyl-CoA” pathway (Fig. 2.2). Not surprisingly, the efficiency of these pathways is not as high as degradation by the aerobic pathways and hence most bioremediation efforts involve an aerobic treatment.

Fig. 2.2. Anaerobic pathway for the transformation of phenol to acetyl-CoA and CO₂ via the central intermediate benzoyl-CoA (Heider and Fuchs 1997; Harwood et al., 1999)

2.3.1.2 Growth kinetics

For non-inhibitory substrates, the specific growth rate (μ , h⁻¹) can be described by the commonly used Monod model where μ asymptotically approaches an upper limit $\mu_{\max}$, as the substrate concentration increases (Bailey and Ollis, 1986).

$$\mu = \mu_{\max} \frac{s}{K_s + s} \quad (2.1)$$

where K_s is the half-saturation constant (mg l⁻¹), s is growth substrate concentration (mg l⁻¹).

Several mathematical models have been developed over the years to describe the inhibitory effect of substrate on growth rate. Many of these models were originally based on reactions involving enzymes. Based on the equation proposed by Aiba et

al. (1968), Edwards (1970) suggested the following equation to predict substrate inhibition:

$$\mu = \mu_{\max} \frac{s}{K_s + s} * \exp\left(-\frac{s}{K_i}\right) \quad (2.2)$$

where K_i is the inhibition constant (mg l^{-1}).

Hill and Robinson (1975) tested the Aiba-Edwards model for the degradation of phenol using *Pseudomonas putida* from both batch and continuous culture experiments maintained at 30°C and at a pH of 6.5. They found that data did not correlate well with the Aiba-Edwards function and suggested that the Haldane model would be more appropriate from a statistical perspective. According to the Haldane model, substrate inhibition of microbial growth is given by Equation (2.3)

$$\mu = \mu_{\max} \frac{s}{K_s + s + (s^2 / K_i)} \quad (2.3)$$

Now the Haldane equation has become the most commonly used model for description of the specific growth rate on the concentration of inhibitory substrates such as phenol. Phenol biodegradation kinetics have been determined for many microorganisms using the Haldane model and listed in Table 2.1. If substrate concentrations are low, the term s^2/K_i in Equation (2.3) is usually less important than the term K_s even for low values of K_i . Under these conditions, the inhibition function reduces to the Monod equation. Under higher substrate concentrations present during the early stages of growth, the term s^2/K_i may be significant even for higher values of K_i , resulting in the decrease in specific growth rate (Andrews, 1968). As shown by Andrews (1968), the maximum specific growth rate attainable in the presence of inhibition occurs when $s = (K_s K_i)^{0.5}$.

Table 2.1 Biodegradation model parameter values for aerobic phenol biodegradation by pure cultures

Microorganism (cultivation type)	Temperature (°C)	$\mu_{\max}$ (h ⁻¹)	K_s (mg l ⁻¹)	K_i (mg l ⁻¹)	Y_{obs} (g biomass g ⁻¹ phenol)	Max. phenol concentration for growth (mg l ⁻¹)	Reference
<i>P. putida</i> NRRL β-14875 (continuous)	25	0.569	18.54	99.37	0.52	400	Seker et al., 1997
<i>P. putida</i> ATCC 17484 (batch)	30	0.534	<1.0	470	0.52±0.08	700	Hill and Robinson, 1975
<i>P. putida</i> Q5 (batch)	10	0.119	5.27	377	0.26-0.55	1000	Kotturi et al., 1991
<i>P. putida</i> ATCC 17514 (continuous)	30	0.567	2.38	106		500	Yang and Humphrey, 1975
<i>P. putida</i> ATCC 17514 (batch)	28.5	0.897	12.2	203.7	0.77	150	Dikshitulu et al., 1993
<i>P. resinovorans</i> ATCC 14235 (batch)	28.5	1.007	13	117.7	0.68	150	Dikshitulu et al., 1993
<i>P. putida</i> ATCC 49451	30	0.9	6.93	284.3	0.4-0.95	800	Wang and Loh, 1999
<i>P. putida</i> Q5	25	0.419	7.09	221	0.4-0.9	600	Onysko et al., 2000
<i>P. putida</i> BCIB 17484		0.443	0.16-5.22	7.5-19.4			Sokol and Howell, 1981

The analysis of growth according to the Haldane model has some complications, however, when considering plots of μ vs. s because this model implies that cells are capable of growing indefinitely. This is not what observed in reality since there is a definite substrate concentration limit above which growth will cease, that is, complete inhibition (Luong, 1987). The Haldane model also assumes that there is only one growth limiting factor, which may not always be the case. In many of the models found in the literature, oxygen was not considered as a limiting factor assuming adequate aeration in the medium.

The growth yield is important as a means of determining the nutrient requirement for the cells. As described in Pirt (1975), the observed growth yield (Y_{obs}) is defined by a quotient:

$$Y_{\text{obs}} = \Delta X / \Delta s \quad (2.4)$$

where ΔX is the increase in biomass consequent on utilization of the amount Δs of substrate. The growth yield is expressed more rigorously by the limit of $\Delta X / \Delta s$ as $\Delta s \rightarrow 0$, that is $Y_{\text{obs}} = dX/ds$. The theoretical yield of cell mass on phenol can be calculated to be 1.67 g Dry Weight g^{-1} phenol based on the assumption that carbon content of dry cells is about 46% (Blank and Clark, 1996). Generally growth yield for phenol as substrate decreases with increasing phenol concentration, because at high concentrations of phenol, inhibition effect are stronger and the energy expended to maintain cell membrane integrity would be expect to be higher (Wang and Loh, 1999; Oynsko et al., 2000).

2.3.1.3 Microbial response to phenol toxicity

The general microbial effect of phenol toxicity is based on the compound's ability to partition into membranes (Heipieper et al., 1991). Since the lipid membrane is the only barrier between the bacterial cytoplasm and the outside world, disruption of membrane function readily causes cell death. While overexposure to phenol is

usually associated with decreases or complete loss in specific growth rate, specific oxygen utilization and enzyme activity (Leonard and Lindley 1998; Watanabe et al., 1999; Kibret et al., 2000), microorganisms are capable of a variety of physiological responses to increase their tolerance towards phenol toxicity. Bacteria have developed mechanisms to resist and survive phenol concentrations that are usually bactericidal. The membrane structure of cells can be modified in response to an increase in phenol concentration. Some adaptive response, for instance, the higher proportion of proteins detected in cell membranes (Keweloh et al., 1990), the isomerization of *cis*-unsaturated fatty acids to the *trans*-configuration displayed by phenol-degrading *Pseudomonas putida* P8 (Heipieper et al., 1992), and increase in the ratio of saturated fatty acids to unsaturated acids in the membrane as described for *Escherichia coli* K-12 (Keweloh et al., 1991) have been reported. These changes in membrane seem to counteract the increased fluidity of the membrane caused by phenol partition.

At high phenol concentrations, the anabolism and catabolism activities can be regulated to confer a selective advantage by shifting energy production from complete to incomplete oxidation to produce higher microbial growth rates. This is accomplished by shifting the expression of phenol-assimilation pathways from the *ortho* cleavage pathway to the *meta* cleavage pathway (Müller and Babel, 1996; Filonov et al., 1997). Differences in the two pathways have been compared and elucidated by following the metabolic intermediates produced and energy generating reactions up to the central carbon precursor 3-phosphoglycerate (PGA) (Kiesel and Müller, 2002). Compared with the *ortho* pathway, the *meta* pathway corresponds to a higher overall growth rate, and produces more energy at the expense of lower biomass yield.

In addition, synthesis of specific proteins was enhanced as a result of exposing

cells to phenolic compounds (Lupi et al., 1995). Exposure of *Acinetobacter calcoaceticus* to phenol caused the predominant induction of heat shock proteins because of high lipophilicity of phenol (Benndorf et al., 2001). A pattern of protein rearrangement with phenol stress response is not unexpected and probably reflects the functions of the proteins involved and the need for an initial rapid adaptation to protect the cell against the damaging influence of the stress followed by a period of functional metabolic enhancement during which the proteins regulate metabolism under the altered physical or chemical conditions imposed.

2.3.2 Conventional activated sludge processes to treat phenol wastewater

The conventional activated sludge process, first developed in England in 1914, has been widely used in municipal and industrial wastewater treatment (Droste, 1997). The activated sludge process belongs to suspended-growth systems, and consists of a single aeration basin or multiple basins in series followed by a clarifier for the separation of the suspended biomass by settling prior to the disposal of the treated wastewater. The settling characteristic of biomass is an important operational parameter in this system. However, several types of sludge separation problems, such as bulking and foaming, are often encountered in the clarifiers (Seviour and Blackall, 1999). Bulking and/or foaming result in poor sludge settleability and washout of biomass in the effluent. This loss of biomass from the system can produce an increase in the sewage substrate loading rate per unit mass of sludge in the plant. The increased loading can lead to a potential loss of nitrification and further deterioration in effluent quality. In extreme cases, a reduction in the oxidation of carbonaceous compound can also occur.

Phenol removal has been carried out for many years by conventional activated sludge systems, while high phenol loading rates and fluctuations in phenol loads have been reported to cause the breakdown of activated sludge processes because

of the toxicity exerted by high concentrations of phenol (Watanabe et al., 1999; Kibret et al., 2000). A phenol loading in excess of $1 \text{ g phenol l}^{-1} \text{ day}^{-1}$ can lead to inhibition of the degradation processes, decrease in the settleability of biomass, washout of large amounts of the effective organisms, and carryover of high phenol concentrations to the effluent, and result in the unrecoverable failure of activated sludge systems with little warning.

2.3.3 Sequencing batch reactor (SBR) technology to treat phenol wastewater

As an alternative form of biological wastewater treatment, SBR technology belongs to the group of the so-called fill and draw reactors. The key feature of SBR technology is the change between feast and famine during the reaction cycle. The typical aerobic SBR process cycle consists of the fill, aeration, sedimentation and draw. The cycle is continuously repeated beginning with the fill phase and ending with the draw phase. After specifying the duration of the fill phase, the times for aeration, settle and draw phases can be selected according to specific process goals. While aeration and sedimentation-clarification take place in two different tanks in conventional activated sludge systems, these two processes occur sequentially in the same tank in SBR technology. SBR technology is flexible, efficient and low cost, and has been gaining popularity through the years in treating different kinds of wastewaters, mainly because of its single-tank design and ease of automation (Mace and Alvarez, 2002). Although it was reported that a SBR with flocculated sludge could treat phenol wastewater at a high phenol loading rate of $3.1 \text{ g phenol l}^{-1} \text{ day}^{-1}$, the settling ability of flocculated sludge in that reactor was generally poor, even when the loading rate was as low as $0.52 \text{ g phenol l}^{-1} \text{ day}^{-1}$ (Yoong et al., 2000). Furthermore, only transient experiments were reported in that article. Whether transient results can be used to interpret the long term operation of reactors needs further study.

2.3.4 Cell-immobilization technology for phenol removal

Immobilization of cells in/on various supports appears an attractive and promising strategy to overcome substrate inhibition. The immobilized cells are less sensitive to phenol and can tolerate higher phenol concentrations than their suspended counterparts. Immobilization of *Rhodococcus erythropolis* UPV-1 on diatomaceous earth resulted in an enhancement of cell respiratory activity by 13% and a shorter lag phase preceding active phenol degradation, thus the immobilization cells were able to completely degrade phenol at a volumetric productivity of 11.5 g phenol l⁻¹ day⁻¹ (Prieto et al., 2002). In the case of suspension cultures, *Pseudomonas putida* ATCC 49451 utilized phenol at concentrations belows 1000 mg l⁻¹, but experienced complete substrate inhibition at higher concentrations. On the other hand, cells immobilized in polysulfone fibers could degrade phenol at concentration of 3500 mg l⁻¹, albeit at relatively slow rates (Loh et al., 2000).

The high tolerance of immobilized cells can be contributed to the development of cell aggregates in/on supports. The concentration gradient that normally develops because of diffusional 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 (Villaverde and Fernández-Polanco, 1999; Rittmann et al., 2000). In addition to limitation 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 (Keweloh et al., 1989; Yap et al., 1999). Extracellular polymers (ECPs) are one of the key components of cell aggregates, and is composed of a mixture of components such as polysaccharides,

protein, nucleic acids and other substances. Besides acting as structural materials, the ECP matrix surrounding the microcolonies within cell aggregates can also physically prevent access of toxic agent by binding the toxic agent or inhibiting diffusion into the colony (Keweloh et al., 1989; Prieto et al., 2002).

Until now, most immobilization experiments used one pure culture or bacterial consortium consisting two or several cultures, and were carried out under sterile (monoseptic) conditions (Mordocco et al., 1999). For wastewater treatment, however, nonsterile conditions are usually required. In addition, for the application of immobilized cells in wastewater-treatment, the support material should be insoluble in wastewater, non-biodegradable, insensitive to abrasion, highly permeable for substrates, ecologically non-hazardous and should be produced under mild conditions in order to enable effective cell immobilization. The demand for a natural or synthetic carrier that meets all these criteria is still relevant (Brányik et al., 2000). However, carriers usually are expensive and they use up a significant amount of reactor space. Therefore, the self-immobilization of mixed culture in environmental conditions without the use of supports, like granular sludge, is strongly desired.

Self-immobilization of anaerobic sludge to form anaerobic granules in upflow anaerobic sludge blanket (UASB) reactors was first reported in the 1970s (Lettinga et al., 1980). Since then, different aspects of granule characteristics as well as the granulation process have been widely studied. The granulation process not only enhances the settleability of biomass in the reactors, but also improves the physiological conditions favorable for interactions between microorganisms. Based on the granular growth course, the granulation process was characterized by three phases: acclimation, granulation, and maturation (Tay and Yan, 1996). In brief, the granulation process starts with the colonization of cells onto a nucleus,

the cells then grow into granules of visible size. Nuclei can be particles consisting of inert material in the original inoculum. The nuclei can also consist of small aggregates of microorganisms. Once the nuclei exist, microorganisms can attach to nuclei, and this attachment is affected by several factors such as extracellular polymers, metal ion and hydrophobicity (Schmidt and Ahring, 1996).

Granular-sludge-based anaerobic technology in the UASB process has gained popularity for treating various wastewater types during the past two decades (Lettinga, 1995). Recent studies have demonstrated that anaerobic granular-sludge-based technology is effective for treatment of industrial wastewater containing phenolic compounds (Tay et al., 2000). UASB reactor could be operated at a steady-state condition under the phenol loading of $2.52 \text{ g l}^{-1} \text{ day}^{-1}$. However, under this condition, effluent phenol concentration was very high and reached to 156 mg l^{-1} . Even under the phenol loading of $0.84 \text{ g l}^{-1} \text{ day}^{-1}$, effluent phenol concentration only dropped to a steady 20 mg l^{-1} level and can not meet the discharge standard. So, it seems that UASB with anaerobic granules can act as a proven sustainable wastewater pre-treatment system for wastewater treatment of toxic/inhibitory compounds. Generally, the anaerobic granulation process from a non-granular inoculum (activated sludge) can be lengthy and using phenol as sole carbon substrate made this process significantly slower. With phenol as substrate, it took 9 months for granulation (Tay et al., 2000).

2.4 AEROBIC GRANULATION AND AEROBIC GRANULES

The production of granules in aerobic SBRs is a recently described phenomenon (Morgenroth et al., 1997; Beun et al., 1999; Tay et al., 2001a, b). Granule size varies from 0.3 mm to 7 mm (Morgenroth et al., 1997; Beun et al., 1999, 2000; Tay et al., 2001a), depending on the operational condition and the substrate used. The average density of the aerobic granules determined by an isopycnic centrifugation procedure ranged from 1.044 to 1.048 g ml^{-1} (Etterer and Wilderer,

2001).

2.4.1 Aerobic granulation process and mechanism

Currently there are two models proposed to describe the aerobic granulation. Based on the experimental observation, Beun et al. (1999) proposed a model in which the aerobic granulation started with fungi (Fig. 2.3). Fungi easily form pellets which settle very fast and can be retained in the reactor. When the pellets grow to a diameter of 5-6 mm, they lyse probably due to oxygen limitation in the inner part of pellets. The pellets break up and only colonies that are dense enough are allowed to settle. These colonies eventually grow to become new granules. A second model suggested that the formation of aerobic granules was a gradual process from seed sludge to compact aggregates, further to granular sludge and finally to mature granules with the sequential operation proceeding (Tay et al., 2001a).

Fig. 2.3 Proposed mechanism of aerobic granulation after the start up of a SBR reactor (Beun et al., 1999)

Recently, Liu et al. (2004) proposed that hydraulic selection pressure is a decisive parameter in the formation of biogranulation, and cell hydrophobicity contributes to the formation of granules. In addition, the development of aerobic granules involves the cooperative effects among different functional groups of microorganisms as well as the interaction between these functional groups and the environment. Accompanied with granulation, a significant shift in the microbial diversity was demonstrated by using amplified ribosomal DNA restriction analysis (ARDRA) (Yi et al., 2003). The bacterial communities of aerobic granules in the initial stage and death phases were much more diverse than those of the mature granules in terms of richness, evenness and the Shannon-Wiener index. Some specific microorganisms were suggested to play an important role in the development of aerobic granules.

Although some mechanisms had been proposed, the fundamentals of granulation are still a subject of much discussion owing to the complexity of aerobic granulation. Recent molecular studies showed that bacteria can sense a large number of environmental signals and process this information into specific transcriptional responses (Loo et al., 2000). It has been shown that quorum sensing is a prominent example of social behavior in bacteria, as signal exchange among individual cells allows the entire population to choose an optimal way of interaction with the environment. Bacteria can communicate with other cells by quorum sensing involving the production and detection of extracellular signaling molecules (Bassler et al., 1999; Elizabeth et al., 2000), and then make decisions about how to coordinate growth, movement, and biochemical activities (Shapiro, 1998). So far, intercellular communication and multicellular coordination have been known as an effective way for bacteria to achieve an organized spatial structure. Genetic analyses also suggest that biofilm formation can proceed by various environmental signals, while the development of the bacterial biofilm has

been shown to require cell-cell signals (Davies et al., 1998). So it is reasonable to assume that aerobic granulation may involve the interaction of both environmental signals and cell-cell signals. Cell-to-cell signaling mechanisms would play a role in developing aerobic granules and organizing the spatial structure of granule-associated bacteria in response to environmental stresses.

2.4.2 Aerobic granule structure

Aerobic granules are merely spherical biofilms where microbes are attached to each other and embedded in an ECP matrix. Transport of nutrients and oxygen can be facilitated by the presence of pores and channels in biofilms (Massol-Deya et al., 1995). In aerobic granules, the presence of pores and channels was also determined by measuring the penetration of 0.1 μm fluorescence beads into the granule interior (Tay et al., 2003). The distributions of pores and channel layers were different in granules with various sizes. For example, a granule with a diameter of 550 μm had a porous layer with a thickness of 250 μm , while a granule with a diameter of 1000 μm had a porous layer with a thickness of 350 μm . Influenced by the distribution of pores and channel layers, the distributions of active biomass in granules were dependent on different granule diameters. Almost all biomass in granules with diameter of approximately 0.6 mm were viable. However, a viable 0.3-0.4 mm thick biomass layer was found in the 1.3 mm-diameter granules, and 0.8 mm thick in the 3 mm-diameter granule. As a result, the extent of porous zones and the amount of microbial biomass, on a per unit volume basis, decreased with increasing granule diameters. Based on calculation, the critical diameter for aerobic granules was 0.5 mm on the assumption that whole granule should consist entirely of a porous and viable biomass matrix.

2.4.3 Effect of operational factors on aerobic granulation and granule structure

The time allowed for settling was the main parameter to select for growth of microorganisms in aerobic granules. Aerobic granules would be formed if flocs are washed out. So, very short settling times can result in washout of slow settling biomass and allow the retention of fast settling granules. Based on this criterion, the settling time was normally set as 1-4 minutes (Morgenroth et al., 1997; Beun et al., 1999, 2000), and aerobic granules have been successfully formed after less than one month of operation.

Shear force strongly affects the aerobic granulation (Tay et al., 2001a, b). Three SBR reactors (R1, R2 and R3) were operated at superficial air upflow velocities of 0.3, 1.2 and 2.4 cm s⁻¹ respectively. Aerobic granules appeared at cycle 42 in R2 and R3 with a mean size of 0.37 mm in R2 and 0.35 mm in R3. The appearance of aerobic granules correlated closely with a significant increase in cellular polysaccharides. However, aerobic granulation was not observed in R1. In the case of R2, aerobic granules disappeared at cycle 90, corresponding to a simultaneous decrease in cellular polysaccharides. It was suggested that the shear force contributed to the formation of aerobic granules through promoting the hydrophobicity of cell surface and stimulating the production of cellular polysaccharides.

The relationship between starvation and cell hydrophobicity has been widely studied and some papers reported that cell hydrophobicity might be increased after experiencing starvation. Periodical aerobic starvation, thus, was suggested to act as an effective trigger for microbial aggregation in the reactor through affecting cell hydrophobicity and further strengthened cell-cell interaction to form dense aggregates and subsequently granules (Tay et al., 2001a). However, until now, this

assumption was not directly confirmed through experiments.

Divalent cations like calcium are regarded as important constituents of microbial aggregates (Flemming et al., 2000). In fact, adequate calcium concentration stimulated the aerobic granulation process (Jiang et al., 2003). The granules appeared from day 16 in R2 with a calcium concentration of 100 mg l^{-1} , while aerobic granules were not observed until day 32 in R1 without augmentation of calcium. The addition of calcium shortened the period required for formation of aerobic granules by approximately 50%. At the same time, the structural characteristics of the granules were also influenced by the calcium concentrations. The granules in R2 showed the better structural characteristics, based on density, settleability and strength measurements.

The types of substrate might also influence to some extent the microbial composition of aerobic granules. With acetate as substrate, the aerobic granules formed consisted mainly of rod-like bacteria in a radial arrangement (Tay et al., 2001a). Granules grown with glucose as substrate consisted mainly of cocci-type bacteria in the internal part of the granules and filamentous bacteria tangled together with rod bacteria on the surface (Tay et al., 2001a).

Granular activated sludge was more resistant against long-term storage than activated sludge (Zhu and Wilderer, 2003). Storage in anaerobic condition at room temperatures for seven weeks did not lead to the change of size, color and sedimentation characteristics of the granular sludge. Although granule activity dropped to very low values due to the long idle time, it took less than a week for granules to recover full substrate degradation capacity. These results imply that aerobic granules can be stored for a considerably long period of time, and brought into service again relatively quickly.

2.4.4 Application of aerobic granules to treat wastewater

As compared with conventional activated sludge, aerobic granules have the regular, dense and strong microbial structure. Granules possessed good settling ability and sludge volume index (SVI) for granules ranged from 50 to 80 ml g⁻¹ SS (Tay et al., 2001a, b), which made it easy to separate biomass from bulk liquid during the settling phase. Due to the compact structure and good settling ability of aerobic granules, the aerobic granulation system can retain high biomass retention and ability to withstand a high organic loading rate. Granules could sustain the high organic loading of 15 g COD l⁻¹ day⁻¹ with glucose as carbon source and no obvious signs of deterioration in granule physiology were observed under this loading (Moy et al., 2002). Recent studies also showed that aerobic granules can be used for phosphate and ammonia removal (Dulekgurgen et al., 2003; Liu et al., 2003b). Thus far, these aerobic granules have been cultivated from activated sludge on simple and relatively benign carbon substrates such as glucose and acetate (Tay et al., 2001a, b; Moy et al., 2002), and little is known about the ability of these granules for high-rate degradation of toxic chemicals such as phenol. In fact, the dense structure of aerobic granules might imply the specific advantages of aerobic granules to treat toxic wastewater. First, the structure of aerobic granules could protect cells in granules as discussed above. Second, activated sludge contains sufficient genetic diversity and aggregation of activated sludge cells into aerobic granules provides an idea environment for establishment of syntrophic relationships, and facilitates horizontal gene transfer, interspecies substrate exchange and removal of metabolic products.

2.5 ANALYSIS OF MICROBIAL COMMUNITY

Biological wastewater treatment systems rely on the interactions and metabolism of microorganisms. Essentially, biological wastewater treatment systems depend on the capacity of the microbial community to recycle elements by ways of

biogeochemical cycles. In the development of biological wastewater treatment systems, scientists have tended to concentrate on the processes rather than on species involved or on microbial community structure. Actually, an understanding of the microbial community of a biological wastewater treatment would assist in many ways in improving system design and performance. Over the years, systems have evolved empirically to what we have today, and we are using community analysis to optimize these systems (Cloete, 1997). Microbial community structure and function has therefore become the driving force in retrofitting, system optimization and system design. The aerobic granulation system is a recently developed system, and the knowledge about microbial community of aerobic granules is very limited. In order to manipulate this system for application of wastewater treatment, studies about microbial community are necessary.

Information that is important for studying microbial ecology may be subdivided into the following categories: diversity, structure and function (Amann and Khl, 1998). These three categories can be used in combination to generate a complete picture of the microbial ecology of a given habitat. It is believed that the patterns in species diversity and microstructure are the products of microbial interaction that vary in relative importance both in time and space, although these interactions are yet not completely understood. Possible interactions between microbial populations can be recognized as negative interactions (competition and amensalism), positive interactions (commensalisms, synergism and mutualism) or interactions that are positive for one but negative for the other population (parasitism and predation) (Atlas and Bartha, 1998). A variety of positive and negative population interactions lead to a stable functional community. The microbial community is structured so that each population contributes to its maintenance. Unfortunately, owing to the lack of suitable technology to determine microbial community structure and function, most biological treatment systems

lack sophistication. Recent developments have seen the integration of microbial ecology and molecular biology, which is giving a new insight into studies of microbial community.

2.5.1 Methods for analysis of microbial community

The application of molecular tools in wastewater microbiology has revolutionized our view on the microbial communities of these systems during the last decade. Although the application of molecular approaches (e.g. rRNA approaches) has indicated that diverse microorganisms are present in polluted environments, these approaches cannot directly establish that detected microorganisms are capable of pollutant degradation. Considering the limitations of molecular-phylogenetic and culture-dependent approaches, the combination of these different methods should be used for identifying and characterizing functionally important populations, and link laboratory genetics and physiology to *in situ* pollutant biodegradation (Watanabe and Hamamura, 2003). Fig. 2.4 and Fig. 2.5 present commonly used approaches in microbial ecology study of pollutant degradation. The methods and the limitations of some molecular techniques [including rDNA sequencing, fluorescence *in situ* hybridization (FISH), and denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE)] and isolation approach are described below.

Fig. 2.4 Commonly used approaches in microbial ecology (Head et al., 1998)

Fig.2.5. A framework for studying the ecology of pollutant degradation (Watanabe and Hamamura, 2003)

2.5.1.1 Molecular techniques

Microorganisms usually do not have enough morphological details for easy identification, although microbiology has traditionally relied on cultivation for identification. In the search for methods to universally identify microorganisms and determine their phylogenetic relationships, researchers have used many new techniques that allow molecular comparisons among organisms, and even among those without common morphological features. The most difficult, and most informative, of these techniques is a comparison of exact nucleotide sequences of DNA or RNA from different sources (Head et al., 1998). Transfer RNAs (tRNAs) are not the most suitable molecules for phylogenetic analysis because: (1) they are short (70-90 nucleotides); (2) they are too conservative with only about 30 to 40% of sequence change between organisms from different kingdoms; (3) they are so constrained in their secondary structures that the change of a single nucleotide can hardly be independent of changes from other positions (Pace et al., 1986). The protein components in ribosomes are not very suitable either, since they are hard to purify and analyze. In contrast, rRNAs are generally considered excellent tools for phylogenetic analysis, because: (1) They are key elements of cells and are functionally and evolutionarily homologous for all organisms; (2) They are extremely conserved in overall structure, and the homologous form can be easily found by the molecular size; (3) They allow phylogenetic analysis and design of probes and primers; (4) They are the major component levels in a cell, and are easy to recover and detect; (5) They are sufficiently long for statistically significant comparisons; (6) They are not transferable between organisms.

Within the three types of rRNAs, 5S rRNA is relatively short and this limits its usage in phylogenetic analysis especially when the sequences are too similar or too dissimilar (Pace et al., 1986). In addition, the usage of 5S rRNA is limited by its high heterogeneity in a given organism. However, in the early stage, when the sequencing of large size rRNA was not practical, 5S rRNA sequencing helped to

determine the microbial composition of some simple communities. The SSU (small subunit, 16S) and LSU (large subunit, 23S) rRNA are sufficiently long and are suitable for phylogenetic analysis. Due to the large collection of SSU rRNA sequences, they are the ones that used most often. However, LSU rRNA should be the better choice if enough sequence data would be available, since SSU rRNA comparison cannot always resolve very closely related sequences. So the phylogenetic classification could be developed based on 16S rRNA sequences, and this allows for the phylogenetic identification of a microorganism purely by its molecular sequences. In other word, the sequence of a microorganism can be retrieved from an environment sample and can be compared to the known sequence database for identification. If the sequences of one group of microorganisms are known, probes can be designed and used to detect and quantify this group of microorganisms in a complex community.

DNA sequencing. Basically the methods of retrieving DNA sequences from one culture include DNA extraction, PCR, DNA sequencing and sequence analysis. Details of these methods and their application can be found in a number of reviews (Amann et al., 1995; Pace, 1997).

A general procedure of DNA extraction from the environment sample consists of three steps: (1) Disruption of the cell wall and release of the DNA into a medium in which it is soluble and protected from degradation; (2) Dissociation of the protein-DNA complexes; (3) Separation of the DNA from other soluble cellular components, for example, RNA can be digested with RNase, protein can be removed by dissolving the spooled DNA in saline medium and repeating the chloroform-isoamyl alcohol treatment until no more denatured protein collects at the interface (Boyer, 2000). The effectiveness of the DNA extraction method can be confirmed by gel electrophoresis and RFLP analysis (Zhu et al., 1993).

Through the proper selection of primers, the specific sequences in the extracted DNA can be amplified using polymerase chain reaction (PCR), leaving all other sequences untouched. Amplification occurs during repetitive cycles of synthesis that repeat the following three steps: (1) denaturation of the double-stranded DNA template; (2) annealing of two primers to the single-stranded template; and (3) extension of the primers by a thermostable DNA polymerase. Each step in the cycle is performed at a different temperature. The temperature of the second step, primer annealing, is crucial and depends on the sequence of the primers, the concentration of the components of the PCR mix and the salt concentration. If the temperature is too high, not enough of the primers anneal to template DNA to have an efficient PCR, but a low annealing temperature allows non-specific priming and results in spurious amplification products (Wilson, 1999). The 16S rDNA sequencing could be obtained from the PCR products using dideoxy chain termination chemistry and automated DNA sequencing systems. Performing an analysis of the retrieved sequences will yield information on the identity or relatedness to new sequences in comparison with the available databases.

However, experimental artifacts can be introduced at each of the above analytical steps, including DNA extraction and PCR (Head et al., 1998). All these might constrain our knowledge of the real species composition of bacterial communities. Problems are encountered with the reliable and reproducible lysis of all bacterial cells as well as with the extraction of intact nucleic acid, and the removal of substances, such as humic acids and bacterial exopolysaccharides, which may inhibit DNA digestion with restriction enzymes and PCR amplification. PCR itself is an important source of errors and biases in molecular studies of environmental samples. Amplification efficiency of genes using whole bacterial cells as template instead of extracted DNA can be affected by the physiological state of the cells (Silva and Batt, 1995). Differential or preferential amplification of rRNA genes by

PCR has been described by Reysenbach et al. (1992). Recently, Suzuki and Giovannoni (1996) found that preferential amplification might be caused by reannealing of the template DNA thereby inhibiting primer binding. Another problem in the use of PCR to amplify mixed target DNAs is the formation of so-called *chimeric molecules* (Kopczynski et al., 1994). Computer algorithms, such as the CHECK CHIMERA option in the Ribosomal Database Project (RDP; Maidak et al., 2001) and the Chimeric Alignment Method (Komatsoulis & Waterman, 1997), have been developed to detect chimeric sequences.

These artifacts may incorrectly be interpreted as an indication of additional biodiversity in the natural sample. The combination of the two different rRNA-based techniques, direct rDNA sequencing retrieval followed by hybridization with probes based on the retrieved sequences, permits the analysis of the extent of these biases and would therefore be the better way to characterize the community structure of microbial ecology (Amann et al., 1995; Snaird et al., 1997).

FISH. Fluorescence *in situ* hybridization (FISH) with fluorescently-labeled oligonucleotide probes for studies in microbial ecology, was first developed in the late 1980s. In recent years, the technique has been used successfully to analyze many ecosystems (Amann et al., 1995). In short, the procedure involves fixing the sample (usually with paraformaldehyde or alcohol) to permeabilize the cells while maintaining their morphological integrity. The paraformaldehyde solution is a good fixative for most Gram-negative bacteria, while the 50% ethanol is preferred for the Gram-positive bacteria (Amann et al., 1995). The fixed cells are immersed in hybridization solution containing a fluorescently labeled oligonucleotide probe and then incubated for 2-16 h to allow the probe to bind to complementary rRNA sequences. The optimal temperature for hybridization must be determined

empirically to avoid binding of the probe to rRNA sequences with some mismatches with the probe. A more convenient method for optimizing probe hybridization is by the inclusion of different concentrations of formamide in the hybridization buffer, with hybridization conducted at a single temperature. Following hybridization, the sample is washed to remove unbound probe. In this way, specific microorganisms can be identified and enumerated *in situ* and their spatial distribution can be elucidated with application of confocal laser scanning microscopy (CLSM) (Amann et al., 1990a, 1995).

The methodological constraints for FISH can be divided into four main categories: cell permeability problems, target site accessibility, target site specificity and sensitivity (Head et al., 1998). The first hurdle that must be overcome for *in situ* whole-cell hybridization to be successful is the entry of the probe into the cell. This is normally achieved by fixation with denaturants such as alcohols, or cross-linking reagents such as formaldehyde or paraformaldehyde. These fixation procedures not only aid in cell permeability, but also help maintain the cells' morphological integrity during hybridization. Simple fixation methods tend to permeabilize 70-90% of microscopically visible cells in aquatic samples (Amann, 1995), but, for some cells, additional treatment with solvents, acid, or enzymes may be required (Head et al., 1998).

Even when cell permeabilization has been achieved, there is no guarantee that probe hybridization to rRNA will occur within the cell. This is believed to be the result of the target sequence in the rRNA being inaccessible due to strong interactions with ribosomal proteins or highly stable secondary structure elements of the rRNA itself. This problem can normally be detected by a strong hybridization signal being obtained with a universal probe that is known to target an accessible site on the rRNA molecule. If another probe does not give a

hybridization signal in the same cell(s), this generally indicates poor accessibility of the target site.

Sensitivity of in situ hybridization is also an issue. In general, probes containing a single labeled molecule give a strong signal only if cells are metabolically active and, hence, contain large numbers of ribosomes and target rRNA. A number of approaches have been taken to improve the sensitivity by using multiple singly labeled probes, multiply labeled probes, and enzyme-linked probes or detection systems that allow signal amplification (Head et al., 1998). In addition, the development of highly sensitive cameras has improved the sensitivity of in situ hybridization assays.

As more rRNA sequences become available in sequence databases, the problem of probe specificity has become apparent, and design of diagnostic probes is becoming more difficult. While this problem has always existed, it is only with the rapidly expanding database of sequences that the problem has become more apparent. These problems are equally relevant to PCR and other oligonucleotide-dependent techniques, not only whole-cell hybridization. It has been pointed out that for a 18mer probe targeting a variable region of an rRNA molecule, there is a $1:4^{18}$ chance of an unrelated target cell being detected (Head et al., 1998). However, because even in variable regions there may be only a few positions that vary between taxa, the probability of detecting an unrelated cell is considerably increased ($1:4^5$, if only 5 positions are variable) (Amann et al., 1995). It has been suggested that this problem can be overcome by using multiple specific oligonucleotide probes targeting different sites on the rRNA molecule and labeled with different fluorochromes. Alternatively, the use of specific PCR primers and confirmation of the identity of amplified sequence(s) by the use of a specific oligonucleotide probe also can reduce the detection of false positives.

Theoretical and practical aspects of DGGE/TGGE. DNA fragments of the same length but with different sequences can be separated through DGGE/TGGE (Fischer and Lerman, 1983; Myers et al., 1987). Separation is based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). The melting of DNA fragments proceeds in discrete so-called *melting domains*: stretches of basepairs with an identical melting temperature. Once a domain with the lowest melting temperature reaches its melting temperature (T_m) at a particular position in the denaturing or temperature gradient gel, a transition of a helical to a partially melted molecule occurs, and migration of the molecule will practically halt. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel. Prior to DGGE/TGGE analysis of DNA fragments, it is necessary to determine the melting behavior of the DNA fragments. Furthermore, to obtain the best separation of different DNA fragments, it is recommended to optimize the gradient and the duration of electrophoresis.

Today DGGE/TGGE is reliable, reproducible, rapid and inexpensive, and has become one well-established molecular tool in environmental microbiology (Muyzer and Smalla, 1998). DGGE/TGGE of PCR amplified 16S rDNA fragments can be first used in analyzing community diversity. For this purpose bacterial genomic DNA was extracted from natural samples, and segments of the 16S rRNA genes were amplified in PCR. This resulted in a mixture of PCR products obtained from the different bacteria present in the sample. The individual PCR products were subsequently separated by DGGE/TGGE. The result was a pattern of bands, for which the number of bands corresponded to the number of predominant members in the microbial communities. To obtain more detailed

information about some of the community members, community members were able to be identified by sequencing of DNA eluted from excised DGGE/TGGE bands. Second, DGGE/TGGE is relatively rapid to perform, and many samples can be run simultaneously. The method is, therefore, particularly useful when examining time series and population dynamics (Santegoeds et al., 1996). Once the identity of an organism associated with any particular band has been determined, fluctuations in individual components of a microbial population, due to environmental perturbations, can be rapidly assessed. This would be of particular use when studying microbial populations in large-scale biotechnological processes such as wastewater treatment, where the microbial population is treated, to a large extent, as a “black box”, but where rapid changes in influent waste composition can have catastrophic effects on the microbiota, and hence the effluent quality. Third, DGGE/TGGE can be used to detect dominant microorganisms in microbial communities (Muyzer and Smalla, 1998; Watanabe et al., 1998). It will be obvious that DGGE cannot separate all of the 16S rDNA fragments obtained from such a variety of microorganisms. In general, these electrophoretic techniques will only display the rDNA fragments obtained from the predominant species present in the community. Several different studies revealed that bacterial populations that make up 1% or more of the total community can be detected by PCR-DGGE (Muyzer et al., 1993; Murray et al., 1996).

Like other molecular methods, DGGE/TGGE is not free from biases and errors. One of the limitations for DGGE is the separation of only relatively small fragments, up to 500 basepairs (Myers et al., 1985). This limits the amount of sequence information for phylogenetic inferences as well as for probe design. It has been demonstrated that it is not always possible to separate DNA fragments which have a certain amount of sequence variation. 16S rDNA fragments obtained

from different methaneoxidizing bacteria could not be resolved by DGGE although they had substantial sequence variation (Vallaey et al., 1997). A similar result was described that it was not possible to separate rDNA fragments differing in two to three nucleotides under the electrophoretic conditions (BuchholzCleven et al., 1997). In contrast to these failures of separation, Nübel et al. (1996) could separate DNA fragments from different *rrN* operons, some of which were only differing in one base pair.

2.5.1.2 Isolation strategies

Studies of microbial communities have traditionally involved the isolation and characterization of pure cultures (Amann et al., 1995). However, the culture-based method has been considered to suffer from several shortcomings (Vallaey et al., 1997). Observations made in the laboratory may not be directly applied to the natural habitats, as laboratory conditions may not adequately simulate condition *in situ*. In addition, it is estimated that so far only a small fraction, possibly below 10%, of the extant microorganisms have been grown in pure culture and characterized (Amann et al., 1995). There are three possible reasons for low diversity revealed by isolation methods: First, a large fraction of the microbial population in their natural environment may be nonviable; second, cells that are viable and active in the natural environment may not be cultivated using conventional methods such as serial dilution and spread plate inoculation methods; and third, some bacterial species are *per se* nonculturable on current available media (Amann et al., 1995).

Although these disadvantages exist, cultivation-based methods such as isolation, however, cannot be totally supplanted. Ecological roles of microorganisms often can not be inferred from a comparison of their 16S rRNA sequences (Heuer et al., 1999). Culture-dependent methods appear to be a more appropriate method for

microbial community analysis than culture-independent approaches in some cases (Ellis et al., 2003). The combination of both culture-independent and culture-dependent techniques can provide very useful and complementary information about the structures of microbial communities. Isolates are certainly needed for a better understanding of their physiology, and new cultures may be discovered that could mineralize recalcitrant compounds. It is also desirable to characterize these recovered bacterial strains for further biotechnological application in bioaugmentation, bioengineering and DNA probe development.

Molecular ecological approaches have detected diverse microbial populations in the natural environment, which make us realize that it would be almost impossible to analyze the behavior of all the individual species in a complex microbial community. To circumvent this difficulty in understanding and manipulating complex microbial communities, there has been increasing interest in isolating dominant or functional important microorganisms in different pollutant-degrading ecosystems (Moller et al., 1996; Watanabe et al., 1998a; Bruns et al., 2001; Whiteley et al., 2001; Tirola et al., 2002). It has generally been considered that functionally important populations in the environment are difficult to isolate using traditional isolation/cultivation methods. Several reports, however, have documented the isolation of pollutant-degrading microorganisms that were quantitatively important using traditional isolation techniques. Identification of the dominant organisms that play a key role in pollutant degradation processes not only help in understanding the substrate degradation processes but is also important in the development of optimal control and management strategies (Watanabe et al., 1998a; Abed et al., 2002; Manefield et al., 2002).

2.5.2 Microbial community in phenol degradation systems

Dominant species were detected by TGGE for activated sludge operated under the

loading of $0.4 \text{ g phenol l}^{-1} \text{ day}^{-1}$ (Watanabe et al., 1998a). Through comparing TGGE band sequences and 16S rDNA sequences of isolates, two dominant bacterial isolates were identified. One of the dominant strains was closely related to *Valivorax paradoxus*. This strain was suggested to be the principle phenol digester in this system as the phenol-oxygenating kinetic constants of this strain was similar to those of the phenol-digesting activated sludge.

The microbial diversity of activated sludge was strongly affected by phenol loadings (Watanabe et al., 1999). Activated sludge was fed phenol as the sole carbon source, and the phenol-loading rate was increased stepwise from 0.5 to $1.0 \text{ g l}^{-1} \text{ day}^{-1}$ and then to $1.5 \text{ g l}^{-1} \text{ day}^{-1}$. Accompanied with the increase in phenol loading, the shift of microbial community of the activated sludge was demonstrated by TGGE of PCR-amplified 16S rDNA fragments, and the population diversity decreased as the phenol-loading rate increased. After the loading rate was increased to $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ from $1.0 \text{ g phenol l}^{-1} \text{ day}^{-1}$, the settling ability of sludge deteriorated and the activated-sludge process broke down within 1 week. Two dominant populations appeared in the sludge during the last several days before breakdown. All of the bacterial isolates affiliated with the two dominant populations corresponded to nonflocculating phenol-degrading bacteria. Thus, an outbreak of nonflocculating catabolic populations was suggested to cause the breakdown of the activated-sludge process. The results also provided one clue that if the growth of nonflocculating phenol-degrading populations could be suppressed and if the flocculating phenol-degrading population could be sustained in the activated sludge, the activity of the activated sludge process would be high enough to cope with phenol even under the high-phenol-loading-rate condition. To achieve this, the following two methods are conceivable: selective biostimulation of the flocculating population (e.g., by adding preferential growth substrates for the flocculating population) and bioaugmentation with flocculating

phenol-degrading bacteria.

The structure of bacterial populations in specific compartments of an operational industrial phenol remediation system was assessed to examine bacterial community diversity, distribution, and physiological state with respect to the remediation of phenolic polluted wastewater (Whiteley and Bailey, 2000). In order to circumvent difficulties in interpreting the changing community composition and its association with process chemistry, a generalized approach which used group-level FISH probes and PCR-DGGE was selected. By using this strategy, the DGGE analyses provided data on the presence and extent of sequence diversity and an indication of approximate community structure, whereas the targeting of communities by whole-cell probing allowed analysis of the actual distribution of component groups within the identified communities. Rapid community fingerprinting by PCR-DGGE of 16S rDNA indicated highly structured bacterial communities residing in all nine compartments of the treatment plant and gross shifts in community structure with phenol loading. At the whole-cell level, the treatment compartments were numerically dominated by cells assigned to the *Cytophaga-Flavobacterium* group and to the γ -*Proteobacteria*. The α subclass of the *Proteobacteria* were of low relative abundance throughout the treatment system whilst the β subclass of the *Proteobacteria* exhibited local dominance in several of the processing compartments. The γ subclass of the *Proteobacteria* had a ribosome content which correlated positively with total phenolics and thiocyanate, indicating that this subclass could contain the dominant process degraders.

2.6 SUMMARY

Phenol wastes are present in effluents with high concentrations from many industries such as petroleum and petrochemical, coal gasification, pharmaceutical, dye manufacturing and pulp and paper. Compared to bulk removal of phenol by

physical or chemical methods, biological degradation is generally preferred due to lower costs and the possibility of complete mineralization.

A wide range of microbes have the ability to utilize phenol as carbon and energy sources at low concentrations. Phenol and its derivatives are aerobically biodegraded by two main metabolic pathways, initiated either by *ortho* or *meta* cleavage. Compared with the *ortho* pathway, the *meta* pathway corresponds to a higher overall growth rate and produces more energy at the expense of lower biomass yield. The growth kinetics of microbes on phenol can be well described using the Haldane equation at many cases. Phenol toxicity appears under slightly high phenol concentrations and increases with increasing phenol concentrations, leading to decrease in specific growth rates. In response to phenol toxicity, microorganisms are able to make physiological adjustments through regulating the anabolism and catabolism activities.

Due to substrate inhibition, phenol-containing wastewater especially with high loads is difficult to biologically treat. Conventional activated sludge processes are known to be sensitive to high phenol loading rates and to fluctuations in phenol loading. Although immobilizations of pure or several cultures in/on various supports were able to completely degrade phenol at higher loadings, sterile (monoseptic) conditions required limited the application of these technologies. Therefore, the self-immobilization of mixed culture in environmental conditions without the use of supports, like granular sludge, is strongly desired. Upflow anaerobic sludge blanket (UASB) reactors with anaerobic granules could be operated at a steady-state condition under the phenol loading of $2.52 \text{ g l}^{-1} \text{ day}^{-1}$. However, effluent phenol concentration was very high even under the phenol loading of $0.84 \text{ g l}^{-1} \text{ day}^{-1}$, due to lower efficiency of anaerobic degradation pathways.

Recently, aerobic granule technology in sequencing batch reactors (SBRs) was developed. Aerobic granules are self-immobilized bacterial aggregates from activated sludge flocs. Compared to conventional activated sludge flocs, aerobic granules had denser and more compact microbial structure and better settling ability, and can lead to higher biomass retention. Aerobic granulation can create a more favorable microenvironment for microorganisms under unfavorable conditions. The dense structure of aerobic granules gives rise to a development of substrate concentration gradient because of diffusional resistance, and then protect the microorganisms in aerobic granules by diluting the toxic chemical below some threshold value to avoid inhibition and allow continued microbial activity and substrate utilization. In addition, aggregation of activated sludge cells into aerobic granules helps establish syntrophic relationships. Because of these characteristics, aerobic granules have potential to treat high-loading industrial wastewaters including toxic and inhibitory substrates such as phenol.

An increase in phenol loading will give rise to metabolic adaptations in aerobic granules including extracellular polymer production and degradation pathways, and lead to changes in structure and activity of granules. Thus, the metabolic response to phenol loadings needs to be examined, in order to understand and exploit the aerobic granule system for treatment of phenol wastewater. Meanwhile, knowledge about the microbial community of aerobic granules would help in improving the system design and performance. The combination of both culture-independent and culture-dependent techniques may reveal useful and complementary information about the structure and function of microbial communities.

CHAPTER THREE

FORMATION OF AEROBIC GRANULES FOR HIGH-RATE BIODEGRADATION OF PHENOL

3.1 INTRODUCTION

Phenol ranks among the top 40 chemicals by production volume in the United States, with 4.77 billion pounds of phenol manufactured in 1998 (van Schie and Young, 2000). Phenol is a commonly found waste by-product in many industries, including petroleum refining, petrochemical, coke conversion, pharmaceutical and resin manufacturing plants. Because of its ubiquity, phenol is a major pollutant of the environment. Phenol can be toxic to some aquatic species at concentrations in the low mg l⁻¹ range (Brown et al., 1967) and causes taste and odor problems in drinking water at far lower concentrations (Rittmann and McCarty, 2001). Hence the removal of phenol from wastewater is of obvious interest.

Phenol can be removed by solvent extraction, adsorption, chemical oxidation, incineration and other non-biological treatment methods, but these methods suffer from serious drawbacks such as high cost and formation of hazardous by-products (Loh et al., 2000). Biological degradation is generally preferred due to lower costs and the possibility of complete mineralization. However phenol-containing wastewater is difficult to treat because of substrate inhibition, whereby microbial growth and concomitant biodegradation of phenol are hindered by the toxicity exerted by high concentrations of the substrate itself. While biological treatment of phenol wastewater has been mostly based on conventional continuous aerobic activated sludge systems, such systems have been known to break down because of fluctuations in phenol loads and high phenol loading rates in excess of 1.0 g phenol l⁻¹ day⁻¹ (Watanabe et al., 1996, 1999; Kibret et al., 2000).

The substrate inhibition difficulties associated with high-strength phenolic wastewaters can be overcome by strategies such as cell immobilization (Keweloh et al., 1989). For instance, cells of *Pseudomonas putida* immobilized in hollow fibre membranes were successful in degrading phenol at concentrations in excess of 500 mg l⁻¹, albeit at

relatively slow rates (Loh et al., 2000). Recent research efforts have focused on aerobic granulation as a new form of cell immobilization for exploitation in biological wastewater treatment (Morgenroth et al., 1997; Beun et al., 1999; Tay et al., 2001a, b; Tay et al., 2002b). Aerobically grown microbial granules are self-immobilized aggregates of microorganisms cultivated in sequencing batch reactors (SBRs) and have a strong, compact microbial structure, good settling ability and high biomass retention. Thus far, these aerobic granules have been cultivated from activated sludge on simple and relatively benign substrates such as glucose and acetate, and have been shown to be able to handle high organic loading rates (Moy et al., 2002). However, little is known about the ability of these granules for high-rate degradation of toxic chemicals such as phenol.

The main objective of this study was therefore to investigate the feasibility of using aerobically grown microbial granules for high-rate phenol biodegradation. During the first phase of this study, aerobically grown microbial granules were cultivated at a loading rate of $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. In the second phase, the loading rate was incrementally raised to a final value of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ and the response of the aerobic granules to the increased loading was evaluated. This work could contribute to a better understanding of the ability of these granules to handle high-strength industrial wastewaters containing chemicals that are inhibitory to microbial growth and biodegradation.

3.2 MATERIALS AND METHODS

Experimental design and set-up

Experiments were performed in a column-type sequencing batch reactor (SBR) (R1) housed at 25°C in a temperature-controlled room. The configuration of the reactor is shown in Fig. 3.1. The reactor had a working volume of 2.0 L, with an internal diameter of 50.0 mm. pH was conditioned at 6.5 to 7.0 with the addition of phosphate buffer (K_2HPO_4 ; KH_2PO_4) in the influent medium mentioned below. Air at a flow rate of 3.5 l min^{-1} was introduced by a fine bubble aerator in the bottom of the column and air flow was controlled by a gas flowmeter. Experiments were conducted in two phases and an

overview of the experiments is given in Table 3.1. The aim of Phase I was to develop stable granules at a loading of $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. During this first phase, the reactor was operated sequentially in 4 h cycles which consisted of 2 min of influent filling, 205-230 min of aeration, 5-30 min of settling and 3 min of effluent withdrawal. Effluent was discharged 50 cm above the reactor bottom at a volume exchange ratio of 50%. The hydraulic residence time (HRT), calculated based on the working reactor volume (2.0 L) divided by influent filling in one cycle (1.0 L in 4 h), was 8 h and an influent phenol concentration of 500 mg l^{-1} was applied. In Phase II, the response of the aerobic granules to an increased loading of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ was evaluated. The increased loading was achieved by increasing the influent phenol concentration to 830 mg l^{-1} and by starting with a cycle time of 8 hours that was decreased to 6 hours and finally to 4 hours. These step changes corresponded to substrate loadings of 1.25 (Phase IIa), 1.66 (Phase IIb) and $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ (Phase IIc), respectively. The influent filling time, settling time and effluent withdrawal time were fixed at 2 min, 5 min and 3 min, respectively, for all cycles in Phase II, and the different cycle times were achieved by adjusting the aeration time.

Fig. 3.1. Schematic diagram of experimental system

Table 3.1. Overview of the operating conditions

	Phase I	Phase IIa	Phase IIb	Phase IIc
Operation time (days)	0-69	69-81	81-97	97-148
Influent phenol concentration (mg l ⁻¹)	500	830	830	830
Cycle time (hours)	4	8	6	4
Hydraulic retention time (hours)	8	16	12	8
Organic loading rate (g phenol l ⁻¹ day ⁻¹)	1.5	1.25	1.66	2.5

Seeding Biomass

Municipal activated sludge was first conditioned as a batch culture for a period of two months by incubating with phenol at concentrations that gradually increased from 50 mg l⁻¹ to 500 mg l⁻¹. The enriched sludge was used as inoculum for the SBR experiments.

Medium

The reactor R1 was fed with phenol as sole carbon source by using a synthetic wastewater with the following composition: phenol, NH₄Cl, MgSO₄·7H₂O, K₂HPO₄ and KH₂PO₄ at a weight ratio of 1:0.4:0.26:3.3:2.7. Phenol concentrations prepared in influent were shown in Table 2. The ratio COD:N was about 100:5. Phosphate buffer (K₂HPO₄: KH₂PO₄) has an acidity constant of 10^{-7.20} (Benjamin, 2002), and was used to condition pH as reported previously (Watanabe et al., 1999).

The medium was supplemented with 1 ml l⁻¹ of trace solution. The composition of the trace solution was: FeCl₃·6H₂O 1.5 g l⁻¹, H₃BO₃ 0.15 g l⁻¹, CuSO₄·5H₂O 0.03 g l⁻¹, KI 0.03 g l⁻¹, MnCl₂·4H₂O 0.12 g l⁻¹, Na₂MoO₄·2H₂O 0.06 g l⁻¹, ZnSO₄·7H₂O 0.12 g l⁻¹, CoCl₂·6H₂O 0.15 g l⁻¹ (Beun et al., 1999).

Volatilization

Volatilization or air stripping is known to be a significant removal mechanism for some xenobiotic pollutants (Liao et al., 1997). Tests to determine the potential for phenol

stripping under sustained aeration were carried out with phenol concentration at 1000 mg l⁻¹ in a 2-l reactor (R2) identical to reactor R1. The tests were conducted under similar aeration conditions as employed in the reactor R1, but with the absence of biomass or inoculum in the reactor. The duration of the air stripping test was generally 4 h. The phenol concentrations at the start and at regular intervals throughout the test period, was used to establish any loss of phenol due to volatilization. Under such conditions any loss in phenol would be attributed to air stripping from aeration.

Analytical Methods

Wastewater samples in the reactor were periodically analyzed for pH, sludge volume index (SVI), suspended solids (SS), volatile suspended solids (VSS) and specific oxygen utilization rate (SOUR) using standard methods (APHA, 1998).

For SOUR determinations, a certain amount of biomass sample taken from the reactor was transferred into a pre-cleaned BOD bottle. Then, the standard BOD (biochemical oxygen demand) bottle was fully filled in with the pre-aerated nutrient and phenol solution, and the oxygen-sensing probe (YSI 5000, 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⁻¹ min⁻¹ was determined from the slope of the line of best fit to a plot of DO against time. The following equation was used to calculate SOUR (in term of mg DO g⁻¹ VSS h⁻¹):

$$\text{SOUR} = (\text{oxygen consumption rate, mg l}^{-1} \text{ h}^{-1}) / (\text{VSS, g l}^{-1}) \quad (1.1)$$

A phenol concentration of 500 mg l⁻¹ was used in the SOUR assays. As biomass concentrations may affect the substrate biodegradability rates, biomass concentrations of 0.30-0.35 g SS l⁻¹ were employed.

Phenol was measured spectrophotometrically by the 4-aminoantipyrine method (APHA, 1998). Samples were centrifuged (14000 × g) at 4°C for 15 min and 5 ml of supernatant withdrawn. 125 µl of 0.5 M NH₄OH was added to the supernatant and the pH was adjusted to 7.9 ± 0.2 with phosphate buffer before addition of 50 µl of 2% (w/w) 4-aminoantipyrine and 50 µl of 8% (w/w) K₃Fe(CN)₆. The contents were well mixed

and allowed to stand for 15 minutes before measuring absorbance at 500 nm. The phenol concentration was determined by reference to a standard curve. To measure soluble TOC (total organic carbon), samples were filtered using 0.45 μm filter paper and analyzed with a TOC analyzer (TOC-Vcsh, Shimadzu).

Biomass size and granule morphology were measured by either a laser particle size analysis system (Malvern Mastersizer 2600) or an image analysis system (Quantimet 500 image analyser, Leica Cambridge Instruments) (Yan and Tay, 1997). Granule strength was measured by following the method of Ghangrekar et al. (1996) and expressed as an integrity coefficient (%), which is defined as the ratio of the residual VSS to the total VSS after a certain period of shaking. For observations with scanning electron microscopy (SEM), granules were prepared by first fixing for 1-4 hours in 2% (v/v) glutaraldehyde. Fixed granules were washed 20 minutes three times with 0.10 M sodium cacodylate buffer, dehydrated in a graded ethanol series (25, 50, 75, 95, and 100% v/v) and dried with a critical point dryer. Dried samples were then sputter-coated with gold to increase their electrical conductivity, at 20mA in a high vacuum (2.8×10^{-6} Torr), and granule microstructure was observed with a Stereoscan 420 (Leica Cambridge Instruments).

3.3 RESULTS

Phase I: Development of phenol-degrading aerobic granules

A settling period of 30 min was applied during the initial phase in order to allow retention of acclimated biomass. Image analysis of the time dependent development of the granules, extending from the seed sludge to granules, is shown in Fig. 3.2. Granules first appeared on day 9 of reactor operation, by which time the mean biomass size for total biomass had exceeded 0.25 mm and the SVI value had decreased to less than 95 ml g^{-1} SS (Fig. 3.3(a)). By day 20, the SVI value had declined to 40 ml g^{-1} SS, indicating the formation of more compact granules, which co-existed with flocculent sludge. Because of the good settleability of biomass in the reactor, the settling period was reduced to 10 min on day 20 and to 5 min from day 35 to end of experiment. The granules eventually displaced the activated sludge flocs to become the dominant form

of biomass within the reactor. The rapid transition from activated sludge to granular sludge shows that the chosen operating strategy was successful. The mean biomass size gradually increased to reach a steady state value of 0.52 mm from day 33. At steady state, 80% by volume of the granules were between 0.35 mm and 0.60 mm in size.

The step-wise decreases in settling periods resulted in stable granules in the reactor and were accompanied by a concomitant increase in biomass concentration in the reactor (Fig. 3.3(b)). The biomass concentration stabilized at 6.8 ± 0.6 g SS l⁻¹ at the end of Phase I, compared to a biomass concentration of 3.0 g SS l⁻¹ for the seed sludge. The granular biomass successfully removed most of the phenol fed into the reactor. Compared to a phenol concentration of 500 mg l⁻¹ in the influent, the phenol concentration in the effluent decreased to below 0.2 mg l⁻¹ beyond day 32 of reactor operation. After day 21, effluent TOC was less than 10 mg l⁻¹ (Fig. 3.3(c)) and TOC removal efficiency reached 98.1 ± 0.5 % (Table 3.2).

Phase II: Response of aerobic granules to step increases in phenol loading

In Phase II of this study, the substrate loading was increased from 1.25 (Phase IIa) to 1.66 (Phase IIb) and finally to 2.5 g phenol l⁻¹ day⁻¹ (Phase IIc) in order to evaluate the response of the formed aerobic granules to increased phenol loading. The phenol concentration in the influent was increased to 830 mg l⁻¹.

During day 69 to day 81, the reactor was operated with an 8 h cycle time at a loading of 1.25 g phenol l⁻¹ day⁻¹. The loading was then increased to 1.66 g phenol l⁻¹ day⁻¹ by decreasing the cycle time to 6 h. Fig. 3.3(a) shows that this loading increase did not significantly affect the settleability of the granules, as the SVI values were maintained between 35 and 45 ml g⁻¹ SS. Phenol was almost completely degraded. TOC removal efficiency in the reactor was high, and effluent TOC concentrations in the reactor were less than 9 mg l⁻¹ (Fig. 3.3(c)).

Fig. 3.2. Image analysis of biomass at different operational time. (a) inoculated activated sludge; (b) 12 days; (c) 20 days; (d) 68 days (at the end of Phase I).

Fig. 3.3. Performance of reactor during 147 day operation: (a) mean biomass size and sludge volume index (SVI); (b) biomass concentration, and phenol and TOC removal efficiency; (c) phenol concentrations in influent and phenol concentration and TOC in effluent

On day 97, phenol loading was increased to $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ by decreasing the cycle time to 4 h. The increased phenol loading was accompanied by an increase in SVI (Fig. 3.3(a)). Unlike the previous step increase in phenol loading, this increase in phenol loading was accompanied by an increase of granule size from 0.52 mm to 1.2 mm. The biomass concentration also increased (Fig. 3.3(b)), and stabilized at $8.2 \pm 0.8 \text{ g SS l}^{-1}$ after about one month into Phase IIc (Table 3.2). At this loading, the oxygen concentrations during aeration period in one cycle were higher than 3.0 mg l^{-1} .

The increase of loading to $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ did not diminish the ability of the granules to remove phenol. The phenol concentrations in the effluent were less than 0.2 mg l^{-1} , even for those periods immediately after an increase in phenol loading was imposed. TOC concentrations in the effluent were less than 14 mg l^{-1} (Fig. 3.3(c)) and TOC removal efficiency at the end of Phase II was as high as that at the end of Phase I (Table 3.2).

Table 3. 2. Characteristics of aerobic granules at the end of Phase I and Phase II

	End of Phase I	End of Phase II
Biomass concentration in reactor (g SS l^{-1})	6.8 ± 0.6	8.2 ± 0.8
TOC removal efficiency (%)	98.1 ± 0.5	98.0 ± 0.3
Phenol removal efficiency (%)	99.96 ± 0.01	99.96 ± 0.01
F/M ratio or biomass loading ^a (g phenol $\text{g}^{-1} \text{ SS day}^{-1}$)	0.221	0.305
Mean biomass size (mm)	0.52 ± 0.5	1.2 ± 0.4
SVI (ml g SS^{-1})	40.0 ± 5.6	60.5 ± 5.0
Integrity coefficient (%)	99.4 ± 0.2	96.4 ± 0.3
Aspect ratio ^b	0.71 ± 0.8	0.84 ± 0.6

Note: ^a F/M: food to microorganisms

^b Refers to roundness of the granule (0 = line, 1 = circle)

Monitoring biomass activity during Phase I

Oxygen uptake by the biomass was measured to monitor changes in biomass activity during the experiment. Biomass growth and substrate removal are linked to batch measurements of specific oxygen utilization rate (SOUR), expressed as milligrams of

oxygen consumed by a gram of VSS per hour. A phenol concentration of 500 mg l⁻¹ was used in the batch SOUR assays with biomass concentrations of 0.30-0.35 g SS l⁻¹. SOUR values increased sharply after the onset of granulation on day 9 and stabilized thereafter at 110 mg DO g⁻¹ VSS h⁻¹ (Fig. 3.4). The SOUR values were multiplied by the corresponding amount of biomass in the reactor to estimate the total oxygen utilization rate (TOUR) for the reactor (Fig. 3.4). TOUR values also increased sharply after the first granules appeared. However, unlike the SOUR values which stabilized within two weeks of reactor startup, TOUR values continued to increase from day 9 and only stabilized after day 30. The TOUR observations are associated with the observed increases in biomass concentration as the granules continued to build up in the reactor (Fig. 3.3b). SOUR values at the end of the experiment were approximately twice as large as the initial values, while TOUR values were approximately four-fold higher because of the increased amounts of biomass.

It should be mentioned that SOUR measured in batch test was not *in situ* respirometry of biomass in the reactor, and just reflected the initial activity of biomass in specified batch-test conditions. So, TOUR, calculated based on SOUR, serves only as an indication of the potential capacity of substrate degradation in the reactor, and can not be used to predict the actual oxygen consumption rate in the reactor. Firstly, ratios of initial phenol concentration (S_0) to initial biomass concentration (X_0) in batch experiment and reactor were different. Ratios of S_0/X_0 for batch SOUR test were about 1.52 g phenol/g biomass, and much higher than ratios of S_0/X_0 in the reactor. In addition, endogenous respiration of granules became a dominant metabolic function at low food availability in one cycle in SBRs (Zhu and Wilderer 2003). So, *in situ* oxygen consumption in the reactor is expected to be less than the TOUR calculated based on batch SOUR assay.

Fig. 3.4. Profiles of SOUR and TOUR for phenol-degrading granules during Phase I

Phenol and TOC removal in one cycle at the end of Phase I and Phase II

Phenol and TOC concentrations were measured during one cycle in the reactor at the end of Phase I and Phase II (Fig. 3.5). The time zero coincided with the start of aeration. Because the volumetric exchange ratio for the reactor is 50% and effluent phenol and TOC concentrations were insignificant compared to influent phenol and TOC concentrations, the phenol and TOC concentrations in the reactor at time zero were approximately half of the influent phenol and TOC concentrations. While 30 minutes were required for complete removal of phenol and TOC at the end of Phase I (Fig. 3.5(a)), phenol and TOC were almost completely removed within the first two hours into the cycle at the end of Phase II and the granules experienced a period of starvation for the rest of the cycle (Fig. 3.5(b)). Results also showed that TOC concentrations were less than phenol concentrations during the initial phenol degradation period in the cycles. Later in the cycles, TOC concentrations were greater than the phenol concentrations, probably due to the presence of some microbial products.

Fig. 3.5 Profiles of phenol and TOC removal in one cycle (a) at the end of Phase I; (b) at the end of Phase II

Volatilization

Air stripping tests were carried out at 1000 mg l⁻¹ phenol concentrations in reactor R2 with configuration identical to R1 using for formation of aerobic granules. Liquid volume in the reactor was 2 liters, same as the working volume of R1. Diffused air aeration in reactor R2 was maintained at the flow meter rate of 3.5 l min⁻¹. In the absence of an inoculum, any loss in phenol in the reactor would be attributed to air stripping from aeration. The data in Fig. 3.6 show that there is little or no stripping of phenol remaining in the reactor. Similar results were also reported in a 4-l batch reactor (Yoong et al., 2000), and are partly related to the high solubility of phenol in aqueous phase (about 80,000 mg l⁻¹) (Yaws, 1999). This confirms that the pathway for phenol removal in reactor R1 was wholly through biodegradation.

Fig. 3.6 Phenol concentration versus time in volatilization experiment

Characteristics of aerobic granules at the end of Phase I and Phase II

SEM showed that the phenol-degrading granules at the end of Phase I had a smooth outer surface with a compact appearance (Fig. 3.7(a)). A close examination of the granule surface revealed the presence of a large diversity of microbial morphotypes, including bacterial rods and cocci, and fungi (Fig. 3.7(b)). These microorganisms are embedded in an extracellular polymeric matrix. Biomass sampled at the end of Phase II still maintained a granular structure (Fig. 3.7(c)). Compared to granules at the end of Phase I, granules at the end of phase II included more filamentous microorganisms.

These appeared only at the surface, while the granule interior was dominated by cocci and rod-shaped bacteria that clustered tightly together in an extracellular polymeric matrix (Fig. 3.7(d)).

The physical properties of the aerobic granules showed no significant deterioration when the phenol loading increased from 1.5 to 2.5 g phenol l⁻¹ day⁻¹. The strength of granules at the end of Phase II decreased slightly compared to that at the end of Phase I (Table 3.2), and was still high. Although the settling ability of the aerobic granules decreased with increased phenol loading, an SVI of 60.5 ml g⁻¹ SS could be maintained in the reactor at the end of the experiment, indicating a satisfactory biomass.

Fig. 3.7. Scanning electron micrographs of aerobic granules: (a) and (b) at the end of Phase I; (c) and (d) at the end of Phase II.

3.4 DISCUSSION

Development of a high-rate reactor for phenol biodegradation

Phenol exerts a toxic or inhibitory effect on bacteria because it can partition into cell membranes and disrupt membrane function (Keweloh et al., 1989). There is therefore considerable interest in the development of biological wastewater treatment systems for high-strength industrial wastewaters containing phenol. In this study, aerobically grown microbial granules were successfully cultivated in a SBR and were able to sustain the maximum loading rate of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. At the maximum loading rate employed, phenol was completely removed by stable granules with a mean size of 1.2 mm and an SVI of $60.5 \text{ ml g}^{-1} \text{ SS}$ that were maintained in the reactor from day 127 to the end of the study on day 148. While phenol removal has been carried out for many years by conventional continuous activated sludge systems, these systems are sensitive to fluctuations in the phenol loading and high phenol loading rates exceeding $1.0 \text{ g phenol l}^{-1} \text{ day}^{-1}$ have been reported to cause system failure (Watanabe et al. 1996, 1999; Kibret et al. 2000). As an alternative form of biological wastewater treatment, SBR technology has been gaining popularity through the years in treating different kinds of wastewaters mainly due to its operational flexibility (Wilderer et al., 2000). Although it was reported that a SBR with flocculated sludge could treat phenol wastewater at a high phenol loading rate of $3.1 \text{ g phenol l}^{-1} \text{ day}^{-1}$, the settling ability of flocculated sludge in that reactor was generally poor, even when the loading rate was as low as $0.52 \text{ g phenol l}^{-1} \text{ day}^{-1}$ (Yoong et al. 2000). Furthermore, Yoong et al. (2000) did not mention how many days the reactor operated under the high loading. So, it was not known whether data in that article were obtained under transient or stable state.

For evaluating treatment capacity of SBR systems, effect of initial phenol concentrations in one cycle may also be involved as phenol toxicity becomes stronger under higher concentrations. Although influent COD concentrations including phenolic waste were as high as 7750 mg l^{-1} in a conventional SBR (Brener et al., 1992), the low loading of about $0.67 \text{ g phenol l}^{-1} \text{ day}^{-1}$ was applied, which made it non-comparative with this study. Based on cycle times and HRT reported by Yoong et al. (2000), the exchange ratio was calculated to be 40% and highest phenol concentrations in one cycle

in that study were less than 522 mg l^{-1} . Due to long filling time, the highest COD concentration detected in one cycle was about 800 mg l^{-1} (Yoong et al., 2000). Assuming that all COD were attributed to phenol substrate, the corresponding phenol concentration was about 333 mg l^{-1} , which was less than the initial phenol concentration of about 417 mg l^{-1} in one cycle in Phase II of this study. In addition, batch experiments show that phenol-degrading aerobic granules can completely degrade phenol at an initial phenol concentration of 3000 mg l^{-1} (Lim, 2003). Taken together, compared to these existing processes, the aerobic granule process developed in this study was a better alternative for treatment of phenol wastewater.

Effect of settling time on aerobic granulation

To date, the mechanisms behind the formation of aerobic granules have not yet been understood. In view of sludge settleability, activated sludge can be roughly characterized as good settling sludge and poor settling sludge. In SBR, the time allowed for sludge settling was the main operating parameter that selects for the growth of good settling bacteria, and is one of the most important parameters influencing aerobic granulation (Buen et al., 1999; Qin et al., 2004). Based on this criterion, the settling time was normally set as 1-4 minutes (Morgenroth et al, 1997; Beun et al, 1999, 2000), and aerobic granules had been successfully formed. In this study, a settling period of 30 min was applied during the initial phase in order to allow retention of acclimated biomass. Results show that granules appeared with a settling period of 30 min, and co-exist with flocculent sludge. Furthermore, aerobic granules in SBR could be formed at 20 min settling time (Zhu and Wilderer, 2003) or even longer settling time (2.5 hours) (Peng et al, 1999) or at lower settling velocity (0.7 m/h) (de Villiers and Pretorius, 2001) compared to $12\text{-}24 \text{ m/h}$ applied by Beun et al. (1999). Although it was argued that selection by settling is not the important parameter for granule preservation (de Villiers and Pretorius, 2001), long settling time may not select a dominant granular sludge with good settleability (de Villiers and Pretorius, 2001; Peng et al., 1999; McSwain et al., 2004). In addition, shortening the settling time significantly improved the settleability of sludge and resulted in aerobic granules with a more compact microbial structure (Qin et al., 2004). So, the settling time decreased to 10 min and then to 5 min in this study for the purpose of maintaining dominant granules in the reactor, although granules formed and co-existed with activated sludge flocs at a settling time of 30 min.

However, reduced settling time did not affect significantly properties of aerobic granules, which was some different from what was reported by Qin et al. (2004). This difference may be attributed to different reactor operation conditions and medium composition applied. First, periods of the reactor operation in this study under settling times of 30 and 10 min may not be long enough to interpret data about granule properties. For example, biomass in two reactors operated at settling times of 10 and 2 min, respectively, possessed similar characteristics on day 56, although significant difference developed over a long period in terms of biomass concentration, SVI and biomass size (McSwain et al., 2004). Second, other parameters such as energy sources for biomass growth may need to be considered when comparing the effect of settling times. It was reported that nitrifying granules could be formed at the settling time of 30 min (Tay et al., 2002a). Although how phenol-degrading aerobic granules response to different settling times was not known, the strategy of initially using long settling time and then decreasing settling time applied in this study seems to be successful for developing stable and compact phenol-degrading granules in the reactor.

Operational strategy to achieve high phenol loading rates

The aerobic granulation phenomenon in SBRs has never previously been reported in the conventional activated sludge process. One unique feature of SBRs different from continuous systems is that the cells in SBRs are exposed to alternating feast and famine conditions. Fig. 3.5 shows that the phenol was consumed rapidly within each cycle and this was followed by a starvation period that spanned several hours. Microorganisms can make physiological changes to survive such starvation conditions (Chiesa et al. 1985; Watanabe et al. 2000). Starvation has been reported to increase hydrophobicity of cell surfaces and enhance sludge adhesion capacity (Watanabe et al. 2000), and these changes can facilitate microbial adhesion and aggregation and the eventual formation of granules. The microbial aggregates are more easily retained in the SBR than light and dispersed particles over the many cycles of the SBR operation, and this selection process confers on these aggregates an advantage for growth. However, negative effect of starvation on change in cell surface hydrophobicity was also reported. It was found that starvation was not a major factor in inducing changes in the cell surface hydrophobicity of *Azospirillum lipoferum* (Castellanos et al., 2000). So, the effect of

starvation on cell hydrophobicity is still debatable, and further research on the role of starvation in aerobic granulation must be conducted (Liu et al., 2004)

Cycle time is another distinctive feature of the SBR, and sludge wasting is achieved through a cyclic withdrawal of effluent. The daily frequency of sludge discharge is inversely related to the cycle time, which is considered to represent the hydraulic selection pressure that is exerted on the microbial community. It has demonstrated that aerobic granulation is a process driven by selection pressure in terms of settling time and hydraulic retention time in SBR (Liu et al., 2004). In the case of nitrifying and heterotrophic aerobic granules, granule formation, or granulation, does not occur if the hydraulic selection pressure is weak or if the cycle time is too long (more than 12 hours), but occurs provided an adequate hydraulic selection pressure is present (Tay et al. 2002a; Pan et al., 2004).

Previous experiments show that acetate-fed aerobic granules strength decreased after increasing organic loading higher than $6.0 \text{ g COD l}^{-1} \text{ day}^{-1}$, although settling ability was not affected (Liu et al., 2003a). So this study was concerned with understanding how aerobic granules responded to a loading of $6.0 \text{ g COD l}^{-1} \text{ day}^{-1}$ (about $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$) using phenol rather than acetate as carbon source. According to literature review, activated sludge was sensitive to increase of phenol loading, and a loading of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ is too high for conventional activated sludge process (Watanabe et al., 1999; Kibret et al., 2000). A phenol loading in excess of $1 \text{ g phenol l}^{-1} \text{ day}^{-1}$ can lead to inhibition of the degradation processes, and result in the unrecoverable failure of activated sludge systems with little warning. Although a high phenol concentration is toxic to most bacteria, a careful operating strategy which includes a judicious choice of cycle times can lead to the successful cultivation of microbial granules in the SBR for high-rate phenol removal.

To achieve the increase in loading from 1.5 to $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ in this study, influent phenol concentrations was increased to 830 mg l^{-1} . Meanwhile, a longer cycle time of 8 hours was initially imposed that gradually shortened to 4 hours. Cycle times of between 4 and 8 hours can impose a hydraulic selection pressure that is adequate for

maintenance of granule structure (Tay et al. 2002a, Pan et al, 2004). Extension of cycle time to 8 hours led to the decrease of volumetric loading and then mediated the phenol toxicity resulted from increased influent phenol concentration. In addition, the longer cycle time of 8 hours gave rise to a longer period of starvation. Although the effect of starvation time on cell surface hydrophobicity and subsequently cell aggregation is not well understood, and long starvation may enhance cell hydrolysis or consumption of ECPs and then lower the structural integrity of aerobic granules, those negative effects do not seem significant at a cycle time of 8 hours as there were not much differences in structural properties of aerobic granules even at a cycle time of 12 hours as compared to those at a cycle time of 6 hours (Pan et al., 2004). By adopting an operational strategy with step changes in cycle time, adequate selection pressures were applied that facilitated the maintenance of the granule structure, and the granular structure in turn afforded enough protection against the inhibitory effects of phenol toxicity to allow the granules to remove phenol completely up to a loading of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. As reactor operation periods at cycle times of 8 and 6 hours were not long enough to get representative parameters for stable conditions, effect of cycle time on phenol-degrading aerobic granules was not assessed. In fact, these works had been investigated for nitrifying and heterotrophic aerobic granules (Tay et al., 2002; Pan et al., 2004), and was not attempted in this study.

While HRT or cycle time is adopted as an operational parameter for aerobic granule systems, and calculated based on the working reactor volume divided by volume of influent added in one cycle, not much is known at this point in time about the effect of MCRT (or sludge age) on aerobic granulation and granule structure. MCRT is an equally important parameter in biological wastewater treatment systems. Preliminary results suggested that long MCRT was accompanied by high specific gravities and low SVI (Pan 2003). However, control of MCRT in aerobic granule systems should be a subject to be studied in future.

Function of granule structure

The self-immobilization of microbial cells into granules likely improved the ability of cells inside the granules to tolerate high phenol concentrations. The oxygen uptake

measurements during the Phase I showed that the granules possessed a higher degree of microbial activity compared to the activated sludge flocs from which they originated (Fig. 3.4). SOUR values increased with a concomitant increase in granule size during the first 15 days of reactor operation and stabilized thereafter. In fact, in phenol medium, cells of *Rhodococcus erythropolis* UPV-1 immobilized in the diatomaceous earth had specific respiratory rate 13% higher than suspended cells (Prieto et al., 2002). This is contrary to observations of biofilms growing at the expense of benign substrates, where an increase in biomass size is usually associated with a decrease in specific biomass activity because of mass-transfer limitation (Tijhuis et al., 1995; Villaverde and Fernández-Polanco, 1999; Gonzalez-Gil et al., 2001). Situations involving toxic organic compounds may need to incorporate other physiological factors to accurately explain biomass activity and performance.

Cell immobilization creates a diffusional resistance and establishes a concentration gradient that shelters the microbial cells beneath the protective barrier by diluting the toxic chemical below some threshold value to allow continued microbial activity and substrate utilization (Villaverde and Fernández-Polanco, 1999; Rittmann et al., 2000). The application of immobilized cells in wastewater treatment permits the degradation of higher concentrations of toxic pollutants than can be achieved with free cells. Immobilized cells are better protected against phenolic and other similarly inhibitory compounds, and this is well documented for many microbial systems, including alginate-encapsulated cells that tolerated higher phenol concentrations than free cells (Bastos et al., 2001), and cells entrapped in hollow-fibre membranes that mitigated the effects of phenol inhibition (Loh et al., 2000). With the protection offered by immobilization, the need for the adaptation period normally required by free cells is eliminated, and the uptake of substrate is enhanced compared with free cells in the bulk liquid (Moslemy et al., 2002). In addition to diffusional resistance, other possible mechanisms for tolerance against elevated phenol concentrations include binding of phenol by cells on biofilm exteriors that allowed internal bacteria to multiply without any inhibition, and modification in lipid composition of cell membranes to compensate for the increase in membrane fluidity induced by phenol (Keweloh et al., 1989; Yap et al., 1999). Although the underlying mechanism for phenol tolerance in the granules has

not been pinpointed, and may be the result of several synergistic mechanisms, the formation of dense, compact granules can facilitate survival of microorganisms in the bioreactor.

Besides serving a function in protecting microorganism within the granules against high phenol concentrations, granule structure allows retention of high biomass concentration in the reactor to improve the system degradation capacity. The biomass loading for aerobic granules under a loading of $2.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ was only about $0.305 \text{ g phenol g}^{-1} \text{ SS day}^{-1}$ (Table 3.2), while biomass loadings for activated sludge were as high as about 0.5 and $0.56 \text{ g phenol g}^{-1} \text{ SS day}^{-1}$ respectively in conventional system under a loading of $1.0 \text{ g phenol l}^{-1} \text{ day}^{-1}$ (Watanabe et al., 1999) and in a conventional SBR under a loading of $2.08 \text{ g phenol l}^{-1} \text{ day}^{-1}$ (Yoong et al., 2000). These seemed to suggest that low biomass loading may also contribute to high treatment capacity of aerobic granule-based systems. However, the simple and direct comparison of biomass loading among these systems may exaggerate the role of high biomass concentrations in aerobic granule reactors, as limitation from internal mass transfer impact should be incorporated for the accurate description of aerobic granule treatment process (Gapes et al., 2004). Biomass loading based on the total biomass should be cautiously interpreted when understanding the performance of aerobic granule reactors.

Although granule structure can protect cells inside granules, ironically, this protection may also create problems associated with diffusion limitation, such as slow diffusion of nutrients and oxygen into and waste metabolites out of the granules. The pulse feeding and high phenol loading regime used in this study created a situation of high phenol concentration in the bulk liquid during the initial part of each SBR cycle, and this probably enhanced the penetration of phenol substrate into the granule interior. On the other hand, the limited solubility of oxygen vis-à-vis organic substrates such as phenol and acetate means that these substrates can penetrate more deeply into the biofilm than oxygen (Beun et al. 2002). Therefore, problems that may be caused by the onset of diffusion limitation are likely to be oxygen-related. Profiles of oxygen concentrations within the granules were not measured in this study. However, previous investigations into the microstructure and ecology of aerobic granules (Tay et al. 2002b, 2003)

support the view that oxygen diffusion may not be limiting for the Phase I granules which had a mean size of 0.52 mm. Anaerobiosis and cell death from diffusion limitation might have occurred in the interiors of larger granules such as those cultivated in Phase IIc which had a mean size of 1.2 mm, although the slightly looser structure of these granules would have allowed them to have better access to oxygen and nutrients. Diffusion limitation can pose a problem in large granules, since not all the microorganisms can actively carry out the biodegradation of target substrates. In order to exploit aerobic granulation technology for treatment of high-load wastewaters, operating controls may need to be imposed to limit the granule size and ensure that the granules consist entirely of actively biodegrading cells.

3.5 CONCLUSIONS

This study demonstrates that the advantages of the SBR technology and the granular form of cell immobilization can be successfully combined to cultivate aerobic granules capable of handling high phenol loading rates. The aerobic granulation system showed good stability as changes in settling time and cycle time did not result in any operational upsets. At a loading of 2.5 g phenol l⁻¹ day⁻¹, phenol was completely degraded and high biomass concentration with good settling ability was maintained in the reactor. The granules remained stable and continued to degrade phenol efficiently for several weeks after attaining the maximum loading, without compromising granule integrity and function. Compared to conventional activated sludge systems and SBR with flocculated sludge, the aerobic granule process was a better alternative.

The compact structure of aerobic granules offers several advantages, namely easy separation of biomass from bulk liquid during settling phase, high biomass retention and protection of cells against the effects of phenol toxicity. Therefore, phenol-degrading aerobic granules can be exploited to design compact, high-rate aerobic granulation systems for the treatment of industrial wastewaters containing high concentrations of phenol and other inhibitory chemicals.

CHAPTER FOUR

EFFECT OF PHENOL LOADINGS ON STRUCTURE, ACTIVITY AND METABOLISM OF AEROBIC GRANULES

4.1 INTRODUCTION

Aerobic granules have been successfully cultivated from flocculated activated sludge fed with phenol as the sole carbon sources (Chapter Three). Phenol-degrading aerobic granules possessed high activity, compact structure and good settleability, and had the potential to treat wastewater with high phenol loading.

Phenol loading is expected to have a profound effect on the activity and structure of microorganisms. While overexposure to phenol is usually associated with decreases or complete loss in specific growth rate, specific oxygen utilization and enzyme activity (Leonard and Lindley, 1998; Watanabe et al., 1999; Kibret et al., 2000), microorganisms are capable of a variety of physiological responses to increase their tolerance towards phenol toxicity. At high phenol loading, the anabolism and catabolism activities can be regulated to confer a selective advantage by shifting energy production from complete to incomplete oxidation to produce higher microbial growth rates. This is accomplished by shifting the expression of phenol-assimilation pathways from the *ortho* cleavage pathway to the *meta* cleavage pathway (Müller and Babel, 1996). The membrane structure of cells can also be modified in response to an increase in phenol concentration. For instance, the higher proportion of proteins detected in cell membranes served to increase the rigidity of the lipid fatty acyl chains (Keweloh et al., 1990). In addition, synthesis of specific proteins was enhanced as a result of exposing cells to phenolic compounds (Lupi et al., 1995)

Although the impacts of phenol loading on pure cultures and activated sludge have

been widely studied, little is known about how aerobic granules respond to different phenol loads. The main objective of this study was to investigate how phenol loading affected the structure, activity and metabolism of aerobic granules. This work is expected to be useful in understanding how this novel aerobic granulation can be exploited for industrial application.

4.2 MATERIALS AND METHODS

Reactor operation

Experiments were performed in parallel in four identical column-type sequential aerobic sludge blanket reactors (5 cm diameter) with a working volume of 2.0 liter. Reactors were operated sequentially in 4-h cycles (2 min of influent filling, 230 min of aeration, 5 min of settling and 3 min of effluent withdrawal) with a hydraulic residence time (HRT) of 8 hours. The reactors were housed in a temperature-controlled room at 25°C. Effluent was discharged 50 cm above the reactor bottom at a volumetric exchange ratio of 50%. Fine air bubbles for aeration were supplied through a dispenser at the reactor bottom at an airflow rate of 3.5 l min⁻¹. The influent phenol concentrations were 330, 500, 670 and 830 mg l⁻¹ in reactors R1, R2, R3 and R4, respectively, corresponding to substrate loadings of 1.0, 1.5, 2.0 and 2.5 g phenol l⁻¹ day⁻¹, respectively.

Seed biomass and medium

The reactors were inoculated with phenol-degrading aerobic granules cultivated at a loading of 1.5 g phenol l⁻¹ day⁻¹ as described previously (Chapter Three). The total inoculum concentration was 3.0 g SS l⁻¹ in each reactor. Phenol was supplied as sole carbon source by using a synthetic wastewater with the following composition: phenol, NH₄ Cl, MgSO₄ · 7H₂ O, K₂ HPO₄ and KH₂ PO₄ at a weight ratio of 1:0.4:0.26:3.3:2.7. The medium was supplemented with 1 ml l⁻¹ of micronutrients, as described previously (Chapter Three)

Analytical methods

Wastewater samples in the reactor were periodically analyzed for pH, specific

gravity, SVI, SS, VSS using standard methods (APHA, 1998). Cell hydrophobicity was determined with the method described by Rosenberg et al. (1980). Hexadecane was used as the hydrophobic phase. Hydrophobicity was expressed as the percentage of cells adhering to hexadecane after 15 min of partitioning (Tay et al., 2001). Phenol was measured spectrophotometrically by the 4-aminoantipyrin method as described previously (Chapter Three). To measure soluble TOC (total organic carbon), samples were filtered using 0.45 μm filter paper and analyzed with a TOC analyzer (TOC-Vcsh, Shimadzu).

Extracellular polymers (ECPs) were extracted using the cation exchange resin (Dowex 50 $\times$ 8, Fluka) method (Frolund et al., 1996). A phenol-sulfuric acid method was used to quantify polysaccharides (Dubois et al., 1956), with glucose as the standard. Proteins were measured as described previously (Bradford, 1976), with Bovine Serum Albumin (BSA) as the standard. The specific oxygen uptake rate (SOUR) was determined in batch cultures incubated at 500 mg phenol l^{-1} with biomass concentration of 0.3 g SS l^{-1} by measuring dissolved oxygen (DO) at time intervals of 10 seconds over a 15 minute period or until DO became limiting, and calculated by dividing the oxygen consumption rate by the VSS concentration (Chapter Three).

For enzyme activity assays of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O), granules were washed with cold potassium phosphate buffer (0.1 M, pH 7.5) before re-suspension in 10 ml of the same buffer. Samples were sonicated on ice in ten 30-s treatments at 30-s intervals at a vibration amplitude of 8 μm (Sonicator, Misonix Incorporated). The crude cell extract was centrifuged at 14000 rpm for 25 min (4°C). The supernatant was immediately used for enzyme assays. Acetone was added (10%, v/v) to preparations for C23O measurement as this enzyme is easily inactivated by oxidizing agents (Nozaki 1970). Determinations of C12O and C23O activities were performed by the methods described by Nakazawa and Nakazawa (1970) and Nozaki (1970) respectively. Catechol (sublimed grade, Sigma Chemical Co., USA) was added to the assays, and C12O and C23O activities were measured spectrophotometrically

(Lambda Bio20, UV/visible Spectrometer, Perkin-Elmer Corporation, USA) at 260 nm (for detection of *cis, cis*-muconic acid) and 375 nm (for detection of 2-hydroxymuconic semialdehyde), respectively. Spectrophotometric measurements were auto-recorded every 2 s for 6 min using UV Winlab Software package (Perkin-Elmer Corporation, USA). Enzyme activities were determined from the initial gradients of the absorption curves. Specific activities of C12O and C23O were expressed as nmol catechol converted $\text{min}^{-1} \text{mg}^{-1}$ protein using molar absorption coefficients for *cis, cis*-muconic acid and 2-hydroxymuconic semialdehyde of $16\,000 \text{ M}^{-1} \text{cm}^{-1}$ and $44\,000 \text{ M}^{-1} \text{cm}^{-1}$ respectively (Nakazawa and Nakazawa, 1970; Nozaki, 1970). All assays were conducted in triplicate.

The net biomass yield in a reactor is usually estimated by monitoring the substrate reduction as well as the biomass contents in both the reactor and the effluent. The amount of biomass accumulated inside the reactor, plus that washed out, divided by the total substrate removed during the same period equals the observed net biomass yield. After all reactors reached steady state, biomass concentrations in reactors and effluent, and phenol concentration in effluent were measured continuously for four days. For each reactor, the daily biomass yield was calculated. The biomass yield was averaged over the four days and this average value was regarded as the net biomass yield for that reactor at the steady state.

4.3 RESULTS

In this experiment, four reactors (R1-R4) were operated at phenol loadings ranging from 1.0 to 2.5 g phenol $\text{l}^{-1} \text{day}^{-1}$ with influent phenol concentrations from 330 to 830 mg l^{-1} . After about two months of operation, all four reactors reached a steady state, as evidenced by stable biomass concentrations and constant phenol removal efficiencies.

Effect of phenol loading on reactor performance

Table 4.1 shows the relationship between the stable biomass concentration in reactor and phenol loading. The biomass concentrations increased with phenol loading to peak at a loading of 2.0 g phenol $\text{l}^{-1} \text{day}^{-1}$, and declined thereafter. The

ratios of VSS/SS in all four reactors ranged from 0.8 to 0.85.

The ability of the granules to removal phenol was not impaired at high phenol loading. Both phenol and TOC were removed during the initial one hour of each operational cycle for reactors R1 to R3, and within two hours in reactor R4. Effluent phenol concentrations in all four reactors were less than 0.2 mg l⁻¹. Effluent TOC values were also very low and less than 15 mg l⁻¹ (Table 4.1). TOC removal efficiencies in the four reactors were 95.9-98.5 %.

It was reported that the ratio of initial phenol concentration (S_0) to initial biomass concentration (X_0) may be used to describe the real inhibitory strength of phenol imposed on biomass (Liu et al., 2002). Due to very low phenol concentrations in effluents, initial phenol concentration in one cycle in reactors were estimated about half of phenol concentration in influents. Then ratios of S_0/X_0 were calculated for four reactors and listed in Table 4.1. Ratios of S_0/X_0 increased with increment in phenol loadings. While step increases in ratio of S_0/X_0 were only 0.006 and 0.003 mg mg⁻¹ respectively as accompanied by increasing phenol loading from 1.0 to 1.5 and to 2.0 g phenol l⁻¹ day⁻¹, S_0/X_0 increased by 0.014 mg mg⁻¹ after further increase of phenol loading to 2.5 g phenol l⁻¹ day⁻¹.

Effect of phenol loading on the structure of granules

Fig. 4.1 shows the effect of phenol loading on the SVI of granules. The SVI values ranged from 40 to 45 ml g⁻¹ SS for phenol loadings between 1 to 2.0 g phenol l⁻¹ day⁻¹. This indicated that these step increases in phenol loading did not influence the settling ability of granules. However, the SVI increased to 65 ml g SS⁻¹ when the phenol loading increased to 2.5 g phenol l⁻¹ day⁻¹.

Fig. 4.1 also shows the effect of phenol loading on the specific gravity of granules, which represents the compactness of the microbial aggregate. When phenol loading was less than or equal to 2.0 g phenol l⁻¹ day⁻¹, specific gravities of granules were high and not affected by the loading. Only after increasing the phenol loading to 2.5 g phenol l⁻¹ day⁻¹ was the specific gravity of granules

observed to decrease. The trends for specific density and SVI of granules are consistent with each other. High specific gravities correlated with low SVI values. Based on the SVI and specific gravity results, phenol loading higher than 2.0 g phenol l⁻¹ day⁻¹ had adverse effects on granule structure and settleability.

Table 4.1 Reactor performance under different phenol loadings. SS Suspended solids, TOC total organic carbon

	R1	R2	R3	R4
Biomass concentration (g SS l ⁻¹)	5.53 ± 0.42	7.0 ± 0.63	8.54 ± 0.74	7.87 ± 0.83
Phenol concentration in influent (mg phenol l ⁻¹)	330 ± 10	500 ± 15	670 ± 17	830 ± 19
Ratio of initial phenol concentration (S ₀) ^a in one cycle to biomass concentration (mg mg ⁻¹)	0.030	0.036	0.039	0.053
TOC concentration in influent (mg TOC l ⁻¹)	255 ± 13	387 ± 11	516 ± 12	651 ± 16
Phenol concentration in effluent (mg phenol l ⁻¹)	< 0.2	< 0.2	< 0.2	< 0.2
TOC concentration in effluent (mg TOC l ⁻¹)	7.8 ± 4.2	8.7 ± 2.7	9.1 ± 3.0	12.9 ± 3.5
Phenol removal efficiency (%)	100	100	100	100
TOC removal efficiency (%)	98.2 ± 0.4	98.5 ± 0.3	98.1 ± 0.4	95.9 ± 0.5

Note: ^a initial phenol concentrations in one cycle in reactors were estimated about half of phenol concentration in influent

Fig. 4.1 Effect of phenol loading on sludge volume index (SVI) and specific gravity of granules. (●) SVI, (○) specific gravity

Fig. 4.2 shows the effect of loading on ECPs of granules, while Fig. 4.3 shows the effect of phenol loading on the ratio of polysaccharides (PS) to proteins (PN) in ECPs. The amounts of polysaccharides and proteins in the granules were similar in R1-R3 but increased in R4. In addition the increased amount of proteins was significantly larger compared to that of polysaccharides in R4. The increase in phenol loading from 2.0 to 2.5 g phenol l⁻¹ day⁻¹ was associated with a three fold increase in the amount of proteins in ECPs from 24.1 to 76.3 mg g⁻¹ VSS, while the amount of polysaccharides increased by 33% or 20.7 mg g⁻¹ VSS. As a result, the amount of ECPs in granules in R4 was much larger than those in R1-R3, but the PS/PN ratio in R4 was significantly lower.

The effect of phenol loading on hydrophobicity is also shown in Fig. 4.3. Cell hydrophobicity is known to play an important role in the self-immobilization and attachment of cells to a surface. The hydrophobicity of granules was around 55-60% in R1-R3, but dropped significantly to 40.3% in R4.

Effect of phenol loading on activity of granules

The effect of phenol loading on C12O and C23O enzyme activities is shown in Fig. 4.4. C12O is associated with the *ortho* cleavage pathway, while C23O is linked to the *meta* cleavage pathway. C12O and C23O enzyme activities showed similar trends, and peaked at the loading of 2.0 g phenol l⁻¹ day⁻¹. In addition, C23O activities in all four reactors were significantly higher than C12O activities. C12O activity was not detected at the highest loading of 2.5 g phenol l⁻¹ day⁻¹. C23O as a percentage of the total enzyme activity (C12O and C23O) reached 84-85% in R1, R2 and R3, and increased to 100% in R4 (Fig.4.5).

Fig. 4.5 also shows the effect of phenol loading on SOUR of granules. Biomass growth and substrate removal are linked to batch measurement of SOUR. SOUR peaked at the phenol loading of 2.0 g phenol l⁻¹ day⁻¹, and declined thereafter. Catechol dioxygenase activity and SOUR are related to oxygen utilization. Linear regression analysis of data in Fig 4.4 and Fig 4.5 showed that linear coefficients were only 0.736 and 0.757 between SOUR and total catechol dioxygenase activity, and between SOUR to C23O, respectively. In fact, for phenol-grown cells, metabolic flux calculation have shown that approximately 40% of oxygen consumption observed in cells catabolizing phenol can be attributed to respiratory consumption, the remainder being directly associated with phenol hydroxylase and catechol dioxygenase activities (Komen et al, 1991).

Fig. 4.2 Effect of phenol loading on the amount of polysaccharides, proteins and extracellular polymers (ECPs). (white bars) proteins, (gray bars) polysaccharides, (black bars) ECPs.

Fig. 4.3 Effect of phenol loading on the ratio of polysaccharides (PS) to proteins (PN) and hydrophobicity of granules. (●) PS/PN, (○) hydrophobicity

Fig. 4.4 Effect of phenol loading on specific enzyme activity of granules. (●) C23O enzyme activity, (○) C12O enzyme activity

Fig. 4.5 Effect of phenol loading on SOUR and the ratio of C23O to total enzyme activities. (●) SOUR, (○) the ratio of C23O to total enzyme activities

Effect of phenol loading on biomass yield

Observed biomass yield is expressed as an increase in microbial biomass at the expense of a growth-limiting substrate. Observed biomass yields based on phenol removal (Y_{obs}) equals the amount of biomass accumulated inside the reactor, plus that washed out, divided by the total phenol removed during the same period. In theory, one gram phenol corresponds to 2.38 gram COD. Then observed biomass yields based on COD removal ($Y_{obs/COD}$) can be estimated by dividing Y_{obs} using a value of 2.38. The effect of phenol loading on observed biomass yield is shown Fig. 4.6. Biomass yields increased with phenol loading increasing from 1.0 to 2.0 g phenol $l^{-1} day^{-1}$, and reached the peaked values, namely, 0.47 g VSS g^{-1} phenol and 0.20 g VSS g^{-1} COD for Y_{obs} and $Y_{obs/COD}$, respectively. Then biomass yield decreased with further increase in phenol loading to 2.5 g phenol $l^{-1} day^{-1}$.

Fig. 4.6 Effect of phenol loading on observed biomass yield. (gray bars) yield based on phenol removal (Y_{obs}) (g VSS g^{-1} phenol), (black bars) yield based on COD removal ($Y_{obs/COD}$) (g VSS g^{-1} COD).

4.4 DISCUSSION

Microbial degradation of phenol is often hindered by toxicity effects exerted at high concentrations. The ability to tolerate and degrade high phenol loadings is therefore desirable in biological wastewater treatment systems as it can facilitate the treatment of high-strength industrial wastewaters containing inhibitory compounds by using compact reactors with small footprints. Aerobic granules were able to sustain the high phenol loadings tested in this study, and displayed good structural and metabolic characteristics that peaked at a loading of 2.0 g phenol l⁻¹ day⁻¹. When the loading was increased to 2.5 g phenol l⁻¹ day⁻¹, significant increases in the amount of ECPs and decreases in PS/PN (polysaccharides/proteins) ratios were observed (Fig. 4.2 and Fig. 4.3). ECPs are the construction materials for microbial aggregates and are responsible for the structure of these aggregates. Polysaccharides and proteins play different roles within the ECP matrix (Flemming and Wingender, 2001), and the stability of the matrix will depend on the interactions between polysaccharides and proteins and the other macromolecules present (Sutherland, 2001). The PS/PN ratio can impact the structural and settling characteristics of the microbial biomass (Tay et al., 2001b). In the case of aerobic granules cultivated on acetate, the PS/PN ratio was directly correlated to structural and settling characteristics. High PS/PN ratios corresponded to high specific gravities and low SVI values (Tay et al., 2001b). Similar observations also held true for stable phenol-degrading granules, in which the amount of polysaccharides was generally higher than the protein content. However, exposure of granules to the highest phenol loading tested of 2.5 g phenol l⁻¹ day⁻¹ clearly triggered a significant decrease in the PS/PN ratio, attributed largely to a sharp increase in protein production (Fig. 4.2).

Microorganisms are known to regulate ECP synthesis and modify ECP properties as a microbial response against the effect of antimicrobial agents (Allison et al.,

2000). ECPs could form a protective shield for the cells against the adverse influences of the external environment, and delay or prevent toxicants from reaching microbes by acting as a diffusion limitation barrier (Wingender et al., 1999). While the aggregation of microbial cells into compact granules served as an effective protection against phenol in reactors R1-R3, this strategy was obviously inadequate at the highest phenol loading employed in R4. The microorganisms apparently sought additional protection via a sharp increase in protein production. In fact, this preferential production of proteins over polysaccharides in ECPs has also been observed in other biofilms exposed to phenol (Fang et al., 2002). Possible explanations for the elevated production of proteins include induction of heat shock-like proteins as a defense mechanism against high phenol concentrations, and induction of special proteins that could be involved in the catalytic degradation of phenol and other potentially toxic compounds (Benndorf et al., 2001).

The overstimulation of protein production in R4 granules was accompanied by a decrease in granule stability as reflected in the decline in cell hydrophobicity. Previous studies have demonstrated that granule stability was positively correlated to cell hydrophobicity (Tay et al., 2001b). The hydrophobicity of the cell surface is an important affinity force in the self-immobilization and attachment of cells. Based on thermodynamic considerations, an increase of the cell hydrophobicity causes a corresponding decrease in the excess Gibbs energy of the surface, and serves as a driving force for bacteria to self-aggregate out of the liquid (hydrophilic) phase. Increased hydrophobicity and the ensuing microbial auto-aggregation are microbial strategies to increase tolerance against toxicity, and have been shown for cells of *Pseudomonas putida* CP1 during growth on mono-chlorophenols (Farrell and Quilty, 2002). A high cell hydrophobicity would promote stronger cell-to-cell interactions, and produce a denser and more stable granular structure. The high

hydrophobicity observed in R1-R3 granules played such a role in maintaining the structural integrity of the granules. On the other hand, R4 granules showed signs of weakening, as evidenced by the reduction in hydrophobicity and specific gravity and the increase in SVI. The fact that there is a decrease in hydrophobicity in R4 granules should not be unexpected. A similar decrease in hydrophobicity observed in activated sludge biomass exposed to phenol shock loading was attributed to the amphiphatic characteristics of phenol to create a more hydrophilic biomass residue with a poorer ability for self-aggregation (Schwartz-Mittelmann and Galil, 2000).

The metabolic characteristics of the granules followed the same trend as the structural properties over the range of phenol loadings tested. SOUR, C12O and C23O activities were highest in R3. For benign substrates such as acetate and glucose, an organic loading rate of 4-6 g COD (chemical oxygen demand) $\text{l}^{-1} \text{day}^{-1}$ could be considered optimal for developing compact and high strength aerobic granules (Moy et al., 2002). Higher organic loadings were possible without compromising granule integrity. So the deterioration in structure and metabolic activity of the phenol degrading granules cultivated at 2.5 g phenol $\text{l}^{-1} \text{day}^{-1}$ or 5.9 g COD $\text{l}^{-1} \text{day}^{-1}$ can be mainly attributed to phenol toxicity. It was reported that the ratio of S_0 to X_0 may be used to describe the real inhibitory strength of phenol imposed on biomass (Liu et al., 2002). The ratio of S_0/X_0 was 0.039 mg mg^{-1} in R3 with a phenol loading of 2.0 g phenol $\text{l}^{-1} \text{day}^{-1}$, while the ratio of S_0/X_0 increased to 0.052 in R4 operated in a loading of 2.5 g phenol $\text{l}^{-1} \text{day}^{-1}$. This suggested that the toxic effect of phenol on aerobic granules seems not obvious at S_0/X_0 not larger than 0.039 mg mg^{-1} . However, this value may be applicable to aerobic granules cultivated using phenol as sole carbon and energy source, as batch culture test showed that a S_0/X_0 ratio of about only 0.012 mg mg^{-1} caused significant decrease in zero-order reactor rate of acetate in acetate-fed aerobic granules (Liu et al., 2002).

Microorganisms can develop resistance to antimicrobial or toxic chemicals through a diversity of physiological mechanisms (Caldwell, 1995). The responses involve metabolic adaptations upon the part of the microorganisms and are reflections of metabolic changes. With phenol, degradation may proceed via either the *ortho* (C12O) or the *meta* (C23O) cleavage pathway, which are often found to occur simultaneously in the same strain (Kiesel and Müller, 2002). C23O activities were much larger than C12O activities for all reactors (Fig. 4.4), suggesting that the biodegradation of phenol by aerobic granules proceeded mainly via the *meta* pathway. Previous studies on growth kinetics have pointed to a correlation between the growth rate and the assimilatory pathway employed in phenol utilization. The induction of the *ortho* or *meta* pathway was mainly triggered by substrate concentration (Müller and Babel, 1996; Filonov et al., 1997). Generally, the *ortho* pathway dominated the *meta* pathway at low growth rates due to affinity reasons, whereas the *meta* pathway attained the highest growth rates. Half-saturation constants (K_s) are also usually higher for the *meta* pathway than for the *ortho* pathway (Müller and Babel, 1996). Thus high-affinity/low-rate properties are found at low substrate concentrations in contrast to low-affinity/high-rate properties in situations with increased levels of substrate. From a kinetics point of view, the high phenol concentration used in R1-R4 might help explain the observed predominance of the *meta* over the *ortho* cleavage pathway.

The choice of cleavage pathways is also mediated by metabolic factors. Differences in the two pathways have been compared and elucidated by following the metabolic intermediates produced and energy generating reactions up to the central carbon precursor 3-phosphoglycerate (PGA) (Kiesel and Müller, 2002). With the *ortho* pathway, acetyl-coenzyme A and succinate are generated in the primary assimilatory steps, and PGA synthesis proceeds via the gluconeogenic route, accompanied by the liberation of relatively low quantities of energy

equivalents. In contrast, the *meta* pathway generates acetaldehyde, pyruvate and formate during primary assimilation, and precursor synthesis for carbon assimilation proceeds via the glyoxylate cycle, with the liberation of a substantial number of reduction equivalents. For kinetics reasons, the shorter route for energy production through the *meta* pathway corresponds to a higher overall growth rate. This may be considered a selective advantage when alternative metabolic routes have to compete successfully for a common carbon/energy source whenever there is excess substrate, but the rate increase is obtained at the expense of a lower efficiency of carbon conversion into biomass. Compared to the *ortho* pathway, more of the energy generated by catabolism in the *meta* pathway was directed away from biomass synthesis. The *ortho* pathway was completely depressed and phenol degradation was degraded exclusively via the *meta* pathway in R4 granules (Fig. 4.5). It is very likely that the selection pressure exerted by the high phenol loading drove the microbial community to regulate its metabolic pathways so as to maintain a balance with the external pressure by consuming non-growth-associated energy, thus avoiding inhibition of cellular activity and deterioration in granule structure. Part of non-growth-associated energy produced by metabolism might be used to maintain the integrity of cell membranes, since energy expended for this purpose would be expected to be higher at higher phenol concentrations (Onysko et al., 2000). Some energy was also directed towards the production of ECPs (Robinson et al., 1984), as pointed out earlier for R4 granules (Fig. 4.2).

Because higher energy is needed at higher phenol concentrations to address the stronger inhibition effects, more phenol would be assimilated for energy production at higher phenol concentrations, and this exerts a pressure on biomass yields (Wang and Loh, 1999; Onysko et al., 2000). In addition, observed biomass yield is influenced by the biomass loading or feed/microorganism (F/M) ratio (Goltara et al., 2003). Higher biomass loading is generally associated with higher

observed biomass yield. This means that increasing phenol concentration may cause both positive and negative effects on biomass yield, as biomass loading and toxicity are both elevated.

While linear regression analysis showed that linear coefficients were 0.386 and 0.766 between biomass loading and biomass yield, and between biomass loading to reciprocal of influent phenol concentration, respectively, the relationship between observed biomass yield and ratio of biomass loading to phenol concentrations possessed a linear coefficient as high as 0.904 (Fig. 4.7). This suggested that the ratio of biomass loading to phenol concentration is related to biomass yield. It should be pointed out that other parameters can affect biomass yields. Compared to the biomass yield of 1.02 g VSS g⁻¹ phenol in phenol-degrading activated sludge process (Rozich and Gaudy, 1985), the observed biomass yields for four reactors in this study were quite low, and less than 0.48 g VSS g⁻¹ phenol.

Fig. 4.7 Observed biomass yield (Y_{obs}) versus ratio of biomass loading (g phenol g⁻¹ VSS day⁻¹) to influent phenol concentration (g phenol l⁻¹)

This study demonstrates that the microbial community in the aerobic granules was

capable of adaptive changes to tolerate the range of phenol loadings tested. Remarkable shifts in structural and metabolic attributes were detected at the maximum loading of 2.5 g phenol l⁻¹ day⁻¹. Evidence of phenol toxicity became more apparent in R4 granules, which had lower hydrophobicity and a weakened structure. The microbial response to phenol toxicity included the exclusive reliance on the *meta* pathway for phenol degradation, and the concomitant overstimulation of ECP and protein production. The ability of aerobic granules to completely remove phenol and TOC compares favorably with activated sludge systems, where deterioration in enzyme and specific phenol-oxygenation activities at a phenol loading rate of 1.0 g phenol l⁻¹ day⁻¹ led to TOC and phenol accumulation in the effluent (Watanabe et al. 1999; Kibret et al. 2000). While R1-R4 operated under identical conditions, save for the differences in phenol loading, it would be interesting to determine if these operating conditions can be judiciously manipulated to select for microbial granules capable of handling higher phenol loading rates.

CHAPTER FIVE

STRUCTURE AND FUNCTION OF THE MICROBIAL COMMUNITY IN PHENOL- DEGRADING AEROBIC GRANULES

5.1 INTRODUCTION

Phenol wastes are present in effluents from industries such as petroleum and petrochemical, coal gasification, pharmaceutical, pesticide, and dye manufacturing (Loh et al., 2000; van Schie and Young, 2002). It was demonstrated that aerobic granules can treat wastewater with higher phenol loading compared with activated sludge processes, and thus could be exploited to design compact, high-rate aerobic granulation systems for the treatment of industrial wastewater containing high concentrations of phenol and other inhibitory chemicals (Chapter Three and Four).

An understanding of the microbial community of a biological wastewater treatment would assist in improving system design and performance (Cloete, 1997). Traditionally, research into the microbiology of phenol degradation has relied purely on classical microbiological techniques as a way of characterizing and identifying microbes that biodegraded phenol. It is now well recognized among microbiologists that only a small fraction of all bacteria have been isolated and characterized (Ward et al., 1990), because of the lack of knowledge of the real conditions under which most of the bacteria are growing in their natural environment. So, the application of molecular biological techniques to detect and identify microorganisms by certain molecular markers, such as 16S rRNA, is now more and more frequently used to explore the microbial diversity and to analyze the structure of microbial communities (Olsen et al., 1986; Amann et al., 1995).

There have been several types of rRNA-based studies on microbial community in the last few years. Hybridization techniques using group- and genus specific

oligonucleotide probes have been used to directly analyze the community structure of activated sludge. Through this method, cells assigned to the *Cytophaga-Flavobacterium* group and to the γ -*Proteobacteria* were found to be numerically dominant within bacterial populations in specific compartments of an operational industrial phenol remediation system (Whiteley et al., 2000). However, the probes used in these studies were still based on publicly available rRNA sequences of cultured bacteria. Denaturing gradient gel electrophoresis (DGGE) of PCR amplified ribosomal DNA fragments has also been introduced into microbial ecology (Muyzer et al., 1993). The technique is reliable, reproducible, rapid and inexpensive (Muyzer 1999), so the microbial diversity in a microbial system can be determined rapidly using DGGE (Muyzer and Smalla, 1998). Furthermore, combined with PCR amplification of marker genes or rRNA, DGGE can give a direct display of the predominant constituents in microbial assemblages (Muyzer and Smalla, 1998), such as phenol-degrading activated sludge (Watanabe et al., 1998a).

Although the application of molecular tools in wastewater microbiology has revolutionized our view on the microbial ecology of these systems during the last decade, cultivation-based methods such as isolation, however, cannot be totally supplanted. Ecological roles of microorganisms often can not be inferred from a comparison of their 16S rRNA sequences (Heuer et al., 1999). Culture-dependent methods appear to be a more appropriate method for microbial community analysis than culture-independent approaches in some cases (Ellis et al, 2003). The combination of both culture-independent and culture-dependent techniques can provide very useful and complementary information about the structures of microbial communities. Isolates are certainly needed for a better understanding of their physiology. It was also desirable to characterize these recovered bacterial strains for further biotechnological application in bioaugmentation and DNA/RNA probe development. More interestingly, functionally important populations in the environment can be successfully isolated using traditional isolation/cultivation techniques (Juretschko et al., 1998; Watanabe et al., 1998a; Tirola et al., 2002). Recent studies stressed the importance of gaining an understanding of the functions

of microbial communities and revealed that population diversity alone does not drive ecosystem stability (Briones and Raskin, 2003). Functional redundancy and functional niche complementation usually contribute to process performance and stability. Recognizing the diversity and the links within each functional group of a system can lead to better ways to model diversity and function as well as helping to improve process stability and system treatment capacity (Bond et al., 1995; Hulot et al., 2000; Kasai et al., 2002; Kong et al., 2002; van der Gast et al., 2003).

In this study, DGGE and culture isolation were combined to study the microbial community of phenol-degrading aerobic granules and identify ecologically relevant organisms from aerobic granules. Physiology and biochemical diversity of isolates were characterized, and some physiological traits helpful for maintenance and growth of organisms in aerobic granules were discussed. In situ distribution of one dominant strain with high phenol-degrading ability was observed and its important role in phenol degradation was also analyzed. Based on the complementary functional roles of several dominant populations in the aerobic granules, a model was proposed to link the microbial community with function of aerobic granules. This work is expected to be useful in understanding the microbial community of aerobic granules and developing optimal control and management strategies for aerobic granule systems.

5.2 MATERIALS AND METHODS

Sampling of phenol-degrading aerobic granules

The phenol-degrading aerobic granules with diameters from 0.4 to 0.6 mm were taken from the reactor at the end of Phase I (Chapter Three). The reactor was fed with phenol as sole carbon source and operated under an organic loading rate of $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$ with an influent phenol concentration of 500 mg l^{-1} .

Isolation procedure

The culture medium used for isolation and growth on phenol was prepared using modified MP medium containing (l^{-1}) 1.0 g of $(\text{NH}_4)_2\text{SO}_4$, 0.2 g of $\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$, 0.1 g of NaCl, 0.02 g $\text{FeCl}_3 \cdot 6 \text{ H}_2\text{O}$, 0.01 g CaCl_2 , and phosphate buffer (1.35 g of

KH_2PO_4 and 1.65 g of K_2HPO_4) (Watanabe et al., 1998a), supplemented with trace elements and vitamins (Table 5.1). The medium was sterilized by autoclaving for 20 min at 121°C. Phenol solution was sterilized by sterile filtration (0.2 μm) and added to the medium after autoclaving.

Table 5.1. Trace element and vitamin solutions for use in bacterial growth medium (Cote, 1994).

Trace element solutions		Vitamin solution	
H_3BO_3	2.85 g	P-Aminobenzoic acid	5.0 mg
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.80 g	Folic acid	2.0 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1.36 g	Biotin	2.0 mg
Sodium tartrate	1.77 g	Nicotinic acid	5.0 mg
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	26.90 mg	Calcium pantothenate	5.0 mg
ZnCl_2	20.80 mg	Riboflavin	5.0 mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	40.40 mg	Thiamin HCl	5.0 mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	25.20 mg	Pyridoxine HCl (vitamin B6)	10.0 mg
Distilled water	1.0 liter	Cyanocobalamin (vitamin B12)	100.0 mg
Note: dissolve chemicals one at a time, and adjust solution to pH 4.0 with H_2SO_4 to retard precipitation. Use 1.0 ml of trace element solution per liter of medium.		Thioctic acid (lipoic acid)	5.0 mg
		Distilled water	1.0 liter
		Note: dissolve vitamins one at a time in distilled water; adjust to pH 7 with NaOH, if necessary, to dissolve biotin and folic acid. Filter sterilize stock solution to retard fungal contamination during long-term storage, and store under refrigeration at 4°C in the dark. Use 10 ml of vitamin solution per liter of medium.	

Portions of granule samples were added to 15 ml of MP medium as described above, aseptically mixed in a sterilized beaker in order to detach granules. All operations were performed in a biohazard flow cabinet (ESCO, Singapore). The supernatant was then diluted with MP medium (10^1 - to 10^7 -fold dilutions), and 150 μl of each dilution was spread onto a MP medium (supplemented with 500 mg phenol per liter) agar plate solidified with 1.2% Bacto Agar (Difco, Detroit, Mich.). Plates were inverted and incubated in a 25°C incubator (Sanyo, Japan), and monitored over 4 weeks. Visible colonies were observed after 1 weeks of incubation. Pure cultures of phenol-degrading bacteria were isolated by cycles of replating onto MP/phenol agar plates. Purity was confirmed by microscopic

examination with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM) (Stereoscan 420, Leica Cambridge Instruments) as described previously in Chapter Three. Pure cultures were stored in 50 mM KH_2PO_4 : K_2HPO_4 buffer (pH 7.2) containing 20% (v/v) glycerol at -70°C .

Morphological and phenotypic characterizations

Cells were observed with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM). Gram-stain test was performed as previously described (Smibert and Krieg, 1994). A non-staining Gram-stain method was also performed to validate the Gram-stain result (Maszenan et al., 1997). Dioxygenase activity and catalase activity was performed as previously (Abd-El-HaleemD et al., 2002). Dioxygenase activity was performed using the indo test. Bacterial colonies were pre-grown on YEPG (Table 5.2) agar plates, and then indol crystals (Aldrich Chem. Co., USA) were plated on the lid of the petri dish. After 1-2 day incubation at room temperature, colonies that produce a blue color were scored positive. The activity of catalase was determined by the appearance of air bubbles after addition of a drop of 30% hydrogen peroxide solution to an overnight grown single bacterial colony.

Table 5.2. YEPG medium by DSMZ, Braunschweig, Germany

(website:<http://www.dsmz.de/media/med662.htm>)

Glucose	1.0 g
Polypeptone	2.0 g
Yeast extract	0.2 g
NH_4NO_3	0.2 g
Distilled water	1 liter
Adjust pH to 7.0	

Specific growth rate

Cells used in the kinetics experiment were exponential phase cultures grown with phenol as sole carbon and energy source. The MP medium was used with addition of trace element and vitamin solution (Table 5.1). Experiments were performed in triplicate in 500 ml serum bottles. The medium solution volume in each bottle was 100 ml with the initial phenol concentrations of 250 mg l⁻¹. The individual strain was then introduced to liquid solution with optical density (OD) of 0.025-0.03, and all reagent bottles were capped immediately and covered with aluminum foil to prevent photo-oxidation. The culture mediums were incubated under aerobic conditions and shaken in the dark at 25°C on a shaker at 150 rpm. Oxygen concentrations were maintained above 5 mg l⁻¹ through regular sterile aeration in a biohazard flow cabinet (ESCO, Singapore). Phenol concentrations were assayed at regular intervals of incubation. Cell growth was monitored by OD measurement using a spectrophotometer (Perkin-Elmer, USA) at a wave-length of 600 nm. The linear relationship between OD values and the total cell numbers counted using DAPI stain was confirmed through experiments on the two strains (Chapter Six). The specific growth rates were calculated from cell growth measurement during the initial period (calculation method and data are listed in Appendix A and Appendix B, respectively).

Growth on phenolic compounds

A MP medium as described above was used to provide inorganic supplements for the various strains of pure cultures to be tested. A total of ten aromatic compounds were used, including benzene, toluene, ethyl benzene, *m*-xylene, *o*-xylene, *p*-xylene, catechol, *m*-cresol, *o*-cresol, *p*-cresol and *p*-hydroxybenzoate. Solutions for each compound with concentrations of 10 and 40 mg l⁻¹ were prepared in 50-ml Teflon-stoppered serum bottles. The high volatile substrates of benzene, toluene, ethyl benzene, *m*-xylene, *o*-xylene, *p*-xylene, were prepared based on Henry's law (Tay et al., 1998). The Henry's law constants and preparation method are listed in Appendix C. Each serum bottle was inoculated with 0.2 ml cells of isolates harvested in exponential phase cultures grown in MP/phenol medium, and then incubated at 25°C in the dark on a shaker at 150 rpm. Control experiments were

performed with autoclaved cell suspensions. The serum bottles were assayed weekly by measuring the increase of turbidity at 600 nm with a UV-visible spectrophotometer (Perkin-Elmer, USA). Experiments were carried out in duplicate for each isolate and aromatic compound. Growth was considered to have occurred when the observed OD value exceeded twice the initial value (Zhuang et al., 2002).

Repetitive extragenic palindromic -polymerase chain reaction (REP-PCR)

The primer used for the REP-PCR reaction was BOX AIR (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic et al., 1991). DNA amplification was carried out as described by Versalovic et al. (1991) in a thermocycler (Mastercycler, Eppendorf, Germany) using approximately 100 ng genomic DNA. The amplification program involved an initial denaturation cycle for 7 min at 95°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 53°C, 8 min extension at 65°C, and 16 min final extension at 65°C. The reaction mixture contained 1.5 U *Taq* DNA polymerase (Promega Corporation, Madison, USA), 300 ng μl^{-1} of primer, 0.875 mM μl^{-1} of each dNTP, 1×reaction buffer (Invitrogen) and 6 mM MgCl_2 (Invitrogen), in a final volume of 25 μl . The PCR product underwent 1.5% agarose gel electrophoresis for 18 h under constant 70 V in 0.5×TBE, and visualized under UV light after being stained with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$). Fragments were photographed and analyzed using Kodak 1D image Analysis software.

Direct-lysis PCR amplification method

A whole cell direct lysis PCR amplification method was used to amplify 16S rDNA as described previously (Maszenan et al., 1997). Cells were cultured on MP/phenol agar plates at 25°C for 4-6 days. A single colony was aseptically collected with a sterile pipette tip and smeared onto the bottom of a PCR tube. 10 μl of 100mM Tris-HCl (pH 8.3), 8 μl 25 mM MgCl_2 , 5 μl 1% NP40 and 64 μl sterilized Milli-Q water were added, and heated at 98°C for 30 minutes in a thermal cycle (Mastercycler, Eppendorf, Germany) for cell lysis to occur. The nearly full-length 16S rRNA gene was amplified by PCR with forward primer

Eubac27F and reverse primer Universal 1492R1 (Lane, 1991). 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 was continued for 10 minutes and followed by cooling at 4°C. The PCR products were purified with Qiagen PCR purification kit (Qiagen, Germany) according to manufacturer's instructions, and stored at -20°C before sequencing. The volume of purified PCR product selected as template for subsequent sequencing was determined by estimating its concentration after gel electrophoresis, and comparing its brightness with concentrations of DNA standards under UV light.

16S rRNA gene sequencing

The nucleotide sequences of 16S rDNA from the isolates were determined using the dideoxy chain termination chemistry and the ABI model 310A DNA sequencer (Applied Biosystems, Perkin-Elmer). The ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (version 3.0) (Applied Biosystems, Perkin-Elmer) was used as specified by manufacturer. The double stranded 16S rDNA fragments from isolates were sequenced using the primers listed in Table 5.3. These target the conserved region of the 16S rDNA of the domain *Bacteria* (Lane, 1991).

Phylogenetic analyses of 16S rDNA data

Almost complete 16S rDNA sequences were compiled by using BioEdit program (Tom Hall, Department of Microbiology, North Carolina State University [<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>]), and checking the overlapping positions between sequences obtained from different primers and previously aligned sequence data sets. The probable identity of the query sequence was determined using a BLAST search (Alschul et al., 1990) program from the database maintained by National Centre for Biotechnology Information (NCBI) (National Institutes of Health, USA), which compares the query sequence with all other sequences available in NCBI database and ranks them from the highest to lowest using similarity scores. A data set containing sequences of closely related organisms obtained from the Ribosomal Database Project (RDP) (Maidak et al.,

2001) or GenBank was established, and re-aligned according to the secondary structure of the 16S rRNA molecular using BioEdit program.

Table 5.3. The sequences of primers used for sequencing 16S rDNA of isolates in the domain Bacteria (Lane, 1991).

Primers	Sequence (5'-----> 3')
27F	AGA GTT TGA TCM TGG CTC AG
343R	CTG CTG CSY CCC GTA G
357F	CTC CTA CGG GAG GCA GCA G
519R	GWA TTA CCG CGG CKG CTG
530F	GTG CCA GCM GCC GCG G
691R	TCT ACG CAT TTC AC
787R	CTA CCA GGG TAT CTA AT
803F	ATT AGA TAC CCT GGT AG
907R	CCG TCA ATT CMT TTR AGT TT
926F	AAA CTY AAA KGA ATT GAC GG
1100R	GGG TTG CGC TCG TTG
1114F	GCA ACG AGC GCA ACC C
1230F	TAC ACA CGT GCT ACA ATG
1392R	ACG GGC GGT GTG TGT TRC
1492R	TAC GGY TAC CTT GTT ACG ACT T
1525R	AAG GAG GTG WTC CAR CC

Note: where S = G:C; Y = C:T; W = A:T; K= G:T; M = C:A; R = A:G in equal proportion.

Phylogenetic trees were constructed with the PHYLIP package (version 3.51c) (Felsenstein, 1993). For the distance matrix method, the DNADIST program was used to compute the distance matrix, using Jukes-Cantor's model. The phylogenetic trees were constructed from evolutionary distances by using the FITCH program. For the maximum-likelihood method (Felsenstein, 1981), fastDNA ml (version 1.1) (Olsen, 1994) was used. Bootstrap confidence values were obtained by resampling of 100 trees. The bootstrap values indicate the

resampling percentages that supported a specific branching pattern. The consensus trees were determined with the CONSENSE program.

DNA extraction from seeding activated sludge, aerobic granules and isolates

The DNA was extracted from seeding activated sludge and granule samples by a modified physical disruption of cells involving direct-lysis procedure (Eichner et al., 1999). Approximately 200-300 mg (wet weight) activated sludge samples or granule samples were suspended in 800 µl sterile MilliQ water in a 2 ml microcentrifuge tube. 800 mg sterile baked glass beads and 50 µl of 20% SDS (200g SDS in 1 liter distilled water pH7.2 [Sigma, USA]) were added into the suspension. The tube was nearly topped up with saturated phenol (0.1 M Tris-HCL, pH8.0 [Sigma, USA]). The suspension was shaken for 5 min with a Mini BeadBeater (Biospec products, USA) at room temperature. Then the suspension was supplemented with 0.05 g of lysozyme (20,000 units/mg, USB, Amersham Life Science, Germany), and incubated at 37°C for 20 min. After incubation, the liquid phase was then extracted three times with phenol, followed by phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamylalcohol (24:1, vol/vol). Nucleic acids were precipitated with 0.1 volumes sodium acetate (pH4.5-5.2, 20%) and 2 volumes ice-cold 95% ethanol overnight. The DNA precipitate was washed with 500 µl of ice-cold 70% ethanol. The DNA pellet was dissolved by soaking in 100 µl Milli-Q water overnight. The concentration and purity of the genomic DNA was assessed by absorbance measurement and by agarose gel electrophoresis. Extracted DNA was stored at -20°C. Isolates were cultivated in MP/phenol liquid medium and cells were harvested by centrifugation for DNA extraction. Genomic DNA of isolates were extracted as described above, except without incubation with supplement of lysozyme after bead beating.

The effectiveness of the cell lysis procedure was confirmed by microscopic examination of samples taken before and after lysis treatment. Preparation were smeared onto microscope slides, dried and stained for 2 min with 0.05% acridine orange solution. The slides were observed by epifluorescence microscopy.

DGGE-PCR amplification

For analysis of the total bacterial community, the V3 region (Muyzer et al., 1993) of the 16S rRNA-encoded gene between positions 341 and 534 (*Escherichia coli* numbering [Brosius et al., 1981]) was amplified by PCR. The primers used were forward primer P2 with a GC clamp comprising 40 GC-rich bases (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CA-3') and reverse primer P3 (5'-ATT ACC GCG GCT GCT GG-3').

Amplification was performed with Eppendorf master cycler gradient using a 50 µl (total volume) mixture containing 1.25 U Taq polymerase (Promega, USA), 10mM Tris-HCl (pH 8.3), 50mM KCl, 2mM MgCl₂, 200µM Deoxynucleotide triphosphate (dNTPs), 25 pmol of each primer and 1µl of DNA solution (20ng/µl). The touch down PCR was employed (Muyzer et al., 1993) and this involves 10 min of activation of the polymerase at 94°C before two cycles consisting of 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. The annealing temperature was subsequently decreased by 1°C for every second cycle until it reached 55°C, at which point 20 additional cycles were carried out; finally, a 10-min extension step at 72°C was performed. The amplicon obtained was confirmed by electrophoresis through 2% agarose gel in 1×TAE buffer stained with ethidium bromide.

DGGE and band sequencing

DGGE was performed using a DCode universal mutation detection system (BioRad Laboratories, USA) in accordance to manufacturer instruction, as described by Muyzer et al. (1993) but with the following specifications. The 25 ml 30% to 70% urea-formamide denaturant gradient gel [10% (w/v) acrylamide solution (40% acrylamide:bisacrylamide, 37.5:1 stock solution, BioRad Laboratories) in TAE pH 8.0 (40 mM Tris base, 20 mM sodium acetate, 1 mM Na₂-EDTA)] was covered by a 4 ml acrylamide stacking gel (10%) without denaturant. 20 µl of PCR amplicon from DNA of pure cultures, or 40 µl of PCR amplicon from DNA extracted from aerobic granule or activated sludge, was loaded in each well of the gel with 20 µl loading dye. The gel was placed in TAE buffer at 40 V at 60°C for 30 min and then at 85 V at 60°C for 14 hours. After electrophoresis the gel was

stained with ethidium bromide for 30 minutes, viewed and photographed with Kodak EDAS 290 gel imaging system.

A gel slice containing the desire DNA band was excised and transferred into a sterile Eppendorf tube containing 50µl sterile MilliQ water. The tube was left to stand at 30°C overnight and subsequently stored at -20°C. 1µl of DNA solution was subjected to a second PCR performed under the same condition as the first PCR except primer GC-2 (5'-GAA GTC ATC ATG ACC GTT CTG GCA CGG GGG GGC CTA- 3') was used instead of primer P3. The sequence of the last 15 bases of primer GC-2 is similar to the middle section of primer P3. The amplicon from the second PCR was confirmed by electrophoresis through 2% agarose gel in 1×TAE buffer and stained with ethidium bromide. The amplicon was then purified by Promega Wizard® SV Gel and PCR clean-up system. GC-1S primer (5'- GAA GTC ATC ATG ACC GTT CTG- 3') which is identical to the first 21 bases of primer GC-2, is used for sequencing as described above. DGGE fragment sequences were assembled using BioEdit software. A search on the identity of the DGGE fragment was performed using BLAST.

Oligonucleotide probes

To detect the in situ distribution of two fast-growing isolates within the granules, two oligonucleotide probes designed to target the 16S rRNA of isolates PG-01 and PG-03, respectively, were generated after comparison of aligned sequences of PG-01 or PG-03 with the sequences of other bacteria by using BioEdit (Tom Hall, Department of Microbiology, North Carolina State University [<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>])). The common principle of probe design is to select an oligonucleotide sequence completely specific (complementary) to a region of the target sequences and with at least one mismatch to the same region in all other (nontarget) sequences. The detailed procedure about probe design can be obtained in ARB website (<http://rtfm.are-home.de/>) and Ribosomal Database Project (RDP) website (<http://rdp.cme.msu.edu/html/>).

The sequences of two probes (Table 5.4 and Table 5.5), were verified by using the CHECK_PROBE program of the Ribosomal Database Project (RDP) (Maidak et al., 2001). The probe signatures which are a sequence of bases from 5' to 3' end are reverse complementary to the target sites. The probe designed for isolate PG-01 was named as S-St-Pand-0822-a-A-20 (Pand822) according to nomenclature of Alm et al. (1996). The name of the probe designed for isolate PG-03 was S-G-Rhod-0168-a-A-25 (Rhod168).

In addition, EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') and Arch915 (5'-GTGCTCCCCCGCCAATTCCT -3'), which target highly conserved regions of most bacterial 16S rRNA molecules and most archaea 16S rRNA molecules, respectively (Amann et al., 1990), were also used. All probes were made and labeled by Genset (Paris, France). Probes Pand822 and Rhod168 were labeled at the 5' end with the indocarbocyanine dye Cy5, while probes EUB338 and Arch915 were labeled with FITC and TRITC, respectively.

Table 5.4. Sequences of Pand822, its target, and the corresponding small-subunit rRNA of selected organisms

Organism or sequence	Probe or target sequence ^a																									
Probe-----	3'-	a	g	g	t	g	a	t	c	g	a	c	g	a	c	c	c	c	c	c	c	t	a	a	-	5'-
Target sequence-----	5'-	u	c	c	a	c	u	a	g	c	u	g	c	u	g	g	g	g	g	g	a	u	u	-	3'-	
Isolate PG-01 -----	5'-	u	c	c	a	c	u	a	g	c	u	g	c	u	g	g	g	g	g	g	a	u	u	-	3'-	
<i>Pandoraea apista</i> strain LMG 16407-----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pandoraea</i> sp. AU1775-----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pandoraea norimbergensis</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pandoraea</i> sp. G8107-----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pandoraea pnomenusa</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pandoraea pulmonicola</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pandoraea sputorum</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Burkholderia cepacia</i> strain LMG 14294-----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Burkholderia pseudomallei</i> (Ara+ biotype)-----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Burkholderia</i> sp. MN 182.2-----	5'-	-	-	a	-	-	-	-	-	u	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Burkholderia phenazinium</i> -----	5'-	-	-	a	-	-	-	g	-	u	-	-	u	c	-	-	-	-	-	u	c	-	-	-	3'-	
<i>Burkholderia sacchari</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	c	-	-	-	3'-	
<i>Burkholderia stabilis</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Burkholderia vietnamiensis</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	-	-	-	-	3'-	
uncultured eubacterium WD258-----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Pseudomonas cepacia</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>P. caryophylli</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Ralstonia pickettii</i> -----	5'-	-	-	a	-	-	-	-	-	u	-	-	u	-	-	-	-	-	-	-	-	-	-	-	3'-	
<i>Alcaligenes</i> sp.-----	5'-	-	-	-	-	-	-	-	-	-	-	-	-	-	c	u	-	-	-	-	-	u	-	-	3'-	

^a Only mismatching nucleotides are indicated.

Fixation and cryosectioning of aerobic granules

For fixation of pure culture PG-01, cells at the exponential phase were centrifuged at $13,000 \times g$ for 3 min, and pellet was washed in 1 ml of 1×PBS. The cells were fixed by using 3 volumes of fresh prepared paraformaldehyde (PFA) (Sigma, USA) solution (4%, wt/vol) for each volume of cell suspension at 4°C for 3 h, and then stored in 1×PBS: ethanol (1:1) at -20°C (Amann et al., 1990). As gram-positive bacteria, cells of isolate PG-03 were fixed in 50% ethanol at 4°C for 24 hours (Head et al., 1998), then stored in 1×PBS/ethanol (1:1) at -20°C. For fixation of granules, intact aerobic granules were firstly washed in 1×PBS (phosphate buffered saline, 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2), and then fixed by using 4% PFA and 50% ethanol at 4°C for 6 h and 24 h respectively, followed by storage in 1×PBS: ethanol (1:1) at -20°C.

The fixed granules were embedded for optimum cutting temperature (OCT) compound (Miles, Elkhart, USA) by immersion. Embedded samples were then frozen at -20°C for overnight and 20 µm sections were cut on a cryomicrotome (CM3050S, Leica, German) and mounted to a gelatin (0.1% gelatin and 0.01% chromium potassium sulfate)-coated microscopic slide. The OCT was removed by immersion in autoclaved Milli-Q water. For fixed pure culture cells, a 5-µl aliquot was directly immobilized on a gelatin-coated microscopic slide and air dried. The pure culture cells and granule sections were then dehydrated by sequential immersion of the slides for 3 min in 50%, 80% and 98% ethanol, and air-dried.

Pretreatment of fixed cells

Several permeabilization protocols were incorporated to ensure that probes could penetrate cells, and thus eliminating the possibility of false negatives. Pre-treatment protocols tested included exposing pure culture to cell wall degrading agents such as lysozyme and mutanolysin (Schuppler et al., 1998), and HCl (Davenport et al., 2000). After dehydration, immobilized cells were treated by adding 5 µl lysozyme (20,000 units/mg, USB, Amersham Life Science, Germany) solution in TE buffer (100 mM Tris, 50 mM EDTA, pH 8.0) with three different concentrations, and/or 5 µl mutanolysin (5,000 units/ml, Fluka, Australia) solution

in 0.1 M phosphate buffer (pH 6.8), or HCl solution (Table 5.6), followed by incubating at room temperature for some time. To remove enzyme solution, cells were rinsed with Milli-Q water and air-dried.

Fluorescence in situ hybridization (FISH) and optimization of hybridization conditions

Whole-cell hybridization with fluorescently labeled oligonucleotides was performed as described by Manz et al. (1992). Hybridizations for EUB338 and Arch915 probes were performed with the hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS)) containing 5 ng probe μl^{-1} for 2.5 h at 46°C in an isotonically equilibrated humidity chamber. The hybridization stringency was adjusted by adding formamide (Promega, Promega Corporation, Madison, USA) to the hybridization buffer (10% for EUB338 and 30% for Arch915) (Amann et al., 1990). This was followed by a 20-min washing step at 48°C in wash buffer before a final wash in Milli-Q water. To achieve the same stringency during washing as that during hybridization, NaCl concentrations in the washing buffer depended on the formamide concentration in hybridization buffer and are listed in Table 5.7 (according to the formula of Lathe [1995]). Wash buffer was prepared in a fresh 50-ml polypropylene tube, containing 1 ml of 1 M Tris-HCl (final concentration 20 mM, pH 7.2), 50 μl of 10% SDS (final concentration 0.01%), some volume of 5 M NaCl corresponding to formamide concentration used in hybridization buffer, with Milli-Q water up to 50 ml.

Table 5.6. Effectiveness of permeabilizing agents as determined by whole-cell hybridization with strains PG-01 and PG-03

Relative fluorescence of hybridized cells with ^a :												
Strain	0.1% lysozyme	0.05% lysozyme	0.025% lysozyme	1% mutanolysin	0.5% mutanolysin	0.1% mutanolysin	1 M HCl	0.5 M HCl	0.05% lysozyme +0.5% mutanolysin	0.05% lysozyme + 0.1% mutanolysin	0.1% mutanolysi n + 0.05% lysozyme	Negative control
PG-01	-	+	+	-	-	-	-	-	-	-	+	++
PG-03	+	++	+	-	-	±	-	-	-	+	+	±

^a -, no fluorescence observed; ±, poorly fluorescent; +, good fluorescence; ++, good to strong fluorescence.

Table 5.7. Formamide concentrations in hybridization buffer and NaCl concentrations in wash buffer

Percent of formamide in hybridization buffer (%)	NaCl concentration in wash buffer (M)	Volume of 5 M NaCl in 50 ml wash buffer (μ l)
0	0.900	9000
5	0.630	6300
10	0.450	4500
15	0.318	3180
20	0.215	2150
25	0.149	1490
30	0.102	1020
35	0.070	700
40	0.046	460
45	0.030	300
50	0.018	180

Hybridization conditions for designed probes Pand822 and Rhod168 were optimized. First, hybridization temperatures were increased from 37 to 65°C in 2 or 5°C steps without formamide in the hybridization buffer to determine the adequate hybridization temperature. Then, effect of pretreatment was evaluated. After that, hybridization stringency conditions were improved empirically by increasing the formamide concentration in the hybridization buffer (Manz et al., 1992). Optimal stringencies for the designed probe were adjusted by hybridization against cells of reference strains with stepwise, increasing amount of formamide (5% each step). Between 200 and 400 cells were analyzed for each value. Probe-conferred signal intensities of the cells were divided by the cell area to determine the intensity values independent of cell size. Mean values of signal intensities between different strains were normalized relative to the signal of the probe EUB338 hybridized at 0% formamide concentration to correct for the different contents of ribosomes (Neef et al., 1996; Friedrich et al., 1999). Sufficient

stringency for total discrimination of non-target sequences was regarded to be achieved when the fluorescence intensity of non-target cells was in the background range.

In addition, a negative control (lacking a probe) was prepared to monitor auto-fluorescence. For combinations of probes with different optimal hybridization stringencies, two hybridizations were done successively. The first hybridization was done with the probe which required the higher formamide concentration and higher hybridization temperature, and this was followed by a second hybridization at a lower stringency.

Confocal Laser Scanning Microscopy

Images of the granules were acquired using a Fluoview300 confocal laser scanning microscope (CLSM) (Olympus, Japan). The green fluorescence of the FITC-labeled EUB338 probes was detected by excitation with a 10mW argon laser at 488 nm and a longpass filter (530 nm). The red fluorescence of Cy5-labeled Pand822/Rhod168 probe was detected by excitation by a helium-neon red laser at 632.8 nm and measured combined with a longpass filter (660 nm). A helium-neon green laser at 543 nm with a longpass filter (560 nm) was used to detect FRITC-labeled Arch519 probe.

Total and probe-conferred cell counts

It is difficult to obtain accurate total cell counts in cell clusters. Therefore, granule samples were converted as efficiently as possible to a suspension of individual cells, or at least to smaller cell clusters, by suspending 200 mg (wet weight) granules in a 2 ml microcentrifuge tube topped up with autoclaved Milli-Q water, and then shaking the tube for 10 min with a Mini BeadBeater (Biospec products, USA) at high speed, followed by vortexing sample thoroughly for 10 min in a 15 ml tube. Subsequently, the sample was diluted 10 times using Mill-Q water and a 5- μ l aliquot was just directly immobilized on a gelatin-coated microscopic slide for hybridization firstly with Cy5-labeled probe Pand822 or probe Rhod168, and then with EUB338. After hybridization, the samples were observed with CLSM. Cell

counts were determined by evaluating at least 20 microscopic fields with at least 100 cells per field for both EUB338-labeled and Pand822 (or Rhod168)-labeled cells. So, the ratio of the Pand822 (or Rhod168) probe-labeled cells to the EUB338 probe-positive cells was calculated.

Analytical methods.

Wastewater samples in the reactor were periodically analyzed for SS and SVI using standard methods (APHA, 1998). The measurement of phenol and TOC concentrations, the extraction of extracellular polymers (ECPs), the qualification of polysaccharides and proteins, and enzyme activities (C23O and C12O) were described previously in Chapter Four. To determine flocculation activities of isolates grown on phenol as the sole carbon source or YEPG medium (Table 5.2), isolates were incubated for one day by reciprocal shaking at 60 rpm and then settled for one minute (Watanabe et al., 1999). Flocculation activity was judged based on whether flocculated biomass can be observed.

Biomass concentrations on a dry weight (DW) basis were determined by filtering the cell suspension through a 0.2 μm filter (cellulose acetate membrane filter, Advanten MFS, Inc, CA, USA) and drying the filter and cells to a constant weight for 24 h at 80°C (Onysko et al., 2000). To determine the oxygen utilization rates of bacteria, a DO meter with Clark-type polarographic oxygen electrodes (YSI 5300A, YSI Incorporated, Yellow Springs, OH, USA) was used to measure the oxygen concentrations. A range of initial phenol concentration from 5 to 1000 mg l^{-1} was used as suggested by Marangoni et al. (2003). Specific oxygen utilization rates were calculated from oxygen concentration curves obtained (calculation method and data listed in Appendix D and Appendix E, respectively). A kinetic analysis of the data was performed based on Haldane's formula for an inhibitory substrate, $Q = Q_{\text{max}} s / [K_s + s + (s^2/K_i)]$, where Q and Q_{max} are the specific and the maximum specific oxygen utilization rates ($\text{mg DO g}^{-1} \text{DW h}^{-1}$), respectively, and s , K_s and K_i are the substrate concentration, half-saturation constant and inhibition constant (mg phenol l^{-1}), respectively. The kinetic parameters were fitted and

standard errors were obtained using JMP 5.0 statistical software (SAS Institute inc., NC, USA). All assays were conducted at least in duplicate.

5.3 RESULTS

Reactor performance and cultivation of phenol-degrading aerobic granules

The sequencing batch reactor was operated under an organic loading rate of 1.5 g phenol l⁻¹ day⁻¹ with an influent phenol concentration of 500 mg l⁻¹ (Chapter Three). Aerobic granules first appeared on day 9 of reactor operation and quickly grew to displace the seed flocs as the dominant form of biomass in the reactor. These granules were compact and consisted of bacterial rods and cocci embedded in an extracellular polymeric matrix. After about two months of operation, the reactor reached a steady state, as evidenced by stable biomass concentrations and almost complete phenol removal. Then granule samples were taken out for isolation, DNA extraction, and fixation for hybridization.

Isolation of bacteria from aerobic granules

A total of 18 different colonies based on colony morphology, color and size were isolated from the phenol-digesting activated sludge obtained by direct isolation technique on MP medium (supplanted with 500 mg phenol l⁻¹) agar plates. The purified colonies were subjected to REP-PCR analysis and partial 16S rDNA sequencing to identify identical strains. As shown in Fig. 5.1, 10 distinct REP-PCR patterns were obtained from the 18 colonies. Representative isolates were given by the designation PG-01 to PG-10. Epifluorescence microbiology and scanning electronic microscopy (SEM) were used to visualize the morphotypes. Three isolates (PG-03, PG-06 and PG-10) are rod-type bacteria, while the other seven are cocci-type (Table 5.8). All isolate colonies appeared on plates with dilution factors higher than 10³ times, as colonies could not be observed on plates with lower dilution factors. Only colonies associated with PG-01 appeared on plates with dilution factors higher than 10⁷ times, indicating PG-01 had the highest number of active cultureable cells in aerobic granules compared to the other nine isolates. Isolate PG-01 was estimated to be present in the phenol-degrading aerobic granules at a concentration of $5.64 \pm 0.87 \times 10^{10}$ cells g VSS⁻¹ of granules, suggesting that

strain PG-01 constituted a significant fraction of the total bacteria residing in the granules.

Fig. 5.1. REP-PCR profiles of bacterial strains isolated from phenol-degrading aerobic granules. Lane M: 1 kb DNA ladder; lane 1: negative control (PCR reaction without DNA template); lanes 2-11: PG-01, PG-02, PG-03, PG-04, PG-05, PG-06, PG-07, PG-08, PG-09 and PG-10.

Phylogenetic analysis of isolates

To evaluate the phylogenetic diversity represented by the 10 isolates, the 16S rDNA genes were fully sequenced. The putative division and nearest relatives of these 10 sequencing types were investigated by BLAST and the results were summarized in Table 5.9. The isolates fell into three major lineages of the Bacteria domain: the β - and γ -*Proteobacteria*, and the Gram-positive bacteria with high G + C content (Fig. 5.2). The majority of isolates (6/10) were placed in the β subclass of *Proteobacteria*, three in the Gram-positive bacteria with high G+C, and the remaining one isolate was assigned to the γ subclass of *Proteobacteria*. Table 5.9 shows the nucleotide identities of individual isolates to the closest validly

identified phylogenetic neighbor in the genebank database as compared by full 16S rRNA gene sequences.

Table 5.8. Phenotypic characterization of isolates. CFU, colony forming unit

Isolates	No. of dilution plate of colony appearance	Minimum Cell density (CFU g VSS ⁻¹)	Morphology	Gram stain ^a	Indigo test ^a	Catalase activity ^a
PG-01	10 ⁷	5.64 ± 0.87 × 10 ¹⁰	Cocci	-	+	+
PG-02	10 ⁵	1.01 ± 0.92 × 10 ⁸	Cocci	+	-	+
PG-03	10 ⁴	5.49 ± 1.80 × 10 ⁶	Rod	+	-	+
PG-04	10 ⁴	3.05 ± 1.42 × 10 ⁶	Cocci	+	-	-
PG-05	10 ⁵	1.53 ± 1.37 × 10 ⁷	Cocci	-	+	+
PG-06	10 ⁵	2.55 ± 1.32 × 10 ⁶	Rod	-	+	-
PG-07	10 ⁴	1.93 ± 0.72 × 10 ⁵	Cocci	-	-	-
PG-08	10 ⁵	3.56 ± 1.52 × 10 ⁶	Cocci	-	+	-
PG-09	10 ⁴	7.62 ± 2.80 × 10 ⁶	Cocci	-	+	+
PG-10	10 ⁴	4.56 ± 1.72 × 10 ⁶	Rod	-	+	-

^a Negative results (-), positive results (+);

From the six isolates belonging to the β subclass of *Proteobacteria*, isolate PG-01 matched sequences of *Pandoraea apista* species with a sequence identity of 98.7%, isolate PG-05 had a 98.8% identity to *Xenophilus azovorans* species, isolate PG-06 a 97.9% identity to *Acidovorax avenae*, and isolate PG-08 a 97.0% identity to *Comamonas sp. D22*, isolate PG-09 a 99.6% identity to *Pigmentiphaga*, and isolate PG-10 a 98.5% identity to *Hydrogenophaga palleronii*. The Gram-positive bacteria with high G+C include PG-02 with a sequence identity of 93.5% to *Propioniferax innocua*, isolate PG-03 with a 99.8% identity to *Rhodococcus erythropolis*, and isolate PG-04 with an 87.7% identity to *Propionibacterium cyclohexanicum*. Isolate PG-07 was 98.1% identical with the type strain of *Xanthomonas axonopodis* belonging to the γ subclass of *Proteobacteria*. PG-02 and PG-04 matched the closest identified strain with less than 97% identity, suggesting that they may represent novel or unknown bacterial strains.

Detection of dominant species by DGGE.

DGGE was employed here to generate genetic fingerprints that could provide information on the diversity of microbial communities in seeding acclimated activated sludge and mature aerobic granules. The bacterial populations in acclimated activated sludge and phenol-digesting aerobic granules were detected by isolating DNA from acclimated activated sludge, mature granules and cultured isolates, and then performing a DGGE analysis of the 16S rDNA fragments amplified from the DNA. Fig. 5.3 shows the DGGE profiles for acclimated sludge, granules and isolates. It was found that about ten dominant populations were present in both acclimated sludge and granules. However, there was no band in the activated sludge lane that could match bands in the granule lane in terms of position. This indicated that major populations in seeding sludge were totally different from those in mature granules. Two new discernable bands were present in the aerobic granules at higher denaturant concentrations.

Fig.5.2 Phylogenetic tree for isolated strains (in boldface) and relatives. The numbers at the branch nodes are bootstrap values based on 100 resamplings for maximum likelihood. Scale bar represents nucleotide

Table 5.9. Phylogenetic characterization of isolates

Isolates	Closest relatives	Taxon. affiliation	Identity value (%)	Number of bases used in establishing identity
PG-01	<i>Pandorea apista</i> (strain LMG 16407)	β -proteobacteria	98.7	1326
PG-02	<i>Propioniferax innocua</i>	Actinobacteria, HGC Gram positive bacteria, Propionibacteriaceae	93.5	1315
PG-03	<i>Rhodococcus erythropolis</i>	Actinobacteria, HGC Gram positive bacteria, Nocardiodaceae	99.8	1433
PG-04	<i>Propionibacterium cyclohexanicum</i>	Actinobacteria, HGC Gram positive bacteria, Propionibacteriaceae	87.7	1370
PG-05	<i>Xenophilus azovorans</i>	β -proteobacteria	98.8	1437
PG-06	<i>Acidovorax avenae</i>	β -proteobacteria	97.9	1437
PG-07	<i>Xanthomonas axonopodis</i>	γ -proteobacteria	98.1	1409
PG-08	<i>Comamonas</i> sp. D22	β -proteobacteria	97.0	1408
PG-09	<i>Pigmentiphaga</i>	β -proteobacteria	99.6	1432
PG-10	<i>Hydrogenophaga palleronii</i>	β -proteobacteria	98.5	1483

Four DGGE fragments from the granule lane, designated G1, G2, G3 and G4 (Fig. 5.4), were isolated and reamplified by PCR for DNA sequencing. Before DNA sequencing, the recovered DGGE bands were run on a DGGE gel to confirm their positions relative to the original sample. This step was repeated at least two times to obtain a pure DNA product for sequencing, since reamplification of a (presumably) single fragment often resulted in the formation of multiple amplicons from the adjacent bands. Partial 16S rDNA sequences of approximately 180-215 nucleotides were obtained from the four dominant bands. These sequences were compared with the sequences available in GenBank. Database searches performed with the nucleotide sequences determined indicated that G1 was identical to a member of the β subclass of the class *Proteobacteria*, exhibited 95% homology with *Comamonas* sp. strain 23310 (Table 5.9). G2 exhibited 98% homology with *Pandora sp.* LY, a member of the β subclass of the *Proteobacteria*. The organisms represented by band G3 belonged to the class of the High G+C Gram positive bacteria, most closely affiliated with *Propioniferax innocua* (97%). G4

was also identical to the High G+C Gram positive bacteria, and exhibited 90% homology with *Salinospora* sp. CHN964.

In order to identify some of the bands and compare the DGGE profile to the diversity assessments based on isolation, the individual isolates were amplified with the same primer pair and separated via DGGE. Of the 10 isolates, ten distinct bands were identified on the gel in Fig. 5.3. Of ten isolates, three isolates corresponded to three different DGGE bands from the granules (bands G1, G2 and G3). The G1 band corresponded to isolate PG-08. Isolate PG-01 corresponded to band G2. Isolate PG-02 corresponded to G3. The sequences of isolated pure culture were compared with the sequences of the dominant populations in the granules that were detected by the DGGE analysis. This comparison revealed that the sequences of isolate PG-01 were identical to the sequence of the G2 band, while the sequences of isolates PG-02 and PG-08 were identical to the sequences of the bands G3 and G1, respectively (Table 5.10).

Fig. 5.3. An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30-70%) with DGGE profiles of the fragments of 16S rDNA after PCR amplification from acclimated activated sludge, matured aerobic granules and isolates. The designations of the gel patterns are indicated above the lanes.

Fig. 5. 4. Drawing of DGGE gel from panel (in Fig. 5.3), showing the bands excised for sequence analysis.

Table 5.10. Sequence analysis of some DGGE bands for granules

DGGE band	Length (bases)	Phylogenetically related organism (accession no.)	% Identity
G1	215	<i>Comamonas</i> sp. Strain 23310 (AJ251577.1)	95
G2	180	<i>Pandoraea</i> sp. LY (AF532595.1)	98
G3	199	<i>Propioniferax innocua</i> (AF227165)	97
G4	180	<i>Salinospora</i> sp. CHN964 (AY040623.1)	90

Physiological analyses of isolates from aerobic granules

The indigo test for six strains, PG-01, PG-05, PG-06, PG-08, PG-09 and PG-10, showed positive results, and five strains, PG-01, PG-02, PG-03, PG-05 and PG-09, showed positive catalase activity (Table 5.8). Considering that aerobic granulation represents a form of cell self-immobilization, flocculation abilities of these isolates were determined. Generally, strains with flocculation ability will contribute to the structural stability of cell aggregates. Table 5.11 shows that most of the isolates belong to non-flocculated strains. PG-02, PG-04 and PG-06 showed flocculation activity as floc-like biomass was visualized. Out of all isolates, PG-08 possessed the strongest flocculation ability. Auto-aggregation took place when PG-08 was cultivated in shaker in phenol medium or YEPG medium and cell aggregates with a compact structure and a mean size of 0.45 mm were formed (Fig. 5.5c, d). Both polysaccharides contents and polysaccharide to protein ratios were high in the ECP of auto-aggregates grown in either phenol medium or YEPG medium, were 152.3 mg g DW⁻¹ and 7.5 respectively in phenol medium, and 137.6 mg g DW⁻¹ and 6.7 respectively in YEPG medium. Cell aggregates possessed good settleability as these aggregates settled to the bottom of reagent bottle after shaking was ceased for only one minute. Optical density of supernatant was very low, indicating most cells were within the aggregates.

Table 5.11. Flocculated activities, oxygen utilization kinetics and enzyme activities of isolates

Isolates	Flocculated activity		oxygen utilization kinetics			Enzyme activity ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$)	
	Growth in phenol ^a	Growth in YEPG medium ^a	Q_{max} ($\text{mg DO mg}^{-1} \text{DW h}^{-1}$)	K_s (mg l^{-1})	K_i (mg l^{-1})	C23O	C12O
PG-01	-	-	1.143 ± 0.086	36.2 ± 5.8	60.5 ± 5.2	2.17 ± 0.16	0.25 ± 0.08
PG-02	+	+	0.553 ± 0.012	7.1 ± 0.4	50.6 ± 3.1	1.81 ± 0.05	0
PG-03	-	-	0.479 ± 0.037	12.8 ± 1.7	150.1 ± 12	0.22 ± 0.03	1.72 ± 0.19
PG-04	+	-	0.369 ± 0.049	70.5 ± 6.2	165.2 ± 20.1	0.61 ± 0.06	0.36 ± 0.10
PG-05	-	-	0.344 ± 0.086	45.6 ± 7.3	60.5 ± 4.9	0.31 ± 0.06	0.25 ± 0.06
PG-06	+	+	0.074 ± 0.009	3.5 ± 0.8	42.0 ± 3.7	0	0.32 ± 0.06
PG-07	-	+	ND	ND	ND	ND	ND
PG-08	++	++	0.037 ± 0.012	47.2 ± 7.8	20.2 ± 6.9	0.05 ± 0.01	0.03 ± 0.02
PG-09	-	-	0.270 ± 0.012	0.4 ± 0.02	58.6 ± 2.2	0.48 ± 0.03	0.72 ± 0.04
PG-10	-	+	0.344 ± 0.074	8.3 ± 0.2	70.3 ± 5.5	0.21 ± 0.05	1.32 ± 0.09

^a No flocculation activity (-), flocculation activity (+), strong flocculation activity (++)

Phenol concentrations in the reactor were approximately to 250 mg l^{-1} at the initial time in one cycle (Chapter Three). So, the specific growth rate and phenol degradation rate were compared for the ten isolates at a phenol concentration of 250 mg l^{-1} . Fig. 5.6 shows that PG-01 had the highest specific growth rate, reached 0.185 h^{-1} . The specific growth rates of PG-02, PG-03, PG-05 and PG-09, ranged between 0.06 - 0.11 h^{-1} . The rest of the isolates had specific growth rates less than 0.05 h^{-1} .

Phenol and its derivatives are aerobically biodegraded mainly by two main metabolic pathways, initiated either by *ortho* or *meta* cleavage (Müller and Babel 1996; Leonard and Lindley 1998). C12O is associated with the *ortho* cleavage pathway, while C23O is linked to the *meta* cleavage pathway. Enzyme activities of

C23O and C12O were measured for nine isolates (Table 5.11), as isolate PG-07 could hardly grow on phenol liquid medium. While isolate PG-02 had only C23O activity and PG-06 had only C12O activity, the other seven isolates contained both C23O and C12O activities. In four isolates out of the seven isolates, C23O and C12O activities for each isolates did not show much difference. However, C23O activity was much higher than C12O activity for PG-01. On the contrary, PG-03 and PG-10 possessed C12O activities higher than C23O activities.

Fig.5.5. Gram-staining picture of PG-02 (a) and phase contrast picture of PG-02 floc (b); stereomicroscope image (c) and scanning electron micrograph (d) of PG-08 aggregates

Fig. 5.6 Comparison of specific growth rate at 250 mg l⁻¹ phenol concentration for all isolates

In addition, oxygen utilization kinetics for the nine isolates were measured as oxygenation is essential for the initial step in the aerobic phenol biodegradation (Watanabe et al., 1996). In this study, Kinetics parameters were fitted through determining specific oxygen utilization rates at different initial phenol concentrations. Specific oxygen utilization rates of eight isolates are shown in Fig.5.7, Fig. 5.8, Fig. 5.9 and Fig. 5.10. Although PG-08 can grow fast in YEPG liquid medium, the oxygen utilization rate for this strain seems lowest as confirmed by the lowest $Q_{\max}$ (Table 5.11). Compared to PG-08, PG-01 possessed the highest phenol-degrading activity. The $Q_{\max}$ of PG-01 was about 31 times that of PG-08. It is also seen that isolates expressed different K_s values based on oxygen utilization kinetics (Fig. 5.11). The K_s value for PG-09 was as low as 0.4 mg l⁻¹, while K_s value for PG-04 was as high as 70.5 mg l⁻¹.

Isolated bacteria were screened for growth on various organic chemicals. Benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene, *p*-xylene, catechol, *m*-cresol, *o*-cresol, *p*-cresol and *p*-hydroxybenzoate were evaluated for their potential to serve as growth substrates. More organisms were able to grow on *p*-cresol (80%) than any

other compounds (Table 5.12 and Fig. 5.12). *m*-cresol supported growth for 70% of the isolates. Benzene, catechol and *o*-cresol supported growth for 60% of the bacteria. 40% of isolates can grow on toluene and *p*-hydroxybenzoate, while 20% of isolate can grow on ethylbenzene. However, no isolate can grow on xylene isomers. It should be mentioned that the cultures were grown on phenol before experiment and not acclimated previously using corresponding compounds. It may be interesting to see whether acclimatation of cultures can broaden degradation capacities.

Fig. 5.7. Specific oxygen utilization rates of PG-01 and PG-02

Fig. 5.8. Specific oxygen utilization rates of PG-03 and PG-04

Fig. 5.9. Specific oxygen utilization rates of PG-05 and PG-06

Fig. 5.10. Specific oxygen utilization rates of PG-09 and PG-10

Fig. 5.11. Half-saturation constants (K_s) for isolates

Fig. 5.12. Percent of bacterial isolates showing growth on the individual compounds (calculated according to Table 5. 12). Benzene (B), toluene (T), ethylbenzene (EB), m-xylene (MX), o-xylene (OX), p-xylene (PX), catechol (CA), m-cresol (MC), o-cresol (OC), p-cresol (PC) and p-hydroxybenzoate (PH)

Table 5.12. Growth on aromatic compound of isolates

Isolates	Compounds ^a										
	Benzene	Toluene	Ethyl- benzene	m- xylene	o- xylene	p- xylene	catechol	m- cresol	o- cresol	p- cresol	p- hydroxy- benzoate
PG-01	+	-	++	-	-	-	++	+	++	++	+
PG-02	-	-	-	-	-	-	-	++	++	+	-
PG-03	-	-	-	-	-	-	+	-	-	+	-
PG-04	++	+	-	-	-	-	-	++	++	++	+
PG-05	+	+	-	-	-	-	-	-	-	++	-
PG-06	-	-	-	-	-	-	-	+	+	-	-
PG-07	+	+	-	-	-	-	++	++	++	++	-
PG-08	+	-	+	-	-	-	+	++	++	+	+
PG-09	++	++	-	-	-	-	+	++	-	++	++
PG-10	-	-	-	-	-	-	+	-	-	-	-

^a No growth (-), growth (+), obvious growth (++).

Probe Pand822 design and hybridization optimization.

A probe targeting a region of the 16S rRNA specific for isolate PG-01 based on a comparative analysis of aligned sequences was designed by using the Bioedit program, recently available sequences, and the CHECK_PROBE program of the Ribosomal Database Project. The resultant probe, Pand822, was shown in Table 5.4 along with their target sequence and the sequences of representative target and reference organisms exhibiting one or more mismatches. The probe Pand822 sequence fully matched the sequence of isolate PG-01 but exhibited at least three mismatches to all other currently available 16S rRNA reference sequences (Table 5.4).

The hybridization temperature was first determined by increased hybridization temperature from 37 to 67°C without adding formamide. Washing temperature was set 2 degree higher than hybridization temperature. Mean values of signal intensities between different strains were normalized relative to the signal of the probe EUB338 hybridized at 0% formamide concentration to correct for the different contents of ribosomes (Neef et al., 1996; Friedrich et al., 1999). Fig 5.13

showed that there was partial hybridization at hybridization temperatures lower 44°C than or higher than 63°C. It suggested that while hybridization for some cells might not happen in lower temperature, some hybridized probe might be washed-out due to high washing temperature in higher hybridization temperature. In addition, cells were found to be deformed after hybridization temperature increased beyond 58°C, indicating that it seemed not appropriate to increase probe stringency through increasing hybridization temperature. In fact, although stringency can be adjusted via temperature, the method in which stringency is varied using formamide concentration is often used (Manz et al., 1992; Amann et al., 1995; Neef et al., 1996; Friedrich et al., 1999). Based on these analysis, hybridization was set as 50°C, and increase in probe stringency was hoped to be achieved through formamide concentrations.

Then the effect of pretreatment was investigated using lysozyme and mutanolysin and HCl. Results showed lysozyme pretreatment can not increase the fluorescence density, and mutanolysin treatment resulted in the decrease in fluorescence density (Table 5.6). Thus, pretreatment was not adopted.

The optimum hybridization conditions for discrimination of target organism were evaluated by increasing the formamide concentrations in the hybridization buffer, which increased the stringency of hybridization (Stahl et al., 1991). *Burkholderia vietnamiensis* ATCC BAA248 and *Burkholderia cepacia* ATCC 10856 were chosen as negative control strains. The effect of increased formamide concentrations on the fluorescence intensity conferred by Cy5-labeled probe Pand822 hybridized with a target organism, isolate PG-01, and two nontarget organisms is shown in Fig 5.14. Probe binding to the target organism decreased as formamide concentrations exceeded 20%, attaining background intensities at about 40% formamide. Probe-conferred fluorescence of the nontarget organisms was in the background range, at 15% formamide. Nontarget organisms can thus be discriminated at 15% formamide. However, since there were no intensity losses, a concentration of 20% formamide was used for all subsequent hybridization.

Fig. 5.13. Effect of hybridization temperature on hybridization of Cy5-labeled Pand822 probe to isolate PG-01.

Fig. 5.14. Signal quantification of Cy5 labeled Pand822 probe to reference organisms. Fixed cells were hybridized with increasing concentrations of formamide with 5% increments. Fluorescence intensities were normalized relative to the signal strength of EUB338 at 0% formamide. Brightness of isolate PG-01 exhibiting maximal probe binding is set as 100%. (●) isolate PG-01; (■) *Burkholderia vietnamiensis* ATCC BAA248; (▲) *Burkholderia cepacia* ATCC 10856.

Probe Rhod168 design and hybridization optimization

A probe targeting a region of the 16S rRNA specific for strain PG-03 based on a comparative analysis of aligned sequences was designed by using the Bioedit program, recently available sequences, and the CHECK_PROBE program of the Ribosomal Database Project. The resultant probe, Rhod168, is shown in Table 5.5 along with its target sequence and the sequences of representative target and reference organisms exhibiting one or more mismatches. The probe sequence fully matched the sequences of *R. erythropolis* and *R. erythreus* species. The probe sequence exhibited two mismatch with the sequences of one *R. zopfii*, and exhibited at least five mismatches to *R. wratislaviensis*, *R. opacus*, *R. fascians*, *R. luteus*, *R. globerulus* and *R. equi* species. Although the probe Rhod168 was not only specific to target organism PG-03, this was the best choice as other probe regions were less specific and fully matched the sequences of more species.

The hybridization temperature was first determined by increased hybridization temperature from 37 to 65°C without adding formamide. Cell signal began to appear at hybridization temperature of 50°C and signal densities increased with increase in hybridization temperature. Based on these results, hybridization was set as 50°C. Because fluorescence signal densities of cell hybridization were still not strong enough, the effect of pretreatment was investigated using lysozyme, mutanolysin and hydrochloric acid (Table 5.6). In fact, additional pre-treatment of the ethanol-fixed genus *Rhodococcus* cells is often needed, in order to improve probe accessibility and enhance fluorescence signals (Schuppler *et al.*, 1998; Davenport *et al.*, 2000).

Table 5.6 shows that 0.05% lysozyme pretreatment results in the highest fluorescence density for cells of strain PG-03. The lysozyme incubation time also affected the hybridization signal densities. The incubation time shorter than 6 min was not ineffective for probe permeabilizing. Extension of incubation times to larger than 12 min was not found to be suitable for hybridization as pretreatment might damage target cells. So, pretreatment of 0.05% lysozyme for 10 min was adopted to improve probe accessibility to cells of strain PG-03.

The optimum hybridization conditions for discrimination of target organism were evaluated by increasing the formamide concentrations in the hybridization buffer, which increased the stringency of hybridization (Stahl et al., 1991). *Nocardia corynebacterioides* ATCC 31130, *Rhodococcus* sp. ATCC 15108 and *Rhodococcus rhodochrous* ATCC 13808 were chosen as control strains. The effect of increased formamide concentrations on the fluorescence intensity conferred by Cy5-labelled probe Rhod168 hybridized with a target organism, isolate PG-03, and three control organisms is shown in Fig 5.15. Probe binding to the target organism decreased at formamide concentrations above 25%, attaining background intensities at about 45% formamide. Thus, a concentration of 25% formamide was used for subsequent hybridization for probe Rhod168. However, the three control organisms can not be discriminated completely under this condition (Fig. 5.15).

Hybridization for probe EUB338 was performed at 46°C with formamide concentration of 10%. Compared to those for probes Pand822 and Rhod168, hybridization stringency for probe EUB338 was lower. Thus, the first hybridization was done with the probe Pand822 or Rhod168, followed by a second hybridization with the probe EUB338, if the two probes were combined in one sample. The effect of hybridization process with the probe EUB338 on the signal density of probe Pand822 or Rhod168 was evaluated (Fig.5.16). Results show that there was strong signal for probe Pand822 or Rhod168 through the observation of CLSM, when the two probes were used sequentially. Within same field of view, the number of probe Pand822 or Rhod168-labelled cells almost equal to that of EUB338-positive cells, as shown in Fig. 5.17.

Fig. 5.15. Signal quantification of Cy5 labeled Rhod168 probe to reference organisms. Fixed cells were hybridized with increasing concentrations of formamide with 5% increments. Fluorescence intensities were normalized relative to the signal strength of EUB338 at 0% formamide. Brightness of strain PG-03 exhibiting maximal probe binding is set at 100%. (▲) strain PG-03; (Δ) *Nocardia corynebacterioides* ATCC 31130; (◆) *Rhodococcus* sp. ATCC 15108; (◇) *Rhodococcus rhodochrous* ATCC 13808.

Fig. 5.17 Counting number of EUB338-labelled cells to number of Pand822-labelled cells (a), and number of Rhod168-labelled cells (b).

Fig. 5.16 CLSM images of isolates PG-01 and PG-03. (a) cells of PG-01 hybridization with probe EUB338; (b) cells of PG-01 hybridization with probe Pand822; (c) combination of the two images; (d) cells of PG-03 hybridization with probe EUB338; (e) cells of PG-03 hybridization with probe Rhod168; (f) combination of the two images

Distribution and function of PG-01 within aerobic granules

To elucidate the abundance and spatial distribution of isolates PG-01 and PG-03 in the aerobic granules, the fluorescence in situ hybridization technique combined with confocal laser scanning microscopy was applied to thin sections of the granules. In situ hybridization was performed with thin (20- μ m) sections using the FITC-labeled EUB338 probe, TRITC-labeled Arch915 probe and the Cy5-labeled Pand822 probe. The granules consisted of a dense layer of cells, surrounding a central region with low cell density (Fig. 5.18). This structural pattern was repeatedly observed in granule sections. FISH quantification showed that these granules were dominated mainly by bacterial cells. While bacteria hybridized with EUB338 probe distributed around the whole section, archaeal signals resulted from Arch915 probe was not detected even in the core of granules.

The application of the designed Pand822 probe to the aerobic granule sections showed that a number of cocci-shaped cells, showing a morphology similar to that of strain PG-01, were detected in the granules (5.18). While some single cells of PG-01 can also be detected in the inner layer, most PG-01 cells distributed in the outer layer of granules as aggregates (Fig. 5.19). FISH combined with CLSM can be used for quantitation (Davenport et al., 2000). Direct in situ hybridization counting after dispersing the granules revealed a significant relative abundance (approximately 4.1 ± 3.2 % of the EUB338 probe-positive cells) of isolate PG-01 present in the granule sample analyzed. On the contrary, cells binding *Rhodococcus*-specific probe Rhod168 were rarely detected.

Fig. 5.18 CLSM images of granule section (a) hybridization with probe EUB338 and (b) hybridization with probe Pand822; (c) combination of the two images.

Fig. 5.19 CLSM image of outer layer of granule section at higher magnification (1000 $\times$). (a) hybridization with probe EUB338; (b) hybridization with probe Pand822; (c) combination of the two images.

5.4 DISCUSSION

Microbial community of phenol-degrading aerobic granules

Aerobic granule-based systems have the potential to treat high phenol loading wastewater due to the compact structure and good settling ability of aerobic granules (Chapters Three and Four). In this study, 16S rDNA DGGE for total-community fingerprints and isolation were used to assess the community structure and investigate the functional diversity of microbial community in phenol-degrading aerobic granules to exploit these kinds of systems. The DGGE analyses provided data on the presence and extent of sequence diversity and an indication of approximate community structure. In the highly specialized communities studied in this investigation, DGGE analyses highlighted the relatively low diversity of acclimated activated sludge and aerobic granules as compared to nutrient removal activated sludge (Govoreanu et al., 2003). The DGGE microbial diversity community fingerprint for acclimated phenol-degrading activated sludge and aerobic granules using general bacterial primers indicated that the bands detected were dominated by about 10 bands. DGGE results for microbial communities in phenol remediation systems also confirmed the existence of low diversity (Whiteley et al., 2000). Although there was not much difference in the numbers of DGGE fragments for acclimated activated sludge and granule samples, the positions of the DGGE fragments for enriched activated sludge were totally different from those for aerobic granules. This indicated major difference in the microbial communities of acclimated activated sludge and granules.

Compared to only one isolate belonging to γ -*Proteobacteria*, six isolates were affiliated to beta subclass of the *Proteobacteria* in this study. Bacteria from the beta subclass of the class *Proteobacteria* have recently been observed by fluorescent in situ hybridization or cloning to be dominant in activated sludge communities (Wagner et al., 1993, 1994; Bond et al., 1995; Snaird et al., 1997). Representatives of the beta subclass of *Proteobacteria* are thought to play important roles in aspects of activated sludge such as degradation of organic material, removal of nutrients, and formation of floc structure (Bond et al., 1995).

The importance of members of the beta subclass of the *Proteobacteria* for degradation of phenol in activated sludge has also been demonstrated in isolation experiments (Watanabe et al., 1998a).

Three phenol-degrading gram-positive bacteria with high G+C were isolated from phenol-degrading aerobic granules, and DGGE also confirmed two dominant populations with high G+C, suggesting an important role of high G+C populations. However, Gram-positive high G+C bacteria could not be isolated from phenol-degrading activated sludge systems or phenol bioremediation system (Watabane et al., 1998a, 1999; Whiteley et al., 2000; Abd-El-Haleem et al., 2002). Bacteria with high G+C preferred to grow in biofilms compared to suspended state (Lehman et al., 2001). *R. erythropolis* also favored growth in styrene-degrading biofilms rather than a free-floating state (Tresse et al., 2002). It seems that the potential for spatial isolation provided by the structure of granules provides an advantage for growth of Gram-positive bacteria with high G+C. In addition, several Gram-positive bacteria with high G+C content are known to consume soluble COD rapidly and store them as storage polymers such as glycogen (Liu et al., 2001; Maszenan et al., 2000). This competitive advantage allows them to thrive in environments with a low food-to microorganism (F/M) ratio. Actually, *Rhodococcus* strains were found to be abundant in a crowded and carbon resource-restricted environment (Juteau et al., 1999). In this study, phenol could not be detected after first 30 min of each operating cycle, suggesting a low F/M for aerobic granules (Chapter Three). The low F/M of the reactor in this study might provide an additional reason for the growth of Gram-positive bacteria with high G+C.

Diversity of physiology of isolates

Based on the C23O and C12O activities, four catabolic types were found among the nine isolates. Type 1, present in one isolate (PG-02), was characterized by degradation of phenol via the *meta* pathway through the induction of C23O. Type 2, present in one isolate (PG-01), was characterized by degradation of phenol mainly via the *meta* pathway as C23O was much higher than C12O. Type 3, present in two isolates (PG-03 and PG-10), was characterized by degradation of

phenol mainly via the *ortho* pathway as C12O was much higher than C23O. Five isolates belonged to Type 4. For strains in Type 4, phenol was degraded via both *meta* and *ortho* pathways and degradation rates through the two pathway appeared similar because C12O and C23O did not show much difference. In the aerobic granule system, the *meta* pathway was strongly induced and C23O activity was higher than C12O activity (Chapter Four). So, Type 1 and Type 2 might be selected with better chances of survival. Isolates PG-01 and PG-02 which corresponded to Type 1 and Type 2, respectively, belonged to members of the dominant populations in aerobic granules. However, strains without this advantage could still grow in aerobic granules, as confirmed by seven isolates in Types 3 and 4.

Substrate gradient also affect to some extent selection of bacteria with oxygen utilization kinetics constants values fitted for specific oxygen utilization rates (Table 5.11 and Fig. 5.11). The operating condition and substrate concentrations strongly selected microorganisms based on K_s values (Massol-Deya et al., 1997). It was reported that strains isolated from a continuous suspended reactor enriched by a low phenol concentration expressed phenol-oxygenating activities with low apparent K_s , while the strains isolated from a batch system enriched by relatively high concentrations of phenol expresses phenol-oxygenating activities with relatively high apparent K_s (Watanabe et al., 1996). However, strains with either high or low apparent K_s were isolated from aerobic granules cultivated in an SBR with relatively high initial phenol concentrations. Obviously, it was necessary to incorporate other factors to accurately explain these observations. The structure of aerobic granules led to the development of a concentration gradient because of diffusional resistance and might provide different niches for growth of different bacteria. Although the granule surface faced high phenol concentrations during the initial part of the SBR cycle, phenol concentrations would be always low in the granule interior. Thus, both bacteria with high K_s and low K_s can grow in aerobic granules. The K_s value (the affinity of a bacterium for a substrate) is of practical importance because a bacterium with a lower K_s value can efficiently remove the pollutant down to lower concentrations, while a bacterium with a higher K_s value

can efficiently compete for substrate at high substrate concentrations. The potential for spatial isolation provided by aerobic granules offers the advantage of increasing the functional redundancy of the microbial community. In fact, biofilm structures offer more microbial diversity and functional genes than suspended enrichment (Stach and Burns, 2002). Functional redundancy and functional niche complementation usually contribute to process performance and stability.

Inhibition constant, K_i , reflects inhibitory effect of a given substrate to microorganisms. It was reported that there was some relationship between K_i and K_s values for isolates from phenol-degrading activated sludge (Watanabe et al., 1996), namely, low K_i correlated with low K_s for bacteria from a continuously cultivated activated sludge and high K_i correlated with high K_s for bacteria from batch culture. This relationship was not observed among isolates from phenol-degrading aerobic granules. We cannot explain these inconsistencies at present. Kinetic parameters could reflect some properties of phenol hydroxylase and other enzymes for phenol degradation. In fact, some bacteria such as *P. putida* ATCC 17484, have kinetic properties with low K_s and high K_i (Table 2.1). Comparing kinetic parameters in Table 2.1 and Table 5.11 reveals that K_i values for isolates in this study were not high but in the normal range. This suggests that aerobic granulation may not select bacteria with superior kinetic characteristics against phenol inhibition. On the contrary, the structure of granules itself may play an important role to diminish the toxicity of phenol, as discussed in Chapter Three.

Ten isolates belong to ten genera based on the phylogenetic tree. Five genera namely *Rhodococcus*, *Acidovorax*, *Xanthomonas*, *Comamonas* and *Hydrogenophaga*, where five isolates (PG-03, PG-06, PG-07, PG-08 and PG-10) were placed respectively, contain species for degrading phenolic compounds (Zhao and Ward, 1999; Poelarends et al., 2000; Contzen et al., 2001; De et al., 2003; Heiss et al., 2003). Although phenolic degradation for *Pandoraea*, *Propionibacterium*, *Xenophilus* and *Pigmentiphaga*, is still not known, some species in these genus can degrade recalcitrant compounds (Zou et al., 2000;

Blumel et al., 2001a, b; Okeke et al., 2002). However, not much is known about *Propioniferax*, particularly about its substrate preferences.

Microorganisms recovered from environmental samples, including soils and sediments, possess broad biochemical and biodegradative capabilities (Stapleton et al., 2000). This biochemical diversity includes physiological profiles that enable bacteria to survive in environments affected by anthropogenic contamination. Microbial isolates recovered from phenol-degrading aerobic granules demonstrated the ability to mineralize several different aromatic compounds, including benzene, toluene, ethylbenzene, catechol, cresol and p-hydroxybenzonate, although xylene could be not degraded by these isolates. Some isolates would be interesting for further biotechnological applications in bioaugmentation and bioengineering. The ability to degrade one kind of aromatic compound means for one bacteria that that microorganisms includes catabolic genes specific for degradation of this compound. So, the gene pool in phenol-degrading aerobic granules possessed high diversity abundance and wide distribution of catabolic genes. In aerobic granules, the close proximity of cells that are attached to each other can facilitate the horizontal gene transfer. It is clear from this research that there remains significant potential for hydrocarbon degradation in phenol-degrading aerobic granules.

Physiological traits facilitating growth of microorganisms in aerobic granules

Under certain conditions, dominant species may possess strong competitive advantages and demonstrate adaptive responses in kinetics and metabolism. An important question that should be addressed is why (or how) the identified organisms have become the important players in the ecosystem. Such information could be used for understanding the microbial interaction within microbial community and manipulating some specific microorganisms to improving the operation of treatment systems. To date, little is known about the specific properties that make up the competitiveness of a degrader in a natural or technical environment. The substrate uptake rates and degradation pathways for the microorganism are of major importance for its competitive behavior and play a significant role (Duetz et al., 1994; Filonov et al., 1997; Krooneman et al., 1998).

Ability of aggregation may also help organisms to be retained within aerobic granules.

Table 5.13. Analysis of competitive characteristics of isolates

Isolates	Specific growth rate ^a	Strong C23O activity ^b	Flocculation activity ^c	Identical DGGE band
PG-01	Very High	+		G2
PG-02	high	+	+	G3
PG-08			++	G1
PG-03	high			
PG-04			+	
PG-05	high			
PG-06			+	
PG-09	high			
PG-07				
PG-10				

^a Five isolates possessed relatively high specific growth rates;

^b Two isolates possessed higher C23O activity compared to C12O activity;

^c Four isolates possessed flocculation activity. Weak flocculation activity (+), very strong flocculation activity (++).

Here, three physiological traits, namely, specific growth rate, flocculation activity, and enzyme activity, related to retain and growth of bacteria in aerobic granules, were selected and compared so as to probe the competitive behavior of the various isolates. For simplification, five isolates with higher specific growth rates compared to the other five isolates were regarded to have “high” specific growth rate, respectively. High specific growth rate, strong flocculation activity and high C23O activity appear to facilitate the maintenance and growth of bacteria. Only these positive traits were indicated in Table 5.13 for clear comparison. Table 5.13 shows that isolates PG-01 and PG-02 possessed three advantages for survive in aerobic granules. Although isolate PG-08 only had one advantages, its exceptionally strong flocculation activity may be an important attribute for successful growth within the granule community. Interestingly, all the three isolates belonged to members of three dominant populations in aerobic granules. Five among the other seven isolates possessed one advantages. However, they did

not become dominant. From Table 5.13, it can be inferred that it seem not enough to judge the possibility for one species to become dominant in microbial community just based on one physiological trait. Advantages in several physiological traits or extreme advantage in at least one physiological trait may be required for organisms to become the important players in the ecosystem

Functional analysis of microbial community in aerobic granules

Recent studies stressed the importance of gaining an understanding of the functions of microbial communities and revealed that population diversity alone does not drive ecosystem stability (Briones and Raskin, 2003). High microbial diversity does not mean good stability. For example, PCR-clone libraries showed that the bacterial community of mature and stable aerobic granules was much less diverse than those of crushed granules in terms of microbial richness and evenness (Yi, 2003). Recognizing the diversity and the links within each functional group of a system can lead to better ways to model diversity and function as well as helping to improve process stability and treatment capacity (Bond et al., 1995; Hulot et al., 2000; Kong et al., 2002; van der Gast et al., 2003). Molecular ecological approaches have detected diverse microbial populations in the natural environment, which makes us realize that it would be almost impossible to analyze the behavior of all the individual species in a complex microbial community. To circumvent this difficulty in understanding and manipulating complex microbial communities, some practical approaches such as determining major functions included in the microbial community for group-specific monitoring and identifying the functionally important populations, were adopted and demonstrated to be useful for developing new strategies for manipulating microbial communities for engineering consideration (Watanabe et al., 2002; Egli et al., 2003).

Isolation, DGGE and hybridization with specific probe confirmed that isolate *Pandoraea* sp. PG-01 was numerically abundant in the aerobic granules. In addition, isolate PG-01 had the highest growth and phenol degradation rate among all isolates at a phenol concentration of 250 mg l⁻¹. Considering the high abundance of active cells and high phenol removal rate, isolate PG-01 could be

regarded as one of the functionally dominant strains and may have contributed significantly to phenol degradation in the granules. It should be mentioned that *Pandoraea* sp. was not isolated in other phenol-degrading microbial communities and not much is known about the environmental role of *Pandoraea*, although a species has been isolated that is capable of degrading 100 mg l⁻¹ endosulfan, an organochlorine pesticide with extremely high acute toxicity (Siddique et al., 2003).

As a strain of a dominant genus in aerobic granules, isolate PG-08 had the low specific oxygen utilization activity, growth and phenol degradation rate among all isolates. However, in contrast to its phenol degradation activity, PG-08 exhibited strong flocculation activity. This strain forms auto-aggregates easily and has high extra-polysaccharide content. In fact, auto-aggregation mostly was correlated with high hydrophobicity (Del Re et al., 2000; Zavaglia et al., 2002), and hydrophobicity is regarded as a triggering force of biogranulation (Liu et al., 2004). In addition, polysaccharides are known to contribute to the formation and stability of aerobic granules (Tay et al., 2001a). So it seems reasonable to suggest that the role of isolate PG-08 may be mainly related to granulation and maintaining the structure of phenol-degrading aerobic granules. Actually, strong autoaggregation of PG-08 made it difficult to detach PG-08 from biomass particles for isolation and then may be one of reasons resulting in the low CFU number (Table 5.8), although other conditions also affect the outcome of isolation (Vallaey et al., 1997).

Phenol degradation and granule structure stabilization are two basic functions for the stability of granule systems exposed to high phenol concentrations. A significant diversity would exist within each functional group of bacteria (Wagner et al., 2002). Actually, the arrangement of different microorganisms responsible for various functions within the granule matrix is not surprising as granules are similar to biofilms, which represents highly differentiated, well-organized, multicultural microbial communities, and the bacteria in a multispecies biofilm can best meet the overall needs of the communities present (Watnick and Kolter, 2000). This kind of

model has important implications for system management. First, it provides a clue to design more acceptable granule-based systems for engineering. Recent studies suggested that environmental selection pressures such as shear force were required for granule formation (Beun et al., 1999; Tay et al., 2001a). However, the high shear force is generally associated with high energy requirement. If some conditions are applied to stimulate the growth of those microorganisms responsible for production of polysaccharides and then cell aggregation, aerobic granules may be formed even in the presence of weaker environmental selection pressures.

The second benefit of the result described is that it provides a basis for a more knowledge-driven treatment of system failures. One strategy to improve a particular aspect of process performance in a wastewater treatment system, for example after its breakdown, is bioaugmentation with specialized microorganisms or activated sludge from another wastewater treatment plant (Rittmann and Whitman, 1994). It is important to identify what kind of function needs to be strengthened prior to any bioaugmentation exercise. If problems arise, these results can be used to provide guidance to select appropriate bacterial additives (or culturable microorganisms). An outbreak of nonflocculating bacteria after an episode of increased phenol loading was suggested to cause the breakdown of a phenol-degrading activated sludge process (Watanabe et al., 1999). And this was also true for aerobic granule systems. These studies indicate that bacteria with strong flocculating function and high phenol tolerance would be the first choice for bioaugmentation in order to exploit systems to treat higher phenol substrate loadings.

In summary, 16S rDNA DGGE and isolation methods were combined in this study to assess the community structure and the functional diversity of microbial community in phenol-degrading aerobic granules cultivated at a loading of 1.5 g phenol l⁻¹ day⁻¹. The DGGE fingerprints pointed to the presence of ten dominant populations in the aerobic granules. Ten pure cultures were also isolated from phenol-degrading aerobic granules, and characterized. Three isolates PG-01, PG-02 and PG-08 had identical 16S sequences to three dominant populations in

granules. Both DGGE and isolation demonstrated that beta subclass of the *Proteobacteria* and high G+C Gram-positive bacteria had a significant presence in phenol-degrading aerobic granules. The isolates showed different physiological traits, in terms of specific growth rates, kinetics, enzyme activities and flocculated activities. All isolates belonging to dominant populations were found to possess more physiological properties that conferred competitive and growth advantages. Isolation, DGGE and hybridization with specific probe confirmed that isolate PG-01 was numerically abundant in the aerobic granules. Combined with its highest growth and phenol degradation rate among all isolates, isolate PG-01 could be regarded as one of the functionally dominant strains and may have contributed significantly to phenol degradation in the granules.

CHAPTER SIX

KINETIC AND METABOLIC COMPETITION

BETWEEN TWO STRAINS IN

PHENOL-DEGRADING AEROBIC GRANULES

6.1 INTRODUCTION

Aerobic granules can be used to treat wastewater with high phenol loading rates (Chapter Three and Four). The high tolerance of aerobic granules to phenol loading may be related to the effective protection afforded to cells by the dense structure (Chapter Three), and the acquisition of new genetic traits through easier horizontal gene transfer (Davey and O' Toole, 2000; Molin and Tolker-Nielsen, 2003). The microbial community that arises from the selection of granulation process may also play an important role. An understanding of the microbial community of aerobic granules would assist in many ways in improving system design and performance.

It is now clear that, even with advanced molecular techniques, the identification of every species in any environment is a daunting task (Curtis et al., 2002). There is therefore an increasing interest to identify the dominant organisms that play a key role in the pollutant degradation processes (Juretschko et al., 1998; Abed et al., 2002; Manefield et al., 2002; Tirola et al., 2002). However, little is known about why (or how) these identified organisms have become the important players in the ecosystem. In fact, such information, if available, could be used to manipulate the microbial community structure for enhancement of system treatment capacity, and to develop optimal control and management strategies.

Interactions between populations are a driving force in the evolution of community structure (Atlas and Bartha, 1998). Of the various types of interactions between

microbial populations, competition for carbon occurs most commonly and is often the major determination of abundance and distribution of microorganisms, and species diversity in the microbial community (Dikshitulu et al., 1993; Ka et al., 1994; Brown et al., 2001). To date, little is known about the specific properties that make up the competitiveness of a degrader in a natural or engineered environment. It is thought that the properties of the uptake mechanisms of the microorganism in question are of major importance for its competitive behavior (Atlas and Bartha, 1998). The nature of the degradation pathways and the biosynthesis of antimicrobial compounds may also play significant roles in impacting the interactions among competing microorganisms (Duetz et al., 1994; Andel et al., 1997; Filonov et al., 1997; Krooneman et al., 1998; Chen and Weimer, 2001; Slattery et al., 2001; Riley and Wertz, 2002).

Most studies on microbial competition used model systems with low complexity that involved experiments under controlled conditions (Lawton, 1995; Sonderkamp et al., 2001). Experiments in batch or chemostat systems using two or several cultures are necessary for analyzing the microbial physiologies and some key determinants of competition (Treves et al., 2003). To further investigate the relationships and behaviors between these microorganisms, quantitative measurements of the occurrence of microorganisms within those natural environment are required (Watanabe et al., 1998b). The determinations may employ culture-independent methods such as fluorescence in situ hybridization (FISH) using fluorescent-labeled oligonucleotide probes coupled with confocal laser scanning microscopy (CLSM) (Amann and Ludwig, 2000).

In this study, we selected two fast-growing isolates from phenol-degrading aerobic granules for further characterization. Then batch experiments were carried out to investigate co-culture competition and competition behavior was analyzed from

kinetic and metabolic considerations. Finally, the two strains were bioaugmented into activated sludge in a sequencing batch reactor and the abundance of each strain was monitored with molecular methods. This study could contribute to a better understanding of the microbial community of these aerobic granules and provide information useful for manipulation of the aerobic granulation process and optimization of microbial community.

6.2 MATERIALS AND METHODS

Bacterial strains isolated from aerobic granules

The aerobic granules were produced using a medium containing phenol as the sole source of carbon in a sequencing batch reactor as described in Chapter Three. The reactor was inoculated with flocculated activated sludge and operated with an influent phenol concentration of 500 mg l⁻¹. After the reactor reached a steady state, the matured granules were sampled and used for further study. The two strains described in this study, *Pandoraea* sp. PG-01 and *Rhodococcus* sp. PG-03, were both isolated from matured phenol-degrading aerobic granules, as described in Chapter Five.

Cell counting using DAPI stain

Cell counts were performed using DAPI stain (Kuwae and Hosokawa, 1999). 1.0 ml culture medium was taken and diluted 50–250 times with particle-free sterilized water, and the diluted samples were stained with DAPI (4',6-diamidino-2-phenylindole) to a final concentration of 5 µg ml⁻¹. After more than 30 minutes of staining, 0.5–2.0 ml of the sample was filtered through polycarbonate black filters (0.2 µm pore size) and then rinsed with particle-free sterilized water. The filters were immersed in non-fluorescent oil on microscopic slides and covered with coverslips. Bacteria retained on the filters were examined within 24 hours after dispersion under a light microscope (UV excitation)

(Olympus BX60) equipped with a 100× magnification oil immersion objective lens. Cells were then counted using a minimum of 10 different microscopic fields of view.

Growth Kinetics

Cells used in the kinetics experiment were exponential phase cultures grown with phenol as sole carbon and energy source. Experiments were performed in triplicate in 500 ml autoclaved reagent bottles. Different concentrations of phenol in MP medium (described in Chapter Five) were then prepared in bottles. The individual strain was then introduced into the phenol solutions, and all reagent bottles were capped immediately and covered with aluminum foil to prevent photo-oxidation. The culture mediums were incubated under aerobic conditions and shaken in the dark at 25°C on a shaker at 150 rpm.

Phenol concentrations in the kinetic experiments were assayed at regular intervals of incubation. Cell growth was monitored by OD measurement using a spectrophotometer at a wave-length of 600 nm. The linear relationship between OD values and the total cell numbers counted using DAPI stain was confirmed through experiments on the two strains. For strain PG-01, the total cell number concentrations were calculated from optical density measurements using the relationship $\text{Num l}^{-1} = 5.63 \times 10^8 \text{ OD}_{600}$ ($R^2 = 0.9914$). For strain PG-03, the total cell number concentration were calculated using the relationship $\text{Num l}^{-1} = 1.61 \times 10^8 \text{ OD}_{600}$ ($R^2 = 0.9917$). Information on the initial rate of phenol disappearance and total cell numbers (listed in Appendix F and Appendix G) were combined to establish the specific growth rates and specific phenol degradation rates.

Biomass concentrations on a dry weight (DW) basis were determined by filtering the cell suspension through a 0.2 µm filter (cellulose acetate membrane filter,

Advantem MFS, Inc, CA, USA) and drying the filter and cells to a constant weight for 24 h at 80°C (Onysko et al., 2000). A linear relationship between OD below 0.35 and dry weight was observed. The biomass concentration (X), expressed as dry weight (g DW l⁻¹), was calculated from optical density measurements using the relationship $X = 0.574 \text{ OD}_{600}$ ($R^2 = 0.984$) for strain PG-01, and $X = 0.531 \text{ OD}_{600}$ ($R^2 = 0.980$) for strain PG-03.

Measurement of respiratory activity

The oxygen utilization rates of cells were determined through a DO meter with Clark-type polarographic oxygen electrodes (YSI 5300A, YSI Incorporated, Yellow Springs, OH, USA) as described in Chapter Five. Oxygen consumption and carbon dioxide production were automatically determined and recorded with a computer-controlled Micro-Oxymax Respirometer with CO₂ and O₂ sensors (Columbus Instruments, Columbus, OH, USA). The respirometer pumped the air from each sample chamber through a drying column packed with magnesium perchlorate, then through a CO₂ sensor, and finally through an O₂ sensor before returning the air to the sample chamber. Sensors were purged with dried filtered outside air after each reading. The system automatically pumped dried air from outside into the sample chamber if O₂ concentration decreased below 19.5% or CO₂ increased above 0.9%. Three SCHOTT 500 ml reagent bottles were used as three chambers. A reference chamber was used to recalibrate the sensors.

Inhibitor screening

Inhibitory activity was measured using a modified plate assay (Chen and Weimer, 2001). A lawn of indicator strain (PG-01 or PG-03) was first seeded by mixing 0.2 ml of an overnight culture with 15.0 ml melted (~45 °C) solid medium [MP medium supplemented with (l⁻¹): 15 g agar and 500 mg phenol]. This suspension was plated on sterile 100×15 mm Petri dishes. A drop (~50 µl) of autoclaved

distilled water or test strain (PG-03 or PG-01) with different cell concentrations from either exponential-phase or stationary-phase cultures was spotted onto the lawn as it solidified. The plate was constantly examined for zones of inhibition for two weeks after overnight incubation in the chamber at 25°C.

Batch culture experiments

Nine autoclaved 500 ml reagent bottles which served as batch systems were run in parallel at the same time. Six bottles were inoculated with cultures of either strain PG-01 or PG-03 for triplicate experiments. In each of the last three bottles for competitive experiments, cultures of strains PG-01 and PG-03 with same biomass content were mixed before inoculation. Inoculated cultures were harvested in exponential growth phase with phenol as sole carbon source. The same concentrations were maintained in the first monoculture systems. In the mixed culture systems, the biomass concentration of each culture was equal to that in the monoculture growth experiments.

The medium solution volume in each bottle was 100 ml and the initial phenol concentration was 250 mg l⁻¹. All reagent bottles were capped immediately and covered with aluminum foil to prevent photo-oxidation. The batch cultures were incubated on a shaker at 150 rpm at 25°C. The densities of cells of each strain were monitored over a period of three days by spreading diluted samples on R2A agar plates. The two strains in competitive systems were distinguished on R2A plates by colony morphology, size and color. Strain PG-01 formed white and transparent colonies with a diameter of 2 mm on R2A agar plates, while colonies of cells of strain PG-03 appeared yellowish white and mycolic with a diameter of 4 mm. For each assay, 1.0 ml of cell suspension was sampled and serially diluted (10⁻² to 10⁻⁶). A quantity of 100 µl of each dilution was spread onto a R2A agar plate. The plates were incubated for 3-8 days at 25°C and then counted. All experiments were

performed in triplicate.

Augmentation of two strains into activated sludge in a sequencing batch reactor

The cells of the two strains were bioaugmented into activated sludge in a column-type sequential aerobic sludge blanket reactor, as described in Chapter Three, to investigate the cell growth of the two strains in mixed biomass. The reactor was fed with phenol as the sole carbon source at a concentration of 500 mg l⁻¹ by using a synthetic wastewater with the same composition as used in Chapter Three. The reactor was operated sequentially in 4-h cycles (2 min of influent filling, 230 min of aeration, 5 min of settling and 3 min of effluent withdrawal). Effluent was discharged 50 cm above the reactor bottom at a volumetric exchange ratio of 50%. The hydraulic residence time (HRT) was 8 h and the phenol loading rate was 1.5 g phenol l⁻¹ day⁻¹. The pH was maintained at 6.7 ± 0.2 with the addition of phosphate buffer. Fine air bubbles for aeration were supplied through a dispenser at the reactor bottom at an airflow rate of 3.0 l min⁻¹. The reactor was housed in a temperature-controlled room at 25°C and inoculated directly with activated sludge taken from Jurong municipal wastewater treatment plant in Singapore. At the same time, cells of the two isolates individually pre-grown in MP medium with 250 mg phenol l⁻¹ were mixed and then introduced into the reactor. The initial cell number densities for the two strains in the reactor were almost equal at approximately 1.0 × 10⁶ l⁻¹ (based on the reactor working volume).

To analyze changes of cell number of two strains with the reactor operation, sludge biomass samples were collected one or two times per week and fixed using 4% PFA and 50% ethanol, respectively, and stored in 50% ethanol at -20°C. Then strain-specific Pand822 probe and genus-specific Rhod138 probe were used to detect strains PG-01 and PG-03, respectively, through fluorescence in situ

hybridization (FISH) and confocal laser scanning microbiology (CLSM). EUB 338 probe was also used for detection of Eubacteria.

Hybridization conditions for the three probes were described in Chapter Five. Hybridizations for EUB338 probes were performed at 46°C with a formamide concentration of 10% in the hybridization buffer (Amann et al., 1990). Probes Pand822 and Rhod168 were hybridized at 50°C with formamide concentrations of 20% and 25%, respectively (Chapter Five). For combinations of probes with different optimal hybridization stringencies, two hybridizations were done successively. The first hybridization was done with the probe which required the higher formamide concentration and higher hybridization temperature, and this was followed by a second hybridization at a lower stringency.

Analytical methods

DNA extraction from activated sludge and granule and pure culture samples, and DGGE were performed as described in Chapter Five. Gram-stain test was performed as previously described (Smibert and Krieg, 1994). Enzyme activities of catechol 1, 2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) were measured as described in Chapter Four. Wastewater samples in the reactor were periodically analyzed for SS using standard methods (APHA 1998). Phenol was measured spectrophotometrically by the 4-aminoantipyrin method as described previously in Chapter Three.

Calculation of biomass cellular yield

Mathematically, the growth rate of microbial cells is frequently expressed as (Rittmann and McCarty, 2001; Shuler and Kargi, 2002):

$$\frac{dX}{dt} = Y \left(\frac{-ds}{dt} \right) - k_d X \quad (6.1)$$

where dX/dt represents the net growth rate ($\text{mg l}^{-1} \text{h}^{-1}$) of active organism (X, mg

l^{-1}), $-ds/dt$ represents the rate of disappearance ($mg\ l^{-1}\ h^{-1}$) of substrate (s , $mg\ l^{-1}$), k_d is the endogenous metabolism or decay rate (h^{-1}) of the organism, and Y is the true yield of microorganism ($mg\ biomass\ mg\ substrate^{-1}$).

Equation (6.1) can be changed as follows:

$$\frac{dX}{Xdt} = Y \left(\frac{-ds}{Xdt} \right) - k_d \quad (6.2)$$

As $\frac{dX}{Xdt} = \mu$ (specific growth rate, h^{-1}), and $\frac{-ds}{Xdt} = q$ (specific phenol degradation rate, $mg\ substrate\ mg\ biomass^{-1}\ h^{-1}$), then

$$\mu = Y q - k_d \Rightarrow Y = \frac{\mu}{q} + \frac{k_d}{q} \quad (6.3)$$

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

$$Y = Y_{obs} + \frac{k_d}{q} \quad (6.4)$$

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

Percentage of total phenol converted to energy production

Phenol was consumed mainly for assimilation into biomass and for energy for cell growth and maintenance (Bailey and Ollis 1986; Shuler and Kargi, 2002).

$$\Delta s = \Delta s_1 + \Delta s_2 \quad (6.5)$$

where, Δs is total disappear of phenol ($mg\ l^{-1}$), Δs_1 is phenol for assimilation into biomass ($mg\ l^{-1}$), and Δs_2 is phenol transformed for energy production ($mg\ l^{-1}$).

$$Y_{obs} = \Delta X / \Delta s \Rightarrow \frac{1}{Y_{obs}} = \Delta s / \Delta X \quad (6.6)$$

$$Y_E = \Delta X / \Delta s_2 \Rightarrow \frac{1}{Y_E} = \Delta s_2 / \Delta X \quad (6.7)$$

where, ΔX is net growth of biomass ($mg\ l^{-1}$), and Y_E represents the yield of cell

mass on phenol consumed for energy (mg biomass mg substrate⁻¹).

$$\frac{1}{Y_{obs}} - \frac{1}{Y_E} = (\Delta s - \Delta s_2) / \Delta X = \Delta s_1 / \Delta X = \frac{1}{Y_C} \quad (6.8)$$

here, Y_c represents the theoretical yield of cell mass on phenol (mg biomass mg substrate⁻¹); Y_c can be calculated theoretically to be 1.67 g biomass g⁻¹ phenol based on the assumption that the carbon content of dry cells is about 46% (Blank and Clark, 1996). Then,

$$\frac{1}{Y_E} = \frac{1}{Y_{obs}} - \frac{1}{Y_C} \quad (6.9)$$

Y_E can therefore be determined according to Equation (6.9). The percentage of the total substrate carbon converted to energy for cell growth and maintenance ($\Delta s_2 / \Delta s$) can be obtained as Y_{obs} / Y_E , and the percentage of the total substrate carbon assimilated into biomass ($\Delta s_1 / \Delta s$) can be obtained as Y_{obs} / Y_c .

6.3 RESULTS

Characteristics of two strains isolated from phenol-degrading aerobic granules

Strains PG-01 and PG-03 were among ten isolates from matured phenol-degrading aerobic granules (Chapter Five). PG-01 is rod-shaped and Gram negative, while PG-03 is a coccus and Gram positive (Fig. 6.1). Two strains did not show any flocculation activity. The 16S rDNA gene sequences of PG-01 and PG-03 were 98.7% and 99.8% identical to those of *Pandoraea apista* and *Rhodococcus erythropolis*, respectively. The two strains grew faster in liquid phenol medium than the other eight isolates.

Fig. 6.1 Gram-staining of stains PG-01 (a) and PG-03 (b).

The bacterial populations in the seeding activated sludge and mature granules were detected by extracting DNA and then performing a DGGE analysis of the 16S rDNA fragments amplified from the extracted DNA (Fig 6. 2). The strains PG-01 and PG-03 was not among the dominant microorganisms in the seeding activated sludge. However, strain PG-01 was determined to be a dominant microorganism in the aerobic granules as the DNA fragment for PG-01 had an identical sequence to one of the DGGE bands from the mature granules. The dominance of strain PG-01 in granules was also confirmed through FISH and CLSM experiments. Direct in situ hybridization counting after dispersing the granules revealed strain PG-01 had a relative abundance of $4.1 \pm 3.2\%$ relative to number of cells hybridized by EUB338 probe, while strain PG-03 had a relative abundance of less than 0.1% relative to number of cells hybridized by EUB338 probe (Chapter Five).

Biomass growth, phenol degradation and enzyme activities for the two strains at a phenol concentration of 250 mg l^{-1}

With a volumetric exchange ratio of 50%, phenol concentrations in the reactors at time zero were approximately half the sum of the influent and effluent phenol concentrations. Because influent phenol concentration was 500 mg l^{-1} and effluent phenol concentrations in the reactor were less than 0.2 mg l^{-1} , the initial phenol concentration in the reactor was about 250 mg l^{-1} (Chapter Three). Thus, characteristics of phenol degradation process for these two strains were investigated using an initial phenol concentration of 250 mg l^{-1} .

Cells in the exponential growth phase with phenol as sole carbon and energy source were used. At regular intervals, cell growth of the strains was monitored by OD with the UV-visible spectrophotometer at 600 nm, and phenol concentrations were measured. Prior to the initiation of experiments, the correlations between the DAPI counts and OD values for both strains PG-01 and PG-03 were evaluated.

The good linear relationships between OD values and the total cell numbers counted using DAPI stain were confirmed at OD values less than 0.4.

Fig. 6.2. DGGE profiles of 16S rDNA fragments after PCR amplification of extracted nucleic acids from flocculated activated sludge, matured aerobic granules, and strains PG-01 and PG-03. The sources of the DNA fragments are indicated above the lanes.

Biomass growth and phenol degradation processes for strains PG-01 and PG-03 at an initial phenol concentration of 250 mg l^{-1} are shown in Fig. 6.3 and Fig. 6.4. The inoculated biomass contents for the two cultures were almost the same. Fig. 6.3 and Fig. 6.4 show that the cells of strain PG-01 grew faster and degraded more phenol than those of strain PG-03 during the initial periods. Based on the information on the initial rate of biomass growth, phenol disappearance and total cell numbers, specific biomass growth rate (μ) and phenol degradation rate (q) at the initial time were determined and listed in Table 6.1. While the specific biomass growth rate of PG-01 was higher than that of PG-03 by 20%, the specific phenol degradation for PG-01 was about 1.5 times higher than that of PG-02. So, the yield (Y_{obs}) at the initial time calculated for PG-01 was 20% less than that for PG-03. Y_{obs} calculated for PG-01 and PG-03 at the initial time were 0.637 and $0.792 \text{ g DW g phenol}^{-1}$, respectively.

Table 6.1. Specific growth and phenol degradation rates, biomass yields, and specific oxygen utilization rate of strains PG-01 and PG-03 at the initial phenol concentration of 250 mg l^{-1} .

	Specific growth rate (μ) (h^{-1})	Specific phenol degradation rate(q) (g phenol $\text{g}^{-1} \text{ DW h}^{-1}$)	Biomass yield ^a (μ/q) (g DW g^{-1} phenol)	Specific oxygen utilization rate (mg DO $\text{mg}^{-1} \text{ DW h}^{-1}$)
PG-01	0.186 ± 0.004	0.292 ± 0.011	0.637	0.225 ± 0.016
PG-03	0.116 ± 0.005	0.146 ± 0.007	0.795	0.186 ± 0.010

^a calculated for the initial time.

According to equation (6.9), the values of Y_E for strains PG-01 and PG-03 at the initial time were calculated to be 1.03 and $1.52 \text{ g DW g}^{-1} \text{ phenol}$, respectively. Then the percentages of the total substrate carbon converted to energy for cell growth and maintenance (Y_{obs}/Y_E) for strains PG-01 and PG-03 at the initial time were 61.9 and 52.4% respectively, while the percentages of the total substrate carbon assimilated into biomass (Y_{obs}/Y_c) for strains PG-01 and PG-03 at the initial time were 38.1 and 47.6% , respectively.

Fig. 6.3 Biomass growth for strains PG-01 and PG-03 at the initial phenol concentration of 250 mg l^{-1} .

Fig. 6.4 Phenol degradation for strains PG-01 and PG-03 at the initial phenol concentration of 250 mg l^{-1} .

The initial physiological responses of the microorganism to environmental conditions are important for its competitive behavior (Ka et al., 1994). This was investigated by examining the phenol degradation pathways employed by the two strains. Aerobic degradation of phenol by microorganisms is mainly based on the *ortho* and *meta* cleavage pathways, with the help of the C12O and C23O enzymes, respectively (Müller and Babel, 1996). The enzyme activities of C12O and C23O for the two strains during the initial 6 hours were listed in Table 6.2. C12O activities of PG-01 were completely inhibited during the initial five hours, suggesting that phenol was almost degraded through the *meta* pathway by PG-01. On the contrary, C12O activities were much higher than C23O activities for strain PG-03, and C23O accounted for less than 18% of total enzyme activity (Fig. 6.5).

Table 6.2. Enzyme activities for strains PG-01 and PG-03 during the initial period at the initial phenol concentration of 250 mg l⁻¹.

Operation time (h)	C12O (μmol mg ⁻¹ protein min ⁻¹)		C23O (μmol mg ⁻¹ protein min ⁻¹)	
	PG-01	PG-03	PG-01	PG-03
0.5	0	0.53 ± 0.06	0.42 ± 0.03	0
1.0	0	0.21 ± 0.02	0.22 ± 0.10	0.03 ± 0.01
1.5	0	0.3 ± 0.02	0.24 ± 0.05	0.05 ± 0.02
2.0	0	0.25 ± 0.03	0.32 ± 0.05	0
4.5	0	0.38 ± 0.07	0.43 ± 0.02	0.08 ± 0.01
6.0	0.07 ± 0.01	0.43 ± 0.04	0.39 ± 0.06	0.05 ± 0.01

Fig. 6.5 Ratios of C23O activity to total enzyme activities for strains PG-01 and PG-03

Respiratory activity for the two strains at a concentration of 250 mg l⁻¹

Specific oxygen utilization rates of cells were determined through a DO meter with Clark-type polarographic oxygen electrodes. At the phenol concentration of 250 mg l⁻¹, the specific oxygen utilization rates of cells of strains PG-01 and PG-03 were 0.225 and 0.186 mg DO mg⁻¹ DW h⁻¹, respectively (Table 6.1).

The respiratory activities in terms of O₂ consumption and CO₂ production, were also measured through a computer-controlled respirometer. The biomass concentrations and liquid volumes for PG-01 and PG-03 were similar to those used in the biomass growth experiments. Experiments were performed in duplicate. Fig. 6.6 shows that both O₂ consumption and CO₂ production by strain PG-01 were higher than those by strain PG-03 during the initial 32 hours. The cumulative O₂ consumption and CO₂ production by strain PG-03 exceeded that for strain PG-01 during the next eight hours. Beyond 40 hours, strain PG-01 consumed more O₂ and produced more CO₂ than strain PG-03. While phenol was completely removed in the first 25 hour (Fig 6.4), both CO₂ production and oxygen consumption for both

strains continued to increase for a period as shown in Fig. 6.6. The dissociation between phenol degradation and CO₂ production may imply that cells can utilize stored polymers or other cellular substances.

Fig 6.6. Respiratory activity at the initial phenol concentration of 250 mg l⁻¹. (a) accumulated CO₂ production content; (b) accumulated O₂ consumption content.

Ratios of CO_2 production to O_2 consumption by the two strains were calculated and shown in Fig. 6.7. For both strains, the ratios increased gradually with time, and stabilized after about 40 hours. The ratios for PG-01 were larger than those for PG-03 during the initial 32 hours. After that, the ratios for both strains were almost similar at 0.8.

Fig 6.7. Ratio of CO_2 production to O_2 consumption at the initial phenol concentration of 250 mg l^{-1} .

Growth and phenol degradation kinetics at various phenol concentrations

The specific growth and phenol degradation rates for cells of the two strains in the exponential growth phase were measured at various initial phenol concentrations ranging from 10 to 1000 mg l^{-1} (Fig. 6.8a, b). Strains PG-01 and PG-03 were capable of degrading phenol at phenol concentrations as high as 1000 mg l^{-1} . The specific growth rates for the two strains increased with phenol concentration from 0 to $50 \text{ mg phenol l}^{-1}$, and declined with further increases in phenol concentrations as substrate inhibition effects became important. In general, changes of specific phenol degradation rates with initial phenol concentrations followed the trend of changes of specific growth rates. While the specific growth and phenol degradation rates for PG-03 were higher than those for PG-01 at phenol concentrations less than 50 mg l^{-1} , the reverse was observed at phenol concentrations above 50 mg l^{-1} .

Fig. 6.8. Specific growth rates (a) and phenol degradation rates (b) of strains PG-01 and PG-03 at different phenol concentrations.

Although kinetics parameters can be determined from the disappearance of substrate ($-ds/dt$) or appearance of biomass (dX/dt) as a function of time, the validity of the results obtained can be questionable (Marangoni 2003). Therefore, in this study, Kinetics parameters were fitted using the Haldane equation as described in Chapter Five through determining specific rates at different initial phenol concentrations. The $\mu_{\max}$, K_s and K_i of strain PG-01 for growth kinetics were found to be 0.45 h^{-1} , 30.4 mg l^{-1} and 205.9 mg l^{-1} , respectively. Compared to strain PG-01, strain PG-03 showed a 1.5-fold higher $\mu_{\max}$ (0.65 h^{-1}) but 2.9-fold and 4.5-fold lower values of K_s (10.4 mg l^{-1}) and K_i (30.9 mg l^{-1}), respectively (Table 6.3). The ratio ($\mu_{\max}/K_s$) for strain PG-01 was 0.015, lower than the value of 0.065 for strain PG-03. The $q_{\max}$, K_s and K_i of strain PG-01 for phenol degradation kinetics were calculated to be $0.91 \text{ g phenol g DW}^{-1} \text{ d}^{-1}$, 68.5 mg l^{-1} and 150.2 mg l^{-1} , respectively (Table 6.3). The phenol degradation kinetic parameters for strain PG-03, with $q_{\max}$ of $0.7 \text{ g phenol g DW}^{-1} \text{ d}^{-1}$, K_s of 18.7 mg l^{-1} and K_i of 48.7 mg l^{-1} , were all lower than those for PG-01. However, K_i for strain PG-03 for oxygen utilization kinetics was higher than that for strain PG-03 (Table 5.11). One possible explanation was that strain PG-03 may adjust its metabolism to oxidize other substances for maintenance or growth under high phenol concentrations.

The degradation kinetics of phenol have been widely investigated under mesophilic conditions, both for mixed and pure cultures. Most investigators found that the Haldane equation fitted to their data satisfactorily described the growth kinetics (Yang and Humphrey, 1975; Onysko et al., 2000). However, measured growth rates for strain PG-01 at phenol concentrations higher than 600 mg l^{-1} were much lower than those calculated from Haldane equation (Fig. 6.8a). This suggested that additional inhibition parameters should be incorporated into the Haldane model under high phenol concentrations.

Table 6. 3. Parameters for growth and phenol degradation kinetics of strains PG-01 and PG-03

<i>Growth kinetics for strains PG-01 and PG-03</i>				
	$\mu_{\max}$ (h ⁻¹)	K_s (mg l ⁻¹)	K_i (mg l ⁻¹)	$\mu_{\max} / K_s$
PG-01	0.45 ± 0.06	30.4 ± 6.1	205.9 ± 45.8	0.015
PG-03	0.65 ± 0.04	10.4 ± 2.9	45.9 ± 12.5	0.063
<i>Phenol degradation kinetics for strains PG-01 and PG-03</i>				
	$q_{\max}$ (g phenol g DW ⁻¹ h ⁻¹)	K_s (mg l ⁻¹)	K_i (mg l ⁻¹)	$q_{\max} / K_s$
PG-01	0.91 ± 0.08	68.5 ± 9.2	150.2 ± 23.7	0.013
PG-03	0.70 ± 0.06	18.7 ± 3.6	48.7 ± 9.4	0.037

Inhibitory screening

Inhibitory screening experiments using a plate assay show that there was no evidence for release of antimicrobial agents from the two cultures. For each test culture, five different cell concentrations in exponential-phase or stationary-phase for PG-01 or PG-03 were prepared and spotted onto agar plates seeded with an indicator strain PG-03 or PG-01. After 3-14 days of incubation, zones of inhibition were not observed for both cultures. In addition, cells of the two strains in exponential-phase or stationary-phase were mixed and diluted, and spread onto MP medium (supplemented with 500 mg phenol per liter) agar plates and R2A agar plates, respectively. After 3-10 days of incubation, colonies of PG-01 and PG-03 were observed to grow side by side on either MP medium (supplemented with 500 mg phenol per liter) agar plates or R2A agar plates.

Competition in batch culture at a phenol concentration of 250 mg l⁻¹

Strains PG-01 and PG-03 were grown either as monocultures (Fig. 6.9a) or together in a mixed culture in batch culture systems (Fig. 6.9b). Although the outcome of competition is independent of inoculum size (Shi et al., 1997), the initial biomass content for each culture was set at similar levels in this study. As

shown in Fig. 6.9a, cell densities for each culture increased after 9 hours of incubation, and peaked at about 35 hours. When two strains were mixed together, the growth of PG-03 was inhibited (Fig. 6.9b). From the beginning, cell densities of PG-03 decreased. No PG-03 colony could be found on plates even with low dilution factors after 12 hours of incubation. On the contrary, there was not much negative effect on growth of PG-01. Growth of PG-01 peaked at about 35 hours after the start of incubation.

Cell growth after augmentation in a sequencing batch reactor

The reactor was operated about two months. Although the inoculated activated sludge was not acclimated, the phenol in influent was completely degraded after two days of operation. With reactor operation, biomass concentrations in the reactor and biomass settleability increased, and stabilized after about one month of operation (Fig. 6.10). Aerobic granules were observed in the reactor after about ten days.

Fig. 6.9. Time course analysis of the cell densities of strains PG-01 and PG-03 collected from batch culture systems where the strains were established either as monospecies cultures (a) or as mixed cultures (b). CFU densities were enumerated with R2A plates.

To detect the change of cell number for strains PG-01 and PG-03 with reactor operation, sludge biomass samples were fixed and hybridized firstly with Pand822 probe or Rhod168, then with EUB338 probe. Cells that hybridized with Pand822 probe, Rhod168 and EUB338 probes were counted through CLSM. The competitive advantage of strain PG-01 over strain PG-03 was evident. After one day of operation, cells binding *Rhodococcus*-specific probe Rhod168 were rarely detected. On the contrary, the relative abundance of cells binding probe Pand822, which was strain PG-01-specific, increased quickly with reactor operation. As inoculated biomass was not acclimated, the relatively high concentration of phenol introduced into the reactor was likely degraded mainly by the bioaugmented strain PG-01. So, cells of strain PG-01 grew fast during the initial period. At the same time, some microorganisms might be washed out, while other microorganisms in the inoculated biomass might successfully adapt to the new environment and thrive. Under effects of environment conditions and various interactions among these organisms, a microbial community would evolve that was different from the initial one.

Fig. 6.10. Reactor operation after bioaugmentation

6.4 DISCUSSION

Kinetic and metabolic analysis of competition in batch culture systems

A prominent concept in microbiology has been to inoculate a habitat with a strain that has desired properties to initiate or enhance a desired process, or to manipulate microbial diversity to increase functional redundancy (Massol-Deyá et al., 1997; Briones and Raskin, 2003). Whether the inoculated strain can establish a high level of abundance mainly depends on its physiological traits for competitive advantages. So, knowledge of these properties is necessary and important prior to carrying out experiments. In this study, the interaction experiment between two fast-growing strains isolated from phenol-degrading aerobic granules was firstly carried out in batch cultures, to analyze the physiological properties that may contribute to microbial dominance. Results show that there exists competitive interaction between strains PG-01 and PG-03, and the strain PG-01 outcompeted the strain PG-03.

The outcome of a competition experiment between different bacterial strains is mainly determined by the relationship between the growth rate and the residual substrate concentration. The properties of the initial growth and substrate uptake kinetics of the microorganism are important for its competitive behavior (Ka et al., 1994). Strain PG-01 had higher specific growth and phenol degradation rates than strain PG-03 at phenol concentrations larger than 80 mg l⁻¹. Therefore, kinetics advantages for PG-01 may be one of explanation for batch culture competition experiments with initial phenol concentration of 250 mg l⁻¹.

The biosynthesis of antimicrobial compounds can mediate microbial interactions (Chen and Weimer, 2001; Slattey et al., 2001; Riley and Wertz, 2002), but there is no evidence for release of antimicrobial agents in these experiments. Difference in degradation pathways for aromatic substrate can also affect the competition

outcome (Duetz et al., 1994; Filonov et al., 1997; Krooneman et al., 1998). With phenol, degradation may proceed via either the *ortho* (C12O) or the *meta* (C23O) cleavage pathway (Leonard and Lindley, 1998; Kiesel and Müller, 2002). Microorganisms can degrade phenol via one or two of these pathways. Table 6.2 shows that only *meta* degradation pathway for strain PG-01 was applied during the initial period, while strain PG-03 degraded phenol mainly based on the *ortho* pathway as C12O activities were much larger than C23O activities. Previous studies on growth kinetics have pointed to a correlation between the growth rate and the assimilatory pathway employed in phenol utilization. High-affinity/low-rate properties are found for *ortho* pathway in contrast to low-affinity/high-rate properties in situations with *meta* pathway (Filonov et al., 1997). As a result, the bacteria grow faster catabolizing phenol via the *meta* pathway rather than the *ortho* pathway at high phenol concentrations.

Besides growth rates, biomass yield and energy production were influenced by degradation pathways employed. As discussed in Chapter Four, compared to the *ortho* pathway, the *meta* pathway leads to lower biomass yields and production of higher energy production. It seems that the *meta* pathway applied for strain PG-01 may partially explain the measured lower biomass yield at the initial time as shown in Table 6.1. Meanwhile, the lower biomass yield at the initial time for strain PG-01 also corresponded to its respiratory activity. Phenol was consumed mainly for assimilation into biomass and for energy production. Energy is generated by respiration, in which process electrons are transported through the electron transport system from an electron donor (phenol) to an electron acceptor (O_2). Accompanied by the production of energy, phenol was transformed into CO_2 . It is possible to calculate the biomass yield through distinguishing between the fraction of phenol that is channeled into the biosynthesis function by anabolism and the fraction of phenol that is assimilated into energy function through catabolism. Fig.

6.7 shows that the ratios of CO₂ production to O₂ consumption for PG-01 were higher than those for PG-03 during the initial period. Namely, biochemical reactions in cells of PG-01 produced more CO₂ when using the same amount of oxygen compared to those of PG-03. This meant that cells of strain PG-01 assimilated higher fraction of phenol into CO₂ for energy production. Then, biomass yield decreased, and the percentage of phenol converted to energy ($Y_{\text{obs}}/Y_{\text{E}}$) increased.

It is obvious that strain of PG-01 was characterized with high growth rates and low biomass yields during the initial period. For fundamental thermodynamic reasons, there is always a trade-off between yield and rate of energy production in heterotrophic organisms. In fact, a rapidly growing cell population will use more substrate for assimilation and growth energy (Bailey and Ollis, 1986). Cells with a higher rate but lower yield may gain a selective advantage when competing for shared energy resources (Pfeiffer et al., 2001).

As discussed above, the preference for *meta* pathway leads to a higher value of $Y_{\text{obs}}/Y_{\text{E}}$, a higher oxygen utilization rate and a higher respiratory activity that directly correlated with the electron transport system activity, and results in more energy production in strain PG-01. Energy is normally used for cell growth and maintenance. More energy is needed to drive higher growth rates. In addition, energy could be used for cell adaptive responses. Microorganisms are known to respond swiftly and appropriately to sudden environmental changes by changing genomic expression programs, in an attempt to set better structural and functional conditions to face adverse environments (Gasch et al., 2000). Regulated and coordinated gene expression provides competitive advantages in highly variable environments (Cases et al., 2003). A high rate of energy consumption is needed for gene expression and transcription under environmental stress (Alexandre et al.,

2001; Varela et al., 2003). According to the results about growth and phenol degradation kinetics, phenol toxicity to cell growth was evident at phenol concentration of 50 mg l⁻¹, and became stronger with increasing phenol concentrations. So, it is very likely that high amount of energy available for PG-01 might enhance gene expression for quick adaptive response when facing toxicity of high phenol concentrations.

Comparison of batch culture and bioaugmentation experiments

The bioaugmentation experiment was mainly consistent with batch culture experiments. This was not surprising as the more competitive strain within natural ecological systems is the one that has the highest affinity and is able to adapt quickly to changes in environmental conditions (Lenski et al., 1998; Pfeiffer et al., 2001). It was known through analysis of physiological characteristics that strain PG-01 possessed superior competitive advantages under batch culture systems. The reactor for bioaugmentation was operated under the sequencing batch mode with six cycles in one day. The constant selective pressure resulting from reactor operation then favored the promulgation of the added PG-01 culture.

However, there was an interesting anomaly between batch culture and bioaugmentation experiments. While no colony of strain PG-03 could be found on plates after 10 hours of incubation in batch culture, cells of PG-03 could be isolated from aerobic granules (Chapter Five), albeit at low cell densities. In fact, a biofilm can serve as a refugium for a competitively inferior population (Massol-Deya et al., 1997). The structural complex of aerobic granules is important for population-level diversification because it allows resources to be partitioned and creates new niches, thereby enhancing microorganism speciation and division into distinct ecological species. Thus, the potential for spatial isolation provided by granule structure may provide a mechanism for controlling microbial

diversity in granules that differs from suspended cultures.

It would be premature to draw general conclusions about the microbial interactions in the microbial community of aerobic granules based on the batch culture experiments. Nevertheless, our results provide a basis to manipulate the microbial community of aerobic granules, and can help to establish laboratory evaluation methods for selecting strains for bioaugmentation. Bioaugmentation can enhance the microbial functions of microbial communities by introducing microorganisms with the desired catabolic traits. However, the inoculated strains are usually of little effectiveness once they are augmented into an established microbial community as bioaugmented strains cannot maintain a high level of abundance in most experiments. Although some strategies were investigated to increase the successful possibility of bioaugmentation (Boon et al., 2002), it is desirable to develop laboratory evaluation methods to select suitable strains for bioaugmentation (Watanabe et al., 1998b). Here, batch competition experiments between/among candidate strains in the microbial community are recommended before bioaugmenting strains into aerobic granules or other microbial communities. Suitable inocula can be developed by designing appropriate competition experiments.

In summary, the overall success of strain PG-01 in batch culture experiment may be due to the combination of several factors: (1) high growth and phenol degradation rate; (2) *meta* degradation pathway employed; (3) high respiratory activities; (4) high amount of energy produced at the expense of low biomass yield. These physiological properties as well as the constant selective pressure resulting from reactor operation then favored the promulgation of the added PG-01 culture in SBR after bioaugmentation, and helped to maintain its numerical dominance in aerobic granules. The consistence between batch culture and SBR operation

experiments suggested that the analysis of physiological traits for dominant species in microbial community of aerobic granules revealed information useful for manipulation of the aerobic granulation process and optimization of microbial community.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

As a relatively new form of cell immobilization, aerobic granules are self-immobilized bacterial aggregates from activated sludge. Compared to conventional activated sludge flocs, the advantages of aerobic granules are denser and more compact microbial structure, better settling ability and higher biomass retention. Using phenol as a surrogate, this study demonstrates for the first time that aerobic granules can tolerate and degrade high loads of toxic and inhibitory substrates. The formation process of phenol-degrading aerobic granules, the metabolic responses of granules to phenol loadings, and the microbial community of granules have been systematically investigated, through monitoring the reactor performances as well as employing traditional cultivation and molecular methods.

In this study, a column-type sequential aerobic sludge blanket reactor was inoculated with activated sludge and fed with phenol as the sole carbon source, at a rate of $1.5 \text{ g phenol l}^{-1} \text{ day}^{-1}$. Aerobically grown microbial granules first appeared on day 9 of reactor operation and quickly grew to displace the seed flocs as the dominant form of biomass in the reactor. These granules were compact in appearance, and consisted of bacterial rods and cocci and fungi embedded in an extracellular polymeric matrix. The granules had a mean size of 0.52 mm and a sludge volume index of $40 \text{ ml g}^{-1} \text{ SS}$. Specific activities of biomass increased with aerobic granulation and stabilized thereafter at a specific oxygen utilization rate of $110 \text{ mg oxygen g}^{-1} \text{ VSS h}^{-1}$.

The compact structure of aerobic granules offers several advantages, namely easy separation of biomass from bulk liquid during settling phase, high biomass retention and protection of cells against the effects of phenol toxicity. Through the combination of the advantages of the SBR technology and the granular form of cell immobilization, the aerobic granulation system showed good stability under

loading fluctuation and ability to handle high phenol loading rates. At a loading of 2.5 g phenol l⁻¹ day⁻¹, phenol was completely degraded and high biomass concentration with good settling ability was maintained in the reactor. The granules remained stable and continued to degrade phenol efficiently for several weeks after attaining the maximum loading, without compromising granule integrity and function. Compared to conventional activated sludge systems and SBR with flocculated sludge, phenol-degrading aerobic granules can be exploited to design compact and high-rate aerobic granulation systems for the treatment of industrial wastewaters containing high concentrations of phenol and other inhibitory chemicals.

In order to investigate the effect of phenol loading on structure, activity and metabolism of aerobic granules, four identical column-type sequential aerobic sludge blanket reactors were employed with phenol as the sole carbon and energy source and operated at loading rates of 1.0, 1.5, 2.0 and 2.5 g phenol l⁻¹ day⁻¹. Compact granules with good settling ability were maintained at loadings up to 2.0 g phenol l⁻¹ day⁻¹, and structurally weakened granules with enhanced production of extracellular polymers and proteins and lower hydrophobicities were observed at the highest loading of 2.5 g phenol l⁻¹ day⁻¹. Specific oxygen uptake rate, catechol 2,3-dioxygenase (C23O) and catechol 1,2-dioxygenase (C12O) activities peaked at a loading of 2.0 g phenol l⁻¹ day⁻¹, and declined thereafter. Granules degraded phenol completely in all four reactors, mainly through the *meta* cleavage pathway as C23O activities were significantly higher than C12O activities. The microbial community in the aerobic granules was capable of adaptive changes to tolerate the range of phenol loadings tested. At the highest loading applied, the anabolism and catabolism of microorganisms were regulated such that phenol degradation proceeded exclusively via the *meta* pathway, apparently to produce more energy for overstimulation of protein production against phenol toxicity.

16S rDNA DGGE and isolation methods were combined in this study to assess the community structure and the functional diversity of microbial community in

phenol-degrading aerobic granules cultivated at a loading of 1.5 g phenol l⁻¹ day⁻¹. The DGGE fingerprint indicated that about ten dominant populations were detected in aerobic granules. Ten pure cultures were isolated from phenol-degrading aerobic granules, and characterized. Three isolates PG-01, PG-02 and PG-08 had identical 16S sequences to three dominant populations in granules. Both DGGE and isolation demonstrated that beta subclass of the *Proteobacteria* and high G+C Gram-positive bacteria had significant presence in phenol-degrading aerobic granules. The ten isolates showed different physiological traits, in terms of specific growth rates, kinetics, enzyme activities and flocculated activities. All isolates belonging to dominant populations were found to possess more of the physiological properties associated with competitive and growth advantages.

Isolation, DGGE and hybridization with specific probe confirmed that isolate PG-01 was numerically abundant in the aerobic granules. Combined with its highest growth and phenol degradation rate among all isolates, isolate PG-01 could be regarded as one of the functionally dominant strains and may have contributed significantly to phenol degradation in the granules. The physiology of PG-01 was then characterized in detail so as to investigate why the PG-01 had become dominant. The batch culture competition experiment involving strain PG-01 and PG-02 suggested that the numerical dominance of PG-01 in granules may be due to the combination of several physiological traits: (1) high growth and phenol degradation rate; (2) *meta* degradation pathway employed; (3) high respiratory activities; (4) high energy production at the expense of low biomass yield. The favored growth of PG-01 in aerobic granules was further demonstrated through bioaugmenting the two strains into a SBR and monitoring the change in abundances of cells with reactor operation using molecular methods. The analysis of physiological traits for dominant species in the microbial community of aerobic granules revealed information useful for manipulation of the aerobic granulation process and optimization of microbial community.

7.2 LIMITATION OF THIS STUDY

This study focused on development of phenol-degrading aerobic granules and investigation of microbial community within aerobic granules. After analyzing the experimental data available, the following limitations were felt:

There are lots of compounds in industrial wastewaters. Inter-inhibition among compounds on microorganisms may be complex. For simplification, single substrate (phenol) was selected as sole carbon and energy source in this study. However, for consideration of engineering, study on biodegradation of mixed substrates will be practical.

Although aerobic granule-based systems compare favorably with conventional SBRs and activated sludge systems, comparison of phenol degradation rates between granules and activated sludge flocs with same microbial diversity was not made. In addition, effect of different granule size on phenol degradation was not study. These information will help improve the treatment capacity of aerobic granule-based systems.

Although DGGE data in Chapter Five showed that microbial diversities between seeding activated sludge and aerobic granules at the stable state were total different, shift in microbial community during aerobic granules was not investigated in detail. The analysis of change in microbial diversity under selection pressure may help manipulate the microbial community of aerobic granules.

7.3 RECOMMENDATIONS FOR FURTHER STUDY

Aerobic granules can treat phenol wastewater with high organic loading, and lead to design of compact, high-rate aerobic granulation systems for the treatment of industrial wastewaters containing high concentrations of phenol and other inhibitory chemicals. It is possible to exploit this novel system for phenol wastewater treatment with higher organic loadings, if reactor operations are further optimized and in-depth knowledge about the microbial community in the granules is acquired.

To optimize the operational parameters for aerobic granule systems. The effect of external energy input, like those caused by aeration and mechanical stirring, on system performance could be studied. Cycle times and starvation times are important factors that need to be tested. Knowledge about how to adjust reactor operational parameters to treat phenol with very high concentrations is required. It is hoped that optimum operational parameters would be obtained. As a result, higher organic loading will be applied with less amount of energy input.

To analyze interactions among isolates PG-01, PG-02 and PG-08 within aerobic granules. Isolates Pg-01, PG-02 and PG-08 had identical 16S sequences to three dominant populations in granules. Interestingly, the three strains possessed some physiological properties associated with competitive and growth advantages. Although in situ distribution of PG-01 was determined, it is worthwhile to determine the abundance of the two strains (PG-02 and PG-08) within granules using FISH with specific-probes or quantitative PCR with specific-primers, and their spatial distributions within granules with combination of FISH and CLSM. Based on these information, interactions among the three isolates were further analyzed. These information plus their physiological characterization may help in the development of a functional model of microbial community in aerobic granules.

To improve the treatment capacity of the system through bioaugmentation. Increasing loadings to some critical points can lead to changes in function of microbial communities and eventual failure. One strategy to improve a particular aspect of process performance in a wastewater treatment system, for example after its breakdown, is bioaugmentation with specialized microorganisms. Therefore, if microbial communities of aerobic granules are monitored during exposure to increasing phenol loading and changes in functional group of microbial communities are identified, the knowledge could be exploited to treat higher phenol loadings through bioaugmenting microorganisms with special physiological functions.

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APPENDIX A

Calculation of specific growth rate for isolates

An example about calculation of specific growth rate for PG-01 at an initial phenol concentration of 250 mg l⁻¹ is shown as follows:

First, drawing the figure based on data about biomass growth (OD) with time;

Then, through fitting these data using EXCEL software, the equation describing oxygen concentration (X) change with time can be obtained as following:

$$X = 0.005 t + 0.0262$$

where, X is biomass concentration (OD), t is time (hour).

Specific growth rate (μ , h⁻¹) at the initial time can be calculated:

$$\mu = \left. \frac{dX}{Xdt} \right|_{t=0}$$

$$\frac{dX}{Xdt} = \frac{0.005dt}{(0.005t + 0.0262)dt} = \frac{0.005}{0.005t + 0.0262}$$

Then,

$$\mu = \left. \frac{dX}{Xdt} \right|_{t=0} = \frac{0.005}{0.0262} = 0.1908 \text{ h}^{-1}$$

APPENDIX B

Biomass growth of isolates at a phenol concentration of 250 mg l⁻¹

Biomass growth (OD)					Biomass growth (OD)						
Time (h)	PG-01		PG-03		Time (h)	PG-08		PG-09		PG-10	
	1ST	2ND	1ST	2ND		1ST	2ND	1ST	2ND	1ST	2ND
0	0.0252	0.0251	0.0279	0.0276	0	0.0061	0.006	0.0073	0.0072	0.0099	0.0099
1	0.0318	0.0331	0.0322	0.0304	2	0.0052	0.0055	0.0074	0.0074	0.01	0.0102
1.5	0.0351	0.0322	0.0317	0.0342	4	0.0056	0.0057	0.0075	0.0076	0.011	0.0109
3	0.0411	0.0425	0.0374	0.0354	8	0.006	0.0061	0.0084	0.0084	0.0121	0.0121
6	0.0545	0.0521	0.0473	0.0462	11	0.0064	0.0065	0.0104	0.01	0.0145	0.0145
7	0.0627	0.0608	0.0483	0.0499	15	0.0054	0.005	0.0138	0.014	0.0152	0.015
9	0.0719	0.0697	0.0591	0.0567	23.5	0.0042	0.004	0.0269	0.0268	0.0403	0.0403
12	0.1124	0.1091	0.0834	0.0826	27.5	0.0055	0.0054	0.0363	0.0363	0.0446	0.0443
16	0.172	0.1763	0.1042	0.1042	34	0.0042	0.004	0.0509	0.0507	0.05	0.0502
18.5	0.2253	0.2259	0.1709	0.196	48	0.0032	0.0032	0.0824	0.0826	0.0538	0.0543

Biomass growth (OD)										
Time (h)	PG-02		PG-04		PG-05		PG-06		PG-07	
	IST	2ND	IST	2ND	IST	2ND	IST	2ND	IST	2ND
0	0.0037	0.0039	0.0018	0.0018	0.0095	0.0093	0.0083	0.0083	0.0356	0.0355
2	0.004	0.0041	0.0008	0.0008	0.0093	0.0092	0.0106	0.01	0.02	0.02
3.5	0.0053	0.005	0.0008	0.0008	0.0098	0.0097	0.0096	0.0095	0.023	0.024
6.5	0.006	0.0063	0.0008	0.0009	0.0127	0.0125	0.0115	0.0119	0.0083	0.0079
9.5	0.0066	0.007	0.0009	0.001	0.0145	0.0149	0.0189	0.019	0.0055	0.0056
23.5	0.0518	0.00536	0.0021	0.0022	0.0353	0.0355	0.0457	0.0455	0.0033	0.0033
27.5	0.0903	0.0902	0.0028	0.0031	0.0394	0.0393	0.0503	0.0503	0.0047	0.0041

APPENDIX C

Henry's law constant for some aromatic compounds at 25°C (O'Neil, 2001).

Compounds	Benzene	Toluene	Ethylbenzene	<i>m</i> -Xylene	<i>o</i> -Xylene	<i>p</i> -Xylene
Henry's Law constant ^a	4.51	4.12	2.90	3.19	4.80	3.19

^a Henry's Law constant = $C_{\text{liquid}}/C_{\text{gas}}$

These compound solutions with some kinds of concentrations in liquid can be prepared after knowing the Henry's law constants. One example about preparing benzene solution with a concentration of 10 mg l⁻¹ was shown as follows:

Because $C_{\text{liquid}} = 10 \text{ mg l}^{-1}$, then C_{gas} can be calculated based on Henry's Law.

$$4.51 = 10 \text{ mg l}^{-1} / C_{\text{gas}} \Rightarrow C_{\text{gas}} = 10 \text{ mg l}^{-1} / 4.51 = 2.22 \text{ mg l}^{-1}$$

In one 50 ml serum bottle, the volume of liquid (V_{liquid}) is 10ml, then the volume of gas (V_{gas}) is 40ml.

So, the total benzene mass used is

$$\begin{aligned} C_{\text{liquid}} \times V_{\text{liquid}} + C_{\text{gas}} \times V_{\text{gas}} &= 10 \text{ mg l}^{-1} \times 0.01 \text{ l} + 2.22 \text{ mg l}^{-1} \times 0.04 \text{ l} \\ &= \mathbf{0.1888 \text{ mg}} \end{aligned}$$

APPENDIX D

Calculation of specific oxygen utilization rate for isolates

An example about calculation of specific oxygen utilization rate for PG-01 at an initial phenol concentration of 80 mg l^{-1} is shown as follows:

First, drawing the figure based on data about oxygen concentration (%) with time;

Then, the equation describing oxygen concentration (%) change with time can be obtained as following:

$$C_{\text{DO}(1)} = -0.004 t + 17.959$$

where, $C_{\text{DO}(1)}$ is oxygen concentration (%), t is time (second).

Experiments were performed at 25°C . According to YSI 5331 Oxygen Probe Instructions, the oxygen concentration ($C_{\text{DO}(2)}$) in units of milligrams per liter of water (mg DO l^{-1}) can be calculated from $C_{\text{DO}(1)}$.

$$\begin{aligned} C_{\text{DO}(2)} \text{ mg DO l}^{-1} &= C_{\text{DO}(1)} \times 8.6 \text{ mg l}^{-1}/21 = 0.41 C_{\text{DO}(1)} \text{ mg l}^{-1} \\ &= 0.41 (-0.004 t + 17.959) \\ &= -0.00164 t + 7.355 \end{aligned}$$

$$\begin{aligned}\text{So, oxygen utilization rate} &= 0.00164 \text{ mg DO l}^{-1} \text{ second}^{-1} \\ &= 0.00164 \text{ mg DO l}^{-1} \text{ second}^{-1} \times 3600 \text{ second h}^{-1} \\ &= 5.90 \text{ mg DO l}^{-1} \text{ h}^{-1}\end{aligned}$$

The medium volume in the sample chamber was 5 ml, then

$$\begin{aligned}\text{oxygen utilization rate} &= 5.90 \text{ mg DO l}^{-1} \text{ h}^{-1} \times 0.005 \text{ l} \\ &= 0.0295 \text{ mg DO h}^{-1}\end{aligned}$$

The biomass content used was 0.07 mg Dry Weight (DW), so the specific oxygen utilization rate can be calculated:

$$0.0295 \text{ mg DO h}^{-1} / 0.07 \text{ mg DW} = \mathbf{0.421 \text{ mg DO mg DW}^{-1} \text{ h}^{-1}}$$

APPENDIX E

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-01)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations											
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		80 mg phenol/l		150 mg phenol/l		200 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.3	17.6	17.5	17.8	17.9	18.2	18	17.8	17.4	18	16.5	16.6
30	17.2	17.5	17.4	17.7	17.8	18.1	17.9	17.7	17.3	17.9	16.4	16.5
60	17.1	17.4	17.3	17.7	17.7	18	17.8	17.6	17.2	17.8	16.3	16.4
90	17.1	17.4	17.3	17.6	17.6	17.9	17.7	17.5	17.1	17.7	16.3	16.4
120	17.0	17.3	17.2	17.5	17.6	17.8	17.6	17.4	17.1	17.6	16.2	16.3
150	16.9	17.2	17.2	17.4	17.5	17.7	17.5	17.3	17	17.5	16.2	16.2
180	16.9	17.1	17.1	17.3	17.4	17.7	17.4	17.2	16.9	17.4	16.1	16.1
210	16.7	17.0	17.0	17.3	17.3	17.6	17.4	17.1	16.8	17.3	16.0	16.1
240	16.7	16.9	16.9	17.2	17.2	17.5	17.3	17.0	16.7	17.3	16.0	16
270	16.6	16.9	16.9	17.1	17.1	17.4	17.2	16.9	16.6	17.2	15.9	15.9
300	16.5	16.8	16.8	17.0	17.0	17.3	17.1	16.8	16.5	17.1	15.8	15.8
330	16.5	16.7	16.7	17.0	16.9	17.2	17	16.7	16.4	17	15.7	15.8
360	16.4	16.7	16.7	16.9	16.8	17.1	16.9	16.6	16.3	16.9	15.7	15.7
390	16.4	16.6	16.6	16.8	16.7	17.0	16.8	16.5	16.2	16.8	15.6	15.6
420	16.3	16.6	16.5	16.7	16.7	17.0	16.6	16.4	16.1	16.7	15.5	15.5
450	16.2	16.5	16.4	16.6	16.6	16.9	16.5	16.2	16.0	16.6	15.5	15.5
480	16.2	16.4	16.4	16.6	16.5	16.8	16.4	16.1	16.0	16.5	15.4	15.4
510	16.1	16.4	16.3	16.5	16.4	16.7	16.3	16.0	15.9	16.4	15.3	15.0
540	16.0	16.3	16.3	16.4	16.4	16.7	16.2	15.9	15.8	16.3	15.2	15.2
570	16.0	16.2	16.2	16.4	16.3	16.6	16.1	15.8	15.7	16.2	15.1	15.1
600	15.9	16.1	16.1	16.3	16.2	16.5	16.0	15.7	15.6	16.1	15.1	15.0
630	15.8	16.0	16.1	16.2	16.1	16.4	15.9	15.6	15.5	16.0	15.0	15.0
660	15.7	15.9	16.0	16.1	16.0	16.3	15.8	15.4	15.4	15.9	14.9	14.9
690	15.7	15.9	15.9	16.0	16.0	16.3	15.6	15.3	15.3	15.8	14.8	14.8
720	15.6	15.8	15.8	16.0	15.9	16.2	15.5	15.1	15.2	15.7	14.7	14.7
750	15.6	15.8	15.8	15.9	15.8	16.1	15.4	15.0	15.1	15.6	14.6	14.6
780	15.5	15.7	15.7	15.8	15.7	16.0	15.3	14.9	14.9	15.5	14.5	14.5
810	15.4	15.6	15.6	15.7	15.6	15.9	15.1	14.7	14.8	15.4	14.4	14.4
840	15.4	15.6	15.5	15.6	15.5	15.8	15	14.6	14.7	15.3	14.3	14.3
870	15.3	15.5	15.5	15.6	15.4	15.7	14.8	14.4	14.6	15.1	14.3	14.2
900	15.2	15.4	15.4	15.5	15.3	15.6	14.6	14.2	14.4	15.0	14.2	14.1
960	15.0	15.2	15.2	15.3	15.1	15.4	14.4	13.9	14.2	14.8	14.0	13.9
1020	14.9	15.1	15.1	15.1	14.9	15.3	14.1	13.7	14.0	14.6	13.8	13.7
1080	14.7	14.9	14.8	14.9	14.7	15	13.9	13.5	13.7	14.3	13.5	13.5

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-01)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations									
	300 mg phenol/l		400 mg phenol/l		500 mg phenol/l		800 mg phenol/l		1000 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.1	17.1	17.2	17.3	18.1	18.2	17.5	17.6	17.8	17.7
30	17.0	17.0	17.2	17.3	18.1	18.1	17.5	17.6	17.8	17.7
60	17.0	17.0	17.2	17.2	18.0	18.1	17.5	17.6	17.8	17.7
90	16.9	16.9	17.1	17.2	18.0	18.0	17.4	17.5	17.8	17.7
120	16.9	16.9	17.1	17.1	17.9	18.0	17.4	17.5	17.8	17.7
150	16.8	16.8	17.0	17.1	17.9	17.9	17.4	17.5	17.8	17.7
180	16.8	16.7	17.0	17.0	17.9	17.9	17.4	17.5	17.8	17.7
210	16.8	16.7	16.9	17.0	17.8	17.9	17.4	17.5	17.8	17.7
240	16.7	16.7	16.9	17.0	17.8	17.8	17.4	17.5	17.8	17.7
270	16.7	16.6	16.8	16.9	17.7	17.8	17.4	17.5	17.8	17.7
300	16.7	16.6	16.8	16.9	17.7	17.8	17.4	17.5	17.8	17.7
330	16.6	16.6	16.8	16.9	17.7	17.8	17.4	17.5	17.8	17.7
360	16.6	16.5	16.7	16.9	17.6	17.7	17.4	17.5	17.8	17.7
390	16.5	16.5	16.7	16.8	17.6	17.7	17.4	17.4	17.8	17.7
420	16.5	16.4	16.7	16.8	17.6	17.7	17.4	17.4	17.8	17.7
450	16.4	16.4	16.6	16.8	17.6	17.7	17.4	17.4	17.8	17.7
480	16.4	16.3	16.6	16.8	17.6	17.7	17.3	17.4	17.8	17.7
510	16.3	16.3	16.6	16.7	17.5	17.7	17.3	17.4	17.8	17.7
540	16.3	16.3	16.5	16.7	17.5	17.7	17.3	17.4	17.8	17.7
570	16.2	16.2	16.5	16.7	17.5	17.7	17.3	17.4	17.8	17.6
600	16.2	16.2	16.5	16.6	17.5	17.7	17.3	17.4	17.8	17.6
630	16.1	16.1	16.4	16.6	17.5	17.6	17.3	17.4	17.7	17.6
660	16.1	16.1	16.4	16.6	17.5	17.6	17.3	17.4	17.7	17.6
690	16.0	16.0	16.4	16.5	17.5	17.6	17.3	17.4	17.7	17.6
720	16.0	16.0	16.3	16.5	17.4	17.6	17.3	17.4	17.7	17.6
750	15.9	15.9	16.3	16.5	17.4	17.6	17.3	17.4	17.7	17.6
780	15.8	15.9	16.2	16.4	17.4	17.6	17.3	17.4	17.7	17.6
810	15.8	15.8	16.2	16.4	17.4	17.6	17.3	17.4	17.7	17.6
840	15.7	15.8	16.2	16.3	17.4	17.6	17.2	17.4	17.7	17.6
870	15.7	15.7	16.1	16.3	17.4	17.6	17.2	17.4	17.7	17.6
900	15.6	15.7	16.0	16.2	17.4	17.6	17.2	17.4	17.7	17.6
960	15.5	15.6	15.9	16.1	17.4	17.6	17.2	17.4	17.7	17.6
1020	15.3	15.4	15.8	16	17.3	17.5	17.2	17.3	17.7	17.6
1080	15.2	15.3	15.7	15.9	17.3	17.5	17.1	17.3	17.7	17.6

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-02)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations													
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		50 mg phenol/l		80 mg phenol/l		100 mg phenol/l		150 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.7	17.5	17.6	17.4	17.1	17.3	17.2	17.0	17.0	16.8	17.9	18.6	18.3	18.4
30	17.6	17.4	17.5	17.3	17.0	17.2	17.2	16.9	16.9	16.7	17.9	18.5	18.2	18.4
60	17.5	17.3	17.4	17.2	16.9	17.1	17.1	16.8	16.8	16.6	17.8	18.5	18.2	18.3
90	17.4	17.2	17.3	17.2	16.8	17.0	17.0	16.7	16.8	16.6	17.8	18.4	18.1	18.3
120	17.3	17.1	17.2	17.1	16.7	16.9	16.9	16.6	16.7	16.5	17.7	18.4	18.0	18.2
150	17.2	17.1	17.1	17.0	16.6	16.8	16.8	16.6	16.6	16.4	17.7	18.3	18.0	18.2
180	17.1	17.0	17.0	16.9	16.5	16.7	16.7	16.5	16.5	16.3	17.6	18.3	17.9	18.1
210	17.0	16.9	16.9	16.8	16.4	16.6	16.6	16.4	16.5	16.3	17.6	18.2	17.8	18.1
240	16.9	16.9	16.7	16.7	16.3	16.5	16.5	16.3	16.4	16.2	17.5	18.2	17.7	18.0
270	16.8	16.8	16.6	16.6	16.2	16.4	16.4	16.2	16.3	16.2	17.4	18.1	17.7	18.0
300	16.7	16.7	16.5	16.5	16.1	16.3	16.3	16.1	16.2	16.1	17.3	18.1	17.6	17.9
330	16.6	16.7	16.4	16.4	16.0	16.2	16.2	16.1	16.2	16	17.3	18	17.6	17.8
360	16.5	16.6	16.3	16.3	15.9	16.0	16.1	16	16.1	15.9	17.2	18	17.5	17.7
390	16.4	16.5	16.1	16.2	15.8	15.9	16.0	15.9	16	15.9	17.2	17.9	17.4	17.7
420	16.2	16.4	16.0	16.1	15.7	15.7	15.9	15.8	15.9	15.8	17.1	17.8	17.4	17.6
450	16.1	16.3	15.9	16.0	15.6	15.6	15.8	15.7	15.8	15.7	17	17.8	17.3	17.6
480	16.0	16.2	15.8	15.9	15.5	15.5	15.7	15.6	15.7	15.6	16.9	17.7	17.3	17.5
510	15.8	16.2	15.6	15.8	15.4	15.4	15.6	15.5	15.7	15.5	16.8	17.7	17.2	17.4
540	15.7	16.1	15.5	15.7	15.3	15.3	15.5	15.4	15.6	15.5	16.7	17.6	17.1	17.3
570	15.6	16.0	15.4	15.6	15.2	15.1	15.4	15.2	15.5	15.4	16.6	17.5	17	17.2
600	15.5	16.0	15.3	15.5	15.1	15.0	15.3	15.1	15.4	15.3	16.5	17.4	16.9	17.1
630	15.4	15.9	15.2	15.3	14.9	14.9	15.2	15.0	15.3	15.2	16.4	17.3	16.8	17
660	15.3	15.8	15.0	15.2	14.8	14.8	15.1	14.9	15.2	15.1	16.4	17.3	16.7	16.9
690	15.1	15.7	14.9	15.0	14.7	14.7	15.0	14.8	15.1	15.1	16.3	17.2	16.6	16.8
720	15.0	15.6	14.8	14.9	14.6	14.6	14.9	14.7	15.0	15.0	16.2	17.1	16.6	16.7
750	14.9	15.6	14.6	14.8	14.5	14.5	14.8	14.6	14.9	14.9	16.1	17.0	16.5	16.7
780	14.8	15.5	14.5	14.6	14.4	14.4	14.7	14.5	14.8	14.8	16.0	16.9	16.4	16.6
810	14.7	15.4	14.4	14.5	14.2	14.3	14.6	14.4	14.7	14.7	15.9	16.8	16.3	16.5
840	14.6	15.3	14.3	14.3	14.1	14.2	14.5	14.3	14.6	14.6	15.8	16.7	16.2	16.4
870	14.5	15.2	14.1	14.2	14	14.1	14.3	14.2	14.5	14.5	15.7	16.7	16.1	16.3
900	14.4	15.1	14.0	14.0	13.9	14.0	14.2	14.1	14.4	14.4	15.6	16.6	16.0	16.2

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-02)

Time(s)	Oxygen concentrations (%) under different initial phenol concentrations											
	200 mg phenol/l		300 mg phenol/l		400 mg phenol/l		500 mg phenol/l		800 mg phenol/l		1000 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	18.3	18.6	17.8	17.9	18.4	18.4	18.6	18.7	17.4	17.3	18.4	18.3
30	18.2	18.5	17.7	17.8	18.4	18.4	18.6	18.7	17.4	17.3	18.4	18.3
60	18.2	18.5	17.7	17.8	18.3	18.4	18.6	18.7	17.4	17.3	18.4	18.3
90	18.1	18.5	17.7	17.8	18.3	18.3	18.5	18.7	17.4	17.3	18.4	18.3
120	18.1	18.4	17.7	17.7	18.3	18.3	18.5	18.6	17.4	17.3	18.4	18.3
150	18.1	18.4	17.6	17.7	18.2	18.3	18.5	18.6	17.4	17.3	18.4	18.3
180	18.0	18.4	17.6	17.6	18.2	18.2	18.4	18.6	17.4	17.3	18.4	18.3
210	18.0	18.3	17.6	17.6	18.2	18.2	18.4	18.6	17.4	17.3	18.4	18.3
240	17.9	18.3	17.5	17.6	18.2	18.2	18.4	18.5	17.4	17.3	18.4	18.3
270	17.9	18.3	17.5	17.5	18.1	18.2	18.4	18.5	17.4	17.3	18.4	18.3
300	17.8	18.2	17.5	17.5	18.1	18.1	18.3	18.5	17.4	17.3	18.4	18.3
330	17.8	18.2	17.5	17.4	18.1	18.1	18.3	18.5	17.3	17.3	18.4	18.3
360	17.7	18.1	17.4	17.4	18.1	18.1	18.3	18.4	17.3	17.3	18.4	18.3
390	17.7	18.1	17.4	17.4	18.0	18.1	18.3	18.4	17.3	17.3	18.4	18.3
420	17.6	18.1	17.4	17.3	18.0	18.1	18.2	18.4	17.3	17.3	18.4	18.3
450	17.6	18.0	17.3	17.3	18.0	18.0	18.2	18.3	17.3	17.3	18.4	18.3
480	17.5	18.0	17.3	17.3	18.0	18.0	18.2	18.3	17.3	17.2	18.4	18.3
510	17.5	17.9	17.3	17.2	17.9	18.0	18.2	18.3	17.3	17.2	18.4	18.3
540	17.4	17.9	17.3	17.2	17.9	18.0	18.1	18.3	17.3	17.2	18.4	18.3
570	17.4	17.9	17.2	17.2	17.9	17.9	18.1	18.2	17.3	17.2	18.3	18.3
600	17.3	17.8	17.2	17.1	17.8	17.9	18.1	18.2	17.3	17.2	18.3	18.2
630	17.2	17.8	17.2	17.1	17.8	17.9	18.0	18.2	17.2	17.2	18.3	18.2
660	17.2	17.7	17.1	17.1	17.8	17.9	18.0	18.1	17.2	17.2	18.3	18.2
690	17.2	17.7	17.1	17.1	17.8	17.8	18.0	18.1	17.2	17.2	18.3	18.2
720	17.1	17.6	17.0	17.0	17.7	17.8	17.9	18.1	17.2	17.2	18.3	18.2
750	17.1	17.6	17.0	17.0	17.7	17.8	17.9	18.0	17.2	17.2	18.3	18.2
780	17.0	17.5	17.0	17.0	17.6	17.7	17.9	18.0	17.2	17.2	18.3	18.2
810	17.0	17.5	16.9	16.9	17.6	17.7	17.9	18.0	17.2	17.2	18.3	18.2
840	17.0	17.4	16.9	16.9	17.6	17.7	17.8	17.9	17.2	17.2	18.3	18.2
870	16.9	17.4	16.9	16.9	17.6	17.7	17.8	17.9	17.2	17.2	18.3	18.2
900	16.9	17.3	16.8	16.8	17.5	17.6	17.8	17.9	17.2	17.2	18.3	18.2

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-03)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations													
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		50 mg phenol/l		80 mg phenol/l		100 mg phenol/l		150 mg phenol/l	
	IST	2ND	IST	2ND	IST	2ND	IST	2ND	IST	2ND	IST	2ND	IST	2ND
0	18.0	18.4	19	19.3	17.9	17.4	16.7	16.4	17	16.5	16.6	16.9	16.1	15.8
30	17.9	18.3	19	19.3	17.8	17.3	16.5	16.2	16.8	16.3	16.3	16.6	15.8	15.5
60	17.8	18.2	18.9	19.2	17.7	17.3	16.3	16.1	16.5	16	16.0	16.4	15.5	15.2
90	17.7	18.2	18.9	19.2	17.7	17.2	16.1	15.9	16.3	15.8	15.8	16.2	15.3	15.0
120	17.7	18.1	18.9	19.1	17.6	17.2	15.9	15.7	16.2	15.6	15.6	16.0	15.1	14.8
150	17.7	18.1	18.8	19.1	17.6	17.2	15.7	15.5	16	15.4	15.3	15.8	14.8	14.5
180	17.7	18.0	18.8	19.0	17.5	17.1	15.5	15.3	15.8	15.2	15.1	15.6	14.6	14.3
210	17.6	18.0	18.7	19.0	17.5	17.1	15.3	15.2	15.6	15	14.9	15.4	14.4	14
240	17.6	18.0	18.7	18.9	17.4	17.1	15.1	15	15.4	14.7	14.7	15.2	14.2	13.8
270	17.6	18.0	18.6	18.9	17.4	17.0	14.9	14.8	15.2	14.5	14.4	14.9	14	13.6
300	17.6	17.9	18.6	18.8	17.3	17.0	14.8	14.7	15	14.3	14.2	14.7	13.8	13.4
330	17.5	17.9	18.5	18.8	17.3	17.0	14.6	14.5	14.8	14.2	13.9	14.5	13.6	13.2
360	17.5	17.9	18.5	18.8	17.3	16.9	14.4	14.3	14.6	14	13.7	14.2	13.4	13
390	17.5	17.8	18.5	18.7	17.2	16.9	14.2	14.2	14.4	13.8	13.5	14.1	13.2	12.8
420	17.4	17.8	18.4	18.7	17.2	16.9	14.1	14	14.2	13.6	13.3	13.9	13	12.6
450	17.4	17.8	18.4	18.7	17.2	16.9	13.9	13.8	14.1	13.4	13.1	13.7	12.8	12.4
480	17.4	17.8	18.4	18.6	17.1	16.8	13.7	13.7	13.9	13.2	12.9	13.5	12.6	12.2
510	17.3	17.7	18.3	18.6	17.1	16.8	13.5	13.5	13.7	13	12.6	13.3	12.4	12
540	17.3	17.7	18.3	18.6	17.1	16.8	13.4	13.4	13.5	12.9	12.4	13.1	12.2	11.8
570	17.3	17.7	18.3	18.6	17.0	16.7	13.2	13.2	13.4	12.7	12.2	12.9	12	11.6
600	17.2	17.7	18.2	18.5	17.0	16.7	13.1	13.1	13.2	12.5	12	12.7	11.8	11.4
630	17.2	17.6	18.2	18.5	17.0	16.7	12.9	12.9	13	12.4	11.7	12.5	11.6	11.2
660	17.2	17.6	18.2	18.5	16.9	16.7	12.7	12.8	12.8	12.2	11.5	12.3	11.4	11
690	17.1	17.6	18.1	18.5	16.9	16.6	12.6	12.6	12.7	12	11.3	12.1	11.2	10.8
720	17.1	17.6	18.1	18.4	16.9	16.6	12.4	12.5	12.5	11.8	11.1	11.8	11	10.6
750	17.1	17.5	18.1	18.4	16.8	16.6	12.2	12.3	12.3	11.7	10.9	11.6	10.9	10.4
780	17.1	17.5	18.0	18.4	16.8	16.5	12	12.1	12.2	11.5	10.7	11.5	10.7	10.2
810	17.0	17.5	18.0	18.3	16.7	16.5	11.8	12.0	12.0	11.3	10.5	11.3	10.5	10
840	17.0	17.4	17.9	18.3	16.7	16.4	11.7	11.9	11.8	11.2	10.2	11	10.3	9.8
870	17.0	17.4	17.9	18.3	16.6	16.4	11.5	11.7	11.7	11.0	10	10.8	10.1	9.6
900	17.0	17.4	17.8	18.2	16.6	16.4	11.4	11.6	11.5	10.8	9.8	10.6	9.9	9.5
960	16.9	17.3	17.7	18.2	16.5	16.3	11	11.4	11.2	10.5	9.4	10.3	9.6	9.3
1020	16.8	17.3	17.7	18.1	16.4	16.2	10.6	10.9	10.8	10.2	9.0	9.8	9.2	8.7
1080	16.7	17.2	17.6	18	16.3	16.1	10.4	10.7	10.5	9.9	8.6	9.4	8.9	8.4

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-03)

Tme (s)	Oxygen concentrations (%) under different initial phenol concentrations											
	200 mg phenol/l		300 mg phenol/l		400 mg phenol/l		500 mg phenol/l		800 mg phenol/l		1000 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	16.2	16.1	15.9	16.4	17.3	17.7	17.2	17.1	19.7	19.2	17.6	18.2
30	15.9	15.9	15.7	15.9	17.3	17.7	17.2	17.1	19.7	19.2	17.6	18.2
60	15.6	15.5	15.6	15.8	17.3	17.7	17.1	17.1	19.6	19.1	17.6	18.2
90	15.4	15.4	15.5	15.6	17.3	17.7	17.1	17.0	19.6	19.1	17.6	18.1
120	15.2	15.2	15.4	15.5	17.2	17.7	17.0	17.0	19.5	19.0	17.6	18.1
150	15.0	15.0	15.3	15.4	17.2	17.6	16.9	17.0	19.5	19.0	17.6	18.1
180	14.8	14.7	15.1	15.2	17.2	17.6	16.9	17.0	19.4	19.0	17.5	18.0
210	14.6	14.5	15.1	15.1	17.2	17.6	16.8	17.0	19.4	18.9	17.5	18.0
240	14.4	14.3	14.9	15.0	17.1	17.5	16.8	16.9	19.3	18.9	17.5	18.0
270	14.2	14	14.8	14.9	17.1	17.5	16.8	16.9	19.3	18.9	17.5	17.9
300	14.0	13.8	14.6	14.7	17.1	17.4	16.7	16.9	19.3	18.9	17.5	17.9
330	13.8	13.6	14.4	14.5	17.1	17.4	16.7	16.9	19.2	18.9	17.5	17.9
360	13.6	13.4	14.3	14.4	17.0	17.3	16.7	16.8	19.2	18.8	17.5	17.9
390	13.4	13.2	14.2	14.3	17.0	17.3	16.6	16.8	19.2	18.8	17.5	17.8
420	13.2	13.0	14.1	14.1	17.0	17.3	16.6	16.8	19.1	18.8	17.4	17.8
450	13.0	12.8	14.0	14.0	17.0	17.2	16.6	16.7	19.1	18.8	17.4	17.8
480	12.8	12.6	13.8	13.9	17.0	17.2	16.5	16.7	19.1	18.8	17.4	17.8
510	12.6	12.3	13.7	13.7	16.9	17.1	16.5	16.7	19.1	18.7	17.4	17.7
540	12.4	12.1	13.6	13.6	16.9	17.1	16.5	16.7	19.0	18.7	17.4	17.7
570	12.2	11.9	13.5	13.5	16.9	17.0	16.4	16.7	19.0	18.7	17.3	17.7
600	12.0	11.7	13.4	13.3	16.9	17.0	16.4	16.6	19.0	18.7	17.3	17.7
630	11.8	11.5	13.2	13.2	16.8	17.0	16.4	16.6	18.9	18.7	17.3	17.7
660	11.6	11.3	13.1	13.0	16.8	17.0	16.3	16.6	18.9	18.7	17.3	17.7
690	11.4	11.1	12.9	12.9	16.8	16.9	16.3	16.6	18.9	18.6	17.2	17.6
720	11.2	10.9	12.8	12.8	16.8	16.9	16.3	16.5	18.9	18.6	17.2	17.6
750	11.0	10.6	12.7	12.7	16.8	16.9	16.2	16.5	18.8	18.6	17.2	17.6
780	10.8	10.4	12.6	12.5	16.8	16.8	16.2	16.5	18.8	18.6	17.2	17.5
810	10.6	10.2	12.4	12.4	16.7	16.8	16.2	16.5	18.8	18.6	17.1	17.5
840	10.3	10.0	12.3	12.2	16.7	16.7	16.1	16.4	18.8	18.6	17.1	17.5
870	10.1	9.8	12.2	12.1	16.7	16.7	16.1	16.4	18.7	18.5	17.1	17.4
900	9.9	9.6	12.0	11.9	16.6	16.7	16.1	16.4	18.7	18.5	17.1	17.4
960	9.5	9.2	11.8	11.7	16.5	16.6	16.0	16.3	18.7	18.5	17.0	17.4
1020	9.1	8.7	11.6	11.4	16.5	16.6	16.0	16.2	18.6	18.5	17.0	17.3
1080	8.7	8.3	11.3	11.1	16.4	16.6	15.9	16.1	18.6	18.4	16.9	17.3

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-04)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations													
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		40 mg phenol/l		50 mg phenol/l		80 mg phenol/l		100 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.4	17.3	17.1	16.7	17.6	17.2	17.1	17.3	17.4	17.2	17.7	17.6	17.5	17.3
30	17.3	17.2	16.9	16.6	17.4	17	17	17.1	17.3	17.1	17.5	17.4	17.3	17.1
60	17.1	17.0	16.8	16.4	17.3	16.8	16.8	16.9	17.1	16.9	17.3	17.2	17.1	16.9
90	17.0	16.8	16.6	16.2	17.1	16.6	16.6	16.7	16.9	16.7	17.1	17	16.8	16.7
120	16.8	16.6	16.4	16.0	16.9	16.4	16.4	16.5	16.6	16.4	16.9	16.8	16.6	16.4
150	16.6	16.4	16.2	15.8	16.7	16.2	16.2	16.3	16.4	16.2	16.7	16.6	16.4	16.2
180	16.4	16.3	16.1	15.7	16.5	16.0	16.0	16.1	16.2	16	16.5	16.3	16.1	16
210	16.2	16.1	15.9	15.5	16.3	15.8	15.8	15.9	16.0	15.8	16.3	16.1	15.9	15.7
240	16.1	15.9	15.7	15.3	16.1	15.6	15.6	15.7	15.8	15.6	16.1	15.9	15.7	15.5
270	15.9	15.7	15.5	15.1	15.9	15.4	15.4	15.5	15.6	15.4	15.8	15.7	15.5	15.3
300	15.7	15.5	15.3	14.9	15.7	15.3	15.2	15.3	15.3	15.2	15.6	15.5	15.3	15
330	15.5	15.3	15.1	14.7	15.5	15.1	15.0	15.1	15.1	15	15.4	15.3	15.1	14.8
360	15.3	15.1	14.9	14.5	15.3	14.9	14.7	14.8	14.9	14.8	15.2	15.1	14.9	14.6
390	15.1	14.9	14.7	14.3	15.1	14.7	14.5	14.6	14.7	14.6	15	14.9	14.6	14.4
420	14.9	14.7	14.5	14.1	14.9	14.5	14.3	14.4	14.5	14.4	14.8	14.6	14.4	14.2
450	14.7	14.5	14.3	13.9	14.7	14.3	14.1	14.3	14.2	14.2	14.6	14.4	14.2	13.9
480	14.4	14.3	14.1	13.7	14.5	14.1	13.9	14.0	14.0	14.0	14.4	14.2	13.9	13.7
510	14.2	14.1	13.9	13.5	14.3	13.9	13.7	13.8	13.8	13.9	14.2	13.9	13.7	13.5
540	14.0	13.9	13.7	13.3	14.1	13.7	13.5	13.6	13.5	13.5	13.9	13.6	13.5	13.3
570	13.8	13.7	13.5	13.1	13.9	13.5	13.2	13.4	13.3	13.3	13.7	13.4	13.2	13.0
600	13.6	13.5	13.3	12.9	13.7	13.3	13	13.2	13.1	13	13.4	13.2	13.0	12.8

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-04)

Time(s)	Oxygen concentrations (%) under different initial phenol concentrations											
	150 mg phenol/l		200 mg phenol/l		300 mg phenol/l		500 mg phenol/l		800 mg phenol/l		1000 mg phenol/l	
	1ST		2ND		1ST		2ND		1ST		2ND	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.9	17.5	18.4	18.0	17.6	17.4	18.0	17.7	16.8	16.9	16.8	17.0
30	17.7	17.3	18.2	17.8	17.5	17.3	17.9	17.6	16.7	16.8	16.7	16.9
60	17.5	17.1	18.0	17.6	17.4	17.1	17.8	17.5	16.6	16.7	16.7	16.8
90	17.2	16.9	17.8	17.4	17.2	16.8	17.7	17.4	16.5	16.6	16.6	16.8
120	17.0	16.6	17.7	17.3	17.0	16.6	17.5	17.2	16.4	16.5	16.5	16.7
150	16.8	16.4	17.5	17.1	16.9	16.7	17.4	17.0	16.3	16.4	16.4	16.6
180	16.5	16.2	17.3	16.9	16.7	16.5	17.2	16.9	16.2	16.2	16.3	16.4
210	16.3	15.9	17.1	16.8	16.5	16.3	17.0	16.7	16.1	16.1	16.2	16.3
240	16.1	15.7	16.9	16.6	16.3	16.1	16.9	16.6	16.0	16.0	16.1	16.2
270	15.9	15.5	16.7	16.4	16.2	16.0	16.7	16.4	15.9	15.8	16.0	16.1
300	15.6	15.3	16.5	16.2	16.0	15.8	16.5	16.2	15.7	15.7	15.9	16.0
330	15.4	15.1	16.3	16.0	15.8	15.6	16.4	16.1	15.6	15.6	15.8	15.9
360	15.2	14.9	16.1	15.8	15.6	15.4	16.2	15.9	15.5	15.5	15.7	15.8
390	14.9	14.6	15.9	15.6	15.4	15.2	16.0	15.7	15.4	15.4	15.6	15.7
420	14.7	14.4	15.7	15.4	15.2	15.0	15.8	15.5	15.2	15.2	15.5	15.6
450	14.4	14.1	15.5	15.2	15.1	14.9	15.6	15.3	15.1	15.1	15.3	15.4
480	14.2	13.9	15.2	15.0	14.9	14.7	15.5	15.2	15.0	15.0	15.2	15.3
510	14.0	13.7	15.0	14.8	14.7	14.5	15.3	15.0	14.8	14.8	15.1	15.2
540	13.7	13.4	14.8	14.6	14.5	14.3	15.1	14.8	14.7	14.7	15.0	15.0
570	13.5	13.2	14.6	14.4	14.3	14.1	14.9	14.6	14.5	14.5	14.8	14.9
600	13.2	12.9	14.4	14.2	14.1	13.9	14.7	14.4	14.4	14.4	14.7	14.8

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-05)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations									
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		50 mg phenol/l		80 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	18.6	18.2	18.7	18.5	18.7	18.5	18.2	17.7	18.6	18.7
30	18.6	18.2	18.7	18.5	18.6	18.5	18.1	17.7	18.6	18.6
60	18.6	18.2	18.7	18.5	18.6	18.4	18.1	17.6	18.5	18.6
90	18.5	18.1	18.7	18.5	18.6	18.4	18.1	17.6	18.5	18.5
120	18.5	18.1	18.6	18.4	18.6	18.4	18.0	17.6	18.4	18.5
150	18.5	18.1	18.6	18.4	18.6	18.4	18.0	17.6	18.4	18.4
180	18.5	18.1	18.6	18.4	18.5	18.4	18.0	17.5	18.3	18.4
210	18.5	18.1	18.6	18.4	18.5	18.3	17.9	17.5	18.3	18.3
240	18.5	18.1	18.6	18.4	18.5	18.3	17.9	17.5	18.2	18.3
270	18.5	18.1	18.6	18.4	18.5	18.3	17.9	17.5	18.2	18.2
300	18.4	18.0	18.5	18.3	18.5	18.3	17.9	17.4	18.1	18.1
330	18.4	18.0	18.5	18.3	18.4	18.3	17.8	17.4	18.1	18.1
360	18.4	18.0	18.5	18.3	18.4	18.2	17.8	17.4	18.0	18.0
390	18.4	18.0	18.5	18.3	18.4	18.2	17.8	17.4	18.0	18.0
420	18.4	18.0	18.5	18.3	18.4	18.2	17.7	17.3	17.9	17.9
450	18.4	18.0	18.4	18.2	18.3	18.2	17.7	17.3	17.8	17.9
480	18.3	17.9	18.4	18.2	18.3	18.1	17.7	17.3	17.8	17.8
510	18.3	17.9	18.4	18.2	18.3	18.1	17.7	17.2	17.7	17.7
540	18.3	17.9	18.4	18.2	18.2	18.1	17.7	17.2	17.6	17.7
570	18.2	17.9	18.3	18.1	18.2	18.1	17.6	17.1	17.6	17.6
600	18.2	17.9	18.3	18.1	18.2	18.0	17.6	17.1	17.5	17.6

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-05)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations													
	100 mg phenol/l		150 mg phenol/l		300 mg phenol/l		400 mg phenol/l		500 mg phenol/l		800 mg phenol/l		1000 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.8	17.9	17.5	17.6	17.6	18.0	17.9	18.3	18.5	18.2	18.0	18.4	17.9	18.3
30	17.7	17.8	17.4	17.5	17.5	17.9	17.9	18.3	18.5	18.2	18.0	18.4	17.9	18.3
60	17.6	17.7	17.3	17.4	17.4	17.8	17.9	18.3	18.5	18.2	18.0	18.4	17.9	18.3
90	17.6	17.6	17.2	17.3	17.3	17.7	17.9	18.3	18.5	18.2	18.0	18.4	17.9	18.3
120	17.5	17.6	17.2	17.3	17.2	17.6	17.9	18.3	18.5	18.2	18.0	18.4	17.9	18.3
150	17.4	17.5	17.1	17.2	17.1	17.5	17.9	18.2	18.5	18.2	18.0	18.4	17.9	18.3
180	17.4	17.4	17.1	17.1	17.0	17.4	17.9	18.2	18.5	18.2	18.0	18.4	17.9	18.3
210	17.3	17.3	17.0	17.0	16.9	17.3	17.8	18.2	18.5	18.2	18.0	18.4	17.9	18.3
240	17.2	17.3	16.9	16.9	16.8	17.2	17.8	18.2	18.5	18.1	18.0	18.4	17.9	18.3
270	17.1	17.2	16.8	16.8	16.7	17.1	17.8	18.2	18.4	18.1	18.0	18.4	17.9	18.3
300	17.0	17.1	16.8	16.8	16.6	17.0	17.8	18.2	18.4	18.1	18.0	18.4	17.9	18.3
330	17.0	17.1	16.7	16.7	16.5	16.9	17.8	18.2	18.4	18.1	18.0	18.4	17.9	18.3
360	16.9	17.0	16.6	16.7	16.4	16.8	17.8	18.2	18.4	18.1	18.0	18.4	17.9	18.3
390	16.9	17.0	16.5	16.6	16.3	16.7	17.7	18.2	18.4	18.1	18.0	18.4	17.9	18.3
420	16.8	16.9	16.5	16.5	16.2	16.6	17.7	18.1	18.4	18.1	18.0	18.4	17.9	18.3
450	16.7	16.8	16.4	16.4	16.1	16.5	17.7	18.1	18.4	18.1	18.0	18.4	17.9	18.3
480	16.7	16.8	16.3	16.3	16.0	16.4	17.7	18.1	18.4	18.1	18.0	18.4	17.9	18.3
510	16.6	16.7	16.2	16.3	15.8	16.3	17.7	18.1	18.4	18.1	18.0	18.4	17.9	18.3
540	16.6	16.7	16.2	16.2	15.7	16.1	17.7	18.1	18.3	18.0	18.0	18.4	17.9	18.3
570	16.5	16.6	16.1	16.1	15.6	15.9	17.7	18.1	18.3	18.0	18.0	18.3	17.9	18.3
600	16.5	16.6	16.0	16.1	15.5	15.8	17.6	18.1	18.3	18.0	17.9	18.3	17.9	18.3
630	16.4	16.5	15.9	16.0	15.5	15.7	17.6	18.1	18.3	18.0	17.9	18.3	17.9	18.3
660	16.3	16.4	15.8	15.9	15.4	15.6	17.6	18.1	18.3	18.0	17.9	18.3	17.9	18.3
690	16.3	16.4	15.7	15.8	15.2	15.5	17.6	18.0	18.3	18.0	17.9	18.3	17.9	18.3
720	16.2	16.3	15.7	15.7	15.1	15.4	17.6	18.0	18.3	18.0	17.9	18.3	17.9	18.2
750	16.1	16.2	15.6	15.6	15.0	15.3	17.5	18.0	18.2	18.0	17.9	18.3	17.9	18.2
780	16.1	16.2	15.5	15.6	14.8	15.1	17.5	18.0	18.2	18.0	17.9	18.3	17.9	18.2
810	16.0	16.1	15.4	15.5	14.7	15.0	17.5	18.0	18.2	18.0	17.9	18.3	17.8	18.2
840	16.0	16.1	15.4	15.4	14.6	14.9	17.4	17.9	18.2	17.9	17.9	18.3	17.8	18.2
870	15.9	16.0	15.3	15.3	14.5	14.8	17.4	17.9	18.2	17.9	17.9	18.3	17.8	18.2
900	15.8	16.0	15.2	15.2	14.4	14.7	17.4	17.9	18.2	17.9	17.9	18.3	17.8	18.2

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-06)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations									
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		50 mg phenol/l		80 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	15.5	16.5	17.0	17.6	17.9	17.9	17.1	16.9	18.1	16.9
30	15.4	16.5	16.9	17.6	17.8	17.8	17.0	16.9	18.0	16.9
60	15.3	16.4	16.8	17.5	17.8	17.8	16.9	16.8	17.9	16.8
90	15.3	16.4	16.8	17.5	17.7	17.7	16.9	16.8	17.9	16.8
120	15.2	16.3	16.7	17.4	17.6	17.6	16.8	16.7	17.8	16.7
150	15.1	16.3	16.7	17.3	17.6	17.6	16.8	16.7	17.8	16.7
180	15.0	16.2	16.6	17.3	17.6	17.6	16.7	16.6	17.8	16.6
210	15.0	16.2	16.5	17.2	17.5	17.5	16.7	16.6	17.7	16.6
240	14.9	16.1	16.4	17.2	17.5	17.5	16.6	16.5	17.7	16.5
270	14.8	16.1	16.3	17.1	17.4	17.4	16.6	16.5	17.6	16.5
300	14.7	16.0	16.2	17.1	17.3	17.3	16.5	16.4	17.6	16.4
330	14.6	16.0	16.1	17.0	17.2	17.2	16.5	16.4	17.5	16.4
360	14.6	15.9	16.0	17.0	17.2	17.2	16.5	16.3	17.5	16.3
390	14.5	15.8	15.9	16.9	17.1	17.1	16.4	16.2	17.5	16.2
420	14.5	15.8	15.8	16.8	17.1	17.1	16.4	16.2	17.4	16.2
450	14.4	15.7	15.7	16.7	17.0	17.0	16.3	16.1	17.4	16.1
480	14.3	15.7	15.7	16.6	16.9	16.9	16.3	16.1	17.3	16.0
510	14.3	15.6	15.6	16.5	16.8	16.8	16.2	16.0	17.3	16.0
540	14.2	15.6	15.5	16.4	16.7	16.7	16.2	16.0	17.3	16.0
570	14.1	15.5	15.5	16.4	16.7	16.7	16.2	15.9	17.2	15.9
600	14.1	15.5	15.4	16.4	16.6	16.6	16.1	15.9	17.2	15.8
630	14.0	15.4	15.3	16.3	16.5	16.5	16.1	15.8	17.2	15.7
660	13.9	15.4	15.2	16.3	16.4	16.4	16.1	15.8	17.1	15.7
690	13.8	15.3	15.1	16.2	16.3	16.3	16.0	15.7	17.1	15.6
720	13.8	15.3	15.0	16.1	16.2	16.2	15.9	15.7	17.0	15.5
750	13.7	15.2	14.9	15.9	16.0	16.0	15.8	15.7	16.9	15.5
780	13.5	15.1	14.8	15.8	15.9	15.9	15.8	15.6	16.9	15.4
810	13.5	15.1	14.7	15.8	15.8	15.8	15.8	15.6	16.9	15.4
840	13.5	15.0	14.6	15.7	15.7	15.7	15.8	15.5	16.8	15.3
870	13.5	14.9	14.5	15.6	15.7	15.7	15.7	15.4	16.8	15.2
900	13.4	14.7	14.4	15.5	15.6	15.6	15.7	15.4	16.8	15.2

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-06)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations							
	100 mg phenol/l		200 mg phenol/l		400 mg phenol/l		500 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.8	18.4	18.4	18.0	17.4	18.2	17.4	18.0
30	17.7	18.4	18.4	18.0	17.4	18.2	17.4	18.0
60	17.7	18.3	18.4	17.9	17.4	18.2	17.4	18.0
90	17.7	18.3	18.3	17.9	17.4	18.2	17.3	18.0
120	17.7	18.3	18.3	17.9	17.4	18.2	17.3	18.0
150	17.6	18.2	18.3	17.9	17.4	18.2	17.3	18.0
180	17.6	18.2	18.2	17.8	17.4	18.2	17.3	18.0
210	17.6	18.2	18.2	17.8	17.4	18.1	17.2	17.9
240	17.5	18.2	18.2	17.7	17.4	18.1	17.2	17.9
270	17.5	18.1	18.2	17.7	17.4	18.1	17.2	17.9
300	17.5	18.1	18.1	17.7	17.4	18.1	17.2	17.9
330	17.5	18.1	18.1	17.7	17.3	18.1	17.2	17.9
360	17.4	18.1	18.1	17.7	17.3	18.1	17.2	17.9
390	17.4	18.0	18.1	17.6	17.3	18.1	17.2	17.9
420	17.4	18.0	18.1	17.6	17.3	18.1	17.2	17.9
450	17.3	18.0	18.0	17.5	17.3	18.1	17.2	17.9
480	17.3	18.0	18.0	17.5	17.3	18.1	17.2	17.9
510	17.3	17.9	18.0	17.5	17.3	18.0	17.2	17.9
540	17.3	17.9	18.0	17.5	17.3	18.0	17.2	17.9
570	17.2	17.9	17.9	17.5	17.3	18.0	17.2	17.9
600	17.2	17.8	17.9	17.4	17.3	18.0	17.2	17.9
630	17.2	17.8	17.9	17.4	17.2	18.0	17.2	17.9
660	17.1	17.8	17.9	17.3	17.2	18.0	17.2	17.9
690	17.1	17.8	17.8	17.3	17.2	18.0	17.1	17.9
720	17.0	17.7	17.8	17.3	17.2	18.0	17.1	17.9
750	17.0	17.7	17.8	17.3	17.2	18.0	17.1	17.9
780	17.0	17.6	17.7	17.3	17.2	17.9	17.1	17.9
810	16.9	17.6	17.7	17.2	17.2	17.9	17.1	17.9
840	16.9	17.6	17.7	17.2	17.2	17.9	17.1	17.9
870	16.9	17.6	17.7	17.2	17.2	17.9	17.1	17.8
900	16.8	17.5	17.6	17.2	17.2	17.9	17.1	17.8

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-08)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations																							
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		40 mg phenol/l		50 mg phenol/l		80 mg phenol/l		100 mg phenol/l		150 mg phenol/l		200 mg phenol/l		300 mg phenol/l					
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.6	19.6	18.4	18.3	19	18.9	19.1	19.2	18.7	18.6				
30	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.6	19.6	18.4	18.3	19	18.9	19.1	19.2	18.7	18.6				
60	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.6	19.6	18.3	18.2	19	18.8	19.1	19.2	18.7	18.6				
90	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.6	19.5	18.3	18.2	18.9	18.8	19	19.2	18.7	18.6				
120	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.5	19.5	18.3	18.2	18.9	18.8	19	19.1	18.7	18.6				
150	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.5	19.5	18.3	18.1	18.9	18.8	19	19.1	18.7	18.6				
180	19.4	19.5	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.5	19.5	18.3	18.1	18.9	18.7	19	19.1	18.7	18.6				
210	19.4	19.4	19.6	19.5	19.8	19.4	19	19.2	19	18.5	19.5	19.4	18.3	18.1	18.9	18.7	19	19.1	18.7	18.6				
240	19.4	19.4	19.5	19.5	19.7	19.4	19	19.2	19	18.5	19.5	19.4	18.2	18.1	18.8	18.7	19	19.1	18.7	18.6				
270	19.3	19.4	19.5	19.4	19.7	19.4	19	19.2	19	18.5	19.5	19.4	18.2	18.1	18.8	18.6	19	19.1	18.7	18.6				
300	19.3	19.4	19.5	19.4	19.7	19.4	19	19.2	19	18.4	19.5	19.4	18.2	18	18.8	18.6	19	19.1	18.7	18.6				
330	19.3	19.4	19.5	19.4	19.7	19.4	19	19.1	19	18.4	19.4	19.4	18.2	18	18.8	18.6	19	19.1	18.7	18.6				
360	19.3	19.4	19.5	19.4	19.7	19.4	18.9	19.1	18.9	18.4	19.4	19.3	18.2	18	18.7	18.6	19	19.1	18.7	18.6				
390	19.3	19.4	19.5	19.4	19.7	19.3	18.9	19.1	18.9	18.4	19.4	19.3	18.2	18	18.7	18.6	19	19.1	18.7	18.6				
420	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.4	19.3	18.2	18	18.7	18.5	19	19.1	18.7	18.6				
450	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.4	19.3	18.2	18	18.7	18.5	19	19.1	18.7	18.6				
480	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.4	19.3	18.2	17.9	18.7	18.5	19	19.1	18.7	18.6				
510	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.4	19.3	18.2	17.9	18.6	18.5	19	19.1	18.7	18.6				
540	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.4	19.2	18.1	17.9	18.6	18.4	19	19.1	18.7	18.6				
570	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.3	19.2	18.1	17.9	18.6	18.4	19	19.1	18.7	18.6				
600	19.3	19.4	19.5	19.4	19.6	19.3	18.9	19.1	18.9	18.4	19.3	19.2	18.1	17.9	18.6	18.4	19	19.1	18.7	18.6				

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-09)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations											
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		50 mg phenol/l		80 mg phenol/l		100 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.7	18.0	17.5	17.5	17.2	17.6	16.8	16.9	17.7	17.5	17.5	17.6
30	17.4	17.7	17.3	17.2	17.1	17.5	16.7	16.7	17.7	17.4	17.5	17.6
60	17.2	17.5	17.1	17.0	17.0	17.3	16.5	16.5	17.6	17.3	17.4	17.5
90	17.0	17.3	17.0	16.9	16.8	17.2	16.3	16.4	17.5	17.2	17.4	17.5
120	16.8	17.1	16.8	16.7	16.7	17.1	16.1	16.2	17.4	17.1	17.3	17.4
150	16.6	16.9	16.6	16.5	16.6	17.0	16.0	16.1	17.3	17.0	17.2	17.4
180	16.3	16.7	16.5	16.4	16.4	16.8	15.9	16.0	17.2	17.0	17.2	17.3
210	16.1	16.5	16.3	16.2	16.3	16.7	15.7	15.8	17.1	16.9	17.1	17.3
240	15.9	16.4	16.1	16.0	16.1	16.5	15.6	15.7	17.0	16.8	17.0	17.2
270	15.7	16.2	16.0	15.9	16.0	16.4	15.4	15.6	16.9	16.7	17.0	17.1
300	15.5	15.9	15.8	15.8	15.8	16.2	15.3	15.4	16.8	16.6	16.9	17.1
330	15.3	15.7	15.6	15.6	15.7	16.1	15.1	15.2	16.7	16.5	16.9	17.0
360	15.1	15.5	15.4	15.5	15.5	15.9	15.0	15.0	16.6	16.5	16.8	17.0
390	14.9	15.3	15.2	15.3	15.3	15.8	14.9	14.9	16.5	16.4	16.7	16.9
420	14.7	15.1	15.0	15.1	15.2	15.6	14.7	14.8	16.4	16.3	16.6	16.8
450	14.5	14.9	14.8	14.9	15.0	15.5	14.6	14.7	16.3	16.2	16.5	16.7
480	14.3	14.7	14.6	14.7	14.9	15.4	14.4	14.6	16.2	16.1	16.5	16.6
510	14.1	14.5	14.5	14.5	14.7	15.2	14.3	14.5	16.1	16.1	16.4	16.5
540	13.9	14.3	14.3	14.3	14.5	15.0	14.2	14.3	16.0	16.0	16.3	16.5
570	13.6	14.1	14.2	14.2	14.3	14.8	14.1	14.2	15.9	15.9	16.3	16.4
600	13.4	13.9	14.0	14.1	14.1	14.7	13.9	14.0	15.7	15.7	16.2	16.3
630	13.2	13.7	13.8	13.9	14.0	14.6	13.8	13.9	15.6	15.6	16.1	16.3
660	13.0	13.5	13.4	13.6	13.8	14.4	13.7	13.8	15.5	15.6	16.0	16.2
690	12.7	13.2	13.2	13.4	13.6	14.3	13.5	13.7	15.4	15.5	15.9	16.1
720	12.5	13.0	13.0	13.2	13.4	14.1	13.4	13.6	15.3	15.4	15.8	16.0
750	12.3	12.7	12.8	12.9	13.3	14.0	13.2	13.4	15.1	15.3	15.8	15.9
780	12.0	12.5	12.6	12.7	13.1	13.9	13.1	13.3	15.0	15.2	15.7	15.9
810	11.8	12.3	12.4	12.5	12.9	13.7	13.0	13.4	14.8	15.1	15.7	15.8
840	11.6	12.1	12.1	12.3	12.8	13.5	12.9	13.0	14.7	15.0	15.6	15.7
870	11.4	11.8	11.9	12.0	12.6	13.3	12.8	12.9	14.6	14.9	15.6	15.7
900	11.1	11.6	11.7	11.9	12.4	13.1	12.7	12.8	14.5	14.7	15.5	15.6
960	10.6	11.1	11.2	11.4	12.1	12.8	12.4	12.6	14.2	14.5	15.3	15.4
1020	10.2	10.7	10.8	11.0	11.8	12.5	12.1	12.4	14.0	14.3	15.1	15.2

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-09)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations									
	150 mg phenol/l		200 mg phenol/l		300 mg phenol/l		400 mg phenol/l		500 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	17.4	17.6	16.9	17.0	17.7	17.1	16.6	16.7	17.4	17.6
30	17.4	17.6	16.9	17.0	17.7	17.0	16.6	16.7	17.4	17.6
60	17.3	17.6	16.8	16.9	17.6	17.0	16.5	16.7	17.4	17.5
90	17.3	17.5	16.8	16.9	17.6	16.9	16.5	16.6	17.4	17.5
120	17.2	17.5	16.7	16.8	17.5	16.9	16.4	16.6	17.3	17.5
150	17.1	17.4	16.7	16.8	17.5	16.8	16.4	16.6	17.3	17.4
180	17.1	17.4	16.6	16.7	17.5	16.7	16.4	16.6	17.3	17.4
210	16.9	17.3	16.6	16.6	17.4	16.7	16.3	16.5	17.2	17.4
240	16.9	17.2	16.5	16.6	17.4	16.7	16.3	16.5	17.2	17.4
270	16.8	17.2	16.5	16.5	17.3	16.6	16.2	16.5	17.2	17.4
300	16.8	17.1	16.4	16.5	17.3	16.6	16.2	16.4	17.2	17.3
330	16.7	17.0	16.4	16.4	17.2	16.6	16.2	16.4	17.1	17.3
360	16.7	17.0	16.3	16.3	17.2	16.5	16.1	16.4	17.1	17.3
390	16.6	16.9	16.2	16.3	17.1	16.5	16.1	16.3	17.1	17.3
420	16.6	16.9	16.2	16.2	17.1	16.4	16.1	16.3	17.1	17.2
450	16.5	16.8	16.1	16.2	17.0	16.4	16.0	16.3	17.0	17.2
480	16.4	16.7	16.1	16.1	17.0	16.3	16.0	16.2	17.0	17.2
510	16.3	16.6	16.0	16.1	16.9	16.3	15.9	16.2	17.0	17.2
540	16.2	16.5	16.0	16.0	16.9	16.3	15.9	16.2	16.9	17.2
570	16.1	16.4	15.9	15.9	16.8	16.2	15.8	16.1	16.9	17.1
600	16.0	16.4	15.9	15.9	16.8	16.2	15.8	16.1	16.9	17.1
630	15.9	16.3	15.8	15.8	16.7	16.1	15.7	16.1	16.9	17.1
660	15.8	16.3	15.8	15.7	16.6	16.1	15.7	16.0	16.8	17.1
690	15.7	16.2	15.7	15.6	16.6	16.0	15.6	16.0	16.8	17.1
720	15.6	16.1	15.6	15.5	16.5	16.0	15.6	15.9	16.8	17.1
750	15.6	16.0	15.6	15.5	16.4	15.9	15.5	15.9	16.8	17.0
780	15.5	15.9	15.5	15.4	16.4	15.9	15.5	15.8	16.7	17.0
810	15.4	15.8	15.5	15.4	16.3	15.8	15.4	15.8	16.7	17.0
840	15.4	15.7	15.4	15.3	16.3	15.8	15.4	15.7	16.7	17.0
870	15.3	15.6	15.4	15.3	16.2	15.7	15.3	15.7	16.7	17.0
900	15.3	15.5	15.3	15.2	16.1	15.7	15.3	15.6	16.7	17.0
960	15.2	15.4	15.3	15.2	16.0	15.6	15.1	15.5	16.6	16.9
1020	15.2	15.2	15.2	15.1	15.8	15.4	15.0	15.3	16.5	16.8

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-10)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations													
	10 mg phenol/l		20 mg phenol/l		30 mg phenol/l		50 mg phenol/l		80 mg phenol/l		100 mg phenol/l		150 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	16.8	17.0	17.2	17.9	17.3	17.8	16.9	17.6	17.3	17.6	17.8	17.7	16.2	16.5
30	16.7	17.0	17.2	17.9	17.3	17.8	16.9	17.6	17.3	17.6	17.7	17.7	16.1	16.3
60	16.7	17.0	17.2	17.9	17.2	17.7	16.9	17.6	17.2	17.6	17.7	17.6	15.9	16.1
90	16.7	16.9	17.1	17.8	17.2	17.7	16.8	17.5	17.2	17.5	17.7	17.6	15.8	15.9
120	16.7	16.9	17.1	17.8	17.2	17.6	16.8	17.5	17.1	17.5	17.7	17.6	15.7	15.8
150	16.7	16.9	17.1	17.7	17.1	17.6	16.8	17.5	17.1	17.5	17.7	17.6	15.6	15.7
180	16.7	16.8	17.0	17.6	17.1	17.5	16.7	17.4	17.1	17.4	17.6	17.6	15.5	15.6
210	16.6	16.8	17.0	17.6	17.0	17.5	16.7	17.4	17.0	17.4	17.6	17.5	15.4	15.6
240	16.6	16.8	16.9	17.6	17.0	17.4	16.7	17.4	17.0	17.4	17.6	17.5	15.3	15.5
270	16.6	16.7	16.9	17.5	16.9	17.4	16.7	17.3	17.0	17.4	17.6	17.5	15.2	15.4
300	16.6	16.7	16.9	17.5	16.9	17.3	16.6	17.3	16.9	17.3	17.6	17.5	15.1	15.4
330	16.6	16.7	16.8	17.5	16.8	17.3	16.6	17.3	16.9	17.3	17.6	17.5	15.0	15.3
360	16.5	16.6	16.8	17.4	16.8	17.3	16.6	17.3	16.9	17.3	17.6	17.5	14.9	15.2
390	16.5	16.6	16.8	17.4	16.8	17.2	16.5	17.2	16.8	17.3	17.5	17.4	14.8	15.1
420	16.5	16.6	16.7	17.4	16.7	17.2	16.5	17.2	16.8	17.2	17.5	17.4	14.7	15.0
450	16.5	16.6	16.7	17.3	16.7	17.1	16.5	17.2	16.8	17.2	17.5	17.4	14.7	14.9
480	16.5	16.6	16.7	17.3	16.6	17.1	16.5	17.2	16.7	17.2	17.5	17.4	14.6	14.9
510	16.5	16.5	16.6	17.3	16.6	17.1	16.4	17.1	16.7	17.2	17.5	17.4	14.5	14.8
540	16.4	16.5	16.6	17.2	16.5	17.0	16.4	17.1	16.7	17.1	17.5	17.4	14.4	14.7
570	16.4	16.5	16.6	17.2	16.5	17.0	16.4	17.1	16.6	17.1	17.5	17.4	14.3	14.6
600	16.4	16.5	16.5	17.1	16.5	16.9	16.3	17.0	16.6	17.1	17.4	17.3	14.2	14.6
630	16.4	16.4	16.5	17.1	16.4	16.9	16.3	17.0	16.6	17.1	17.4	17.3	14.1	14.5
660	16.4	16.4	16.4	17.0	16.4	16.9	16.2	17.0	16.5	17.0	17.4	17.3	14.0	14.4
690	16.3	16.4	16.4	17.0	16.4	16.8	16.2	17.0	16.5	17.0	17.4	17.3	13.9	14.3
720	16.3	16.4	16.4	16.9	16.3	16.8	16.2	16.9	16.5	17.0	17.4	17.3	13.8	14.2
750	16.3	16.3	16.3	16.9	16.3	16.7	16.1	16.9	16.4	17.0	17.4	17.3	13.7	14.2
780	16.3	16.3	16.3	16.9	16.2	16.7	16.1	16.9	16.4	16.9	17.4	17.2	13.6	14.1
810	16.2	16.2	16.2	16.8	16.2	16.7	16.1	16.9	16.4	16.9	17.4	17.2	13.6	14.0
840	16.2	16.2	16.2	16.8	16.1	16.6	16.1	16.8	16.3	16.9	17.3	17.2	13.5	13.9
870	16.2	16.2	16.2	16.7	16.1	16.6	16.0	16.8	16.3	16.9	17.3	17.2	13.4	13.8
900	16.1	16.1	16.1	16.7	16.1	16.5	16.0	16.8	16.3	16.8	17.3	17.2	13.3	13.7
960	16.1	16.1	16.0	16.6	16.0	16.4	15.9	16.7	16.2	16.8	17.3	17.2	13.1	13.5
1020	16.0	16.0	15.9	16.5	15.9	16.3	15.9	16.7	16.1	16.7	17.2	17.1	12.9	13.3
1080	16.0	16.0	15.8	16.4	15.8	16.3	15.8	16.6	16.1	16.7	17.2	17.1	12.7	13.2

Oxygen concentration change with time under different initial phenol concentrations (for isolate PG-10)

Time (s)	Oxygen concentrations (%) under different initial phenol concentrations											
	200 mg phenol/l		300 mg phenol/l		400 mg phenol/l		500 mg phenol/l		800 mg phenol/l		1000 mg phenol/l	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
0	15.8	16.5	15.5	15.4	15.1	15.4	15.4	15.5	17.4	17.7	17.6	18.0
30	15.4	16.3	15.4	15.2	14.9	15.2	15.3	15.4	17.4	17.7	17.6	18.0
60	15.3	16.1	15.3	15.1	14.8	15.2	15.3	15.4	17.4	17.6	17.6	18.0
90	15.3	15.9	15.3	15.0	14.8	15.1	15.2	15.3	17.3	17.6	17.6	18.0
120	15.2	15.8	15.2	14.9	14.8	15.1	15.2	15.3	17.3	17.6	17.6	18.0
150	15.1	15.7	15.1	14.8	14.7	15.1	15.1	15.2	17.3	17.6	17.6	18.0
180	15.0	15.6	15.0	14.7	14.7	15.1	15.1	15.2	17.3	17.5	17.6	17.9
210	15.0	15.6	15.0	14.6	14.7	15.0	15.1	15.2	17.2	17.5	17.6	17.9
240	14.9	15.5	14.9	14.6	14.6	15.0	15.0	15.1	17.2	17.4	17.6	17.9
270	14.8	15.4	14.8	14.5	14.6	15.0	15.0	15.1	17.2	17.4	17.6	17.9
300	14.7	15.4	14.7	14.4	14.6	15.0	14.9	15.1	17.2	17.4	17.5	17.9
330	14.6	15.3	14.6	14.3	14.5	14.9	14.9	15.1	17.2	17.4	17.5	17.9
360	14.6	15.2	14.6	14.3	14.5	14.9	14.9	15.1	17.2	17.4	17.5	17.9
390	14.5	15.1	14.5	14.2	14.5	14.9	14.8	15.0	17.2	17.3	17.5	17.9
420	14.5	15.0	14.5	14.1	14.4	14.9	14.8	15.0	17.2	17.3	17.5	17.9
450	14.4	14.9	14.4	14.1	14.4	14.8	14.7	15.0	17.2	17.3	17.5	17.9
480	14.3	14.9	14.3	14.0	14.4	14.8	14.7	14.9	17.2	17.3	17.5	17.9
510	14.3	14.8	14.3	14.0	14.3	14.8	14.7	14.9	17.2	17.3	17.5	17.9
540	14.2	14.7	14.2	13.9	14.3	14.8	14.6	14.9	17.2	17.2	17.5	17.9
570	14.1	14.6	14.1	13.8	14.3	14.7	14.6	14.8	17.2	17.2	17.5	17.9
600	14.1	14.6	14.1	13.8	14.2	14.7	14.5	14.8	17.2	17.2	17.4	17.9
630	14.0	14.5	14.0	13.7	14.2	14.7	14.5	14.7	17.2	17.2	17.4	17.9
660	13.9	14.4	13.9	13.6	14.2	14.7	14.5	14.7	17.2	17.2	17.4	17.9
690	13.8	14.3	13.8	13.5	14.2	14.7	14.4	14.7	17.1	17.2	17.4	17.8
720	13.8	14.2	13.8	13.5	14.1	14.6	14.4	14.6	17.1	17.2	17.4	17.8
750	13.7	14.2	13.7	13.4	14.1	14.6	14.3	14.6	17.1	17.1	17.4	17.8
780	13.6	14.1	13.6	13.3	14.1	14.6	14.3	14.5	17.1	17.1	17.3	17.8
810	13.5	14.0	13.5	13.2	14.1	14.6	14.2	14.5	17.1	17.1	17.3	17.8
840	13.5	13.9	13.5	13.2	14.0	14.5	14.2	14.5	17.1	17.1	17.3	17.8
870	13.4	13.8	13.4	13.1	14.0	14.5	14.2	14.4	17.1	17.1	17.3	17.8
900	13.3	13.8	13.3	13.0	13.9	14.5	14.1	14.4	17.1	17.0	17.3	17.8
960	13.2	13.5	13.2	12.8	13.9	14.4	14.0	14.3	17.0	17.0	17.3	17.8
1020	13.0	13.3	13.0	12.6	13.8	14.4	13.9	14.2	17.0	17.0	17.2	17.8
1080	12.8	13.2	12.8	12.5	13.8	14.3	13.8	14.1	17.0	16.9	17.2	17.7

APPENDIX F

Cell density of strain PG-01 with time under different initial phenol concentrations

Cell density (number/ml)																				
Time (h)	10 mg phenol/l			20 mg phenol/l			Time (h)	30 mg phenol/l			50 mg phenol/l			100 mg phenol/l			Time (h)	200 mg phenol/l		
	Average	Standard error		Average	Standard error			Average	Standard error		Average	Standard error		Average	Standard error			Average	Standard error	
0	4.13E+06	2.47E+05	4.83E+06	4.95E+06	4.95E+04	0	1.55E+07	0.00E+00	1.51E+07	2.97E+05	1.79E+07	9.90E+04	0	3.61E+07	4.24E+05					
0.75	4.90E+06	0.00E+00	5.15E+06	9.90E+04	0.75	1.62E+07	9.90E+04	1.67E+07	1.48E+05	1.79E+07	4.95E+04	3	6.07E+07	2.12E+05						
1.5	5.25E+06	4.95E+04	5.92E+06	4.95E+04	1.5	1.77E+07	2.47E+05	2.09E+07	0.00E+00	2.21E+07	2.97E+05	6	8.24E+07	1.27E+06						
2.25	5.53E+06	4.95E+04	6.55E+06	0.00E+00	2.25	2.01E+07	2.47E+05	2.25E+07	0.00E+00	2.31E+07	2.97E+05	9	1.08E+08	3.18E+05						
3	5.81E+06	4.95E+04	7.14E+06	0.00E+00	3	2.28E+07	1.98E+05	2.55E+07	9.90E+04	2.56E+07	3.46E+05	19	1.87E+08	4.24E+05						
3.75	6.79E+06	4.95E+04	8.16E+06	4.95E+04	3.75	2.67E+07	1.98E+05	2.83E+07	1.98E+05	2.94E+07	3.96E+05	22	1.76E+08	8.49E+05						
4.5	7.63E+06	4.95E+04	9.42E+06	9.90E+04	4.5	3.04E+07	8.91E+05	3.19E+07	0.00E+00	3.32E+07	4.95E+05	25	1.65E+08	9.55E+05						
5.25	7.91E+06	4.95E+04	1.06E+07	1.98E+05	6	4.06E+07	2.97E+05	4.24E+07	8.41E+05	3.99E+07	8.91E+05									
6.25	8.82E+06	0.00E+00	1.19E+07	1.48E+05	8	6.21E+07	6.43E+05	6.52E+07	6.43E+05	5.53E+07	1.39E+06									
7.25	1.10E+07	1.48E+05	1.43E+07	1.98E+05	9	6.78E+07	4.95E+04	8.13E+07	2.28E+06	6.94E+07	7.42E+05									
8.25	1.18E+07	4.95E+04	1.72E+07	4.95E+04	9.75	6.81E+07	1.48E+05	1.00E+08	1.48E+05	8.10E+07	3.46E+05									
9.25	1.47E+07	9.90E+04	2.11E+07	1.98E+05	10.75	6.82E+07	1.98E+05	1.24E+08	8.91E+05	9.57E+07	7.42E+05									
10.25	1.86E+07	4.95E+04	2.70E+07	9.90E+04	11.5	6.83E+07	1.48E+05	1.52E+08	4.70E+06	1.16E+08	4.95E+04									
11.25	2.17E+07	1.98E+05	3.43E+07	1.48E+05	12.25	6.85E+07	9.90E+04	1.64E+08	1.34E+06	1.35E+08	1.98E+06									
12.25	2.66E+07	0.00E+00	4.21E+07	3.46E+05	17	6.34E+07	1.24E+05	1.48E+08	3.79E+06	2.28E+08	1.48E+06									
					22	5.83E+07	3.46E+05	1.32E+08	2.37E+05	2.62E+08	5.44E+05									
					23.5	5.95E+07	9.90E+05	1.33E+08	9.70E+04	2.67E+08	1.98E+05									

Cell density of strain PG-01 with time under different initial phenol concentrations

Time (h)	Cell density (number/ml)									
	300 mg phenol/l	500 mg phenol/l	600 mg phenol/l	700 mg phenol/l	800 mg phenol/l	900 mg phenol/l	1000 mg phenol/l	Average	Standard error	Standard error
0	3.70E+07	3.78E+07	3.85E+07	3.89E+07	3.92E+07	3.90E+07	3.18E+05	3.89E+07	3.18E+05	3.89E+07
3	5.75E+07	5.05E+07	4.70E+07	4.47E+07	4.37E+07	4.08E+07	7.42E+05	3.83E+07	7.42E+05	3.83E+07
6	7.54E+07	5.94E+07	5.61E+07	5.20E+07	4.99E+07	4.81E+07	1.06E+06	4.37E+07	1.06E+06	4.37E+07
9	9.86E+07	7.52E+07	6.42E+07	5.25E+07	5.02E+07	4.95E+07	5.30E+05	4.78E+07	5.30E+05	4.78E+07
19	1.79E+08	1.34E+08	1.11E+08	8.18E+07	5.73E+07	4.95E+07	6.05E+06	4.52E+07	5.30E+05	4.52E+07
22	2.07E+08	1.46E+08	1.31E+08	9.24E+07	7.11E+07	5.00E+07	4.24E+05	4.50E+07	5.30E+05	4.50E+07
25	2.41E+08	1.59E+08	1.38E+08	9.97E+07	7.93E+07	5.05E+07	1.06E+05	4.52E+07	2.12E+05	4.52E+07
28	2.58E+08	1.72E+08	1.55E+08	1.09E+08	8.81E+07	6.36E+05	6.36E+05	4.62E+07	9.55E+05	4.62E+07
31	2.33E+08	1.90E+08	1.72E+08	1.25E+08	1.02E+08	1.06E+05	1.06E+05	4.80E+07	8.49E+05	4.80E+07
43		2.83E+08	2.07E+08	1.63E+08	1.43E+08	2.12E+05	2.12E+05	4.75E+07	6.36E+05	4.75E+07
45.75		3.16E+08	2.22E+08	1.73E+08	1.54E+08	6.36E+05	6.36E+05	5.03E+07	5.30E+05	5.03E+07
48.75		3.48E+08	2.49E+08	1.90E+08	1.70E+08	2.12E+05	2.12E+05	5.24E+07	6.36E+05	5.24E+07
51.75			2.95E+08	2.13E+08	1.87E+08	7.42E+05	2.12E+05	5.75E+07	2.12E+05	5.75E+07
54.75				2.43E+08	2.12E+08	3.18E+05	1.80E+06	6.02E+07	1.80E+06	6.02E+07
66.75				4.01E+08	3.17E+08	6.36E+05		8.01E+07	4.24E+05	8.01E+07
69.75								8.56E+07	4.24E+05	8.56E+07
72.75								9.00E+07	1.06E+05	9.00E+07
75.75								9.48E+07	9.55E+05	9.48E+07
88.75								1.13E+08	1.27E+06	1.13E+08
93.15								1.17E+06	1.70E+06	1.17E+06
100.15								1.22E+08	1.70E+06	1.22E+08
117.75								1.33E+08	5.83E+06	1.33E+08
								1.72E+08	6.36E+05	1.72E+08

APPENDIX G

Cell density of strain PG-03 with time under different initial phenol concentrations

Cell density (number/ml)										
Time (h)	10 mg phenol/l			Time (h)	20 mg phenol/l			50 mg phenol/l		
	Average	Standard error			Average	Standard error		Average	Standard error	
0	6.40E+06	0.00E+00	6.90E+06	0	2.22E+07	0.00E+00	1.86E+07	4.24E+05	2.56E+07	1.41E+05
0.75	7.00E+06	0.00E+00	7.80E+06	0.75	2.32E+07	1.41E+05	2.30E+07	2.12E+05	2.55E+07	7.07E+04
1.5	7.50E+06	7.07E+04	9.10E+06	1.5	2.53E+07	3.54E+05	2.73E+07	0.00E+00	3.16E+07	4.24E+05
2.25	7.90E+06	7.07E+04	9.60E+06	2.25	2.87E+07	3.54E+05	3.17E+07	0.00E+00	3.30E+07	4.24E+05
3	8.30E+06	7.07E+04	1.08E+07	3	3.26E+07	2.83E+05	3.60E+07	1.41E+05	3.65E+07	4.95E+05
3.75	9.70E+06	7.07E+04	1.25E+07	3.75	3.82E+07	2.83E+05	4.04E+07	2.83E+05	4.20E+07	5.66E+05
4.5	1.09E+07	7.07E+04	1.44E+07	4.5	4.34E+07	1.27E+06	4.56E+07	0.00E+00	4.74E+07	7.07E+05
5.25	1.13E+07	7.07E+04	1.58E+07	6	5.80E+07	4.24E+05	6.05E+07	1.20E+06	5.70E+07	1.27E+06
6.25	1.26E+07	0.00E+00	1.83E+07	8	8.87E+07	9.19E+05	9.31E+07	9.19E+05	7.90E+07	1.98E+06
7.25	1.57E+07	2.12E+05	2.26E+07	9	9.69E+07	7.07E+04	1.16E+08	3.25E+06	9.91E+07	1.06E+06
8.25	1.69E+07	7.07E+04	2.65E+07	9.75	9.73E+07	2.12E+05	1.43E+08	2.12E+05	1.16E+08	4.95E+05
9.25	2.10E+07	1.41E+05	3.38E+07	10.75	9.74E+07	2.83E+05	1.78E+08	1.27E+06	1.37E+08	1.06E+06
10.25	2.65E+07	7.07E+04	4.32E+07	11.5	9.76E+07	2.12E+05	2.17E+08	6.72E+06	1.66E+08	7.07E+04
11.25	3.10E+07	2.83E+05	5.49E+07	12.25	9.78E+07	1.41E+05	2.34E+08	9.05E+06	1.93E+08	2.83E+06
12.25	3.80E+07	0.00E+00	6.53E+07	17	9.06E+07	1.77E+05	2.11E+08	5.41E+06	3.25E+08	2.12E+06
				22	8.33E+07	4.95E+05	1.88E+08	1.77E+06	3.74E+08	7.78E+05
				23.5	8.50E+07	1.41E+06	1.90E+08	4.24E+05	3.82E+08	2.83E+05

Cell density of strain PG-03 with time under different initial phenol concentrations

Cell density (number/ml)																					
Time (h)	200 mg phenol/l			300 mg phenol/l			400 mg phenol/l			500 mg phenol/l			Time (h)			600 mg phenol/l			700 mg phenol/l		
	Average	Standard error		Average	Standard error		Average	Standard error		Average	Standard error		Average	Standard error		Average	Standard error		Average	Standard error	
0	1.19E+07	3.54E+05		1.30E+07	2.47E+06		2.38E+07	7.07E+05		1.67E+07	1.06E+06		0	2.70E+07	2.83E+05		2.90E+07	0.00E+00		0.00E+00	
2	1.15E+07	2.12E+06		1.41E+07	0.00E+00		2.66E+07	7.07E+05		2.47E+07	3.89E+06		2.5	3.22E+07	0.00E+00		3.24E+07	0.00E+00		0.00E+00	
4	1.19E+07	5.30E+06		1.65E+07	0.00E+00		2.78E+07	0.00E+00		2.65E+07	3.54E+05		5	3.71E+07	2.12E+05		3.57E+07	4.95E+05		4.95E+05	
16	2.55E+07	3.54E+05		2.23E+07	3.54E+05		5.17E+07	5.30E+06		4.70E+07	7.07E+05		7.5	4.37E+07	2.12E+05		4.45E+07	4.95E+05		4.95E+05	
18	4.20E+07	0.00E+00		2.66E+07	7.07E+05		6.15E+07	3.89E+06		5.86E+07	4.24E+06		10	4.92E+07	4.24E+05		4.73E+07	4.95E+05		4.95E+05	
20	5.58E+07	0.00E+00		2.91E+07	1.77E+06		7.32E+07	3.54E+06		6.29E+07	3.54E+05		20.75	7.09E+07	2.12E+05		6.25E+07	2.12E+05		2.12E+05	
22	5.97E+07	3.54E+05		3.08E+07	7.07E+05		8.86E+07	7.07E+05		7.58E+07	6.36E+06		23.25	8.02E+07	2.83E+05		6.71E+07	3.54E+05		3.54E+05	
24	6.65E+07	3.54E+05		3.70E+07	2.12E+06		1.12E+08	3.54E+06		9.31E+07	3.54E+05		25.75	8.24E+07	4.24E+05		6.72E+07	2.83E+05		2.83E+05	
26	7.29E+07	3.54E+05		3.92E+07	0.00E+00		1.39E+08	6.36E+06		1.09E+08	2.47E+06		28.25	9.36E+07	4.24E+05		6.91E+07	3.54E+05		3.54E+05	
28	7.98E+07	7.07E+05		4.29E+07	1.77E+06		1.72E+08	3.54E+05		1.31E+08	3.89E+06		30.75	1.07E+08	2.12E+05		7.29E+07	7.07E+04		7.07E+04	
40	1.89E+08	9.19E+06		7.70E+07	0.00E+00		4.58E+08	0.00E+00		3.75E+08	1.20E+07		33.25	1.25E+08	2.83E+05		7.72E+07	1.41E+05		1.41E+05	
42	2.26E+08	7.07E+05		9.01E+07	1.06E+06		5.40E+08	1.80E+07		4.33E+08	2.12E+06		46.25	2.56E+08	7.78E+05		1.17E+08	0.00E+00		0.00E+00	
44	2.83E+08	1.77E+06		1.09E+08	3.18E+06		6.38E+08	4.24E+06		5.18E+08	2.37E+07		48.75	2.94E+08	7.07E+04		1.30E+08	1.41E+05		1.41E+05	
46	3.48E+08	5.66E+06		1.30E+08	1.41E+06		7.54E+08	7.78E+06		5.92E+08	1.70E+07		51.75	3.50E+08	3.54E+05		1.38E+08	2.12E+05		2.12E+05	
48	4.37E+08	2.83E+06		1.47E+08	0.00E+00		8.77E+08	2.83E+06		6.90E+08	5.30E+06		54.75	4.23E+08	2.83E+05		1.60E+08	6.36E+04		6.36E+04	
													57.75	4.97E+08	5.66E+05		1.87E+08	1.41E+06		1.41E+06	

Cell density of strain PG-03 with time under different initial phenol concentrations

Time (h)	Cell density (number/ml)					
	800 mg phenol/l		900 mg phenol/l		1000 mg phenol/l	
	Average	Standard error	Average	Standard error	Average	Standard error
0	2.89E+07	2.12E+05	3.04E+07	4.24E+05	3.93E+07	3.54E+05
2.5	3.23E+07	7.07E+04	3.09E+07	7.07E+04	4.36E+07	1.41E+05
5	3.53E+07	7.07E+04	3.32E+07	1.41E+05	4.54E+07	0.00E+00
7.5	4.40E+07	1.41E+05	3.83E+07	3.54E+05	5.18E+07	0.00E+00
10	4.55E+07	2.12E+05	4.36E+07	1.41E+05	5.63E+07	2.12E+05
20.75	5.81E+07	2.12E+05	5.36E+07	2.83E+05	6.61E+07	3.54E+05
23.25	6.18E+07	0.00E+00	5.55E+07	7.07E+04	6.62E+07	0.00E+00
25.75	6.21E+07	3.54E+05	5.35E+07	2.12E+05	6.68E+07	4.24E+05
28.25	6.29E+07	7.07E+04	5.56E+07	2.83E+05	6.80E+07	4.24E+05
30.75	6.45E+07	7.07E+04	5.66E+07	4.24E+05	6.64E+07	2.83E+05
33.25	6.86E+07	1.41E+05	5.80E+07	1.41E+05	6.96E+07	0.00E+00
46.25	8.21E+07	3.54E+05	6.67E+07	3.54E+05	7.82E+07	7.07E+05
48.75	8.66E+07	2.83E+05	7.11E+07	7.07E+04	8.06E+07	4.24E+05
51.75	8.60E+07	0.00E+00	6.98E+07	1.41E+05	8.05E+07	7.07E+04
54.75	8.97E+07	3.54E+05	7.27E+07	3.54E+05	7.98E+07	1.41E+05
57.75	9.66E+07	4.24E+05	7.48E+07	2.83E+05	8.14E+07	1.41E+05
70.5	1.27E+08	0.00E+00	8.08E+07	2.83E+05	9.29E+07	2.12E+05
73.5	1.35E+08	1.41E+05	8.78E+07	2.83E+05	1.19E+08	0.00E+00
76.5	1.53E+08	4.24E+05	8.98E+07	4.24E+05	9.61E+07	2.12E+05
79.5	1.52E+08	0.00E+00	8.72E+07	1.41E+05	9.30E+07	1.41E+05
82.5	1.92E+08	2.83E+05	9.05E+07	3.54E+05	1.02E+08	4.24E+05
94.25	3.30E+08	3.54E+05	1.05E+08	3.54E+05	1.01E+08	7.07E+04
98.75			1.09E+08	7.07E+04	1.01E+08	4.95E+05
103.75			1.16E+08	2.12E+05	1.01E+08	2.83E+05
118.5			1.23E+08	2.83E+05	1.05E+08	2.83E+05
122			1.35E+08	3.54E+05	1.13E+08	2.83E+05
126.75			1.93E+08	7.07E+04	1.15E+08	2.12E+05
142.25					1.21E+08	5.66E+05
146.25					1.20E+08	4.24E+05
153					1.30E+08	3.54E+05
166.25					1.27E+08	1.41E+05
170.25					1.39E+08	1.41E+05
176.5					1.40E+08	3.54E+05
190.25					1.73E+08	0.00E+00
194.25					1.79E+08	3.54E+05
198.25					1.79E+08	2.12E+05
214.25					2.16E+08	2.12E+05
218					2.38E+08	4.24E+05
241					3.94E+08	2.12E+05
262.5					8.59E+08	6.36E+05
274.75					1.27E+09	1.41E+05