The Role of Microbial Aggregation in Aerobic Granulation

Luo Yiqun

School of Civil and Environmental Engineering

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SUMMARY

Aerobic granulation is a novel biological technology for the treatment of a wide spectrum of wastewater. It owns several benefits, such as dense and compact microbial structure, good settling capability, high biomass retention etc., when compared with conventional activated sludge. Extensive research efforts have been made to develop aerobic granules in sequencing batch reactor (SBR) for the efficient degradation of various pollutants. However, the mechanisms of aerobic granulation are still unclear, especially with respect to the microbial driving force. Therefore, the present work aims to address the potential role of cell-cell aggregating interactions as a microbial driving force to accelerate the formation of aerobic granules, to improve the SBR performance, and to maintain the integrity of aerobic granules.

In order to evaluate the role of cell-cell aggregation in the initially formed aggregates in flow environment, dispersed aerobic granules bacteria were subjected to selective hydraulic pressure by settling time. The coaggregating bacteria with mild autoaggregating ability were enriched as a result. The aggregating community composition in both granules and enrichment cultures were identified and assessed, respectively. Results suggested that strong autoaggregating bacteria or coaggregating bacteria can occur in aerobic granules sheltered by compact structure. In the formation of relatively loose structured aggregates which could act as precursor for big sized bioflocs or granules, bacteria with both coaggregating and autoaggregating ability was preferred.

The coaggregating behaviors of two coaggregating pairs partnered by granule isolates B6-2 with B6-25 and B6-25 with B7-8 were investigated under different pH value, temperature, sugar content, EDTA concentration, and electrolyte combination.

Both coaggregating pairs were found to be ion-sensitive and the coaggreations were inhibited slightly by extreme pH value and presence of EDTA. Coaggregation between B6-25 and B7-8 was not sensitive to temperature, but inhibited by glucose.

The aggregating community diversity in aerobic granules fed by different substrates was surveyed. 16s rRNA sequencing was used to identify the composition and diversity of aggregating microbes in activated sludge, acetate-fed granule and phenol-degrading granule. DGGE profiles were also studied to track the shift of aggregating populations responding to chemical and toxic shock induced by phenol. The results showed that higher proportion of microbial community members in aerobic granules was involved in cell-cell aggregating interactions than that in activated sludge. The analysis of aggregating community components indicated that the fraction of coaggregating microbes can be increased under the chemical and toxic shock. Coaggregating interactions among most microbial community members were desirable to resist the toxicity imposed by phenol. Filamentous forms of microorganisms, such as fungus and filaments, were shown to be important for the integrity of phenol-degrading granule structure because they not only provide scaffolds for small-sized bacteria to colonize, but work as dynamic part of the aggregating community to interact with other microbial members.

The effect of bacterial aggregation on accelerated formation and enhanced performance of aerobic granules was studied. Granule isolate S35 showing both aggregating and autoaggregation ability was augmented into activated sludge to cultivate fast-forming granules. The bioaugmentation of monoculture (S35) in relatively low amount (10% in dry weight) demonstrated that aggregating isolate S35 can persist and predominate in activated sludge, and accordingly facilitate the co-development of multiple species to form compact granular structure. The feasibility of bioaugmentation of aggregating monoculture to accelerate the

granulation process was supported, implying the critical role of aggregation in the formation of aerobic granules.

The effect of cell-cell aggregating interactions on the recovery of the disintegrated granular sludge was investigated by the employment of two identical column-type SBRs with bioaugmented R1 by granule isolate S15 and non-augmented R2. Herein, S15 with autoaggregating ability was pre-isolated from aerobic granules. Bioaugmentation with S15 significantly improved recovery extent of disintegrated granular sludge in R1 in comparison with reference reactor R2. DGGE profile indicated the elimination of S15 abundance in R1 sludge community within 5 days. However, the hastened and enhanced recovery of granular sludge continued. Reaggregated aerobic granules appeared in R1 on day 5 and quickly grew to displace the loose disintegrated granular sludge as a dominant form in the biomass. The results suggested that S15 was functionally important, other than numerically dominant in granule community. The microbial aggregation might be an integral part for aerobic granulation, and the presence of S15 acted as a trigger to the recovery of disintegrated granular community.

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LIST OF ACRONYMS

APHA	American Public Health Association
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
CLSM	Confocal Laser Scanning Microscopy
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EPS	Extracellular Polymeric Substances
HRT	Hydraulic Retention Time
IA	Image Analysis
MATH	Microbial Adhesion To Hydrocarbon
NCBI	National Centre for Biotechnology Information
OD	Optical Density
OLR	Organic Loading Rate
PAHs	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PN	Protein
PS	Polysaccharide
RNA	Ribonucleic Acid
SBR	Sequencing Batch Reactor
SEM	Scanning Electron Microscopy
SRT	Sludge Retention Time
SS	Suspended Solids
SVI	Settling Volume Index
TOC	Total Organic Carbon
UASB	Upflow Anaerobic Sludge Blanket

CHAPTER ONE
INTRODUCTION

1.1 BACKGROUND

Wastewater treatment originates from the beginning of the twentieth century. With a rapidly expanding world population and industrial revolution, the problems related to the management of wastewater have gained considerable importance. Wastewater treatment involves the use of physical, chemical or biological processes to remove organic matter, nutrients and pathogens. Biological wastewater treatment is an environmental friendly and inexpensive process compared with other methods. It takes advantage of natural microbial activity for the degradation of organic or inorganic matter and removal of nutrients (Grady et al. 1999). In biological treatment, living microorganisms are exploited to transform pollutants through enzymatically catalyzed chemical reactions. The most widely used biological processes in municipal or industrial wastewater treatment are the activated sludge system and the biofilm system.

The activated sludge process was first developed in England in 1914. From then on, many modifications have been made to the activated sludge system. Nonetheless, most activated sludge systems or biofilm systems for wastewater treatment have some disadvantages, such as low flexibility with respect to fluctuating loading rate, high surplus biomass production, a large area requirement for reactors (especially settlers), and a relatively low volumetric conversion capacity (Beun et al. 1999).

In activated sludge systems, several types of sludge separation problems, e.g. bulking, foaming and rising sludge, are commonly encountered. These problems are mainly due to the poor settling properties of biomass, whose washout into the effluent deteriorates the treatment performance. The loss of biomass from the sludge tank also makes the sludge system fragile. Moreover, the design of settlers that demand large areas for reactors is necessary in the activated sludge system.

In contrast, highly compact reactors have been developed for the biogranulation process, for example, 40 kg COD m^{-3} day^{-1} for the up-flow anaerobic sludge blanket (UASB) reactor (Lettinga 1995). Granulation is a physical, chemical, and biological process, and has been described as an aggregation of biomass that results from the self-immobilization of microorganisms under favorable conditions. Aggregated or granulated microbial communities are packed in spherical structure under a high shear force regime, which allow the accumulation of high amounts of active biomass in the reactor.

Thus compared with conventional activated sludge process, the granular sludge system offers several advantages (Beun et al. 1999; Liu et al. 2004a; Logan et al. 1988; Moy et al. 2002; Zhu 2003):

(1) Granules have a strong and compact structure with high sludge retention time, hence less reactor volume is required and sludge production is minimized in the process.
(2) Under conditions where the composition of the bulk solution is unfavorable for microbial growth, a more favorable microenvironment can be created within the aggregate so that active metabolism is still possible.
(3) This system has microbial aggregates with good settling velocities and high metabolism ensuring low solid concentration in the effluent. Sludge separation is integrated in the reactor itself and usually no clarifier is needed.
(4) Granular sludge can tolerate high organic loading rate and withstand high strength wastewater shock loading.
(5) The granules can overcome substrate inhibition effects and protect against the adverse environment imposed by toxic substrates.

Though aerobic granules are regarded as hydrogels recently (Seviour et al. 2009), it is more widely accepted that granules can be considered to be particle-based biofilms in the form of spherical aggregates. A biofilm is a matrix of cells and cellular products attached to a solid surface of substratum, offering positive opportunities in bioremediation and wastewater treatment such as increased process

flow rates without washout of the organisms in the reactor (Brent et al. 1995). Traditional biofilm systems, such as trickling filters and rotating biological disk reactors, are normally used in situations where the treatment efficiency through the usage of suspended organisms is limited by the low biomass concentration and hydraulic residence time (Nicolella et al. 2000). For the slow-growing microorganisms such as nitrifiers whose growth requires long residence time in suspension, a biofilm system is an effective way not only to retain biomass and support the microbial growth on attached surfaces, but also to improve the volumetric conversion capacity. But in normal biofilm systems, the delivery of poorly soluble substrates (e.g. oxygen) to the biofilm surface might become restricted. One of the solutions to enlarge the biofilm specific area and enhance mass transfer and substrate utilization is to develop granulation process, which has both large specific area and good settling velocity.

Compared to other biofilm systems, microorganisms in a biogranulation system are immobilized in compact granules through self-aggregation without carriers which are usually expensive and occupy a significant amount of reactor, so less capital is required and a large biomass content can be maintained. Furthermore, the large specific surface area of granules ensures that the conversions are not strictly limited by the biofilm liquid mass transfer rate.

Both anaerobic and aerobic granules have been reported in the past several decades (Beun et al. 1999; Lettinga et al. 1980; Schmidt et al. 1996; Tay et al. 2001b, 2002). The formation and mechanism of anaerobic granules have been extensively studied especially in the upflow anaerobic sludge blanket (UASB) since 1970s' (Alves et al. 2000; Grotenhuis et al. 1991; MacLeod et al. 1990). Despite the wide application in wastewater treatment plants, this anaerobic granulation technology has some limitations: the start-up period for anaerobic granules can be difficult and long, a relative high operation temperature is needed, and anaerobic granulation process is not suitable to treat low strength organic wastewater, nor to remove nutrients (Liu et al. 2004a).

Aerobic granulation is a novel biotechnology developed recently and was first reported in a continuous aerobic upflow sludge blanket reactor (Mishima et al. 1991). For applications in which the discontinuous process is advantageous, aerobic granules can also be formed in sequencing batch reactors (SBRs) (Beun et al. 1999; Morgenroth et al. 1997; Peng et al. 1999; Tay et al. 2001b).

The development of granules is determined by the aggregation of microorganisms. Though the formation process, structure and the properties of granules have been studied (Etterer et al. 2001; Tay et al. 2001a, c), the mechanism of aerobic granulation and manipulation of granulation process on the basis of cell aggregation knowledge is still a subject of investigation. For a proper evaluation of aggregating interactions in granulation, there are several questions to be answered which are not yet well understood: What is the diversity and compositions of aggregating population in aerobic granules? How do the microbial isolates in aerobic granule community get involved in the initial aggregate formation with respect to the acquisition or loss of aggregation abilities? What are the dynamics of aggregating microbial community of aerobic granules when exposed to the chemical and toxic shock? Is it possible to manipulate the formation and performance of aerobic granules, as well as the recovery of disintegrated granules based on the knowledge of cell aggregation?

1.2 OBJECTIVES OF STUDY

This research aims on the following objectives:

(1) To analyze the microbial community structure with aggregating ability in aerobic granule community;
(2) To identify the aggregating community of enrichment culture in the formation of initial microbial aggregates in dynamic environment;
(3) To study the aggregating behaviors of selected aerobic granule isolates under various physical and chemical conditions;
(4) To survey the aggregating community dynamics of acetate-fed aerobic granules

with the presence of phenol;

(5) To examine the application of aggregating mono-culture on the fast formation of aerobic granules;

(6) To test the strategy to accelerate the recovery of disintegrated aerobic granules by using the selected aggregating culture.

In essence, this research will focus on various aspects related to the cell aggregation in aerobic granules for a better prediction and control of the aerobic granulation process.

This thesis contains seven chapters. Chapter One serves as an introduction to the present study. Chapter Two is a literature review on the fundamentals of aggregating interactions and the possible role in aerobic granulation. Chapter Three contains the studies on diversity of aggregating populations in aerobic granules and the initial aggregates enriched from dispersed granule bacteria, and the aggregating behavior of selected strains under various physical and chemical conditions. Chapter Four investigates the response of granule community with respect to the dynamics of aggregating populations to the presence of phenol. The aggregating community structure and compositions in benign-substrate-fed granules is also studied. Chapter Five focuses on the accelerated formation of aerobic granules by bioaugmentation with aggregating mono-culture isolated from aerobic granules. Chapter Six explores the contribution of one aerobic granule isolate on the reaggregation of disintegrated aerobic granular sludge. Chapter Seven draws conclusions from the experimental results, followed by recommendations for the future works.

CHAPTER TWO
LITERATURE REVIEW

2.1 CELL AGGREGATION

Cell aggregation can be defined as the gathering together of cells to form stable, contiguous, multi-cellular associations (Calleja 1984; Calleja et al. 1984). It is also commonly referred as adherence, adhesion, agglomeration, agglutination, association, clumping, flocculation, or flotation. Aggregation is the clumping together of discrete microbial cells from a dispersed state into aggregates containing many cells linked together through complex interactions.

2.1.1 Coaggregation and Autoaggregation

From the aspects of cells involved in aggregation process, there are two categories of microbial aggregation: coaggregation and autoaggregation. Coaggregation is the interaction between a pair of genetically distinct partners mediated by cell-cell recognition. In contrast, autoaggregation is the interaction between cells of the same strain. These two types of cell aggregation are important in research to enhance biotechnological treatment process, yet coaggregation might be more useful from the practical point of view, because functional consortia of coaggregated cells often possesses a combined metabolic activity which is greater than that of the component species (Moller et al. 1998). In contrast to coaggregation, autoaggregation might be a selfish mechanism in which a strain within the biofilm expresses polymers to enhance the integration of genetically identical strains (Rickard et al. 2003b). Another phenomenon of great interest in coaggregation is the enhanced horizontal transfer of bacterial genes and exopolymers between microbial partners in coaggregates as the response to selective pressures and environmental signals, which indicated the existence of an extensive coordination at a multicellular level for selection of optimal cell-cell interactions in the environment (Osterreicher et al. 2000).

The phenomenon of coaggregation was reported to occur in oral plaque-forming bacteria (Kolenbrander 2000, 2002), and among bacteria isolated from aquatic biofilms (Buswell 1997; Rickard et al. 2002), activated sludge (Malik et al. 2003c, 2003d; Zita et al. 1997), aerobic granules (Jiang et al. 2006b) and marine sediments (Grossart et al. 2003). Coaggregation between *lactobacilli* and *Escherichia coli* or between two *lactobacilli* strains isolated from the intestinal tract of humans (Drago et al. 1997), chickens (Vandevoorde et al. 1992) and pigs (Kmet et al. 1995) were also investigated. The evidences accumulated indicated that coaggregation between genetically distinct strains was a widespread phenomenon.

The cell coaggregation was studied extensively because of its medical significance. Coaggregation interaction was shown to be widespread in bacterial strains isolated from dental plaque (Kolenbrander et al. 1999; Kolenbrander 2000). The cell's ability to coadhere was thought to play a crucial role in microbial infections, spread of cancer, and formation of multi-species biofilms or activated flocs.

The first paper to describe coaggregation in dental plaque-forming bacteria was published in 1970's. Since then, more than 475 research papers described coaggregation between dental plaque bacteria (search in www.pubmed.com in terms of coaggregation and oral or dental in April 2009). It can be generally accepted that coaggregation is extremely important not only in formation of oral plaque, but also to create a more favorable microenvironment under unfavorable conditions (such as extreme pH, temperature) for the growth of some bacteria, so that metabolism can still be possible. For example, Kolenbrander et al. (2000) found that tooth-plaque-colonizing bacteria were able to multiply and aggregate with additional bacterial species and embed themselves in a matrix of extracellular polymers originating from both saliva and bacteria. *Streptococci* and *Actinomyces* were the major initial colonizers on the tooth surface, and the coaggregation between them and their substrata helped establish the early biofilm community. *Fusobacteria* played a central role as physical bridge that mediated coaggregation of cells, and as physiological bridge that promoted anaerobic microenvironments which protected strict anaerobes in an aerobic environment. From the studies of

Shen and coworkers (2004) on coaggregation amongst twenty two different wild types of microbial species, bacterial coaggregation reactions between different species and the autoaggregation of the same species were suggested to be associated with the initiation and development of dental plaque and biofilms. *Actinomyces spp., Veillonella spp., Prevotella spp.* and *Fusobacterium spp.* appeared to play important roles.

In addition to dental plaque biofilm bacteria, there were numerous reports of coaggregation phenomena in biofilm communities in other habitats. Several studies investigated triggers for coaggregation as well as other aspects of the coaggregation process (Bossier et al. 1996b). Intrageneric, intergeneric and intraspecies coaggregating interactions were observed in the sequential integration of bacteria into freshwater biofilms (Rickard et al. 2000). In some cases, the coaggregation interaction was a highly specific process mediated by the recognition of complementary lectin–carbohydrate molecules between the aggregating partners (Cisar et al. 1997; Rickard et al. 2000). This finding was supported by reports that the addition of simple sugars and chelating agents could reverse the lectin-carbohydrate mediated coaggregation (Buswell 1997; Rickard et al. 2003b). Furthermore, the genetic stability of coaggregating properties was shown to depend on either the cell age of coaggregating partners or the type of culture media used (Rickard et al. 2000).

The bacterial biofilm was described as a highly structured community of bacterial cells enclosed in a self-produced polymeric matrix and adhering to an inert or living surface (Jiang et al. 2003). Good coaggregation in biofilm indicated a large variety of microorganisms conglomerated in communities with complex interactions. A current model described the development of biofilm as a succession of cell aggregation and multiplication (Rickard et al. 2003a). The first microorganisms to attach were primary colonies to form the young biofilm, and multiply. The secondary colonies were able to attach in succession. So the biofilm began to develop into a multi-species community. Extracellular polymeric substances (EPS) played an important role in this process; they could strengthen cell aggregates

within biofilm and also act as receptors of other coaggregating cells. Although it is accepted that EPS could act as intercellular 'cement' in the cell coaggregates, its exact role in mediating or enhancing coaggregation in suspended cells remains unclear.

'Bridging organisms' were isolated from freshwater biofims (Rickard et al. 2002). These isolates can coaggregate with many other bacteria, no matter how the latter can coaggregate or autoaggregate. Moreover, coaggregation between freshwater biofilms was mediated by protein-saccharide interactions, which was also found in oral plaque-forming microorganisms (Rickard et al. 2000). Thus there were similar mechanisms to mediate microbial coaggregation in oral plaque forming bacteria and freshwater biofilm bacteria.

However, the freshwater bacteria and dental plaque bacteria were shown to be different in the expression of coaggregation. Time dependent coaggregation was found between bacteria isolated from freshwater biofilms (Rickard et al. 2000). Cell coaggregation was optimally expressed only during the stationary phase in batch culture, while little coaggregation was observed during the exponential phase. Between freshwater microbial coaggregating partners, the acquisition and loss of coaggregation ability appeared at various times during stationary phase. Such time-dependent behavior has not been observed in dental plaque forming bacteria community (Oliveby et al. 1989). This was presumably due to the high shear forces encountered in the buccal environment as loss of adhesion via coaggregation would ultimately result in loss of oral bacteria (Bossier et al. 1996a; Oliveby et al. 1989).

Since coaggregation exerted a significant influence on the development of complex multi-species biofilms, it should also play a major role in the granulation process, as granules were generally regarded as spherical biofilms.

2.1.2 Industrial Applications of Cell Aggregation

It is generally accepted that phenomena of microbial aggregation are widespread in

the natural environment. Over the past several years, aggregation was progressed from a natural phenomenon to being a useful tool in biotechnological practice such as bioreactor design and bioseparation. For decades, microbial aggregation of cells into flocs played an important role in various industrial processes, such as biological wastewater treatment, beer brewing, and enhanced sedimentation (Davis 1995a). Potential biotechnological applications of aggregation included overcoming contamination, maintaining biomass in continuous fermentors, and selectively removing hybridoma cells from culture.

Activated sludge

Activated sludge processes are commonly used in secondary treatment to decompose a variety of soluble or insoluble compounds for municipal and industrial wastewater. The activated sludge system typically comprised a dense microbial community in the form of colloid pellets and suspended particles (Nopens et al. 2007). There are two main types of bacteria in activated sludge: filamentous bacteria and floc-forming bacteria such as *Pseudomonas* sp., and *Flavobacterium* sp., etc. The aggregation and competition between these two bacterial subpopulations were considered to be responsible for the proper settling of sludge and avoidance of filamentous bulking in activated sludge system.

Efficient bacterial aggregation (or bioflocculation) and good settling ability of aggregates are essential for the solid-liquid separation and the generation of good quality effluent, as well as the maintenance of adequate biomass in the system. Therefore, proper coaggregation in activated sludge is expected to be important to improve the performance of activated sludge plant with respect to microbial activities.

Recently, specific cell to cell interaction and aggregation among non-flocculating sludge bacteria isolated from municipal sewage wastewater treatment plant were investigated, confirming the nature occurrence and significance of these microbial aggregating interactions in activated sludge. Studies (Malik et al. 2003a) suggested

the contribution of non-flocculating bacteria to the bioflocculation and diversity of microbial populations in the activated sludge.

Beer fermentation

The beer industry had its beginnings around 4,000 B.C. The aggregating ability of several kinds of species, such as *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, was of extreme importance in beer fermentation process (Davis 1995b). In the case of excessive or insufficient aggregation in yeast cells, incomplete fermentation or secondary fermentation can deteriorate the beer quality. In addition, a high concentration of dispersed yeast cells in the supernatant can reduce the filtration efficiency to clarify the beer. Therefore, the assessment and control of microbial aggregation and sedimentation during beer fermentation were important to produce good quality beer.

2.2 AGGREGATION MECHANISMS

Coaggregation mechanisms can be generally categorized into two types: specific and non-specific interactions. Though all types of coaggregation were mediated by the same physical forces, chemical forces, and biochemical forces between molecules on the cell surface, the distribution of these forces was distinct (Busscher et al. 1987, 1992). In specific coaggregation, these forces worked on the discrete bonds between the receptor molecules on one cell and complementary ligands on another. In non-specific coaggregation, the attractive forces facilitated adhesion of cells continuously over the contact area of interest.

However, for either specific or non-specific coaggregation to occur, one cell must approach sufficiently close (up to several nanometers) to another cell's surface for coaggregation to initiate coaggregation process from initial contact between bacteria through physical movement of individual cells (Busscher et al. 2008), in which several factors may be involved, e. g. hydrodynamic force, diffusion mass transfer, thermodynamic effects, gravity, and cell mobility in liquid media.

2.2.1 Ligand-receptor Bond Formation

It was found that the specific coaggregation was mediated by pairs of molecules with proper shape which fitted each other and formed specific bonds (Clemans et al. 1999). The strength of these bonds would be affected greatly even with slight changes in the shape. The specific interactions were formed between antigens and antibodies, also known as those specific bonds between cell surface proteins, components of extracellular polymeric substances (EPS), carbohydrates and lectins, hormonal receptors and their hormones, and sensory receptors and the target compounds.

The molecule residing in cell membranes is usually called the receptor and the complementary molecule is referred as the ligand. In cell-cell interactions, the molecules on one of the cells were regarded as receptors and the molecules on the other cell were designated as ligands.

Several important systems were reported to carry out multiple ligand-receptor interaction types during the coaggregation process (Busscher et al. 1992; Cisar et al. 1979, 1997; Clemans et al. 1999; Kolenbrander 2002; Malik et al. 2003c, 2007). For instance, primary colonizer *Streptococcus gordonii* DL1 on the dental plaque carried five different proteins on the surface involved in coaggregation interactions, which maximized the coaggregation frequency in the competition for survival in highly selective environments. But the studies on the ligand-receptor system were often restricted to only one type of ligand-receptor interaction with specific kinetic properties for simplicity.

After cell partners encountered at adhesion surface, the receptors and ligands on distinct cells were not likely to be in the appropriate position to form a large number of bonds. They must move into proper position for the sake of discrete bonding. Many of the ligands were found not to be located on the cell wall, but to be associated with external appendages that enabled cells to contact with prospective partners more efficiently (Rickard et al. 2003a). The number of bonds determined

whether the cells coaggregated when subjected to removal forces, and depended on the kinetics of bonds and the time for bond formation. Several factors, such as fluid environment around cells, the properties of cell membranes, characteristics of cell surfaces, and dynamic conditions, were also revealed to be important to influence the kinetics of bond formation (Calleja 1984).

The ligand-receptor bond formation could be represented as a two step process (Roos et al. 1995):

$$R + L \underset{d_-}{\overset{d_+}{\leftrightarrow}} R - L \underset{k_-}{\overset{k_+}{\leftrightarrow}} RL$$

Step one Step two

The first step involved the movement of the receptor (R) and the ligand (L) into the proper position for bond formation. The complex of R and L is termed as R-L. Constants d_+ and d_- represent the formation and breakup rate of the complex, respectively. The second step described the reversible formation process of ligand-receptor bond, and k_+ and k_- are the constants for bond formation and breakup, respectively. In this model, intrinsic rate constants for ligand-receptor binding were affected to a large degree by steric restrictions on the binding sites, which was attributing to the specificity of ligand-receptor bound coaggregation (Roos et al. 1995).

At the atomic level, hydrogen bonding, van der Waals force and electrostatic interations between components of the receptor and ligand contributed to ligand-receptor bond formation, the same as that for non-specific coaggregation. Nevertheless, only a ligand with complementary structure could approach within the distance necessary for a bond to form (Busscher et al. 1992).

Ligand-receptor bonds mediating coaggregation were widely reported in the studies of specific coaggregation. Specific intergeneric and intraspecies coaggregations mediated by lectin-saccharide interactions were described between *M. luteus* 2.13 and four *B. natatoria* strains, which were isolated from aquatic biofilms (Rickard et

al. 2000). Members of the coaggregating pairs carried either a protease- and heat-sensitive protein (ligand), or a saccharide (receptor). Coaggregating pairs mediated by protein-saccharide interactions and blocked by addition of simple sugars were found in bacteria isolated from different ecosystem, such as freshwater biofilms, oral plaque, yeast, and sewage activated sludge (Cisar et al. 1979; Cookson et al. 1995; Ebisu et al. 1988; Malik et al. 2007; Rickard et al. 2002, 2004b; Weiss et al. 1987). Galactosamine-sensitive, galactose-sensitive and lactose-sensitive coaggregations were found in *Blastomonas natatoria* isolated from freshwater biofilms (Rickard et al. 2002, 2003b).

Coaggregation ligands were different in composition and could appear on various locations on cell surface for bond of potential coaggregating partners. The ligands were also different from the coaggregation acceptors, which were limited in diversity. Studies on streptococcal receptors showed that each strain had one major saccharide receptor in cell wall through which these strains could coaggregate with a wide range of partners (Kolenbrander et al. 1999). Each of the six structural types of receptor polysaccharides isolated from streptococci was found to belong to one of only two characteristic groups (Cisar et al. 1997). All these studies on coaggregation receptors indicated that the specific interaction of coaggregation was mainly determined by diverse ligand molecules.

2.2.2 Non-specific Aggregation

Several types of physical forces may facilitate non-specific coaggregation, e.g. coulombic (electrostatic) forces existing between any charged body, van der Waals (or electrodynamic) forces arising between electrically neutral bodies, and steric stabilization forces resulting from the hydrated layers of long-chain polymer molecules on cell membranes (Koller et al. 1995).

Coulombic forces are attractive between particles or cells with opposite charge or repulsive among similarly charged particles, whose magnitude is directly proportional to the product of the charges (Parsegian 1973). Coulombic forces are

decreased exponentially with separation distance. Thus the coulomic forces can be regulated easily by the distances between particles or by the concentration of electrolytes that are present in any cell culture medium. Under most conditions, intact bacteria were negatively charged. In comparison to other physical forces, the coulombic forces were much smaller as shown by theoretical calculations (Parsegian 1973). It was reported that the coaggregation was excited in bacteria that were isolated from sewage activated sludge by addition of positive charged calcium ions (Kakii et al. 1990).

Van der Waals forces can be decayed relatively slowly with the distance of separation as compared with coulombic force. It is raised as a consequence of the spontaneous transient fluctuations of charge distribution in a cell. These charge fluctuations are resulted from thermal motion, and changes in the positions of electrons and/or atomic nuclei. Considering that a large number of electrons and atomic nuclei on cells were involved in interaction and van der Waals forces did not drop as rapidly as coulombic forces did with distance, the van der Waals forces appeared to be more important than coulombic forces in the initial attachment of cells (Parsegian 1973).

The steric stabilization force is well known in the theory of colloid suspension and has been reported to be significant in the approach to a cell. The steric and osmotic effect varying with the number and type of polymeric molecules on cell membranes could assist cells to resist close contact (Neal et al. 1998).

Thus for non-specific coaggregation to occur, hydrophobic surface properties of cells are required to overcome energy barrier resulting from repulsive physical forces among cells. The slow, reversible disaggregation by sodium dodecylsulfate (SDS) on fission yeast suggested the contribution of hydrophobic interactions (Calleja 1984). In the self-immobilizing granulation system, the surface properties of granules were reported to play an important role in the initiation of granulation (Liu et al. 2003c). Other studies of starvation on inducement of hydrophobicity and the changes of surface properties on facilitation of microbial aggregation formation

indicated that the increase of hydrophobicity could favor granulation with compact structure and good settling velocity (Bossier et al. 1996; Liu et al. 2004c).

The effect of repulsive forces on formation of anaerobic granules was also suggested by two thermodynamic models, based on thermodynamic considerations of the interactions among bacterial cells, including the local dehydration model (Wilschut et al. 1986) and the surface tension model (Grootaerd et al. 1997).

2.2.3 Stimulations of Coaggregation by Environmental Signals

Coaggregation is not merely a pure chance process by a physical encounter, but also the response to environment stress and collective actions in microbial community. Microbial attributes and the property of whole microbial community, such as structural properties and extracellular polysaccharide substances (EPS) production, could be modified under selection pressure or other environmental signals to form aggregates as a strategy for microorganisms to survive in harsh environment.

Bossier et al. (1996b) demonstrated that bacteria might sense the substrate gradients in liquid and hence move towards higher substrate concentration around aggregates due to organic macromolecules formed by adsorption of particulate nutrient from liquid, or by the entrapment of nutrient by EPS. The ability of bacteria moving towards the direction of a substrate was well described in *Salmonella typhimurium* (Stock et al. 1991). Similarly, degradation of organic macromolecules by extracellular enzymes might increase the concentration of metabolites around aggregates, thus directing the bacterial motility. Furthermore, the channel- and pore- rich structure of aggregates was found to transport oxygen and nutrient in activated sludge and aerobic granules (Tay et al. 2002a, b). During the process of advection around and through the channels and/or pores, aggregated bacteria can experience much higher substrate availability than free swimming bacteria. Signal transductions present in the bacterial community might direct bacterial response to particular environmental signals or lead to the transcriptional activation of functional genes.

Starvation was widely reported to favor coaggregation in a variety of circumstances (Chiesa et al. 1985; Tay et al. 2001c). It was believed that bacteria could respond to sophisticated integrated intercellular signals induced by starvation, and alter coaggregating performance through changes of cell morphology, surface hydrophobicity or EPS production (Gurdon et al. 2001; Wiegant 1988). Other environmental signals produced by toxicity, physical stress, and predation were reported to lead to gene activation of cells or genetic rearrangements, further facilitating coaggregation (Bossier et al. 1996).

2.2.4 Extracellular Polymeric Substances (EPS)

Extracellular polymeric substances (EPS) are metabolic products accumulating on the bacterial cell surface (Wingender et al. 1999). All EPS are mainly composed of polysaccharides, proteins and humic substances, plus small quantities of uronic acid and DNA. They form an outer layer to protect cells against the harsh external environment. EPS could catch nutrients from liquid surroundings with loose binding and serve as the carbon and energy pool during starvation process. EPS might adhere to the outside of the cell, or be released into the surrounding medium, depending upon the growth conditions (Kaplan et al. 1982). Laspidou et al. (2002a, b) reported that there were two forms of EPS in activated sludge: bound EPS on aggregating biomass (sheaths, capsular polymers, condensed gel, loosely bound polymers, and attached organic material), and soluble EPS (soluble macromolecules, colloids and slimes).

EPS was regarded as an important factor in both specific and non-specific coaggregation to envelop the attached cells into aggregates, strengthen the coaggregation among cells as 'glue', and also play roles of receptors in ligand-receptor coaggregation interactions. EPS is an essential component of biofilms, as organisms are embedded in a matrix of EPS. Furthermore, EPS was also important in metal removal (Brown et al. 1979), as they could keep metals chelated in some cases. These polymers could also bind to the cell surface and change the surface properties of water insoluble organic and inorganic compounds, thus affecting

biotechnological treatment (Malik et al. 2003b).

Microbial cells can normally produce EPS, leading to floc formation by aggregation of bacteria. Urbain et al. (1993) reported that exopolysaccharides were primary constituents of activated flocs. The combined activity of EPS influenced aggregate structure and stability by balancing hydrophobic and hydrophilic interactions, with proteins playing an important role (Sponza 2003). Without EPS, microorganisms might not easily display high flocculation and metal uptake capacity, though the bacteria could interact with each other (Scatt et al. 1998). In granulation process, EPS was also shown to be important in the formation of granules with compact structure and excellent settling properties (Qin et al. 2004b; Tay et al. 2001a, c, 2003).

Though EPS was considered to be essential in the formation of aggregates, too much exopolysaccharide production could negatively influence the settling ability of activated sludge (Urbain et al. 1993).

2.3 FUNDAMENTALS OF AGGREGATION

Though bioaggregation is ubiquitous among microorganisms and bacterial cultures, it remains poorly understood due to lack of direct observation on microbial mechanism. Some studies were based on the knowledge of colloidal aggregation, but cell coaggregation involved less well-defined systems and was comparably more complex. For cells to coaggregate with other genetically distinct cells, a host of complex conditions such as genetic, physiological, environmental, and manipulative in nature (Calleja 1984; Calleja et al. 1984) should be fulfilled. The first two categories comprised the inherent properties of cells which retained coaggregation available genetically and physically, while the external conditions associated with cell coaggregation allowed the expression of that capability for coaggregation.

2.3.1 Genetic Conditions

Although widespread, the capacity of coaggregation was not found in all microorganisms. Strains within a species may vary in the capacity for coaggregation. There were deviants even among members of a large clone. Mutation followed by selection might moderate the genetic property of coaggregation. Extrachromosomal inheritance could also be used to explain the variation of genetic coaggregation capacity. Furthermore, structural genes for coaggregating strains might not be expressed due to the presence of repressive regulatory genes.

Masduki et al. (1995) studied mutants in *Pseudomonas typhimurium* which were not able to swarm without losing the ability to swim, and failed in response to chemical stimuli. It might be resulted from affected genes involved in changing the direction of flagellar rotation, thus impairing the ability of cells to change direction and aggregate at sites of high substrate concentration. It was found that *Phanerochaete chrysosporium* showed a differential gene expression for cellobiohydrolase, when it was cultured in immobilized condition (Vallim et al. 1998). Therefore, it was quite possible that the new gene expression gave rise to change in cell surface properties to help the cells adapt to the new living condition. Several genes were revealed to participate in the synthesis of an exopolysaccharide from *Zoogloea ramigera* (Davis 1995b), which implied the control of microbial coaggregation with use of recombinant DNA technology to manipulate genes to accelerate or inhibit coaggregation.

2.3.2 Physiological Conditions

Coaggregation was also reported to be dependent upon the physiological state in the cell cycle or life cycle of microorganisms. Generally, conditions favoring cell proliferation are not suitable for strong coaggregation. The end of exponential phase and steady phase of cell growth is the time for development of coaggregation in many systems. Rickard et al. (2000, 2004a) investigated the influence of culture age on visual coaggregation performance between bacteria isolated from freshwater

biofilms in detail. It was found that the coaggregation abilities among four strains of *Blastomonas natatoria* and one strain of *Micrococcus luteus* varied with culture age, but all pairs reached maximum coaggregating stage at the stationary phase of growth.

Surface properties of bacteria, such as protruding surface structure and specific cell wall proteins, also had influence on cell coaggregation. Protruding structure showed higher possibility to attach to the surface of other cells by piercing through the energy barrier between cells. Normally the smooth hydrophilic spherical cell is not easily to contact with cell or substratum of smooth hydrophilic surface due to mutual specific repulsion that prevented a close approach. Furthermore, the protruding structure favored specific coaggregation by overcoming steric hindrance in the ligand-receptor bond. Faced with selection pressure, adaptation of bacteria structure occurs to stimulate cell aggregation. James et al. (1995) observed a surface-colonizing *Acinetobacter sp.* changed to a coccoid morphology under starvation and stayed firmly to the surface, and turned to a bacillar morphology with increased availability of nutrients, thus forming a loose surface interaction with commencement of drifting. This observation also indicated the correlation of surface properties and coaggregation capacity.

2.3.3 Environmental Conditions

The observation of coaggregating ability occurring at particular stages of cell growth suggested that the capability of coaggregation was not similarly expressed. Beside the intrinsic traits of cells, physical and chemical effectors in the environmental conditions were equally important for occurrence of cell-cell coaggregation.

Malik et al. (2004) reported the negative influence of extreme pH values (3 and 11 respectively) on the coaggregation between *Acinetobacter johnsonii* S35 and *Oligotropha carboxidovorans* S23 isolated from activated sludge. pH values affected cell surface charge and electrolyte, and could disrupt coaggregating

stability. Inhibition of coaggregation by heat pretreatment of members of coaggregating pairs isolated from freshwater biofilm and dental plaque was demonstrated by Rickard et al. (2000) and Kolenbrander et al. (1999). Stability of coaggregation structure was affected by temperature presumably for the reason of fast growth of culture or the denaturation of enzymes under high temperature. The requirement of aeration in certain system for coaggregation was probably due to the energy requirement of other processes, such as protein synthesis in aerobic bacteria, which facilitated the coaggregation process. The coaggregation ability was also reported to be associated to the surface hydrophobicity (Malik et al. 2003b). Positive correlation among cell surface hydrophobicity, coaggregate size and coaggregating index was observed.

Other environmental factors on coaggregation process, e.g. the ion concentration in liquid, production of EPS, sugars (particularly mannose), energy source, hydrogen binding, selection press (such as shear force, HRT, and settling time) were also documented to affect the expression of cell coaggregation, as described the earlier sections.

2.3.4 Manipulative Conditions

By manipulation, fluctuation of coaggregation from already induced coaggregating pairs might raise from the rate of agitation of liquid culture, temperature, or the cell population density used in the experiment. Rate limits might also exist in coaggregation kinetics during manipulation. Coaggregation could occur almost immediately upon the mixing of bacterial suspensions, forming significantly large coaggregates that can be observed by naked eye, such as the coaggregation between *Acinetobacter johnsonii S35* and *Oligotropha carboxidovorans* (Malik et al. 2003c). In some instances, a long lag might also exist before the appearance of coaggregation after mixing of coaggregating pairs. Malik et al. (2003a) demonstrated the flocculation of coaggregating performance between *A. junii* S33 and a partner strain during 6-hour duration.

2.4 GRANULATION PROCESS

2.4.1 Conventional Aerobic Activated Sludge

The conventional activated sludge was first developed in England, 1914. Now this technology has been widely used in municipal and industrial wastewater treatment. The activated sludge process belongs to suspended-growth systems, and it can be operated in either continuous or sequencing batch reactor modes. Dense microbial suspension is vigorously mixed with the wastewater in a single or multiple basins in series under aerobic conditions. In the presence of sufficient nutrients, and oxygen, microorganisms can utilize the organic matter in the wastewater for the biosynthesis process, and produce oxidized end products such as CO_2, NO_3^-, SO_4^{2-} and PO_4^{3-}. In addition, suspended colloidal and ionic matter in the wastewater can be removed by adsorption and agglomeration. Following the aeration basin, a clarifier is normally used for the rapid and efficient separation of suspended biomass from effluent by sedimentation prior to the treated water disposal (Droste 1997).

In the activated sludge system, the settling characteristic of biomass is an important parameter. However, several problems are often encountered in the clarifier, such as bulking and foaming. Bulking or foaming can probably result in poor settling ability of sludge and abundant washout of biomass in effluent, leading to less biomass in activated sludge system and sharp increase of substrate loading rate per unit sludge. Accordingly, the increased loading rate can induce further deterioration of treatment efficiency.

2.4.2 Anaerobic Granulation

Granulation is the self-immobilized aggregates of cells from activated sludge to overcome the substrate inhibition and achieve good settling ability compared with conventional activated sludge system. Since the first anaerobic granules were cultivated in an upflow anaerobic sludge blanket (UASB) reactor in the 1970's, the mechanism (Gonzalez-Gil et al. 2001; Grootaerd et al. 1997; Liu et al. 2003a;

Schmidt et al. 1996), microbial diversity (Rocheleau et al. 1999; Sekiguchi et al. 1998) and microstructure (Grotenhuis et al. 1991; Lanthier et al. 2002; MacLeod et al. 1990) of anaerobic granules were extensively investigated and well documented.

In the UASB process, wastewater was normally distributed into the tank at appropriately spaced inlets, and then passes were upwarded through an anaerobic sludge bed where the microorganisms in the sludge came into contact with wastewater substrates. The microorganisms naturally formed granules (pellets) of 0.5 to 5 mm in diameter that had high sediment velocity and thus resisted wash-out from the system. The UASB reactor was equipped in the upper part with a three-phase gas-liquid-solid separator since the anaerobic degradation process typically resulted in the production of biogas. The feasibility and modification of UASB reactors were demonstrated in publications, and this process was applied to efficiently remove various kinds of wastewater: denitrification (Lettinga et al. 1980), methanol and alcohols (Lettinga 1995; Lettinga et al. 1981), bean blanching and chemical industry waste (Van den Berg et al. 1979), low fatty acids, and heavy metals.

An anaerobic granule is a dense aggregate composed of millions of microorganisms per gram of biomass. Multiple resident species communicated through coaggregation as well as other physical and metabolic interactions and thus were able to degrade complex industrial wastewaters. Since the granular sludge system was able to retain a great deal of biomass due to the good settling ability and dense and strong microbial structure, the treatment of large volumes of high-strength wastewater in compact reactor and efficient solid-liquid separation in effluent can be easily achieved.

However, anaerobic granulation technology had some shortcomings, including the difficulty and long time to start up the system, high operating temperature required, and unsuitability for treatment of low-strength organic wastewater and so on (Beun et al. 1999; Liu et al. 2004a). Recent researchers reported the production of aerobic granulation in sequencing batch reactors (Beun et al. 1999; Peng et al. 1999; Tay et

al. 2000) to overcome the weaknesses inherent in anaerobic granulation.

2.4.3 Aerobic Granulation

Aerobic granules that form in sequencing batch reactors (SBRs) were first reported by Morgenroth et al. (1997). The SBR was developed in U.S.A. in the late 1960's, and became widely used during the 1980's to 1990's (Tchobanoglous et al. 1991). Nowadays, almost all aerobic granules were currently cultivated in SBRs. The SBR system is a modification of conventional activated sludge process which belongs to the group of fill-and-draw reactors and has been widely used in municipal and industrial wastewater treatment. The wastewater is treated aerobically in a typical process cycle of a few hours consisting of the fill, aeration, sedimentation and draw. The cycle is repeated continuously beginning with the fill of wastewater into the reactor, after which aeration and conversion take place in succession. This aeration phase is followed with sedimentation of aerobic granules for several minutes and drawing of treated effluent from the upper part of reactor. In this way, the removal of targeted compounds and sludge settling take place in the same reactor, resulting in a single reactor with a high concentration of granular sludge and therefore high volumetric conversion rates (Beun et al. 1999).

The SBR is operated on the assumption that aerobic granules can be retained if flocs are washed out during short settling time, since granules have a dense structure and high settling velocity compared with flocs. A short settling period can be eventually used to select for biomass aggregates with a high settling velocity. Thus how to aggregate the microbial communities into dense granules in a short time is the key to speed up the formation of aerobic granules from inocula.

This relatively recent global increase in the use of aerobic granules in environmental engineering applications can be owed to several distinct characteristics compared with other conventional technology (Cai et al. 2004; Etterer et al. 2001; Hu 2005; Tsuneda et al. 2004; Zheng et al. 2005), as described below:

- Regular shape with a smooth outer surface;
- Dense structure with high sludge retention time, hence less reactor volume is required and sludge production is minimized in the process;
- Robust microbial structure consisting in diverse microorganisms, thus under conditions where the composition of the bulk solution is unfavorable for growth, a more favorable microenvironment can be created within the aggregate so that active metabolism is still possible;
- Large enough to be visible as separate entities in the mixed liquor during mixing and settling phase;
- Excellent settling ability to ensure an efficient liquid-solid separation, leaving low solid concentration in the effluent; and
- Ability to tolerate toxicity and high organic loading rates and withstand high strength wastewater shock loading;

Due to the unique attributes of aerobic granules, the aerobic granulation is recently developed for treating various types of synthesized or real industrial wastewater to remove organic matters, nitrogen, phosphorus, toxic compounds and xenobiotics (Adav et al. 2007; Jiang et al. 2006; Lin et al. 2003; Loperena et al. 2007; Moy et al. 2002; Ni and Yu 2008; Wang et al. 2007b,c; Yang et al. 2005; Yilmaz et al. 2008; Zhang et al. 2008a).

Aerobic granules are compact and dense microbial aggregates with a spherical outer shape. But most naturally formed aggregates of cells have loose, branched and porous structures, rather than tight compact structures. Furthermore, larger aggregates are usually more porous. The fractal nature influences the settling rate of cell aggregates and can also inhibit the production of well-formed granules. So a number of conditions have to be satisfied in order to overcome this nature.

Moreover, the numbers and types of microorganisms present in granules were likely to depend upon the type of reactors, operating conditions and the composition of wastewater (Grotenhuis et al. 1991). Several operation factors were studied, including settling time, exchange ratio, shear force, the types and compositions of substrate, starvation and feeding pattern, presence of metal irons, aeration rate, and hydraulic retention time (HRT) etc. (Jiang et al. 2003; Kim et al. 2004; Li et al. 2004; Liu et al. 2002, 2003c, 2005a, b; McSwain et al. 2004; Moy et al. 2002; Pan et al. 2004; Qin et al. 2004a; Su et al. 2005; Tay et al. 2001a).

Generally, the settling time for granule sludge acts as a main hydraulic selection pressure for composition and growth of bacterial community in aerobic granules. The basis of granulation is the continuous selection of sludge inside the reactors. Dispersed and poor settling particles will be washed out with well-structured granules left. A comparably short settling time ensures the retention of granular aggregates with good settling velocity against the poor settling sludge. The settling time was reported to be set as 1-4 minutes in SBR system (Morgenroth et al. 1997). Recent studies on settling time in an aerobic granulation system (Liu et al. 2005, 2006; Qin et al. 2004a) indicated that granules were dominant at a short settling time of 5 minutes, while longer settling times in excess of 10 minutes could result in mixtures of aerobic granules and suspended sludge. Short settling time at initial stage of aerobic granulation was reported to determine the efficiency of subsequent granulation processes (Adav et al. 2009a). When the non-flocculating strains were washed out by a short settling time, then functional strains were enriched in system.

It was reported that hydrodynamic shear force can strongly affect the aerobic granulation in several investigations (Shin et al. 1992; Tay et al. 2001a). High shear force is favorable to the formation and stability of granules along with stimulating the production of extracellular polysaccharides by microorganisms, which can mediate cell aggregation in granules and help maintain the structural integrity. However, it should be pointed out that the shear force in SBR system relies on adjustment of aeration flow rate. Thus differences in shear force may be coupled with different dissolved oxygen (DO) concentrations, which may also influence the

microstructure and microbial activities related to the aerobic granules. It was stated that strong shear force had dual effectives on the granulation process: 1) shear force served as a driving force to 'compact' small aggregates into aerobic granules; and 2) it would provide sufficient oxygen to suppress the overgrowth of filaments, thus benefiting granules' stability (Adav et al. 2008a).

Various substrates including acetate, glucose, phenol, ethanol, synthetic wastewater, synthetic seawater, and industrial wastewater, were used for the cultivation of aerobic granules in SBR (Adav et al. 2007; Belen et al. 2004; Beun et al. 1999; Jiang et al. 2002; Moon et al. 2002; Ni et al. 2009; Peng et al. 1999; Tay et al. 2001a, 2003; Yilmaz et al. 2008). The type and composition of carbon source also appeared to affect granule morphology and microstructure.

Substrate starvation in SBR operation was also investigated. One of the key features of SBR system is the switch between feast and famine during reaction cycle. During an operation cycle, an aerobic substrate starvation period existed after the exogeneous substrate was taken up by the microorganisms (Tay et al. 2001b). Under this starvation environment, the hydrophobicity of the bacteria in the granules was enhanced which facilitated cell aggregation and further influenced the surface characteristics, biomass density, size, and microbial activity. It was reported that the greater number of hydrophobic bacteria in the microbial community, the faster aerobic granulation possessing excellent settleability (Wilen et al. 2007).

Under the long-term starvation condition, part of the extracellular polymeric substances (EPS) present in aerobic granules was found to be consumed by their own producers, when substrates were severely deficient (Wang et al. 2005, 2007). With the increased EPS diffusivity caused by its degradation under starvation, the averaged oxygen penetration depth and oxygen diffusivity were observed to increase in aerobic granules (Chiu et al. 2006, 2007; Pijuan et al. 2009). Therefore, the suitable starving time for aerobic granulation was proposed (Liu et al. 2008a).

Additionally, the formation of compact and dense aerobic granules was favored by

reduction of filling time to several minutes per cycle (Beun et al. 1999), and intermittent feeding strategy (McSwain et al. 2004).

A wide range of organic loading rates (OLR) was used to successfully develop aerobic granules in SBRs (Adav et al. 2009b; Chen et al. 2008; Li et al. 2008), different from anaerobic granulation in UASB systems where a high organic loading was normally required. Though OLR was not decisive in formation of aerobic granules, a decrease in physical strength of granules was reported at high OLR (Moy et al. 2002). The fast growth rate of cells under high OLR could result in loss of integrity in granules, whereas the low OLR resulted in the slow formation of smaller and more compact aerobic granules (Li et al. 2008). In Li's report, the reactor fed with highest OLR had lowest species diversity; and in contrast, the reactor with lowest OLR showed highest species diversity.

Metal irons were thought to play an important role in aggregation of biomass (Ren et al. 2008). Since bacteria had negatively charged surfaces under usual pH conditions, an approach to induce bacterial aggregation was to reduce electrostatic repulsion between negatively charged bacteria. Cations could bind negatively charged sites on bacterial surfaces and on the extracellular biopolymers to decrease the surface charge of negatively charged microbes, thus acting as a bridge of aggregating partners in aerobic granules (Jiang et al. 2003; Keiding et al. 1997). Introducing multi-valence positive ions, such as calcium, ferric, aluminum or magnesium ions, into seed sludge was able to enhance cell aggregation and promote aerobic granulation. E.g. the augmentation with Mg^{2+} was reported to significantly decrease the sludge granulation time together with the appearance of larger, denser and more compact granules possessing higher polysaccharide contents (Li et al. 2009). But the augmentation did not influence the microbial morphology.

Hydraulic retention time (HRT) can be served as a main hydraulic selection pressure on the microbial community in the SBR system (Liu et al. 2004; Tay 2002). HRT was reported to affect the formation and stability of aerobic granules (Beun et al. 1999). A short HRT was able to suppress suspended biomass growth, but an

extremely long cycle time could give rise to poor aggregation and complete wash out of sludge, and accordingly may result in failure of aerobic granulation.

In conclusion, operational and environmental conditions associated with aerobic granulation in SBR system, e.g. settling time, shear force, famine, HRT and other factors, were able to influence the cell aggregation process and thus affect wastewater treatment efficiency.

2.4.4 Granulation Mechanisms

Since the early studies of granulation in the context of methanogenic systems (Lettinga et al. 1981), it has been commonly hypothesized that the bacterial interactions are one of the main causes of granulation (Beun et al. 1999). Important bacteria in granulation was not restricted to methanogens, as the formation of granules by nitrifying bacteria (De Beer et al. 1993), acidifying bacteria (Beeftink 1987), denitrifying bacteria (Van der Hoek 1988), aerobic autotrophs and heterotrophs (Tijhuis et al. 1994; van Benthum et al. 1996; Ni et al. 2008b), hydrogen-producing bacteria (Zhang et al. 2007; 2008b) and some other bacteria had been observed.

Several models and theories were proposed to explain the anaerobic granulation process, such as inert nuclei model (Lettinga et al. 1980), spaghetti model (Wiegant 1980), proton translocation dehydration theory (Tay et al. 2000), Capetown's model (Palns et al. 1987), divalent cation-bridge model (Schmidt and Ahring 1996), and syntrophic microcolony model (Dubourguier et al. 1988). All these theoretical models involved the colonization or coaggregation of microbial cells into granules, although the processes were not studied experimentally:

- Inert nuclei models: inert matters was thought to initial the granulation as precursors;

- Spaghetti model: filamentous microorganisms attached on precursors and

formed a network to entrap bacteria. The 'spaghetti' structured aggregates grew into bigger size and were eventually compacted and shaped by hydraulic shear force;

- H^+ translocation-dehydration theory: in this theory, the bacterial surface dehydration occurred, which was caused by proton translocation activity. The dehydration of membrane surface and decrease in hydration interaction favored the formation of microbial aggregates;

- Extracellular polymeric substances (EPS) bonding model and Capetown's model: the accumulation of EPS served as 'glue' and capsular material to facilitate cell-cell aggregation processes into the formation of floc aggregates;

- Divalent cation-bridge model: divalent cations were demonstrated to stimulate granulation by neutralizing negative charges on bacteria surfaces;

- Syntrophic microcolony model: the various microorganisms involved in biodegradation process must live in a close synergistic relationship to transfer cellular products. As a result, stable microcolonies or consortia were formed as initial granules.

Similar to anaerobic granulation, there were several theories or mechanisms proposed to explain the self-immobilization of aerobic granules.

Based on experimental observation in a reactor which was started up with small amount of suspended cells as inocula, Beun et al. (1999) proposed a model for aerobic granulation, in which fungi predominated in the reactor and pellets were easily formed to settle fast and subsequently retain. Due to the shear force, filaments were further detached from pellets surface. Bacteria immobilized on mycelial pellets and grew to colonies. When the size of pellets increased to 5-6 mm in diameter, lysis occurred probably due to the oxygen limitation in the aggregate interior. After breakage of pellets, bacterial colonies were retained and further developed to aerobic granules.

However, this model would be different if flocs or small aggregates were used as inocula for cultivation of aerobic granules, due to the lack of backbones supplied by filamentous structure of fungi for the attachment of free cells. Furthermore, the size of aggregates formed by flocs or small aggregates could not expand as rapidly as that formed by fungi which led to lysis of loosen structure and reservation of pellet colonies.

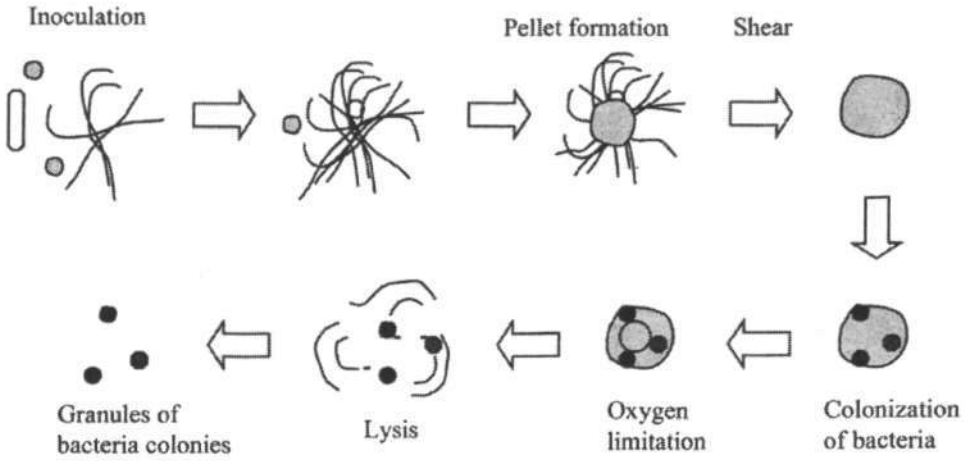

Figure 2.1 Mechanism of aerobic granulation formation in a SBR reactor (Beun et al. 1999)

Peng et al. (1999) also reported that mechanism of granulation fed with acetate as sole carbon source was similar to the organization of activated sludge flocs which was observed by Scuras et al. (1998). At the lowest level, the individual particles in size between 0.5–5 μm were composed of preliminary living cells, decaying cells, non-biodegradable cell debris, and influent solids. At the second level, aggregates consisted in individual particles that were encapsulated in a clearly defined polymer matrix to form microcolonies (5–50 μm in diameter) of roughly spherical shape in flocs. At the third level, the numerous microcolonies accumulated together with exopolymers to form large aggregates.

Regarding to the aerobic granulation process, four steps were proposed by Liu and Tay (2002) in the development of aerobic granules:

- Step 1: cell-cell contact or bacterial attachment onto a solid surface to form small aggregates. The process was mediated by hydrodynamic force, diffusion force, gravity force, thermodynamic forces, e.g. Brownian movement; and/or cell mobility;

- Step 2: initial attractive forces to ensure stable multicellular contacts and/or attachment of bacteria onto solid surface through physical (van der Waals, opposite charge, thermodynamic forces, cell hydrophobicity and/or bridging by filaments), chemical (hydrogen liaison, formation of ionic pairs, and/or formation of ionic triplet), and biochemical (cellular surface dehydration, and/or cellular membrane fusion) forces. In this step, the cell-cell interactions including bacterial aggregation played a crucial role in initiation of aerobic granules;

- Step 3: microbial forces to form mature aggregates by the secretion of extracellular polymeric substances (EPS), growth of cellular cluster, metabolic change and genetic competence in microbial community; and

- Step 4: stabilization of three-dimensional structure of microbial aggregate that were shaped and strengthened by hydrodynamic shear forces.

The confocal laser scanning microscopy (CLSM) images for above mentioned steps 1-3 by using the multicolor fluorescent technique are shown in Figure 2.2. Microbial aggregation was believed to be the initial step in granule formation and crucial to subsequent granulation process, as demonstrated by fluorescent staining and CLSM tests (Adav et al. 2008a).

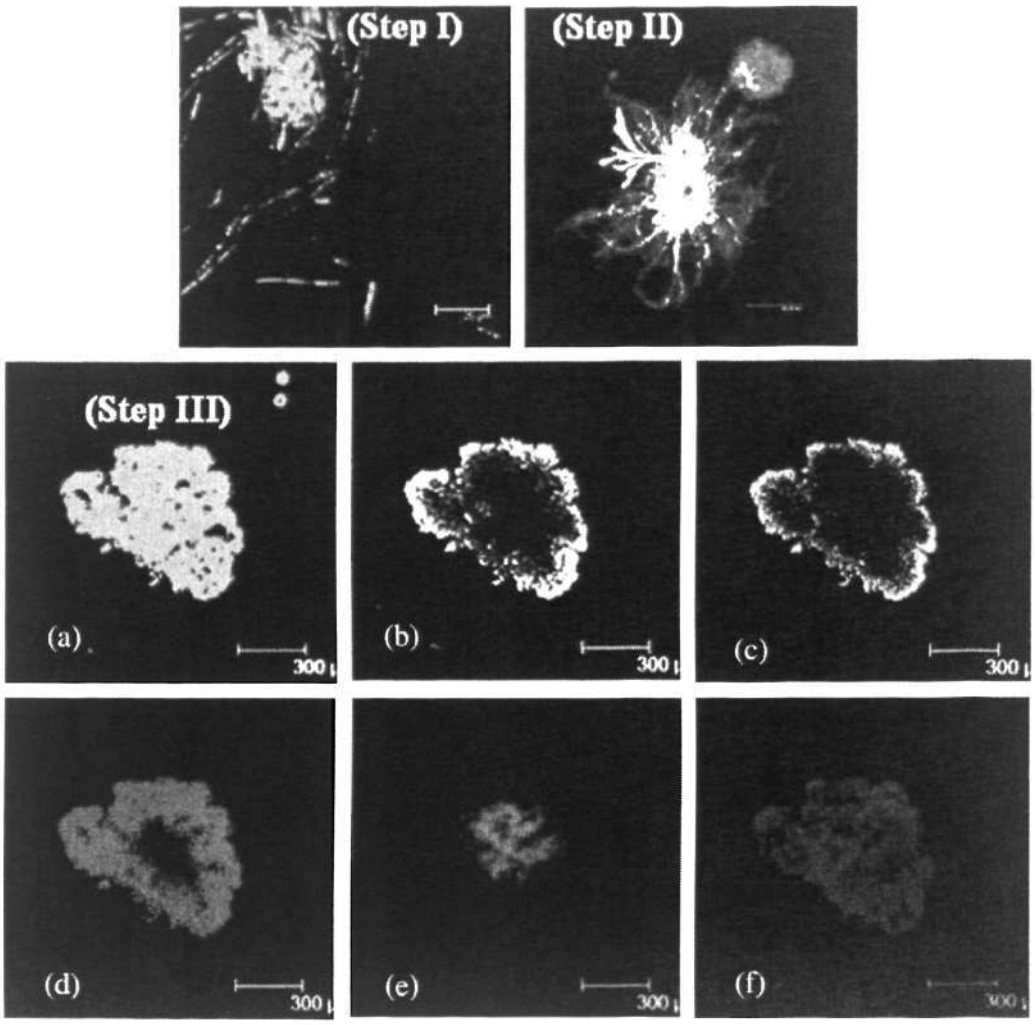

Figure 2.2 CLSM images of microbial aggregates formation in different stages, cultivated in sequential batch reactor with synthetic wastewater containing 500 mg L^{-1} phenol (Adav et al. 2008a)

In these proposed mechanisms for aerobic granulation which starts from fungi or suspended particles, the aggregation of bacterial and particulate matters entrapped into exocellular matrix as well as the subsequent microcolonies formation is possibly one of the key steps for granule formation. The microbial aggregation in self-immobilization process of aerobic granules can be mediated by cell hydrophobicity, filamentous microorganism networks, EPS products, signal exchanging in microbial communities or other mechanisms, in simultaneous

presence of various selective pressures.

Liu et al. (2003c; 2004c, e) proposed that cell hydrophobicity acted a triggering force in aerobic granulation. Cell hydophobicity could be induced by culture conditions, and in turn initiate cell-cell aggregation which was supposed to be a crucial step towards biogranulation. Growth of filamentous microorganism, such as fungi, was commonly observed in aerobic granulation process (Liu et al. 2006b; McSwain et al. 2004; Schwarzenbeck et al. 2005; Tay et al. 2001b). Bacteria were able to colonize and grow on filaments microorganisms, e.g. stalked ciliates of protozoa (Weber et al. 2007). Subsequently, the ciliates were completely overgrown by bacteria and die. Thereby, the remnants of dead ciliates acted as backbone for attached bacteria to form small granules. EPS was widely reported to correspond to aerobic granulation, being especially helpful in initial granulation stage/step (Adav et al. 2008b; Tay et al. 2001c; Yang et al. 2004; Zhu et al. 2008). Liu et al. (2004) hypothesized that EPS bridged bacteria and other particulate matters into aggregates.

Almost all aerobic granules were successfully cultivated in SBRs. It seemed that there was only a narrow range of physical, biochemical and microbiological parameters for aerobic granulation. As discussed in Section 2.4.3, favorable conditions and strategies for aerobic granule formation and stability were extensively studied. These operational conditions served as selection pressures to drive the aerobic granulation. All above mentioned mechanisms of aerobic granulation in this section were correlated to the driving forces, in terms of either hydraulic driving forces or microbial driving forces. Microbial communities (or several functionally important bacteria) were found to respond to combined driving forces, and alter in production of EPS, cell surface hydrophobicity, cell-cell aggregation, and inter-cellular transduction, etc. (Adav et al. 2008c; Xiong and Liu, 2009; Zheng et al. 2006).

The cooperative effects among different functional groups of microorganisms as well as the interactions among those groups and outer environment also contribute into the development of aerobic granules. It was found through molecular studies

that bacteria could sense a large number of environment signals and process these information into specific transcriptional responses (Loo et al. 2000). With the production and detection of extracellular signaling molecules, bacteria could communicate with each other by quorum sensing (Elizabeth et al. 2000). Intercellular communication and multicellular coordinations were known for the bacteria to contribute into the organization of spatial structures (Liu 2004a, b). Thus it is reasonable to assume that interactions of both environmental signals and signals between individual cells play an important role in aerobic granules formation and spatial structure organization.

Quorum sensing was an example of social behavior in bacterial community, as signal exchanging among individual cells allowed the entire population to choose an optimal way to interact with the environment. Xiong et al. (2010) reported that the AI-2-mediated quorum sensing would be essential in the aerobic granule structural integration and stability through signaling-based microbial coordination, whereas it would not be necessary for initial granular aggregate formation.

Besides the initial formation of aggregates, the integration of bacteria into good settling aggregates is also essential, not only for the growth and stability of granules, but also for the maintenance of high microbial activities for biodegradation in wastewater. Moreover, for the sake of well-functioning granules, attachment of different functional microbial groups into compact, strong and stable structure is needed. Furthermore, adhered microorganisms would display a high level of resistance to biocidal compounds, such as antibiotics, and also combine and support some slowly growing bacteria in a community under extreme conditions which sometimes can reach higher metabolic activity of community. All these facts demonstrated that microbial aggregations are necessary throughout aerobic granulation.

Therefore the detailed study on factors to initiate cell aggregation and introduce bacteria into good settling aggregates is desired for the development of aerobic granules, as well as for the enhanced granulation performance.

2.5 BIOAUGMENTATION

The aerobic granule is normally inoculated by fresh suspended activated sludge in SBRs. An adequate adaptation is essential for the change of microbial community under selective pressures. The adaptation of natural microbial communities could be induced by different mechanisms or the combination, such as gene transfer or mutation, enzyme induction and population changes (Spain et al. 1983).

Bioaugmentation was a process to introduce the naturally occurring or genetically modified microorganisms (either pure culture or mixed cultures) into native microbial communities, e.g. sludge or biofilm, to achieve the improved treatment performance (Dabert et al. 2005; Rittman et al. 1994; Stephenson et al. 1992; Van Limbergen et al. 1998). The reason to apply bioaugmentation in bioremediation was simple: for the richness of catabolically-relevant organisms to hasten remediation. This method was reported to be successfully applied in palm oil mill effluent control, treatment of papermaking wastewater, resin acid, 2,4-dichlorophenol, phenol, and dichloroethenes, etc. (Jiang et al. 2006a, b; Olaniran et al. 2006; Onyia et al. 2001; Quan et al. 2003, 2004; Wang et al. 2006; Yu et al. 2001).

The removal efficiency of wastewater treatment system could be improved by seeding superior microorganisms that had high capability to degrade the main ingredients of wastewater (Wang et al. 2006). Bioaugmentation was usually considered to accelerate the removal of undesired compounds in contaminated waste sites or bioreactors by using wild type or genetically modified organisms (Van Limbergen et al. 1998). Extra benefits were addressed on the gene transfer and newly formed functional strains as a result for bioaugmentation. For example, enhanced 2,4-D degradation was achieved through the horizontal gene transfer by 2,4-D degradative plasmid pJP4 from the introduced donor strain to the microbial community of a laboratory wastewater treatment system (Bathe et al. 2004). Therefore, bioaugmentation would be a more successful strategy than biostimulation to enrich the numerical abundance of functional populations (Wuertz et al. 2004).

Bioaugmentation was discovered to be suitable particularly in confined systems, such as SBRs, where the survival and activities of augmented microorganisms were able to be well controlled (Fantroussi et al. 2005). Also abundant microbial populations available in activated sludge conferred the feasibility of the migration and persistence of augmented exotic strains. Aerobic granules are mostly cultivated in SBRs and inoculated from activated sludge. Therefore, in conjunction with the selection pressures manipulated and regulated by operational parameters, bioaugmentation is expected to achieve accelerate aerobic granules formation and elevated performance.

The degradation capacity of the granular microbial community to particular compounds can be enhanced by addition of specific microorganisms. It is also hypothesized in this thesis that if the augmented aggregating microorganisms can persist in system, they may assist the settlement of some species that are less likely to attach to granular sludge. The interactions among aboriginal strains in that microbial community could also be elevated.

However, augmentation is not always successful. According to Rittman et al. (1994), despite the potential for industrial application, more controversies on the applications of bioaugmentation were noticed than some other biotechnologies. Many failures on bioaugmentation were well documented and reviewed (Thompson et al. 2005). Although the inocula used in bioaugmentation were usually highly efficient for the removal of target compounds in laboratory scale studies, these inoculated strains had to compete with the original population in well established microbial community, hence resulting in a decrease of inoculated cells amount (Goldstein et al. 1985). In aquatic surroundings like SBRs, the inoculated organisms' growth rates and adaptation abilities into new habitat should be high enough to avoid quick loss of abundance by washout (Watanabe et al. 2000). So far in wastewater treatment, not many cases of successful bioaugmentation on activated sludge systems were reported. The application of bioaugmentation onto aerobic granulation was commonly challenged by the stability of process performances and the presence of competing bacteria to augmented strains in multiple-culture community (Adav et al. 2007a).

From above discussion, it can be indicated that the selection of appropriate strain for bioaugmentation is one of the key factors in successful bioaugmentation application, the same as Thompson et al. (2005) reported. The selection was normally based on a good understanding of organism abundance, functional activity and population dynamics in the target habitat. Many failures of bioaugmentation were not attributed to the poor identification of possible inhibitions to biodegradation (such as pH value, the presence of toxic contaminants, or the absence of key co-substrate), but because of the insufficient consideration of introduced strains in long-term survival in a given environment.

Thus augmentation of aggregating bacteria had an extra benefit on the survival of augmented strains, which was related to the flocculation ability of inocula in a bacterial community. McClure et al. (1991) found that a sludge isolate AS2 inoculated into activated sludge was able to reach a stable population, different from other inocula tested in the same study. This strain AS2 possessed flocculation ability, which might be important to its survival. It was suggested that the exopolysaccharide (PS) production by cells under stressful conditions contributed to the floc formation (Bossier et al. 1996), which would be relevant to the retention of strains within microbial community.

2.6 DISAGGREGATION

The performance of a biological wastewater treatment plant was especially determined by the metabolic activities of microorganisms and by the efficiency of the solid-liquid separation at the last stage of a treatment process (Schwarzenbeck et al. 2004a, b). In aerobic granulation, mature granules were observed to disintegrate sometimes. The discussions on possible reasons of aerobic granules included the disfunction of energy metabolism (Jiang and Liu 2009), inhibition of microbial respiration activity (Yang et al. 2004), and the unsuitable combined selection pressures (Liu et al. 2006), etc.

In Jiang and Liu's study (2009), a chemical uncoupler 3,3',4',5-tetrachlorosalicylanilide (TCS) was introduced into pre-cultivated mature aerobic

granules. TCS could dissipate the proton gradient, resulting in ATP disruption. Disintegration of granules was observed after exposure to TCS within one month. The results revealed that the failure of energy metabolism could cause the breakage of aerobic granules. Correspondingly, the inhibition of energy generating function was also found to prevent the cell-cell aggregations (Calleja 1984; Calleja et al. 1984).

In the study of Yang et al. (2004), it was reported that microbial respiration activity in terms of SOUR was seriously inhibited by elevated levels of free ammonia. As a result, failure of aerobic granulation was observed.

In some extreme cases, once filamentous growth dominated the reactor, settling ability of aerobic granules became poor, hence subsequent biomass washout and eventual disappearance of aerobic granules occurred, leading to the failure of the whole granulation process (Liu et al. 2006). This instability of aerobic granules related to outgrowth of filamentous microorganisms became one of the major technical problems in operation of aerobic granular sludge SBR. Thereby instability of aerobic granulation was one of the limiting factors in applying this technology for treatment of wastewater.

Disaggregation could occur in nature when aggregates were transferred to fresh growth medium (Calleja 1984). When cells in aggregates began to grow, they tended to break away from the confining aggregate arrangement and to disperse as free cells. On the other hand, the detachment of cells from aggregates was viewed to be influenced by granule size and hydraulic selection pressure. Anaerobes, lysed cells, non-degradable cell debris and influent solids were reported to compose the center inside aerobic granules (Peng et al. 1999; Tay et al. 2002), therefore partial disaggregation of granules sometimes occurred due to the presence of organic acids and gases produced by anaerobes inside big aerobic granules. Though there was no direct evidences from laboratory experiments, the combined hydraulic shear force and organic loading was hypothesized to have significant influence on the stability of aerobic granules. The optimal combination of these two selection pressures was

demonstrated to be essential for aerobic granulation (Chen et al. 2008).

In freshwater biofilm system, coaggregation between strains was expressed only during the stationary phase in batch culture. Furthermore, coaggregating partners were found to gain and lose their coaggregation ability at various times in stationary phase (Rickard et al. 2003b). It was reported that the aggregating interactions of freshwater biofilm were affected by temperature, extreme pH value, deioniation, presence of chelators (e.g. EDTA), periodate or potassium (Rickard et al. 2004a). With the hypothsis that cell-cell aggregations are important in biogranulation and it 's stability, the disaggregation of aggregating partners would help to explain the breakage of mature granules.

Addition of incompetent cells to well structured microbial community sometimes also contributed to the disaggregation. The incompetent cells could weakly aggregate to induced cells, thus disrupting the aggregates by occupying binding sites which should have been taken by competently aggregating cells. Therefore microbial distribution should also be investigated to help the interpretation of formation and breakage mechanisms.

Other possible causes of instability and disintegration of aerobic granule or biofim were related to the insufficiency of oxygen supply, the deterioration of EPS production, and the change of nutrient diffusivity and mass transfer resistance (Adav et al. 2008c; MacSwain et al. 2005; Picioreanu et al. 1998)

The formation of aerobic granules has been extensively studied. The stability of aerobic granulation is now gaining more and more research attentions, since the rapid formation of granule is achieved. Some strategies were developed recently to maintain the integrity and stability of aerobic granules, such as: 1) selection of slow-growing bacteria to form low-growing granules with good settling ability and strong structure (Liu et al. 2004b); 2) stimulation of EPS production by operational control (Liu et al. 2004d); or 3) inducement of cell hydrophobicity through selection pressure (Liu et al. 2004c).

However, so far as I know, there is no reports till now on how to recovery the disaggregated aerobic granules in SBR system, when the integrity of granules in large size is no longer available. The reaggregation of aerobic granules can be of high importance in environmental engineering practice, which is crucial to achieve satisfactory wastewater treatment in long term operation, and to avoid extra economic loss caused by restart of entire aerobic granulation system.

2.7 COAGGREGATION AND PATHOGENICITY

Normally mechanisms for bacterial infections includes the death of cells in host tissues, production of bacterial toxin which results in lysis of host cells, and the host immune responses to bacterial, etc. (Fleming et al. 2000). Though these mechanisms differed, on the initial stage of bacterial infection, a microorganism must be able to gain access to a host through its coaggregative section. The aggregation of microorganism mediated binding to the host and establishing an infection in succession. Therefore, pathogenic bacteria with coaggregating ability may infect humans more easily.

Some bacteria of strong coaggregating properties could get involved into potential pathogenicity due to cell to cell adhesion on human cell monolayers. Some pathogens, such as *Prevotella nigrescens, Prevotella intermedia*, and *Pseudomonas paucimobilis*, were implicated in a variety of nosocomial infections, including periodontal diseases, meningitis, peritonitis, and cutaneous infections (Casadevall et al. 1992; Cookson et al. 1995; Peel et al. 1979).

Along with the growing interests of aggregation on bioengineering aspects, the potential pathogens should be well controlled in laboratory and other working places. Thus the aggregating bacteria of potential pathogenicity will be avoided in this study.

2.8 SUMMARY

Cell aggregation can be categorized into coaggregation and autoaggregation. Coaggregation is the interaction between a pair of genetically distinct partners mediated by cell-cell recognition, while the autoaggregation occurs between identical cells. The phenomenon of aggregation exists ubiquitously in the environment. The microbial ability to aggregate plays an important role in bacterial infections, tooth plaque formation, development of bacterial community in plants, animals and human bins, colonization of cells into different types of multi-species biofilm, activated sludge, and many other biotechnologies, such as beer fermentation and cell separation. Functional groups of microbial cells in aggregates often exhibit a combined metabolic activity and create a more favorable microenvironment. Therefore aggregation is regarded as a good strategy to maintain desired microbial activities and biomass against unfavorable conditions.

As a novel biotechnology recently developed for wastewater treatment, aerobic granulation has attracted increasing research interests as a new form of self immobilization through cell-cell aggregation without carriers. Compared to conventional biofilms and activated sludge technologies, aerobic granule possess several advantages, including compact and strong structure, good settling ability with robust mechanism, tolerance to high organic loading or toxic shock, and adaptability to fluctuated loading rate. In addition, the start-up period for aerobic granulation system can be much shorter than that for anaerobic granulation.

Several models and theories have been developed based on experimental observations in order to explore the granulation mechanisms since the past three decades. It has been regularly hypothesized that the bacterial interactions are the main cause of biogranulation. The attachment process of microbial cells is thought to be an integral component in almost all the proposed mechanisms.

Microbial aggregation is believed to contribute to mass conglomeration that leads to fast settlement in liquid condition, and facilitate the attachment of proper

functioning bacteria into sludge. Therefore, it is assumed that the presence and richness of aggregating bacteria in aerobic granule could result in positive effect on aerobic biogranulation, since the successful granulation process is closely related to good settling ability and solid retention through the formation of dense and compact aggregates.

In addition to the possible contribution in formation of compact and well-functioning aerobic granules, aggregating interactions are also hypothesized to be important for the stability and maintenance of high microbial activities in aerobic granules. Furthermore, cell aggregation is possibly capable of facilitating microorganisms to respond and survive under a range of environmental pressures, thus subsequently affect aerobic granulation system performance.

In spite of the hypothesis as mentioned above on the role of cell-cell aggregation in the aerobic granulation, little study has been carried out on the aggregating community structure and composition in aerobic granules. The dynamics of microbial community with respect to the changes of aggregating population to the external environmental conditions is also unknown. There are few reports on the potential of cell-cell aggregation in fast formation of aerobic granules through manipulation of single aggregating strain. The potential application of aggregating bacteria to hasten the reaggregation of disintegrated granular sludge also requires investigation. All these studies mentioned above will work for the better understanding of the role of cell-cell interactions in aerobic granulation.

Therefore, this study will focus on the screening and characterization of aggregating bacterial populations in aerobic granules. The factors affecting cell-cell aggregating interaction among aerobic granule isolates will also be investigated. The aggregating communities in aerobic granules and initial aggregates formed in flow liquid environment will be compared. The aggregating community dynamics as the response to external environments (chemical and toxic shock) accompanied with the monitoring of reactor performance and granule morphological revolutions will be studied as well. Aggregating bacterial strains will be isolated, identified, and

compared from granules fed by either benign substrates or toxic substrates. One novel aggregating isolate will be augmented to accelerate the formation of aerobic granules and enhance the granulation performance. Then the recovery of disintegrated granular sludge will be enhanced by addition of one autoaggregating bacterial isolate, in order to explore and examine the potential effects of aggregating bacteria on the reaggregation of broken aerobic granules.

CHAPTER THREE
ISOLATION AND IDENTIFICATION OF AGGREGATING BACTERIA IN AEROBIC GRANULES AND ENRICHED GRANULAR SLUDGE

3.1 INTRODUCTION

As a novel environmental biotechnology for wastewater, aerobic granulation has attracted intensive research attentions since the 1990's (Beun et al. 1999; Liu et al. 2005; Liu and Tay 2002, 2004a; Morgenroth et al. 1997; Peng et al. 1999; Tay et al. 2000, 2002a). The successful formation of aerobic granules relies on a repetitive selection of denser aggregates retained in the system, while lighter flocs or microorganisms were washed out by effluent (Beun et al. 1999). However, the fractal nature of microbial aggregates usually inhibits the production of compact granules which contain dense microbial community packed in spherical shape. Therefore, complex combination in environmental and operational conditions is required for the formation and maintenance of aerobic granules. Though operational factors and physical conditions to differentiate the settling properties between retained aerobic granules and discharged loose particles have been well studied previously (Chen et al. 2008; Chiu et al. 2006; Li et al. 2009; Liu and Tay 2009; Ni et al. 2009; Pan et al. 2004; Pijuan et al. 2009; Qin et al. 2004a, b; Shin et al. 1992; Tay et al. 2001a), the aerobic granulation mechanisms are still unclear.

Interactions among bacterial populations were believed to be one of the main forces to drive microbial community evolutions, such as in biofilm and activated sludge (Atlas et al. 1998). The microbial interactions were thought to determine the multi-species community in terms of the microbial structure, bacterial multiplication, species distribution and tolerance to harsh environment (Buswell 1997; Kolenbrander et al. 1999; Malik et al. 2003b), and would further influence the biomass retention and solid-liquid separation which are crucial to the aerobic

granulation.

In order to investigate the microbial interactions in aerobic granule communities under different operational and/or environmental conditions, it is essential to understand the microbial community structures and dynamics. Jiang et al. (2004, 2006) isolated ten bacteria from phenol-degrading aerobic granules. Among these ten isolates, seven belonged to *Proteobacteria* with rest three belonged to Actinobacteria, among which some bacteria were commonly found in phenol-degrading granules cultivated elsewhere (Adav et al. 2007a; Arrojo et al. 2004). In aerobic granules fed with acetate or glucose as a carbon source, bacteria were identified to belong to genus *Epistylis*, *Poterioochromonas*, *Geotrichum*, and *Geotrichum klebahnii* (Williams and de los Reyes III, 2006). *Candida tropicalis* strain was also isolated from phenol-degrading granules, which was reported as a functionally dominant strain for phenol degradation bioactivity (Adav et al. 2007a). Abundant dominance of bacteria in mature glucose-fed granules was examined by denaturing gradient gel electrophoresis (DGGE) (Li et al. 2008). It was shown that the majority of 6 isolates clustered with *Proteobacteria*.

As bacteria from β- and γ-Proteobacteria were also commonly dominated in activated sludge, it was suggested that the presence and dominance of particular bacteria species were not necessary in aerobic granulation (Li et al. 2008). The community structure and intracellular interactions are of greater importance. Cell-cell coaggregations were demonstrated to play an important role in aerobic granulation (Jiang et al. 2006a). It was hypothesized in several mechanisms previously proposed that, as a result of microbial aggregation, the nucleus could be provided for attachment and growth of free cells, which was determining the subsequent aerobic granulation (Adav et al. 2008d, 2009b; Beun et al. 1999; Bossier et al. 1996a; Jiang et al. 2006a; Liu and Tay 2002; Malik et al. 2003b).

Though some of functional bacteria were screened separately in phenol-degrading aerobic granules, glucose- and acetate-fed aerobic granules in independent studies (Adav et al. 2009b; Li et al. 2008; Jiang et al. 2004), the shift of microbial

community structure under various shocks is seldom studied, and the corresponding dynamics of aggregating populations as response of microbial community are also untouched to date. The detailed information with respect to the aggregating bacterial populations is expected to provide information on how the microbial interactions drive the aerobic granulation, which could contribute to the understanding of granulation mechanisms.

Therefore, this Chapter aimed to: (1) provide information on aggregating population diversity in aerobic granules; (2) survey the aggregating ability of the microbial community in aerobic granules and in the initial aggregate formed in flowing environment. In the current study, the fast settling strains isolated from the pre-cultivated aerobic granules were enriched by selection of settling ability. In order to compare the diversity of aggregation populations in aerobic granules and enrichment flocs, the strains were isolated and aggregating bacteria were then identified. The aggregating behaviors of selected aggregators were also studied in response to various physical and chemical conditions.

3.2 MATERIALS AND METHODS

3.2.1 Aerobic Granules Collection

Aerobic granules were collected from a column type of sequencing batch reactor (SBR) that was operated at an average room temperature of 25 ^{o}C. Each reactor had a working volume of 2.0 l, and diameter of 50 mm in internal diameter. Reactors were aerated at superficial upflow air velocity of 3.5 cm s^{-1} by an aerator at the bottom of column. The configuration of the experiment system is shown in Figure 3.1. pH value was controlled around 7.0-8.0. Hydraulic retention time was 8 h. Synthetic wastewater with sodium acetate as a sole carbon source was used for the cultivation of granules. Effluent was discharged at the middle port of the column. The operating time in one cycle applied for the cultivation of granules was 4 h, including 5 min for filling, 229 min for aerating, 2 min for settling and 4 min for withdrawal of effluent.

Seed sludge was obtained from a municipal wastewater treatment plant and inoculated into the reactor. The reactor was fed with sodium acetate as a sole carbon source by using a synthetic wastewater. Influent chemical oxygen demand (COD) concentration of 1000 mg l^{-1} was applied in the influent with composition of: Sodium acetate 1328 mg l^{-1}; NH_4Cl 100 mg l^{-1}; K_2HPO_4 22.5 mg l^{-1}; $CaCl_2 \cdot H_2O$ 15 mg l^{-1}; $MgSO_4 \cdot 7H_2O$ 12.5 mg l^{-1}; $FeSO_4 \cdot 7H_2O$ 10 mg l^{-1}; and trace solution 1 mg l^{-1}. The micro-nutrient solution contained (mg l^{-1}): H_3BO_3 50; $ZnCl_2$ 50; $CuCl_2$ 0.03; $MnSO_4 \cdot H_2O \cdot (NH_4)_6$ 0.05; $Mo_7O_{24} \cdot 4H_2O$ 50; $AlCl_3$ 50; $CoCl_2 \cdot 6H_2O$ 50; and $NiCl_2$ 50 (Moy et al. 2002).

Approximately 100 ml of mature granule samples were collected at the middle and bottom port of one working column and mixed well. After collection, granule samples were separated from suspended flocs and other impurities and washed three times with autoclaved 1× phosphate-buffered saline (PBS, containing 0.13 M NaCl, 10 mM sodium phosphate, pH 7.2). All samples were either used immediately or stored at –20°C in 1× PBS. Granules with diameter of 500-600 μm were selected by analytical sieve (analysensieb, Fritsch GmbH, Industriestrasse 8) for the isolation of aggregating bacteria.

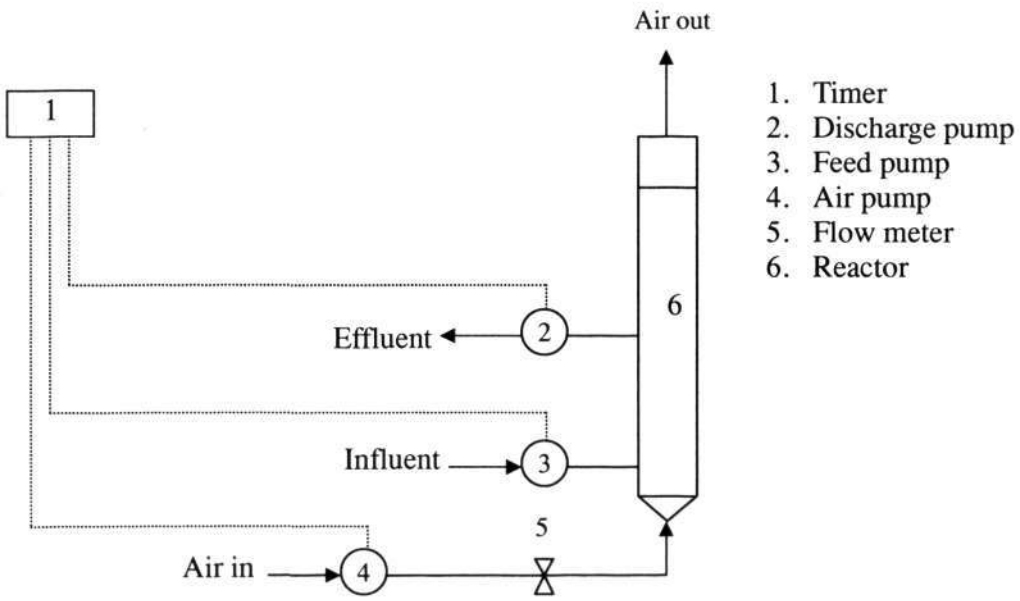

Figure 3.1 Scheme of experiment system

3.2.2 Heterotrophic Bacteria Isolation Procedure

The direct serial dilution and plate spreading was employed to isolate heterotrophic bacteria from acetate-fed aerobic granules before the enrichment, and from the enrichment sludge with highest coaggregating index at the end of enrichment. All equipments and medium used in isolation experiment were sterilized by autoclaving for 20 min at 121 °C or filtered with sterile syringe (latex free syringe, Becton Dickinson, Singapore) through 0.2 μm sterile filter paper (non pyrogenic, Schleicher & Schuell).

The aerobic granules or aggregates of enrichment sludge were harvested by centrifuge (1000×g, 5 min) and broken up with a mini bead beater (Biospec Products, USA) after washing twice with phosphate buffered saline (PBS, pH 7.2). Serial dilutions of grinded granule sludge or grinded enrichment sludge were made in PBS and dilutions (10^{-1} to 10^{-10} fold) were spread on agar plates, followed with incubation at 25°C. Two kinds of solid media were used in isolation: R2A agar (Difco, Detroit, USA), and Bacto agar (Difco, Detroit, USA) with addition of the same chemical composition as the synthetic wastewater used for granule cultivation in SBR. All visible colonies of distinct morphological types appeared after two days to two weeks were separated and repetitively subcultured on R2A or Bacto agar.

Strains isolated after growth on solid agar were subjected to Gram staining and morphologies were observed by light microscope (Olympus, BX60 epifluorescence microscope, Japan). Pure cultures were preserved in KH_2PO_4:K_2HPO_4 solution (pH 7.0) and 20% (v/v) glycerol at -60 °C for subsequent analysis.

3.2.3 Enrichment Procedure

The grinded granule samples were filtered through a 0.45 μm sterile filter paper and suspended in autoclaved distilled water for the enrichment of aggregating bacteria. 1 ml of liquid mixture was transferred to sterile 50 ml micro-centrifuge tube (Axygen scientific, Union City, USA) containing 15 ml growth media (same with

the synthetic wastewater used for granule cultivation in SBR, except for the different concentration of sodium acetate as the sole carbon source), and incubated at 25 °C and 150 rpm on a shaker for different incubation time. The tubes were aerated for 24 h in a biohazard flow cabinet (ESCO, Singapore). Two distinguished incubation times (2 day and 4 days respectively) and four COD concentrations (50 mg l^{-1}, 200 mg l^{-1}, 400 mg l^{-1} and 800 mg l^{-1}, respectively) were applied. After a fixed time span, micro-centrifuge tubes were allowed to settle for 10 min, and 1 ml of enrichment sludge solution on the bottom of tube was transferred to new tubes and incubated by cycles until visible dense aggregates appeared. The aggregation index of each enrichment solution was measured and the one with highest aggregation index was selected for the isolation of aggregating bacteria in succession.

3.2.4 Amplification of 16S rRNA Genes

A whole cell direct lysis polymerase chain reaction (PCR) amplification method was used to amplify the 16S rRNA gene of isolates. Approximate 100 ng strain was picked up from one colony of each pure culture as template DNA. Universal primers of 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used. The mix PCR solution used for amplification of 16S rRNA gene of each culture contained one portion of target DNA, 93 µl PCR buffer (100 µl of thermal polymer buffer, 80 µl of 25 mM $MgCl_2$, and 820 µl of autoclaved distilled water per milliliter), 2 µl dNTP (10 µM), 2 µl of forward and reverse primer (10 µM each) and added to a 0.5 ml microcentrifuge tube. The tube was vortexed and centrifuged briefly, and heated at 98 °C for 30 min in a thermal cycler (Mastercycler, Eppendorf, Germany) before addition of 1 µl *Taq* polymerase (Promega, USA).

Thermal cycling was carried out in the thermal cycler as follows: denaturation at 94 °C for 1.5 min, primer annealing at 60 °C for 1.5min, extension at 72 °C for 1.5 min for a total of 30 cycles. The primers 27F, 357F, 530F, 970F, 1100F, 800R, 907R, 1100R, 1392R and 1492R were used for the full 16S rRNA gene sequencing, while

primers 530F and 800R were used for partial 16S rRNA gene amplification and sequencing (Table 3.1).

Amplification of target DNA was confirmed by agarose gel electrophoresis. The amplified DNA product was purified with Qiaquick PCR purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at –20 °C before sequencing. Agarose gel electrophoresis was used to confirm purity and concentration of the purified DNA products.

Table 3.1 Primers used for sequencing 16S rRNA of isolates (Ivanov et al. 2005; Lane 1991)

Primer	Sequence (5'----------> 3')
27F	AGAGTTTGATCMTGGCTCAG
357F	CTCCTACGGGAGGCAGCAG
530F	GTGCCAGCMGCCGCGG
907F	AAACTCAAAGGAATTGACGC
1100F	CAACGAGCGCAACCCT
800R	CATCGTTTACGGCGTGGAC
907R	CCGTCAATTCMTTTRAGTTT
1100R	AGGGTTGCGCGCTCGTTG
1392R	ACGGGCGGTGTGTRC
1492R	ACGGYTACCTTGTTACGACTT

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

3.2.5 Agarose Gel Electrophoresis

0.5 g agarose (Promega, USA) was molten in 50 ml 1xTAE buffer (242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0) followed with addition of 5 µl ethidium bromide solution (10 mg/ml, BioRad, England) and cast on a horizontal gel slab for solidification. The solidified agarose gel was then immersed

in a mini-subcell GT electrophoresis tank (BioRad, England) containing 250 ml 1 × TAE buffer. 5 µl of DNA product was mixed with 2 µl gel loading dye (0.25% bromophenol blue, 0.25% cylene cyanol FF, 30% glycerol in water), and loaded onto solidified agarose gel. The mixture of 2 µl 100-bp ladder marker (Promega, USA) and 3 µl loading dye was also loaded simultaneously in comparison to assess the concentration of DNA products. The gel was run in succession at 85 mV for 30 min in PowerPac 300 (BioRad, England). After the running, the gel was viewed with a Kodak Digital Science Image Station 440CF (NEN Life Science Products). The existence of DNA product was confirmed by the presence of a DNA band.

3.2.6 16S rDNA Sequencing

The 16S rRNA gene sequences of isolates were determined by using the ABI model 310A DNA sequencer (Applied Biosystems, Perkin-Elmer, Forster, CA, USA) and the ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing kit (version 3.0; Applied Biosystems, Perkin-Elmer) as specified by manufacturer.

For each sequence, the sequences alignments were assembled by the BioEdit software (Hall 1999) and the BLAST search program was used to query the database maintained by National Centre for Biotechnology Information (NCBI) (National Institutes of Health, USA) for an initial determination of the nearest phylogenetic neighbour sequences as described previously (Ivanov et al. 2005; Tay et al. 2002b)

3.2.7 Microscopy Examination

The pure culture cells or coaggregates were washed three times carefully with autoclaved distilled water and then filtered with 0.2 µm filter (cellulose acetate membrane filter, Advanten MFS Inc, CA, USA). The filter and cells were soaked in 250 ml beaker containing 2% glutaraldehyde solution for 2 h. The harvested cells or coaggregates were washed three times (20 min each time) in 0.1 M sodium cacodelate buffer which was prepared by dissolving 21.39 mg cacodylic acid

sodium salt hydrate (98%) in 1 l of DI water and dehydrated in series of 10 min washes in 50%, 70%, 85% and 95% ethanol. The dehydrated cells or coaggregates were stored in 100% ethanol before filter. The dried samples were wrapped in punctured Al foil following critical-point dry with CO_2 (Bal-Tec CPD 030). After sputter coated with Au-Pd at 20 mA in a high vacuum (2.8×10-6 Torr), the samples were viewed in scanning electron microscope (Leica Stereoscan 420, Leica Cambridge Instruments, Cambridge, UK) at 15-20kV.

Gram-stained samples were conducted using a procedure kit of Becton Dickinson Microbiology Systems (Cockeysville, MD, USA) and observed with light microscope (Olympus BX60, Tokyo, Japan).

3.2.8 Aggregation Index Assay

The coaggregation index of pure cultures was examined by a modified method as described by Malik et al. (2003a, b). 50 ml micro-centrifuge tubes (Axygen scientific, union city, USA) containing 20 ml of liquid nutrient broth (Oxid Ltd., Bastingstoke, England; 'Lab-lemco' powder 1.0; yeast extract 2.0, peptone 5.0, sodium chloride 5.0, pH 7.4 ±0.2) were inoculated with pure bacterial isolates. Cultures in broth were incubated in an incubator (Sanyo, Osaka, Japan) at 25 °C and 150 rpm for suitable periods of time, depending on the growth rates of the strains. Cells were harvested at the early stationary growth phase by centrifuge (4,000×g for 20 minutes), washed three times in 3 mM NaCl containing 1 mM $CaCl_2$, and resuspended in the same solution. The cell suspension was allowed to settle for 5 minutes and the remaining cell suspension was used for aggregation experiments. Two coaggregation assays were used: spectrometric method and visual method.

Spectrometric method

The cell suspension density was monitored by optical density (OD) measurement using a spectrophotometer (Perkin-Elmer, USA) at a wave length of 600 nm. The cell suspension of each pure bacterial partner was adjusted to around 0.5 with the same solution and pH value was adjusted to 7.0. Equal volumes (1 ml each) of each

coaggregating partner suspension were mixed in 2 ml micro-centrifuge tube on a vortex mixer and the optical density at a wave length of 600 nm (OD_{600}) of mixed solution was measured immediately. 2 ml of each pure bacterial suspension was added into a 2 ml micro-centrifuge tube simultaneously to measure the autoaggregation index. All the solutions were allowed to stand for 180 minutes and then OD_{600} of supernatants after coaggregation or autoaggregation were measured. The aggregation index was calculated as follows:

$$\text{Aggregation index}\ (\%) = \frac{OD_{total} - OD_{supernatant}}{OD_{total}} \times 100$$

Where OD_{total} and $OD_{supernatant}$ represent the optical attenuance of supernatant in mixing liquid after standing for 0 min and 180 mins, respectively.

Visual scoring method

A visual coaggregation method modified from Cisar et al. (1979) was used to assess the aggregating ability. The degree of coaggregation of each pair was scored as follows: 0, no aggregates in suspension; 1, small aggregates in a turbid suspension; 2, obviously visible aggregates in a turbid suspension; 3, obviously visible aggregates settled down leaving a clean suspension; 4, large aggregates settled down nearly immediately leaving a clean suspension. Autoaggregation of each partner in the coaggregating pair was scored using the same criteria simultaneously as control, and the autoaggregating scores were deducted from the pair's score to assess the coaggregating degree.

3.2.9 Inhibition of Aggregating Behavior by Heat Treatment

Effect on coaggregation by heat pretreatment of bacterial partners was performed using modified method as previously described by Rickard et al. (2004a). Strains were harvested, washed twice in 3 mM NaCl containing 1 mM $CaCl_2$, resuspended in the same solution and heated for 30 min at 80°C. All strains were combined in reciprocal pairs with a pair of untreated cells as control. The change of coaggregation index or visual score was used to assess the effect of heat treatment.

3.2.10 Inhibition of Aggregating Behavior by Simple Sugar

The ability of simple sugar to affect coaggregation was measured as described previously (Malik et al. 2003a, 2003b; Rickard et al. 2004a). Filter sterilized solution of glucose was added to the coaggregation pair suspension to give a final concentration of 50 mM or 250 mM. The mixture was then vortexed for 20 seconds, and coaggregation index was measured using spectrometric method.

3.2.11 Inhibition of Aggregating Behavior by EDTA

The effect of intermittent addition of EDTA to coaggregation was performed as described by Malik et al. (2003b). A set of two coaggregating mixtures was prepared containing 2 mM EDTA (mixture a) which was added at 120 min when the coaggregation pairs reached high coaggregation index. A set of two coaggregation mixtures and the pure culture suspensions without addition of EDTA (mixture b) was measured simultaneously as control test. The intermittent EDTA addition was used to investigate the reversal of coaggregation by EDTA. Subsequently, 3 mM calcium ion was added to mixture at 180 min. The addition of calcium ion aims to study whether the EDTA works to reverse coaggregation through chelation of Ca^{2+}.

3.2.12 Inhibition of Aggregating Behavior by Electrolytes

Alternative Na^{+} and Ca^{2+} concentration were added into coaggregation mixtures at 0 min to measure the effect of electrolytes to the coaggregation index.

All the experiments mentioned above in 3.2.9 to 3.2.12 about the inhibition factors on aggregating behaviors were performed in duplication.

3.3 RESULTS

3.3.1 Cells Isolated from Acetate-fed granules

In total 56 pure cultures were isolated from acetate-fed aerobic granules and 20 distinct isolates were collected after screening with repetitive extragenic palindromic sequence PCR (rep-PCR). Brief descriptions of the 20 strains are listed in Table 3.2. Light microscopy and scanning electronic microscopy (SEM) were used to observe the morphological types of the isolates. The following nomenclature was used to enumerate the different batch reactors: B*.# or En*.#, with 'B' represented direct isolation of granules from reactor "B", while 'En' represented the cell isolation of enriched granules sludge; '*' for the dilution series number in isolation, followed by the '#' representing the sequence in bacterial community on plates of the same dilution series number. For instance, strain B6-14 was isolated directly from aerobic granules, which was observed on plate of dilution 10^6.

Table 3.2 Characteristics of strains isolated from acetate-fed aerobic granules

	Name	Description of Colonies	Size (mm)	Gram stain	Morphological type by Light Microscopy
1.	B6-2	Yellow, round, smooth surface	2	negative	Rods
2.	B6-5	Orange, round, smooth surface	2	positive	Cocci
3.	B6-9	White, semi-transparent, gliding, irregular	10	positive	Rods
4.	B6-10	Light yellow, gliding, round, smooth surface	1.5	positive	Cocci
5.	B6-11	Pink, round, smooth surface	1	negative	Rods
6.	B6-13	Brownish yellow, round, smooth surface	5	positive	Rods
7.	B6-14	White, gliding, round, smooth surface	3	negative	Rods
8.	B6-15	Yellow, gliding, round, smooth surface	2	negative	Cocci
9.	B6-16	White, semi-transparent, round, smooth surface	1	negative	Rods
10.	B6-17	Yellow, round, smooth surface	1.5	negative	Rods
11.	B6-25	Semi-transparent, gliding, irregular, smooth surface	20	positive	Cocci
12.	B6-27	Transparent, gliding, smooth surface	2	negative	Cocci
13.	B7-2	Light orange, irregular, smooth surface	1.5	positive	Rods
14.	B7-7	White, irregular, smooth surface	1	negative	Rods
15.	B7-8	Brownish white, round, rough surface	2	negative	Rods
16.	B7-14	Yellowish white, gliding, round, smooth surface	2.5	negative	Rods
17.	B7-15	White, round, rough surface	2	negative	Rods
18.	B7-18	Light brown, round, smooth surface	4	positive	Cocci
19.	B8-1	Yellowish white, irregular, smooth surface	3	negative	Rods
20.	B8-2	White, gliding, round, smooth surface	6	negative	Rods

3.3.2 Effect of Cultivation Media

Most isolates cultivated on either R2A agar or Bacto agar formed visible colonies from two days to one week at room temperature. Heterotrophic bacterial colonies appeared on 16 R2A agar plates with dilution factors as high as 10^8, whereas only agar plates from 10^1 to 10^4 fold dilution were observed with the growth of colony forming units. Direct plating of acetate-fed aerobic granules on R2A agar plates generated $7.65 \pm 0.2 \times 10^8$ bacterial colonies per milliliter of granule sludge (CFU ml^{-1}) compared to $3.20 \pm 0.15 \times 10^6$ CFU ml^{-1} on Bacto agar plates. Furthermore, the bacterial strains present on R2A agar showed to be diverse with respect to the

morphological types. 20 isolates of distinguished characteristics were found on R2A agar, while 8 isolates originated from Bacto agar. The photographs of bacterial community cultivated on R2A agar or Bacto agar are shown on Figure 3.2.

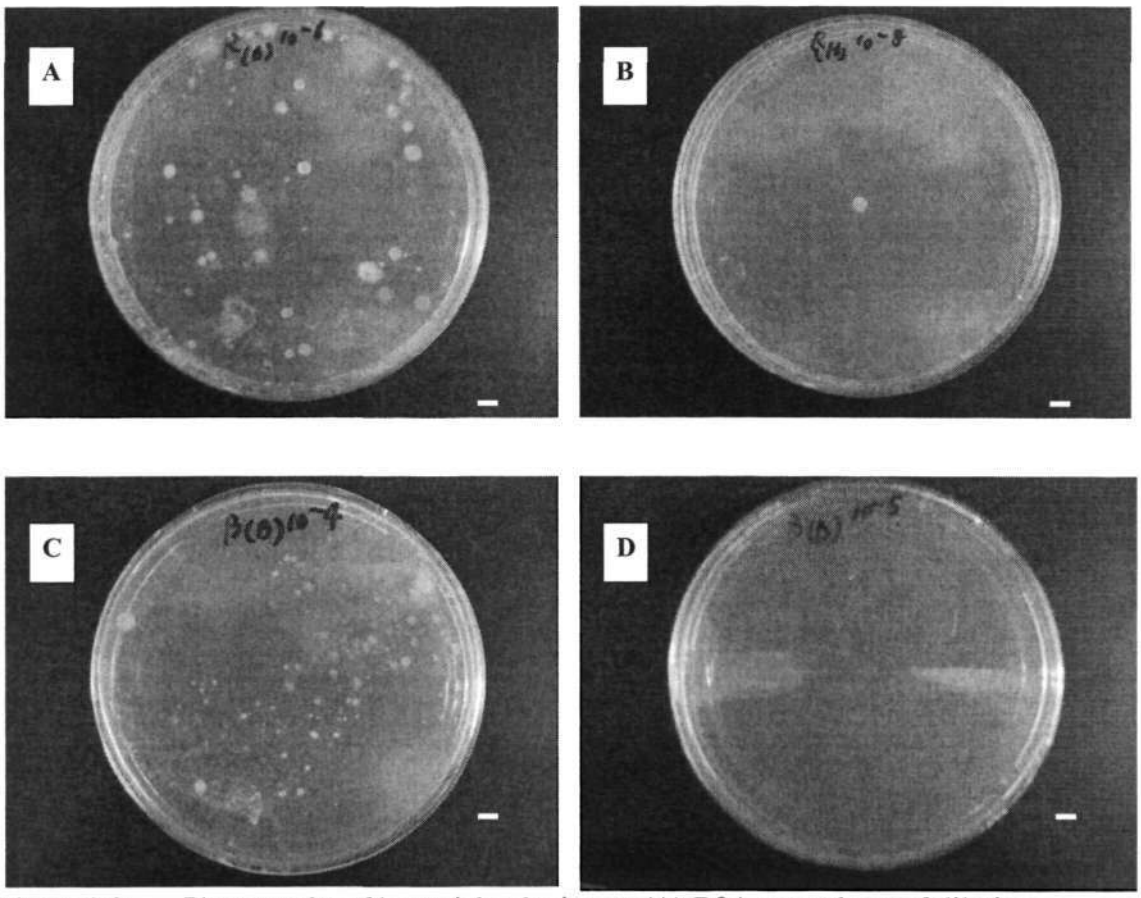

Figure 3.2 Photographs of bacterial colonies on (A) R2A agar plates of dilution series 10^6 (Bar: 5 mm), (B) R2A agar plates of dilution series 10^8 (Bar: 5 mm), (C) Bacto agar plates of dilution series 10^4 (Bar: 5 mm), and (D) Bacto agar plates of dilution series 10^5(Bar: 5 mm).

3.3.3 Enrichment of Granular Sludge

After enrichment for 28 days, visible aggregates were observed in all tubes with combination of two sets of intermittent feeding time (2 and 4 days) in four influent concentrations (50, 200, 400 and 800 mg COD l^{-1}). As shown in Figure 3.3, the

aggregating index of enrichment sludge with feeding interval of 2 days decreased from 6.3% to 1.25% when the influent COD loading increased from 50 to 800 mg COD l^{-1}. In contrast, for the intermittent feeding period of 4 days, the aggregating index of enrichment sludge increased from 2.4% to 12.9% in accordance with the same increase of COD loading. Due to the highest aggregation index, the enrichment sludge with 800 mg COD l^{-1} in influent and 4 days of feeding interval was selected for further isolation to investigate aggregating populations.

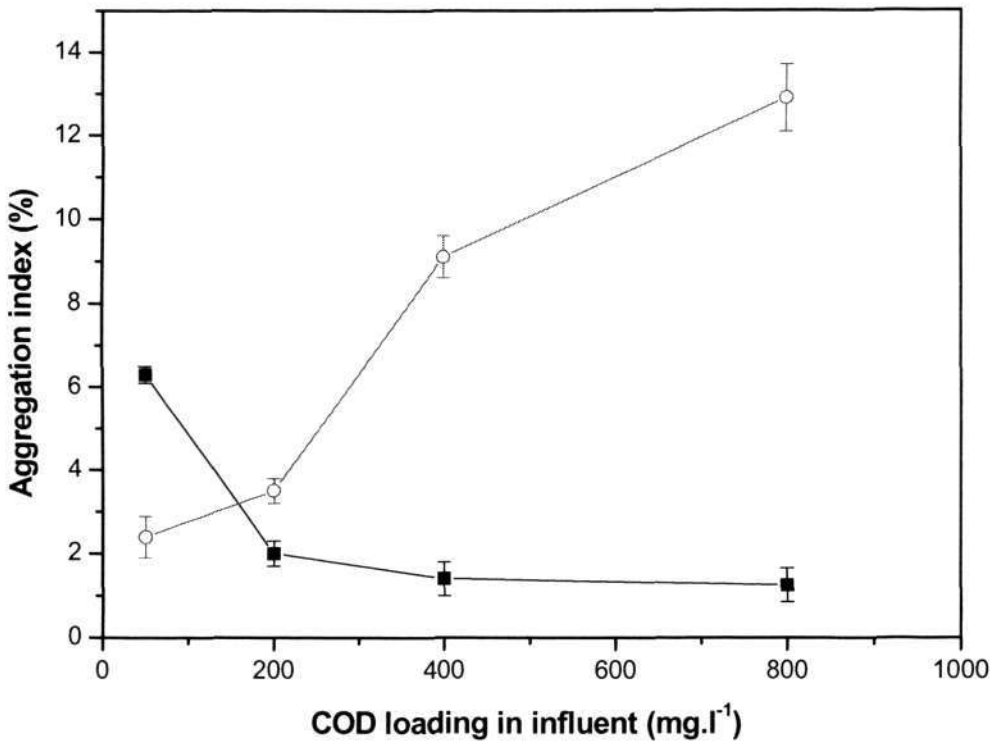

Figure 3.3. Aggregation index of enrichment sludge after operation for 28 days with intermittent feeding time of: 2 days (■) and 4 days (○)

3.3.4 Aggregation Index of Isolates from Aerobic Granules

In total 56 pure cultures were isolated from acetate-fed aerobic granules and 20 distinct isolates were identified after screening with repetitive extragenic palindromic sequence PCR (rep-PCR). 14 of the 20 distinct strains were found to be capable of either coaggregating or autoaggregating (Table 3.3). Of the 20 strains, 8 strains (40%) possessed autoaggregation ability, among which 1 strain was determined with autoaggregating score of 4. It was evidenced by large flocs formation and settled down almost instantaneously, leaving a clear supernatant. The proportion of strong autoaggregating strains even increased with longer incubation period in some cases. 11 (55%) of the 20 aggregating strains appeared to coaggregate with at least one partner. These scores were reproducible after incubation in batch culture for a period of time from three days to one week.

Strain B6-2 had the highest number of coaggregating partners among isolates, showing the ability to interact with half of all the isolates from aerobic granules. Furthermore, B6-25 and B7-8 were also selected besides strain B6-2 for the further investigation of aggregating behaviors under various physical and chemical conditions. They were chosen not only due to the high score of coaggregating interactions, but due to the fact that these three strains can coaggregate with each other. Figure 3.4 shows the photograph of coaggregates formed between strains B6-2 and B6-25 with coaggregation score of 3.

Table 3.3 Coaggregating and autoagregating scores for isolates from aerobic granule (at stationary growth phase cultivated in nutrient broth)

Strains*	Coaggregation partner (coaggregation score)	Autoaggregating Score	Number of coaggregating partners
B6-2	B6-10 (1), B6-14 (1), B6-16 (2), B6-25 (3), B7-7(3), B7-8 (1), B7-14 (1)	0	7
B6-10	B6-2 (1)	2	2
B6-14	B6-2 (1), B6-25 (1)	3	2
B6-15	none	2	0
B6-16	B6-2 (2), B6-17 (3), B7-7 (1)	0	3
B6-17	B6-16 (3), B6-25 (2), B7-15 (2), B8-2 (1)	0	4
B6-25	B6-2 (3), B6-14 (1), B7-8 (3)	0	3
B6-27	none	4	0
B7-7	B6-2 (3), B6-16 (1)	0	2
B7-8	B6-2 (1), B6-17 (1), B6-25 (3)	0	3
B7-14	B6-2 (1)	3	1
B7-15	B6-17 (2)	2	1
B8-1	none	3	0
B8-2	B6-17 (1)	3	1

: The following nomenclature was used to enumerate the different batch reactors: B.# with 'B' represented direct isolation from aerobic granules; '*' for the dilution series number in isolation, followed by the '#' representing the sequence in bacterial community on plates of the same dilution series number. For instance, strain B6-14 was isolated directly from aerobic granules, which was observed on plate of dilution 10^6.

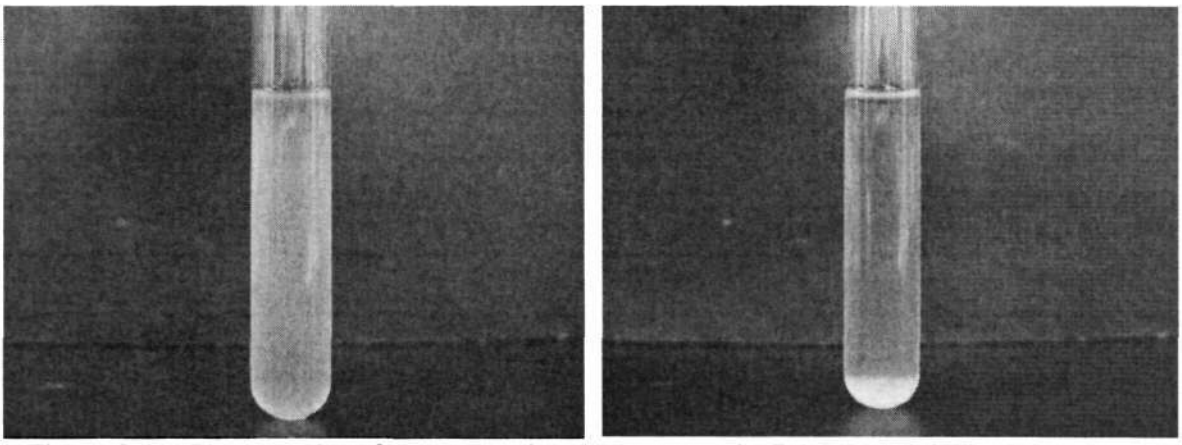

Figure 3.4 Photographs of coaggregation between strain B6-2 and B6-25. Cells were grown at stationary phase.

3.3.5 Aggregation Index of Isolates from Enriched Granule Sludge

Compared with aerobic granules, less isolates were recovered from enrichment sludge, accompanied with symbiotic colonies and gliding colonies observed to dominate in the microbial community on agar plates. In total 13 strains with distinguished morphological characteristics were isolated and 11 of these isolates showed ability to either autoaggregate or coaggregate (Table 3.4). Of the 13 isolates, 10 strains (77%) were able to coaggregate with at least one partner, and none showed high autoaggregating ability (autoaggregating score≥3 or 4). 3 strains (23%) cannot autoaggregate but can only coaggregate. The proportion of autoaggregating strains was 61.5% in the 13 enrichment isolates.

Table 3.4 Coaggregation and autoagregation scores for isolates from enriched aerobic granular sludge (at stationary growth phase cultivated in nutrient broth)

Strains*	Coaggregation partner (coaggregation score)	Autoaggregating Score	Number of coaggreating partners
En5-9	En5-10-1(1), En5-10-2(1), En5-11(1), En5-12-1(1), En5-12-2(1), En5-13(1), En5-18(1), En7-3(1)	1	8
En5-10-1	En5-9(1)	1	1
En5-10-2	En5-9(1), En6-1(1)	2	2
En5-11	En5-9(1)	1	1
En5-12-1	En5-9(1), En5-13 (2), En5-18(2)	0	3
En5-12-2	En5-9(1)	2	1
En5-13	En5-9(1), En5-12-1(2), En5-18(2), En6-1(1)	0	4
En5-18	En5-9(1), En5-12-1(2), En5-13(2), En7-3(2)	0	4
En6-1	En5-10-2(1), En5-13(1)	1	2
En6-2	none	2	0
En7-3	En5-9(1), En5-18(2)	1	2

: the following nomenclature was used to enumerate the different batch reactors: E.# with 'E' represent the cell isolation from enrichment granules sludge; '*' for the dilution series number in isolation, followed by the '#' representing the sequence in bacterial community on plates of the same dilution series number.

3.3.6 16s rRNA Sequencing of Strains Isolated from Acetate-fed Aerobic Granules

To provide information on the composition and diversity of microbial communities in the acetate-fed aerobic granule microbial consortium, the 16s rRNA genes were fully sequenced. Compiled full sequence 16s rRNA gene sequences of approximately over 1,400 bases in length were obtained from strains isolated from acetate-fed aerobic granule and compared to known sequences in the database maintained by National Centre for Biotechnology Information (NCBI). The results

were summarized in Table 3.5.

The isolates fell into three major lineages of the bacteria domain: the α-, β-, and γ-*Proteobacteria*, *Actinobacteria*, and *Cytophaga-Flavobacteria-Bacteroides* (CFB) bacteria. The majority of the bacterial sequences (11 over 13) grouped with members of *Proteobacteria*, with seven in the β subdivision, three in the α subdivision and one in the γ subdivision. One isolate was placed in the Gram-positive bacteria with high G+C content, with the remaining one clustered in CFB group. Table 3.5 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.

From the seven isolates belonging to the β subdivision of *Proteobacteria*, isolate B6-14 matched sequences of *Zoogloea resiniphila* species with identity of 97%, isolate B6-16 with a 97% identity to *Hydrogenophaga sp.* species, isolate B7-7 with a 95% identity to *Phenanthrene-degrading bacteria 90* species, isolate B7-14 with a 94% identity to *Thauera aromatica* species, B7-15 with a 98% identity to *Uncultured bacterium clone* HP1B06 species, B8-1 with a 97% identity to *Zoogloea resiniphila* species, and B8-2 with a 93% identity to *Azoarcus sp.* species.

The bacteria in α subdivision include isolate B6-2 with a sequence identity of 99% to *Sphingomonas sp.* S37 species, isolate B6-17 with a 96% identity to *Monochloroacetic acid degrading bacteria* Species, and isolate B7-8 with a 93% identity to *Sinorhizobium terangae (Ensifer terangae)* Species. Isolate B6-25 was 96% identical with the strain *Pseudoxanthomonas daejeonensis strain TR,* belonging to the γ subdivision. Isolate B6-27 was 90% identical with the *Flavobacterium sp. 'Smarlab BioMol-2300973'* species in CFB group, and isolate B6-10 was 97% identical with strain *Microbacterium sp.* belonging to Gram-positive bacteria with high G+C content.

Table 3.5 Sequence analysis of strains isolated from acetate-fed aerobic granule

Strains	Sequence length (bases)	Closest relatives (accession number)	Identity (%)	Phylogenetic Division
B6-2	1472	Sphingomonas sp. S37 (AF367204)	99	α-Proteobacteria
B6-10	1436	Microbacterium sp. oral strain C24KA (AF287752)	97	Actinobacteria
B6-14	1522	Zoogloea resiniphila (AJ505852)	97	β-proteobacteria
B6-16	1487	Hydrogenophaga sp. TRS-05 (AB166889)	97	β-proteobacteria
B6-17	1527	Monochloroacetic acid degrading bacteria (AF532194)	96	α-Proteobacteria
B6-25	1520	Pseudoxanthomonas daejeonensis strain TR6 (AY550264)	96	γ- Proteobacteria
B6-27	1518	Flavobacterium sp. 'Smarlab BioMol-2300973' (AY230767)	90	Flavobacterium
B7-7	1519	phenanthrene-degrading bacteria 90 (AY177371)	95	β-proteobacteria
B7-8	1481	Sinorhizobium terangae (Ensifer terangae) (AM418766)	93	α-Proteobacteria
B7-14	1511	Thauera aromatica (AM419048)	94	β-proteobacteria
B7-15	1464	Uncultured bacterium PHOS-HC20 (AF314418)	98	β-proteobacteria
B8-1	1432	Zoogloea sp. EMB 62 (DQ413151)	97	β-proteobacteria
B8-2	1518	Azoarcus strain DS 30 (EF494194)	93	β-proteobacteria

3.3.7 16s rRNA Sequencing of Strain Isolated from Enriched Granular Sludge

To evaluate the shift of polygenic diversity in enriched granule sludge community, the 16S rRNA gene was partially sequenced, and the putative bacteria division and nearest relatives were invested by BLAST. The results were summarized in Table 3.6. The isolates fell into four major lineages: α-, β-, and γ- *Proteobacteria*, and *Actinobacteria*. The isolate EN5-13 was investigated to be unclassified. Three of these eleven isolates were assigned to the α subclass of *Proteobacteria*, four to the β subclass, and one to the γ subclass. The remaining two isolates were placed in the *Actinobacteria*.

Table 3.6 Sequence analysis of strains isolated from enriched aerobic granular sludge

Strains	Sequence length (bases)	Closest relatives (accession number)	Identity (%)	Phylogenetic Division
En5-9	962	Rhizobium tropici strain PRF93 (AY117624)	97	α-Proteobacteria
En5-10-1	948	Kartchner Caverns bacterium HI-I1 (DQ205298)	97	α-Proteobacteria
En5-10-2	962	Microbacterium sp. ZD-M2 (DQ417926)	98	Actinobacteria
En5-11	465	Microbacterium oxydans isolate OUCZ46 (AY785738)	97	Actinobacteria
En5-12-1	930	Aminobacter sp. MI-p2a (DQ196478)	97	α-Proteobacteria
En5-12-2	1046	Variovorax paradoxus (AF532868)	98	β-proteobacteria
En5-13	1023	Bacterium 14C13 (DQ298787)	98	Unclassified
En5-18	799	Comamonas testosterone (AY653219)	99	β-proteobacteria
En6-1	1101	Variovorax paradoxus MBIC3839 (AB008000)	98	β-proteobacteria
En6-2	946	Delftia acidovorans (AB074256)	99	β-proteobacteria
En7-3	885	Stenotrophomonas sp. H2 (AY663436)	99	γ- proteobacteria

3.3.8 SEM Observation

The aggregate size and shape of coaggregates among B6-2 and B6-25 or B6-25 and B7-8 were observed using phase contrast microscopic and scanning electron microscope (shown in Figure 3.5). The coaggregates of B6-25 and B7-8 are visible pelleted particles with bigger mean diameter of 60-120 μm compared to coaggregate between B6-2 and B6-25 (40-70 μm).

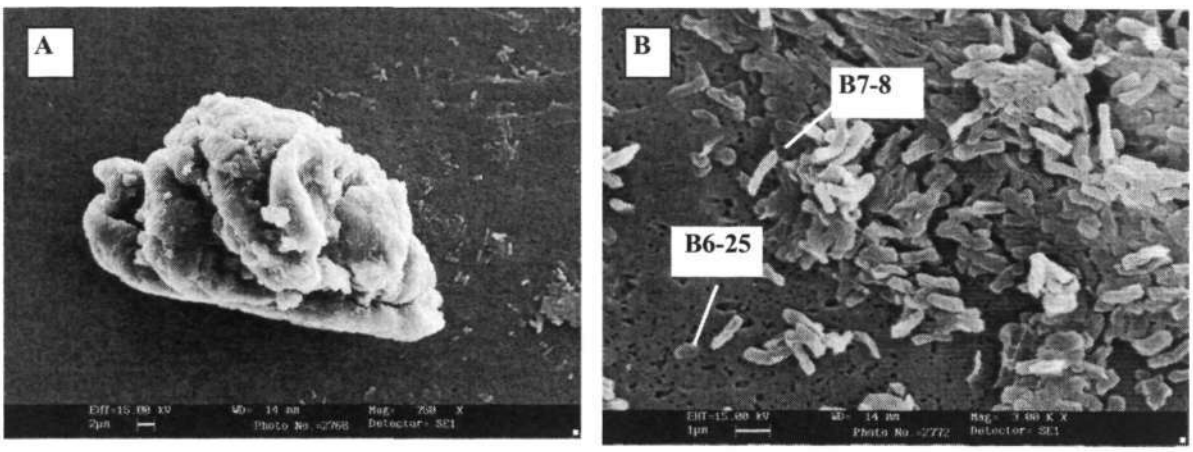

Figure 3.5 Scanning electron micrograph (SEM) of coaggregate between B6-25 and B7-8

3.3.9 Inhibition of Aggregating Behaviors by EDTA, pH Value, Simple Sugars and Heat Treatment

Effect of electrolytes and pH value

Three electrolyte concentrations were used in the experiment. As shown in Table 3.7, change of electrolyte concentration in liquid media had slight influence on coaggregation between B6-25 and B7-8 and the lack of calcium ion caused partial disaggregation, whereas the absence of ions in media led to the complete disaggregation. Similar results were obtained in the case of coaggregating pair of B6-25 and B6-2, as the influence of calcium ion was especially significant to this pair. Thus absence of electrolytes affected both coaggregating pairs studied in this

research. Under the extreme pH value, coaggregates of B6-25 and B7-8 lost the capacity to coaggregate completely. Results are presented in Table 3.7 as well.

Table 3.7 Effect of electrolytes and pH value on coaggregation of B6-25 with B6-2 and B7-8

Condition	*Coaggregation score at 120 min*	
	B6-25 +B7-8	B6-25 +B6-2
Electrolyte concentration		
6mM NaCl +1mM $CaCl_2$	4	3
20mM NaCl	3	1
5mM $CaCl_2$	4	3
MilliQ water	1	0
PH value		
7.0	4	3
3.0	0	0
12.0	0	1

Effect of EDTA

The effect of intermittent addition of EDTA (2mM) and subsequently Ca^{2+} (3mM) on the coaggregation index of coaggregates formed by B6-25 with B6-2 and B7-8 is shown in Figure 3.6. All of these three strains were non-flocculating and showed no strong ability to autoaggregate during the experimental process. The intermittent addition of 2 mM EDTA at 120 min led to the slight decrease of coaggregation index in both pairs, when compared with the control pairs without addition of EDTA. Subsequently, with addition of 3 mM Ca^{2+} at 180 min, increase of coaggregation index was observed on the pair of B6-25 and B7-8 to the similar coaggregation index level as that of the control pair. In contrast, addition of calcium ions did not recover the coaggregation between B6-2 and B6-25.

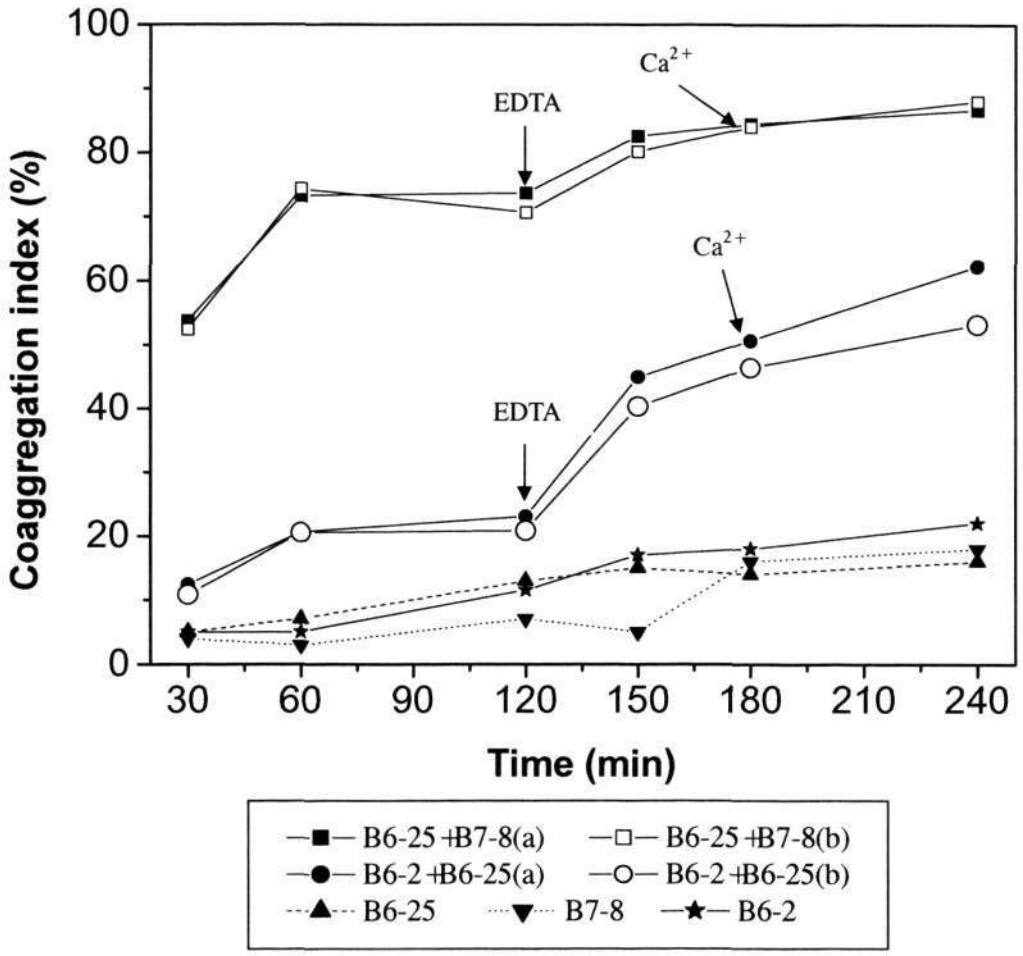

Figure 3.6 Effect of EDTA (2mM) and intermittent addition of Ca^{2+} on coaggregation of B6-25 with B7-8 and B6-2. Solid symbols show coaggregation mixture in absence of EDTA and extra addition of Ca^{2+}; empty symbols show coaggregation mixture with addition of 2mM EDTA at 120 min and 3mM Ca^{2+} at 180 min.

Effect of simple sugar

Glucose was used in the experiment for the study of effect of simple sugar on coaggregating behaviors of coaggregating pair B6-2 and B6-25. The effects of various concentrations of glucose are shown in Figure 3.7. The exposure of coaggregate to 50 mM or 250 mM glucose was shown to inhibit the coaggregation behavior when compared with the coaggregating pair without addition of glucose. With the presence of higher concentration of glucose, the coaggregation index was kept at lower level.

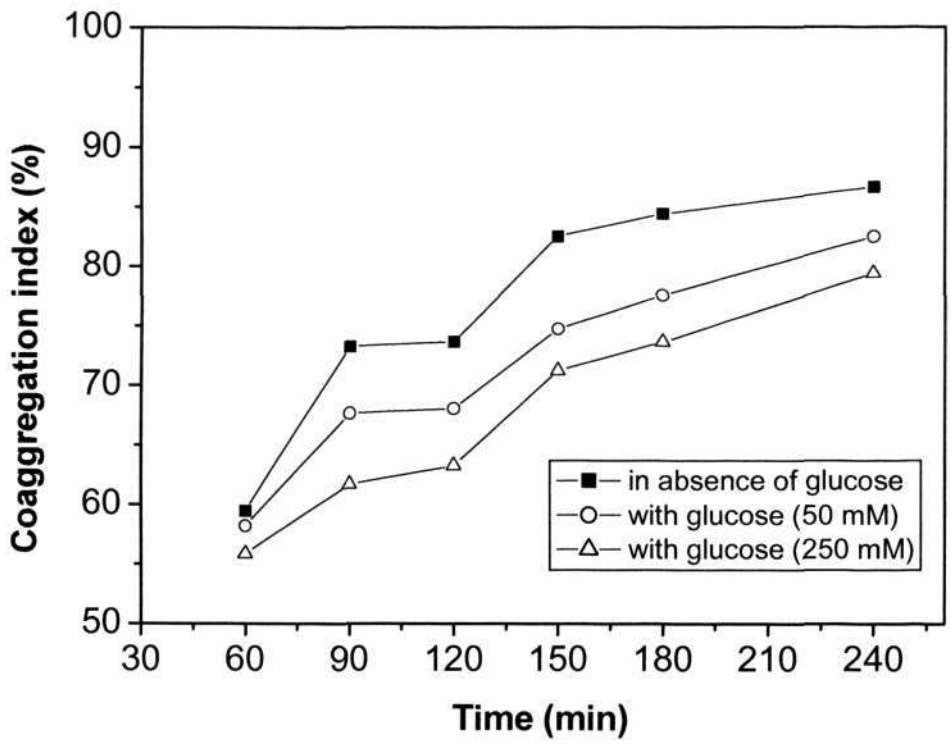

Figure 3.7 Effect of glucose (added at 0 min) on coaggregation of B6-25 and B7-8. Solid symbols show coaggregation mixture in absence of glucose, empty symbol shows coaggregation mixture with addition of glucose.

Effect of heat pretreatment

Both coaggregating pairs were heat-resistant as shown in Table 3.8. The mixture of heat-pretreated strains B6-25 or B7-8 at 80 °C for 30 min with other partners resulted in the increase of visual score compared to the control in short time period (30 min). None of the coaggregation pairs was inhibited when any partner was exposed to high temperature.

Table 3.8 Effect of heat on coaggregation scores of mixtures at 30 min with each partner treated separately (80 °C for 30 min) and mixed up in reciprocal pairs

		Coaggregation scores			
		B6-2		B7-8	
		untreated	treated	untreated	treated
B6-25	untreated	2	1	3	3
	treated	2	2	3	3

3.4 DISCUSSION

3.4.1 Aggregating Community of Enriched Cultures

The incomplete depletion of substrate in each feeding cycle could explain the decrease of aggregation index along with the increased influent COD loading, when the enrichment culture were periodically fed in interval of 2 days. With no or short starvation period, the microbial ability of enrichment cultures would be suppressed and microorganisms tended to grow in dispersed status. Sufficient starvation conditions were reported to be one of the triggers for microbial aggregation (Bossier et al. 1996b). Under relatively long feeding period of 4 days, when facing the starvation surface characteristics of the enrichment sludge would change to facilitate in forming dense aggregates. Furthermore, an increased influent COD loading could raise the growth rate of enrichment biomass. As a result, the

aggregate exhibited comparably high biomass density and specific gravity, which enhanced the settling ability of aggregate in the experiment tube.

Considering the same settling time for all enrichment sludge, the frequency to withdraw the supernatant served as the main determinant hydraulic selection factor imposed on enrichment sludge. This frequency could be related to hydraulic retention time (HRT) at the given exchange ratio of 0.5 in the present study. The enrichment sludge operated at periodic withdrawal interval of 4 days were observed to form visible flocs with comparably higher coaggregating scores than those operated with the withdrawal interval of 2 days. The phenomenon was possibly correlated to the small quantity of inocula used in the experiment, as HRT should be long enough to allow the microbial growth and accumulation. Along with the low shear force in the micro-centrifuge tube, the long feeding periodicity supported the growth of slow-growing bacteria, which was investigated to facilitate the stable granular aggregates (De Kreuk et al. 2004; Liu et al. 2004b).

3.4.2 Comparison of Aggregating Communities in Aerobic Granules and Enriched Cultures

The distribution and characteristics of aggregating population in aerobic granules and enrichment sludge were compared in Table 3.9. Compared to aerobic granule isolates, the proportion of strains with autoaggregating ability in enrichment sludge increased from 40% to 61.5%. Furthermore, the ratio of strains able to coaggregate with at least one partner in enrichment sludge was 22% higher than that in aerobic granules. In comparison, 85% of the enrichment cultures exhibited either moderate coaggregating or autoaggregating scores, while only 70% of the aerobic granule isolates were associated with autoaggregating or coaggregating interactions. However, bacteria possessing high coaggregating ability (visual coaggregating score $\geq$ 3 or 4) and high autoaggregating ability (visual autoaggregating score $\geq$ 3 or 4) were found only in aerobic granule community.

Table 3.9 Comparison of aggregating bacterial population isolated from aerobic granules or from enrichment granule sludge

		Autoaggregation score									
		0		1		2		3		4	
		B[a]	En[b]	B	En	B	En	B	En	B	En
Co-aggregation score	0	6	2	0	1	1	1	1	0	1	0
	1	6	1	0	5	1	3	3	0	0	0
	2	3	1	0	0	1	1	0	0	0	0
	3/4	6	0	0	0	0	0	0	0	0	0

[a], B represents isolates in aerobic granules

[b], En indicates isolates in enrichment granule sludge

The wide distribution of strong aggregator in aerobic granule was also supported by the aggregating ability of numerically significant microorganisms and the bacteria which can coaggregate with largest number of community members. The liquid dilution procedure used for isolation predicts that the highest terminal positive tubes should contain an inoculum consisting of the numerically significant microorganisms presented in the sample (Chin et al. 1999). In acetate-fed granule isolates, strains B8-1 and B8-2 originating from highest terminal dilutions (10^8 times) showed high autoaggregating score of 3, indicating that the numerically significant populations in aerobic granules had high autoaggregating ability (Table 3.3). The strain B6-2 that can coaggregate with 7 members in community was not able to autoaggregate. Different from the finding in aerobic granule, the numerically significant strain En7-3 in enrichment cultures exhibited comparably low autoaggregating capability of 1, while inclining to be of more coaggregating ability (Table 3.4). The strain E5-9 that coaggregated with most members in the community was able to autoaggregate.

The collective results suggest that both coaggregating and autoaggregating interactions are important in formation of initial aggregate, as well as the development and maintenance of compact granule structure in a flowing

environment. However, the non-autoaggregating strains participated in strong coaggregation interactions was only found in aerobic granules, where a dense and compact structure was formed to enclose abundant bacteria by interdigitated extracellular interactions. The abundant diversity of non-autoaggregating bacteria and the proximity of microbial strains in aerobic granule would facilitate the cell-cell interactions. As a consequence, the coaggregation interactions also have positive effects on the enrichment of community diversity and density.

On the other hand, a large proportion of strains with high levels of aggregation could be attributed to the comparably high shear rate in aerobic granule SBRs. It was suggested that the cell hydrophobicity can be stimulated under high shear rate in aerobic granules (Tay et al. 2001a), which enhanced the autoaggregating ability of microorganisms.

The strong autoaggregator or coaggregator lost the advantages in abundance from the enrichment microbial community, even though the dispersed aerobic granule bacteria were subjected to the selection by fast-settling ability through the enrichment process. In contrast, the proportion of strains with either autoaggregating or coaggregating ability increased in the enrichment cultures. These strains were also observed to be slimy on the solidified growth media, implying the excess quantities of slime or extracelluar polymeric substances to be excreted in cultivation, which facilitated the coagulation of strains to form small-sized aggregates. The strong autoaggregation or coaggregation abilities were less desired in the aggregate formation by enrichment cultures, when dispersive forces decreased in accordance with low shear force in the experiment tubes. Therefore, less adhesive forces were required to overcome the dispersive forces during the cell-cell and cell-surface attachment. In the enrichment culture, non-autoaggregating bacteria cannot coaggregate, and no strong autoaggregator existed. These facts indicated that bacteria with moderate aggregating ability were functional strains in forming loose-structured aggregate during enrichment process.

3.4.3 Comparison of Aggregation Assays

Two coaggregation assays have been widely used in the study of co- and auto-aggregating behaviours (Malik et al., 2003a, b, c, d, 2004; Taweechaisupapong et al., 2000), which are spectrometric method and visual scoring method described above. The former method has advantage of accuracy to indicate the slight change of coaggregation index according to experimental process and the difference between coaggregating pairs. However, it is not the same in the case of measurement of autoaggregation index, since strains with strong autoaggregating ability are likely to form pellets and settle down quickly during spectrophotometric measurement, thus leading to the instability and inaccuracy of experimental results.

Another problem of spectrometric method is that it certainly takes longer time when compared to visual scoring method, resulting in pre-settlement of target cultures before testing with spectrophotometer when an large amount of coaggregation pairs are test. In contrast, visual scoring method overcomes the above mentioned shortcoming and is highly operable without requirement of special equipments or limitation of solution volume used for spectrophotometer.

However, the visual scoring method undergoes the underestimation of coaggregation score among partners caused by simultaneous autoaggregation of partner bacteria. The individual scores of each partner are deducted from the coaggregation score of the coaggregation pair, leading to no positive coaggregation scores given for two strains which have sum of individual scores of equal to or above 4. For instance, autoaggregating strains B8-1 and B6-15 have individual visual score of 3 and 2 respectively. When these two strains are mixed, the clean supernatant was left in the coaggregating mixture suspension (score=4). Thus these two strains are not regarded to coaggregate by scoring method, because the net coaggregating score is -1 (coaggregating score of 4 minus autoaggregating scores of 3 and 2). However, these two strains are observed to be able to coaggregate and form netlike structure from the image picture and SEM picture (Figure 3.8).

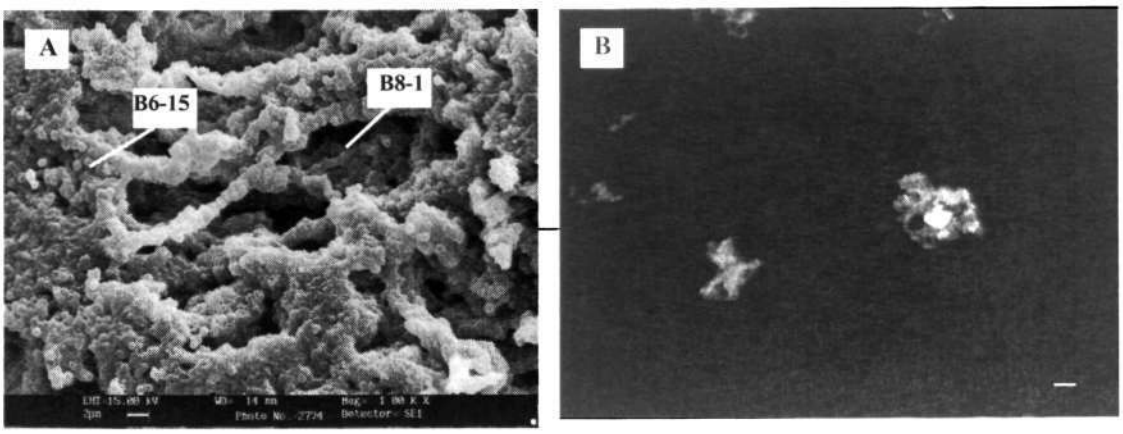

Figure 3.8 (A) Scanning electron micrographs (B) image picture of coaggregation (bar: 1mm) between two autoaggregating bacteria B6-15 and B8-1

From the observation of lab practices, low and/or negative visual coaggregation scores are not necessarily indicating weak interactions between individual ligands on distinct cells surfaces, as Buswell (1997) noted. These visual scores primarily depend on the relative cell sizes and microbial morphologies of microorganisms involved in the interactions, and are influenced by the bacterial density in reaction solution.

According to Buswell et al. (1997), low visual coaggregation scores are not necessarily indicators of weak interaction between cells. The scores detected with this assay are not accurate measures of the relative interaction strength between individual ligands on different cells. Furthermore, these authors proposed that visual coaggregation scores will depend on the relative sizes and morphologies of the bacteria involved and may depend on the densities of interacting ligands on the bacterial surface. Nevertheless, the rapid and simple visual assay provided reproducible results with enough sensitivity to detect significant interactions (Buswell 1997).

3.4.4 16s rRNA Sequencing of Aggregating Isolates from Aerobic Granules and Enriched Cultures

In aerobic granules, the largest fraction of microbial community exhibiting aggregating ability belonged to the subclass β-*proteobacteria* (7 in 13 isolates), while the fraction decreased in enrichment culture (4 in 11 isolates). However, in both aerobic granule and enrichment sludge, the microbial communities were dominated by members of the class *Proteobacteria*. Similar results were reported in the previous study on aerobic granules fed with phenol (Jiang et al. 2004). Furthermore, the growth of bacteria in the genus *Acinetobacter* was favored by enrichment process fed with acetate in this study. Similar phenomena were observed in previous studies on nitrifying SBRs fed with acetate (Bouchez et al. 2000). It was also demonstrated that the importance of *Acinetobacter spp.* in activated sludge was overestimated by spreading the sludge on acetate-rich media (Wagner et al. 1994). Therefore the shift of aggregating population to *Acinetobacter* could be attributed to the exposure of enrichment cultures to acetate-enriched environment during enrichment.

Bacteria of the genus *Sphingomonas* was found to be widely distributed in sea water, sea ice, river water, wastewater and mineral water. It was isolated from freshwater biofilm and had the ability to coaggregate with other aquatic biofilm community members so far tested (Rickard, et al., 2004a, b). The strain *Sphingomonas* exhibiting highly coaggregating ability was found to be quantitatively and functionally important in acetate-fed granules community in this study. Moreover, the ability of *Sphingomonas* to adhere to other strains in granule might give this population a privilege in colonization.

3.4.5 Inhibition of Aggregating Behaviors by EDTA, pH Value, Simple Sugars, Heat Treatment and Electrolytes

In this study, both coaggregating pairs of B6-25 / B6-2 and B6-25 / B7-8 were completely inhibited by the lack of electrolytes, indicating both pairs were ion-

sensitive. The electrolytes were important in overcoming steric hindrance between cells. Extensive discoaggregation occurred in absence of cations as compared to those with presence of Ca^{2+} and Na^{+}. These results contrast with the observation by Malik et al. (Malik et al. 2003a) about strains of non-flocculating sewage sludge bacteria. The coaggregating pair of B6-25 and B6-2 was more sensitive to the lack of Ca^{2+}. Cations could bind negatively charged sites on bacterial surfaces and on the extracellular polymeric substances (EPS) to decrease the surface charge of negatively charged microbes, thus acting as a bridge among partners involved in coaggregation. Electrolyte concentration was reported to interfere with coulombic forces, thus affect the coaggregation process. The discoaggregation phenomenon of coaggregation interaction among aerobic granule bacteria in the absence of Ca^{2+} could be correlated to the findings on aerobic granulation. Jiang et al. (2003) reported that the augmentation of Ca^{2+} could significantly speed up the formation of aerobic granules with better properties. This implies that the inhibition of Ca^{2+} on coaggregation interactions would further affect the granulation process through the influence on the distribution and retention of aggregating population within the microbial community in a dynamic environment.

EDTA is a strong chelating agent and the inhibition on bacterial coaggregation may result from chelation and subsequent unavailability of ions (Malik et al. 2003a). The inhibition of EDTA on both coaggregating pairs under examination was found in this study. However, this inhibition was not as significnat as the observation in the presence of 2 mM (30% to 50%) EDTA reported by Taweechaisupapong et al. (2000) and Malik et al. (2003a). With the intermittent addition of 3 mM calcium ions, the coaggregation index of the pair of B6-25 and B7-8 was recovered to the similar level of the pair in absence of EDTA and Ca^{2+}. Kakii et al. (1990) also documented the reversal of dispersed sludge aggregate by ions and the reaggregation increased with NaCl concentration and especially with Ca^{2+} concentration. It showed that the inhibition of EDTA on the pair of B6-25 and B7-8 was reversible. Meanwhile, the coaggregation index of B6-2 and B6-25 did not reach the similar level when compared with the pair without addition of EDTA and Ca^{2+}, indicating partial loss of coaggregation ability among the coaggregating

partners in this pair.

The inhibition on coaggregation between B6-25 and B7-8 was also found with presence of glucose. Higher extent of inhibition was in line with higher concentration of glucose. Glucose, galactosamine, galactose, mannose and lactose are the common used sugars with respect to the inhibition of simple sugars to coaggregation by blocking the saccharide receptor on cell surface. The experimental results in this study implicated the involvement of protein-saccharide interactions, which are commonly discovered in oral and aquatic biofilm communities (Kolenbrander 2000).

The inhibition of extreme pH to coaggregation was found in both coaggregating pairs in this study. The pH value of growth media could affect cell surface charge, hydrogen ion concentration and ionizable reactive groups on cell surface (Calleja et al. 1984), though Malik et al. (2004) reported that exposure of coaggregates to extreme pH didn't cause significant deflocculation. Coaggregation is highly specific process involving molecules on participating cells surface which act as adhesions and complementary receptors. The adhesions and receptors normally include proteins and carbohydrates (Kolenbrander 2000). The extreme pH value would induce the ionization of charged functional groups such as carboxylic groups in proteins and carbohydrates (Wingender et al. 1999), resulting in strong repulsion among cells and EPS, and subsequently inhibiting the coaggregation interactions.

B6-25 and B7-8 were able to coaggregate by heat pretreatment for 30 minutes at 80 ^{o}C, demonstrating the resistance against thermal agitation under environmental conditions. Even if the microbial activities are perished as that heat does, the cells remain to coaggregate, which shows the nonvitality of coaggregation process. Some coaggregating pairs of freshwater biofilm bacteria or aquatic bacteria were heat-sensitive and coaggregation was completely inhibited as previously demonstrated by Rickard et al. (Rickard et al. 2000, 2004b), yet opposite results were also reported indicating the tolerance of coaggregating pairs to heat (Malik et al. 2004). The reported inhibition of heat on aggregation possibly functions due to the

denaturation of enzymes. The stability of coaggregating interactions between aerobic granule strains demonstrated in the present study might support the feasibility of thermophillic aerobic granulation, which could be applied to treat hot wastewater from industries, e.g. pulp and paper production, and potato processing water (Adav et al. 2008a; Zitomer et al. 2007).

Coaggregation between B6-2 and B6-25 leads to large floc-like aggregates compared to pelleted particle formed from coaggregation of B6-25 with B7-8, which is stronger, more stable with electrolyte concentration, simple sugars, temperature, and chelating agents. Such strong interactions of B6-25 and B7-8 are desirable in the granulation process as they may form steady coaggregation under varied microbial environment and integrate with other bacteria to form big aggregates through the interaction with B6-2, which is able to coaggregate with several strains, thus subsequently prevent the bacteria in granules from wash out as well as achieving better availability of carbon and energy sources.

3.5 SUMMARY

The study on change of microbial community structure and aggregating ability of microbial species from enrichment process was undertaken, for the sake of better understanding on the aggregating populations in aerobic granules and the feasibility of enrichment method on selection of bacteria with distinguished aggregating abilities.

Both R2A agar and Bacto agar with similar growth media as in granulation reactor which the granule samples were taken from were used for the cultivation of granular isolates. More morphologically distinct isolates were recognized from R2A agar plates than from Bacto agar.

Less diversity was found in enrichment community compared with the aerobic granules. Colonies possibly representing the numerically significant population in aerobic granules were strong autoaggregating bacteria. This differed from the

enrichment sludge where the numerically significant population was less autoaggregating but more involved in coaggregating interactions. Under the selective pressure imposed by enrichment process, the proportion of bacteria with intermediate autoaggregating ability which can also coaggregate increased, when compared with aerobic granules. In both granule sludge and enriched sludge, Proteobacteria was dominant in the microbial populations.

Two pairs of coaggregating partners (B6-25 with B7-8, and B6-25 with B6-2) isolated from aerobic granules were subjected to the inhibition testing of EDTA, electrolytes, simple sugar, heat treatment and pH value. Both pairs were shown to be sensitive to electrolyte. Slight inhibition of 2 mM EDTA on both pairs was observed. With the intermittent addition of 3 mM Ca^{2+}, the coaggregation interaction among B6-25 and B7-8 could be completely reversed, while partial loss of coaggregation ability was found among B6-25 and B6-2. Severe inhibition by extreme pH values were observed on both coaggregatig pairs. The inhibition of coaggregation between B6-25 and B7-8 with simple sugar implicated protein-saccharide interactions involved in coaggregating interactions among these two strains. The inhibition testing of electrolytes and EDTA on coaggregation among aerobic granule bacteria was in accordance with findings in aerobic granulation, implying that the EDTA and electrolytes could affect the granulation through effects on cell-cell interaction in microbial community.

CHAPTER FOUR
ANALYSIS OF AGGREGATING POPULATION SHIFT FROM THE ACETATE-FED GRANULES TO PHENOL-DEGRADING GRANULES

4.1 INTRODUCTION

Phenol is an organic component widely found in many industries such as oil refinery, plastic, petroleum refining, petrochemical, dyestuff and pharmaceutical plants. Phenol is highly toxic to the environment and can cause health problems in human beings or animals through food chain (Brown et al. 1967). The removal of phenolic compounds from wastewater was focused on the biological degradation due to lower costs and the possibility of complete mineralization without formation of hazardous byproducts (Adav et al. 2007b; Arrojo et al. 2004; Jiang et al. 2004; Tay et al. 2004a).

Activated sludge is widely used for biological wastewater treatment owing to its abundant microbial diversity. However, the acclimation period is relatively long (more than one month) for the removal of phenol (Jiang et al. 2002). The growth of microorganisms and subsequent biodegradation of phenol are commonly inhibited by toxicity imposed by high phenol concentration. When 1.8 kg m^{-3} day^{-1} phenol was introduced, the biomass concentration of activated sludge dropped sharply, resulting in the failure on biological removal of phenol (Tay et al. 2005b).

As an effective alternative to the conventional activated sludge system, aerobic granulation has been successfully applied for the treatment of high-concentrated phenol of up to 1.5 kg m^{-3} day^{-1} by using the phenol-enriched activated sludge as inoculum (Adav et al. 2007; Jiang et al. 2002; Tay et al. 2004a, 2005a). The cultivation process for aerobic phenol-degrading granules was reported to be shortened to 9 days, by using

acetate-fed granules as microbial seed to degrade phenol (loading rate of up to 2.4 kg m^{-3} day^{-1}) with the presence of acetate as co-substrate (Tay et al. 2005b). The changes on physiological traits were observed during the conversion from acetate-fed granule to phenol-degrading granule.

As one of the microbial cell-cell interactions, microbial aggregations with respect to coaggregation and autoaggregation were crucial for the retention and growth of participating members, and could mediate the spatial locations of bacterial species in a complex biofilm community (Foster et al. 2004; Ishii et al. 2005; Palmer et al. 2003; Schachtsiek et al. 2004). The cell immobilization through aggregating interactions could provide a protection to participating members against the inhibition imposed by phenol, and increase the biodegradation rate of toxic compounds (Jiang et al. 2004; Prieto et al. 2002). As a result, the microbial community can display a high tolerance to toxic or antimicrobial substances (Anwar et al. 1992). The aggregating interactions were suggested to be important for formation of aerobic granules as well as maintenance of stable structure and high metabolism (Adav et al. 2007a, 2009b; Jiang et al. 2004). Therefore, the specific species and respective interactions among aggregating partners may be crucial for acetate-fed granule to convert into phenol-degrading granule in such short time (nine days). However, the detailed role of aggregating populations in the fast conversion process is still unknown.

In this paper, the population dynamics of aggregating bacteria were investigated without the presence of co-substrate during the conversion process of acetate-fed granules into phenol-degrading granules. The composition and diversity of aggregating community were studied to reveal the evolution of granule microbial communities under shock of toxicity. The morphological changes of granules during the conversion process were also observed in details. The findings in this work will broaden the knowledge of aerobic granule microbial communities, and contribute to a deeper understanding on the role of aggregating interactions in the formation of aerobic granules.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Design and Set-up

Experiments were performed in parallel in two laboratory-scale column-type sequencing batch reactors (SBRs) R1 and R2 with the same set-up as described in Chapter Three.

Before the start of the experiment, acetate-fed aerobic granules were cultivated for four weeks in two SBRs and were subsequently harvested from both reactors after reaching steady-stage. The two reactors were fed on an acetate-based synthetic wastewater solution which contained: 1,282 mg l^{-1} sodium acetate, 100 mg l^{-1} NH_4Cl, 22.5 mg l^{-1} K_2HPO_4, 15 mg l^{-1} $CaCl_2 \cdot H_2O$, 12.5 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 10 mg l^{-1} $FeSO_4 \cdot 7H_2O$, and 1 ml l^{-1} micro-nutrient solution as described previously (Tay et al. 2005a). The chemical organic demand (COD) loading was 3 kg m^{-3} day^{-1}. The harvested acetate-fed granules were well mixed before refilling into two SBR R1 and R2 at equivalent volume of 1000 ml. The controlling process was carried out in reactor R2, accompanied with COD loading increased to 5.7 kg m^{-3} day^{-1} by using acetate-based synthetic wastewater, which was composed of 2432 mg l^{-1} sodium acetate, 190 mg l^{-1} NH_4Cl, 42.75 mg l^{-1} K_2HPO_4, 28.5 mg l^{-1} $CaCl_2 \cdot H_2O$, 23.75 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 19 mg l^{-1} $FeSO_4 \cdot 7H_2O$, and 1.5 ml l^{-1} micro-nutrient solution. Phenol was used in the experimental reactor R1 at concentration of 800 mg l^{-1} as the sole carbon and energy source, giving the phenol loading rate of 2.4 kg m^{-3} day^{-1} (or equalized to COD loading rate of 5.7 kg m^{-3} day^{-1}, which was comparable to the COD loading rate in control reactor R2).

4.2.2 Measurement

COD, mixed liquor suspended solid (MLSS) and SVI were determined by Standard Methods (APHA 1998). Soluble total organic carbon (TOC) was filtered through 0.45 μm filter paper and measured using a TOC analyzer (TOC-Vcsh, Shimadzu). Granular

sludge size was measured either by a laser particle size analysis system (MasterSizer Series 2000, Malvern, UK) or by an image analysis system (Quantimet 500 Image Analyser, Leica Cambridge Instruments, Cambridge, UK).

The pure cultures were pretreated with the same procedure, following with coaggregation index determined by the visual aggregating method as described in Chapter Three. The cell suspension was allowed to settle for 5 min and the remaining cell suspension was used for aggregation experiments.

The 16s rRNA gene sequences of strains isolated from both the acetate-fed and phenol-degrading granules were determined as described in Chapter Three.

4.2.3 Isolation Procedure

Microbiological isolation was conducted by standard spread-plate method. The cultures were isolated from acetate-fed aerobic granules at the beginning of the experiment and from phenol-degrading aerobic granules at the stable stage, respectively. Cultures were also isolated from activated sludge sampled from local municipal wastewater plant for comparison. Liquid serial dilution was used to isolate bacteria on two solid media: R2A agar (Difco, approximate formula per liter: yeast extract 0.5 g, protease peptone No.3 0.5 g, casamino acids 0.5 g, dextrose 0.5 g, soluble starch 0.5 g, sodium pyruvate 0.3 g, dipotassium phosphate 0.3 g, magnesium sulfate 0.05 g, agar 15 g), and Bacto agar (Difco) containing the same chemical composition as in experimental reactor R1. Purity of isolates was examined with light microscopy (Olympus BX60, Tokyo, Japan). Genomic fingerprints of isolates from two culture media were compared and obtained by repetitive extragenetic palindromic sequence PCR (rep-PCR) performed as described by Versalovic et al. (1991). The 16S rRNA from the isolated strain was amplified by using the whole cell direct lysis polymerase chain reaction (PCR) amplification method as described in Chapter Three.

4.2.4 DNA Extraction from Seeding Activated Sludge and Aerobic Granules

The DNA was extracted from seeding activated sludge and aerobic granules by a modified physical disruption of cells involving direct-lysis procedure. Approximately 200-300 mg (wet weight) samples were suspended in 800 µl sterile MilliQ water in a 2 ml microcentrifuge tube. 800mg sterile baked glass beads and 50 µl of 20% SDS were added into the suspension. The tube was then topped up with saturated phenol (0.1M Tris-HCl, ph 8.0; Sigma, USA). The suspension was shaken for 3 min with a Mini Beadbeater (Biospec products, USA) at room temperature and then incubated at 60°C water batch for 5 min. After incubation, the liquid phase was extracted three times with phenol, followed by phenol-chloroform (4:1, v/v) and chloroform twice respectively. Nucleic acids were precipitated with 0.1 volumes of 20% (w/w) sodium acetate (pH 5.1) and 2 volumes of 95% ethanol overnight at -20°C. The DNA precipitate was washed with 500 µl of 70% ethanol. Then the DNA pellet was dissolved by soaking in 100 µl MilliQ water. The concentration and purity of the genomic DNA was assessed by both absorbance measurement and agarose gel electrophoresis. DNA extracts were stored at -20°C prior to analysis.

4.2.5 DGGE-PCR Amplification

For analysis of the total bacterial community, two primers were used to amplify the V2-V3 region of 16S rDNA (corresponding to position 339 to 539 of E. *coli.*). Forward primer P2 with a GC clamp comprised 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 was 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), 10 mM Tris-HCl, 50 mM KCl, 2 mL $MgCl_2$, 200 uM Deoxynucleotide triphosphate (DNTPs), 25 pmol of each primer (P2 and P3) and 1 µl of DNA solution (20 ng/µl). The touch

down PCR was employed 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°Cm and 2 min at 72°C. The annealing temperature was subsequently decreased by 1°C for every second cycle until it reached 55°C. Finally a 10 min extension step at 72°C was preformed. Successful PCR products were confirmed by electrophoresis through a 2.0% agarose gel in TAE buffer stained with ethidium bromide.

4.2.6 DGGE and Band Sequencing

DGGE PCR products were analyzed using a DCode universal mutation detection system (Bio-Rad Laboratories, USA) in accordance to manufacture instruction. Polyacrylamide (10%) gels (16 by 16 cm and 1 mm deep) were run with 1×TAE buffer (diluted from 50×TAE buffer: 40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA). A denaturing gradient ranging from 30% to 70% for a parallel DGGE analysis was selected for community analysis. 40 µl of PCR amplicon of DNA extracted from seeding activated sludge or granule was loaded in each well of the gel with 15 ul loading dye. The gel was placed in TAE buffer at 85 V at 60°C for 15 h. After electrophoresis the gel was stained with ethidium bromide for 30 min and destained for 1 h before viewed and photographed with Kodak EDAS 290 imaging system.

Selected DNA bands were aseptically excised and re-amplified by PCR several times as described previously (Watanabe et al. 1998) in order to gain partial sequence information with the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit (version 3.0) and the ABI model 310A sequencer (Applied Biosystems, Perkin-Elmer), according to manufacturer instructions. The partial sequences were assembled by using the BioEdit software and analyzed with BLAST and other algorithms as described previously (Tay et al. 2002).

4.3 RESULTS

4.3.1 Reactor Performance

Both reactor R1 and R2 were initially seeded with aerobic acetate-fed granules. These granules were characterized with biomass concentration of 5 g l^{-1} suspended solid (SS), SVI value of 40 ml g^{-1} and mean biomass size of 0.85 mm. As shown in Fig. 1 and 2, no significant deterioration was observed in biomass properties for control reactor R2 as the influent acetate concentration increased from 3.0 to 5.7 kg COD m^{-3} day^{-1}. The SVI remained below 70 ml g^{-1} and biomass concentration stabilized around 7.5 g l^{-1} within three weeks.

For experimental reactor R1 with the presence of phenol, biomass concentration dropped to 2.65 g l^{-1}, and SVI was observed to peak at 67 ml g^{-1} simultaneously on day 2 and day 6 (Figures 4.1 and 4.2). This indicates that reactor performance fluctuated under phenol concentration of 800 mg l^{-1} at the first operation phase of 6 days. Thereafter, the biomass concentration increased continuously in 30 days and stabilized around 12.1 g l^{-1} within 5 weeks and SVI stabilized at 27 to 35 ml g^{-1} from day 11. This show good biomass retention compared with reactor R2.

Time profile of mean biomass size changes for reactors R1 and R2 are shown in Figure 4.3. Mean biomass size for biomass in R2 were increased rapidly and fluctuated markedly after the increase of influent acetate concentration from 3.0 to 5.7 kg COD m^{-3} day^{-1}. Mean biomass size of acetate-fed granules in reactor R2 increased to 1.5 mm on day 4 and 8, approximately two times of the biomass size on the first day. Following, the mean biomass size decreased to the lowest level (0.95 mm) on day 14. In reactor R1, The increment of mean biomass size was also observed with the peak level of 1.8 mm on day 8 after the alternation of substrate from acetate to phenol as the sole carbon source. After 11 days, the biomass size decreased gradually in R1, and maintained steady around 0.8 mm from day 29.

These evidences indicated that the aerobic granules in R1 were well adapted to phenol at a loading rate of 2.4 kg m^{-3} day^{-1} within 8 days of operation. Biomass in R1 had a more compact structure and better settling ability than those in R2, with the presence of phenol as the sole carbon source.

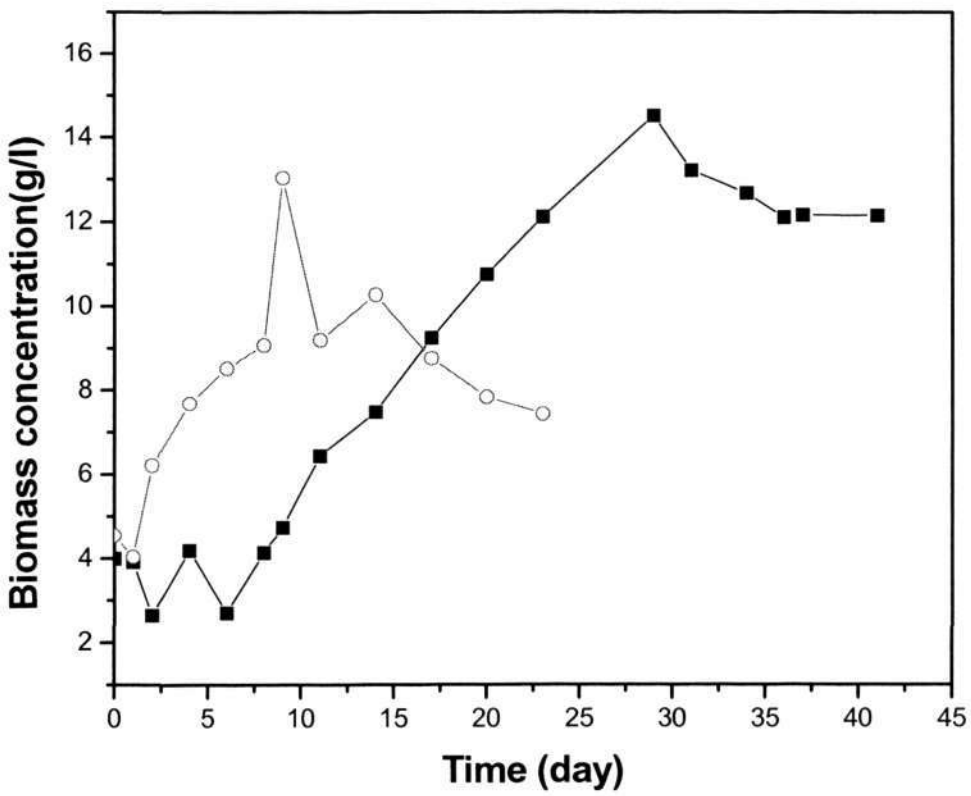

Figure 4.1 Time profiles of biomass concentrations of experimental reactor R1 (■), and control reactor R2 (○).

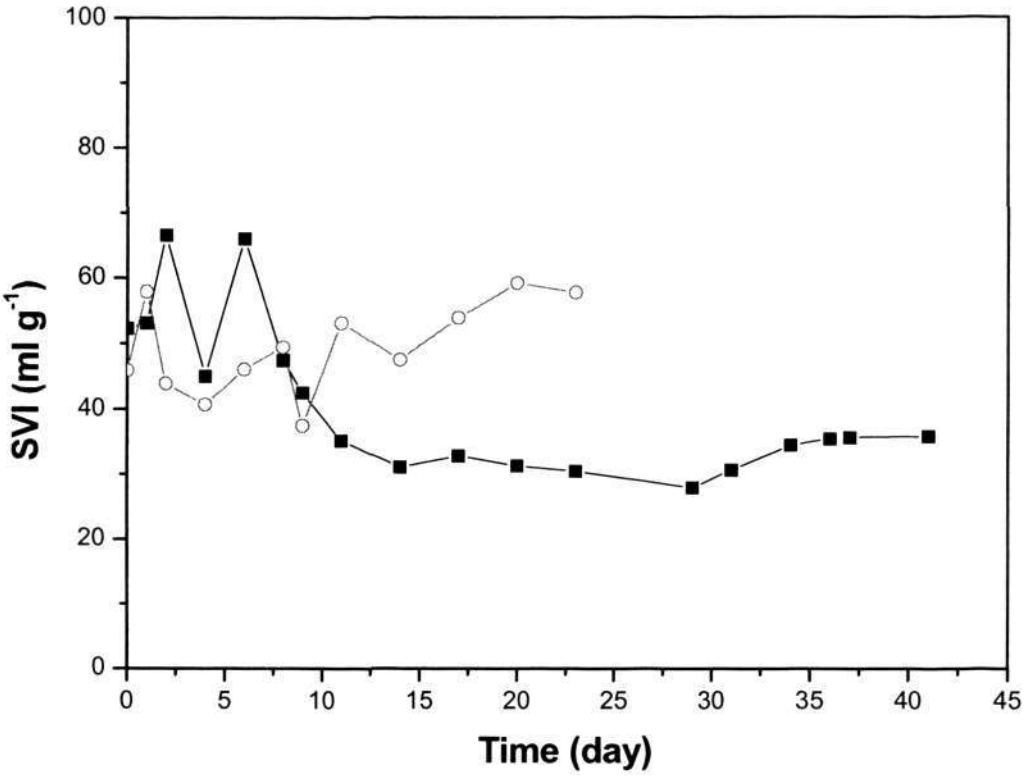

Figure 4.2 Time profiles of sludge volume index (SVI) changes of experimental reactor R1 (■), and control reactor R2 (○).

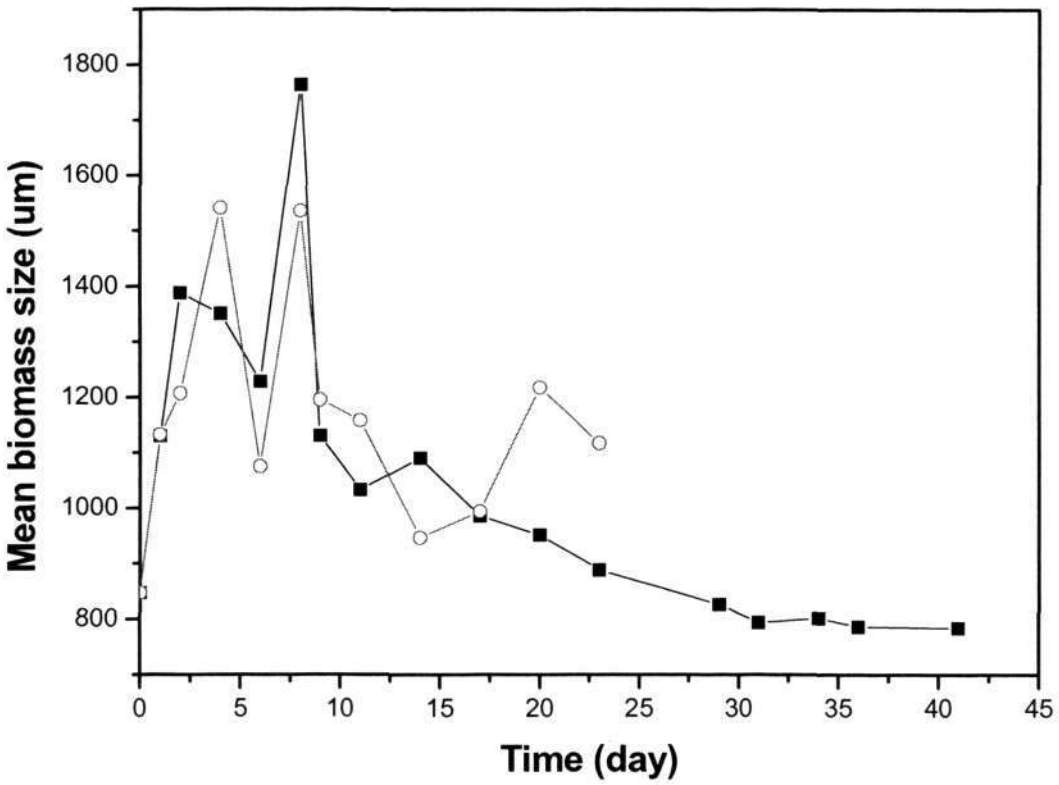

Figure 4.3 Time profiles of mean biomass size of experimental reactor R1 (■), and control reactor R2 (○).

4.3.2 Microscopy and Morphology of Granules

SEM observation was adopted to identify and monitor the morphological evolution of aerobic acetate-fed and phenol-degrading granules during the experiment. As seen in Figure 4.4a and 4.4b, the acetate-fed granules seeded in both reactors R1 and R2 were formed of lumps of microaggregates assembling together with most cells in the shape of rods and cocci on the surface. The cells were embedded in a slime extracellular polymeric net as disclosed by the close observation of acetate-fed granule. After 11

days' cultivation in control reactor R2 with elevated acetate concentration, no significant change on the irregular and ruffled surface structure of granule was observed (Figure 4.4c). However, some secondary aggregate with smooth surface emerged from the principal part of granules. In some regions on granule in R2, the ruffled surface disappeared gradually. Figure 4.4d shows that microorganisms in form of mainly cocci and rod-shape inhabited on the outer surface and linked tightly to compose the firmly ruffled configuration.

The morphology structure change of phenol-degrading granules in R1 was observed after exposure of acetate-fed granule to phenol after 11 days. The compact ruffled structure of acetate-fed granule seeds disappeared gradually. The loose and porous conglomeration emerged, which consisted in small regular-shaped aggregates of microorganisms (Figure 4.5). After 31 days, the granules appeared to be more regular, with fungal organisms and filamentous bacteria partitioned on the surface area (Figure 4.6).

The seeding acetate-fed granules showed a ruffled structure and semi-transparent appearance by image analysis (IA) (Figure 4.7a). After 1 day of exposure, the outmost surface of the granules appeared to be brownish color (Figure 4.7b). After 11 days of cultivation, the color of granules turned into brownish yellow in a form of loosely linked conglomeration (Figure 4.7c). The microaggregates that agglomerated in compact granules demonstrated a trend to detach from the main structure, leading to a loose cluster containing a set of well-defined microbial aggregates. Finally, on day 31 more regular-shaped small granular aggregates separated from the main part of old granules, along with a fluffy surface on granules (Figure 4.7d). The observed morphological changes during cultivation were consistent with the earlier observation by using SEM.

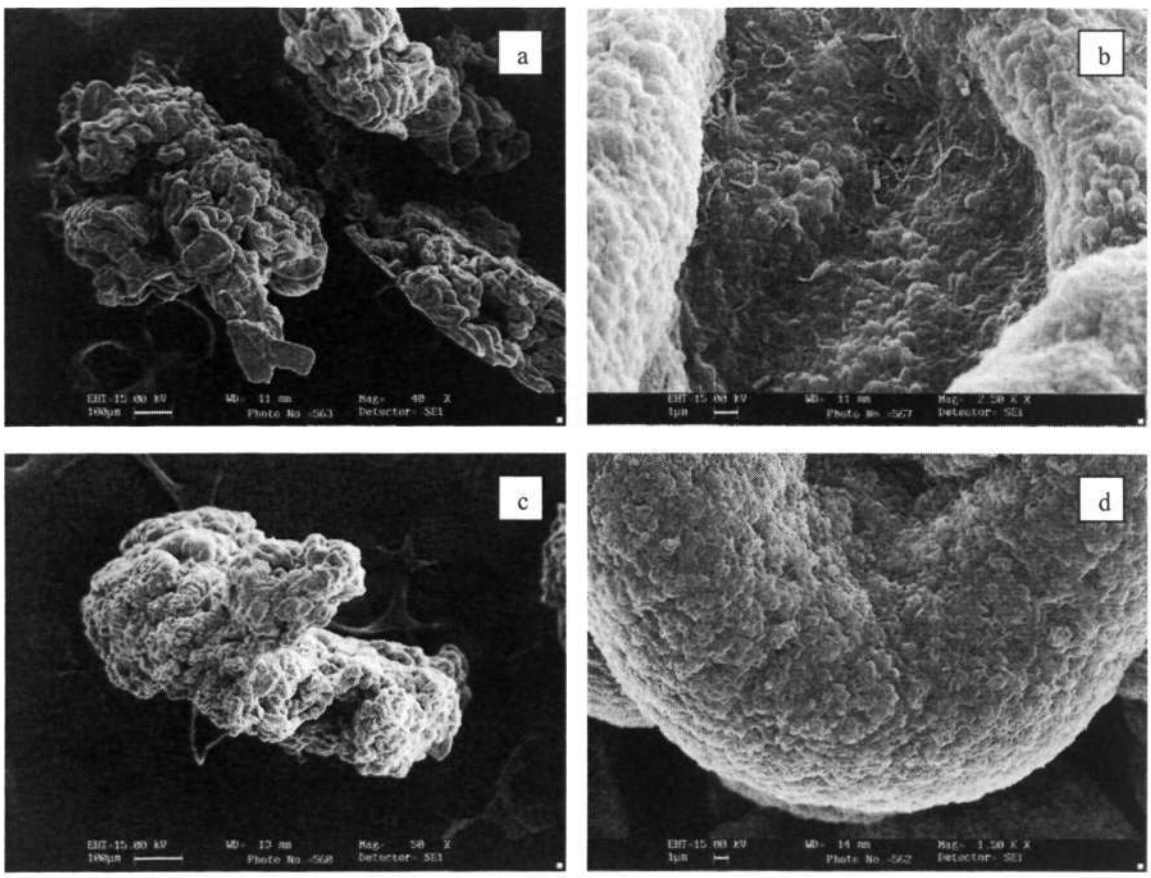

Figure 4.4 Scanning electron microscopy images of granules in control reactor R2. (a) seed granule at magnification of 40 × on day 0; (b) seed granule at magnification of 2500 × on day 0; (c) acetate-fed granule at magnification of 100 × on day 11; (d) acetate-fed granule at magnification of 1500 × on day 11

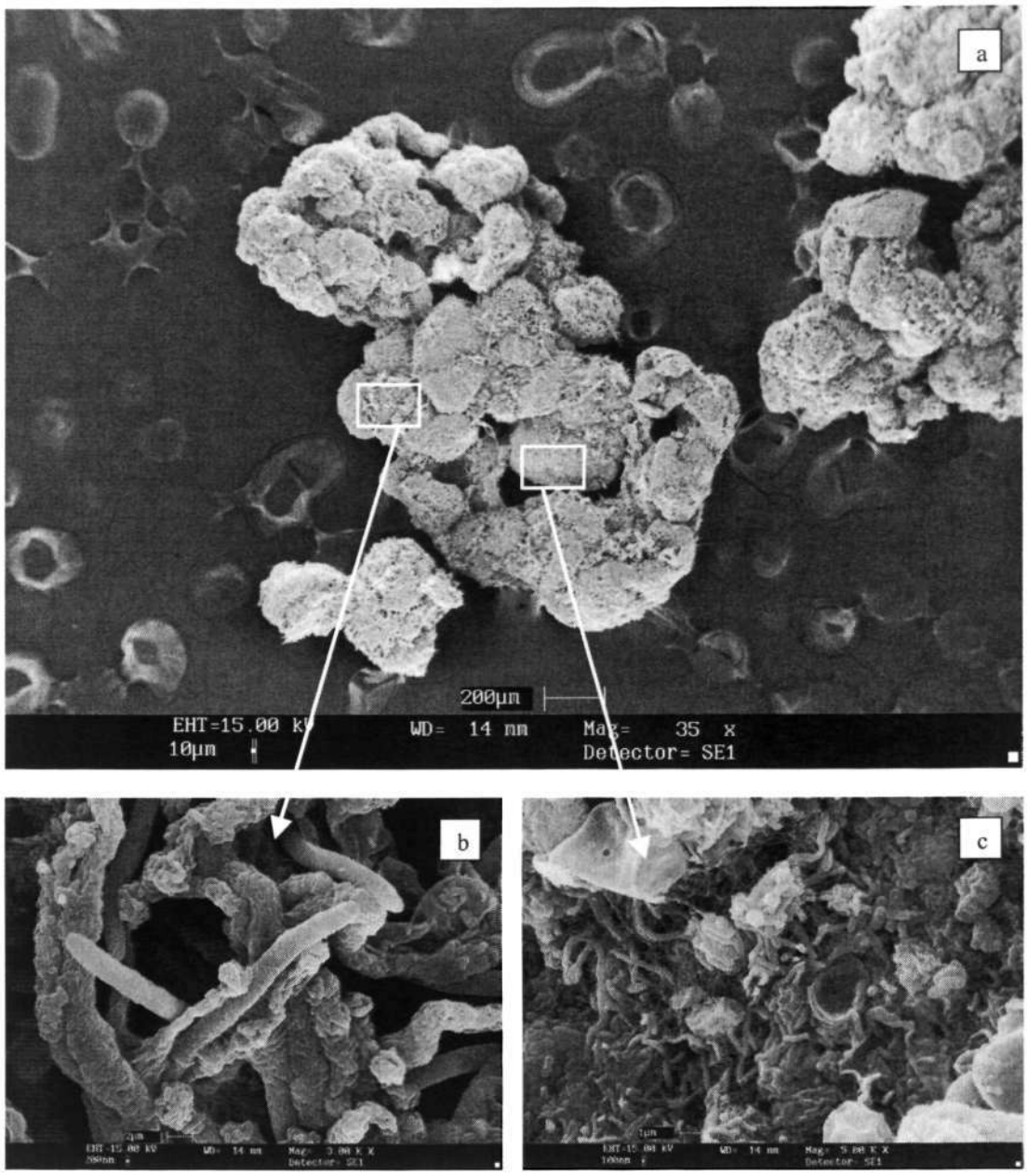

Figure 4.5 Scanning electron microscopy images of granules in experiment reactor R1 on day 11. (a) overall viewing of granule conglomeration; (b) large sized microorganisms attached and enwrapped by small sized bacteria; (c) close shot of microbial community morphologies on granule surface

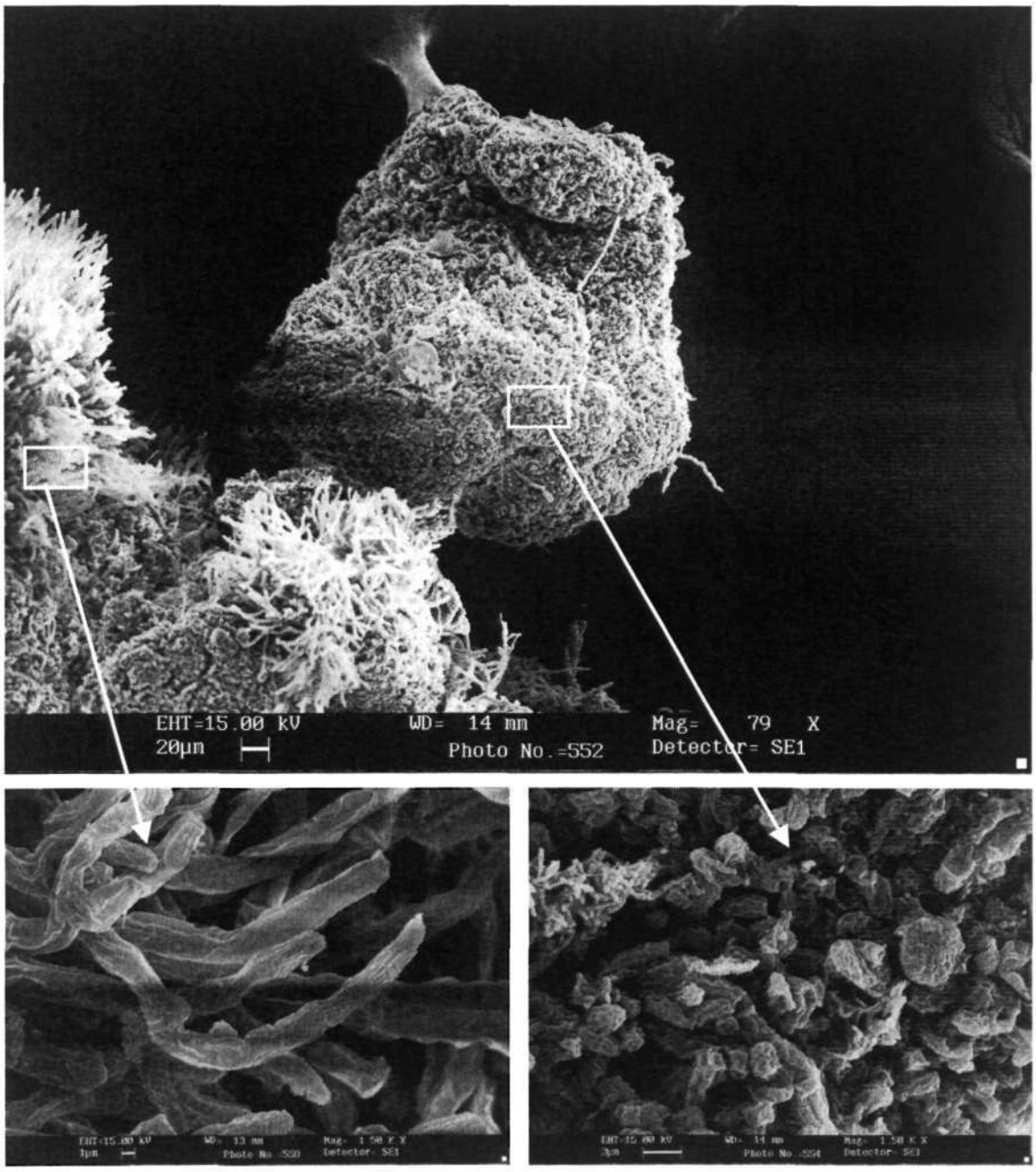

Figure 4.6 Scanning electron microscopy images of granules in experiment reactor R1 on day 31

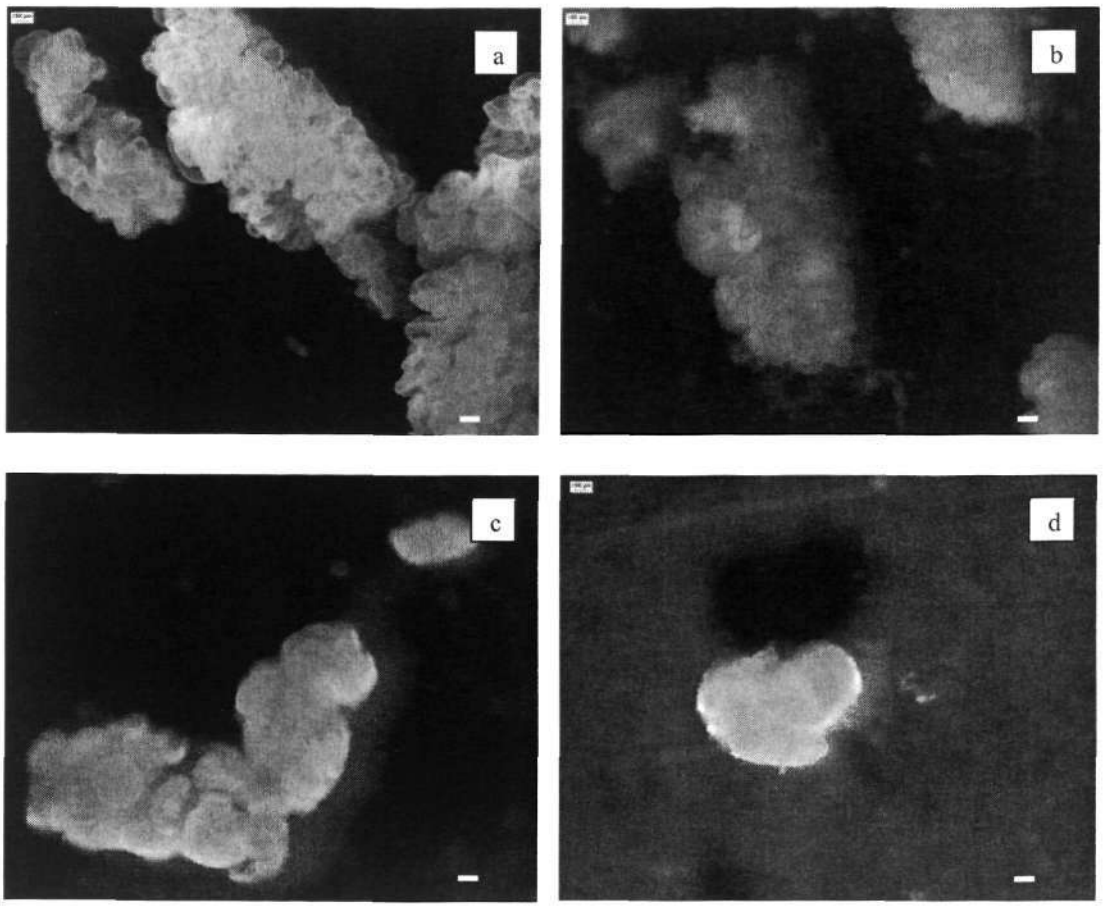

Figure 4.7 Morphological changes of granules in experimental reactor R1 on: (a) day 0 (seed granule); (b) day 1; (c) day 11; and (d) day 31. (Bar: 0.1mm)

4.3.3 Detection of Dominant Species by DGGE

DGGE was employed to generate genetic fingerprints in order to provide information on the constituent and diversity of granular microbial communities during the exposure to phenol. Bacterial populations in biomass of R1 were detected by extracting DNA from granules sampled in predetermined time interval from day 0 to day 43. PCR amplification and DGGE analysis of 16S rRNA fragments were performed on pooled aliquots of extracted nucleic acids. Identical fingerprints were obtained for replicate samples. Figure 4.8 shows the representative DGGE profiles of amplified 16S rRNA genes from reactor R1 biomass. The fingerprint patterns alternated for the different biomass samples during the period of phenol shock, showing the acclimation process of microbial community to the environment.

When phenol was introduced, some dominant strains in acetate-fed granule microbial community, e.g. strain1, lost the advantage for the competition of bacterial populations. DGGE fragment of strain 2 predominated in the entire period of experiment, which showed that it can sustain in both acetate-fed and phenol-degrading granule communities. Several discernable bands, such as strain 3, start to present in the phenol-degrading granules after 11 days. After a cultivation period of more than 400 days, DGGE profile (Figure 4.9) showed a dominant band at the same position as the strain 3 in the granule samples taken on day 426, 456, and 487, respectively, from reactor R1. These results indicated the appearance and persistence of new dominant populations. Recovered bands were re-amplified by PCR and run on a DGGE gel to confirm purity for several times. Pure DNA products were obtained for partial 16S rRNA gene sequencing from three selected different DGGE bands (i.e. strain 1, strain 2, and strain 3).

Reactor performance and biomass characteristics in control reactor R2 varied during the cultivation period using sodium acetate as sole carbon source when chemical organic demand (COD) loading was increased from 3.0 kg COD m^{-3} day^{-1} to 5.4 kg

COD m^{-3} day^{-1}. Figure 4.10 shows representative DGGE profiles of amplified 16S rRNA genes from R2 biomass at day 0 and day 23. On day 23, more bands were present than those on day 0, showing relatively high abundance of microbial populations in aerobic granules fed by acetate of higher concentration. Both bands representing strain 1 and strain 2 were dominant in the microbial community of granules on day 0 and 23, showing a high advantage of the two strains.

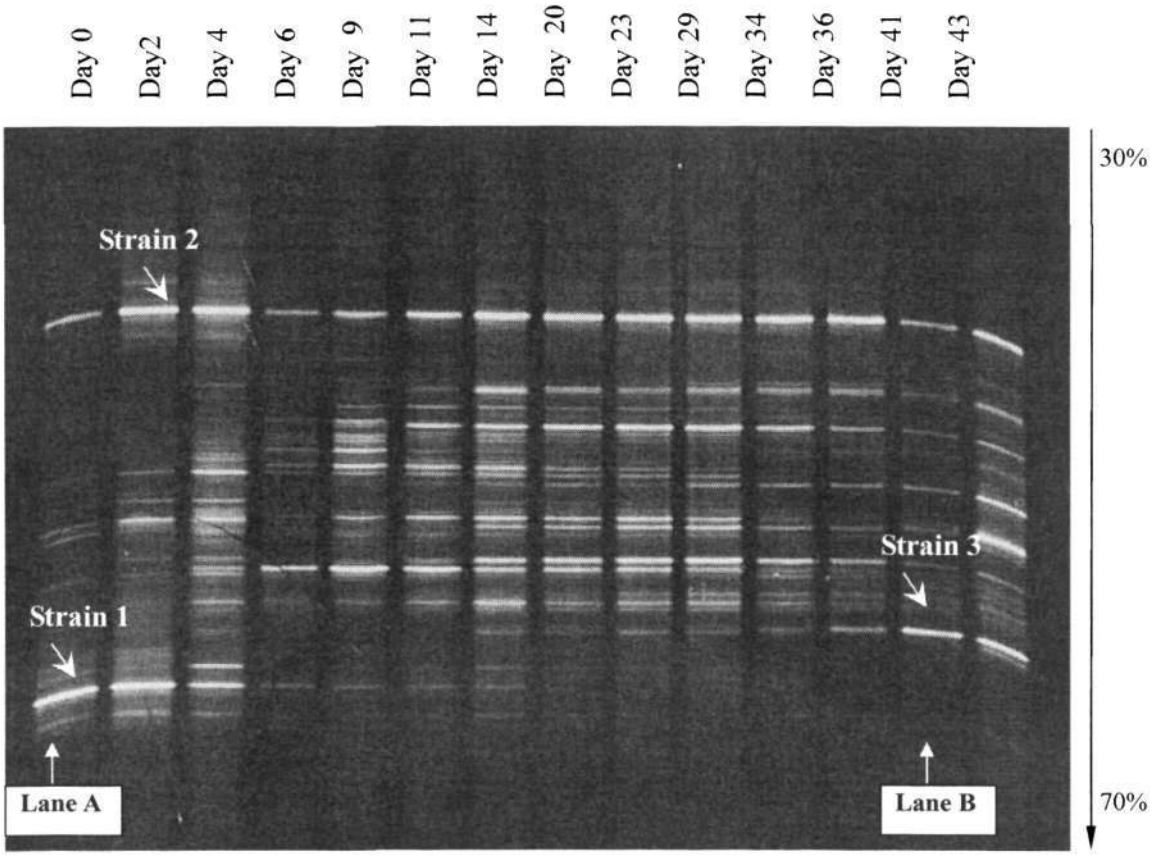

Figure 4.8 Genotypic diversity (shown in DGGE profile) of phenol-fed aerobic granules seeded with acetate-fed granules. Lane A: granule using acetate as sole carbon source (experiment start point); Lane B: phenol-degrading granule (experiment end point). Strain 1: culture dominant in acetate-fed granules; Strain 2: culture persisting in process of phenol shock; Strain 3: culture dominant in phenol-degrading granule.

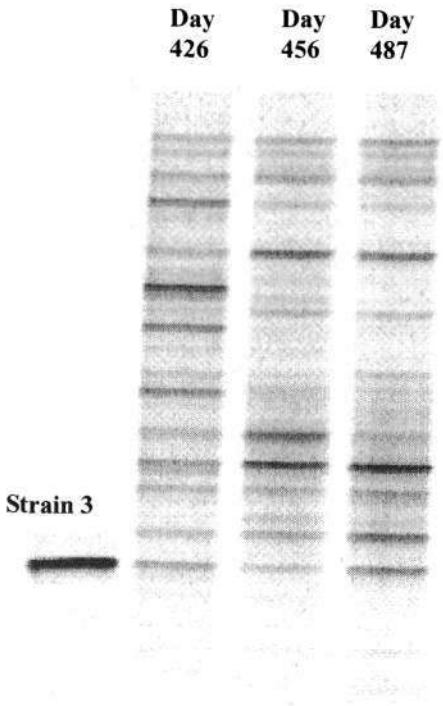

Figure 4.9 Denaturing gradient gel electrophoresis (DGGE) fingerprints of Strain 3 and PCR-amplified 16S rRNA genes from aerobic phenol-degrading granules in R1 after cultivation of 426 day, 456 day and 487 day.

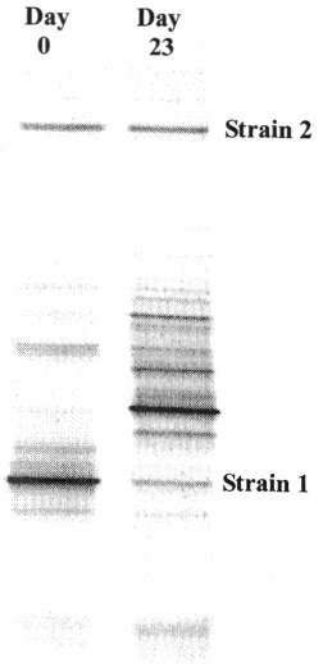

Figure 4.10 Denaturing gradient gel electrophoresis (DGGE) fingerprints of Strain 1, Strain 2 and PCR-amplified 16S rRNA genes from aerobic acetate-fed granules in R2 at day 0 and day 23.

4.3.4 Screening of Aggregation Bacteria in Activated Sludge, Acetate-fed Granule and Phenol-degrading Granule

Table 4.1 shows the results of bacterial isolated from activated sludge, acetate-fed granule and phenol-degrading granules. In total 74 different colonies were isolated from fresh activated sludge collected from municipal wastewater plant, based on the colony morphology, color and size by direct isolation technique by using R2A agar plates as solid media. 37 distinct strains were identified from the 74 isolates by using REP-PCR. 89 and 92 distinct isolates were isolated from acetate-fed aerobic granules and phenol-degrading granules respectively using the same isolation technique and medium. 32 and 42 purified colonies were subsequently distinguished by REP-PCR analysis and partial 16S rDNA sequencing.

No significant differences were detected in the abundance of genetic distinct microbial cultures isolated from activated sludge, acetate-fed granules, and phenol-degrading granules communities.

Table 4.1 Cultivation of isolates from activated sludge, acetate-fed granule and phenol-degrading granules

	Activated sludge	*Acetate-fed Granule*	*Phenol -degrading Granule*
MLSS of isolates (g/ml):	0.0067	0.011	0.017
MLVSS of isolates (g/ml):	0.0052	0.0093	0.014
Medium for cultivation	R2A agar	R2A agar	R2A agar
Colony formed on agar (CFU/ml)	$8.17 \pm 0.75 \times 10^9$	$2.76 \pm 0.04 \times 10^9$	$1.73 \pm 0.04 \times 10^9$
No. of culture isolated	74	89	92
Genetically distinct isolates	37	32	42

Tables 4.2, 4.3, and 4.4 show the interactions among aggregating populations isolated and distinguished from activated sludge, acetate-fed granule and phenol-degrading granules. The proportions of aggregating populations within three bacterial communities are summarized in Table 4.5.

In activated sludge community, the largest fraction of isolates (32.4%) showed strong autoaggregating ability with autoaggregating score higher than 2. The portion of populations of strong autoaggregator decreased to 23.3% in acetate-fed granule. Only 2.4% of phenol-degrading granule populations belonged to the category of strong autoaggregator. The proportions of mild autoaggregating populations (autoaggregating score =1 or 2) that can coaggregate with at least one community member were comparable in three microbial communities of activated sludge, acetate-fed granule and phenol-degrading granule. The proportion of non-autoaggregating populations that can coaggregate with other community members was 73.8% in phenol-degrading granule community, while those proportions in activated sludge and acetate-fed granule community were 18.9% and 43%, respectively.

Most activated sludge populations (59.4%) were able to autoaggregate, and 21.7% of the isolates did not participate into the aggregating interactions among isolated strains. In acetate-fed granule, the proportion of isolates with autoaggregating ability was 50.3%, while the proportion of coaggregating isolates was 70%. Only 6.7% of the strains isolated from acetate-fed granule had neither autoaggregating nor coaggregating ability. In phenol-degrading granule, the largest fraction (97.6%) of the populations was able to participate in the cell-cell coaggregating interactions. Every isolate from phenol-degrading granule demonstrated aggregating ability, indicating the participation in cell-cell aggregating interactions across the entire microbial community.

Four ray fungi strains were isolated from phenol-degrading granules on day 41: F8, F12, F13 and F27. They possessed mild autoaggregating scores of 1, and can coaggregate among each other and with other isolates.

Table 4.2 Aggregating interactions among strains isolated from activated sludge

Strains	Coaggregation partner (coaggregation score)	Autoaggregating score	Number of coaggregating partners
a, strong autoaggregators (12 or 32.4%)			
AS09	None	3	0
AS10	None	3	0
AS14	None	3	0
AS16	None	4	0
AS17	None	3	0
AS19	None	4	0
AS20	None	4	0
AS21	None	3	0
AS35	None	3	0
AS36	None	3	0
AS40	None	3	0
AS43	None	4	0
b, strains with mild autoaggregating ability but cannot coaggregate (1 or 2.7%)			
AS08	None	1	0
c, strains with both autoaggregating and coaggregating ability (10 or 27%)			
AS06	AS22 (1), AS38(1), AS41 (2)	1	3
AS07	AS38 (1)	1	1
AS13	AS 41 (2)	2	1
AS22	AS04 (2), AS06 (1), AS23 (1), AS24 (1), AS32 (1), AS37 (2), AS38 (1), AS41 (2)	1	8
AS23	AS22 (1)	1	1
AS24	AS22 (1)	1	1
AS25	AS15 (1), AS34 (1), AS37 (1), AS41 (1)	1	4

Strains	Coaggregation partner (coaggregation score)	Autoaggregating score	Number of coaggregating partners
AS32	AS22 (1), (also can coaggregate with AS33 which cannot be reflected by visual method)	2	1
AS33	AS34 (1), AS 37 (1), AS39 (1), AS41 (1)	2	4
AS38	AS06 (1), AS07 (1), AS22 (1), AS28 (1), AS41 (1)	2	5
d, non-autoaggregating strains with coaggregating ability (7 or 18.9%)			
AS04	AS22 (2),	0	1
AS15	AS25 (1), AS41 (3)	0	2
AS28	AS38 (1)	0	1
AS34	AS25 (1), AS33 (1)	0	2
AS37	AS22 (2), AS25 (1), AS33 (1), AS41 (3)	0	4
AS39	AS33 (1)	0	1
AS41	AS06 (2), AS15 (3), AS13 (2), AS22 (2), AS25 (1), AS33 (1), AS37 (3), AS38 (1)	0	8
e, non-autoaggregating strains without coaggregating ability (7 or 18.9%)			
AS01	None	0	0
AS02	None	0	0
AS03	None	0	0
AS26	None	0	0
AS29	None	0	0
AS31	None	0	0
AS42	None	0	0

Table 4.3 Aggregating interactions among strains isolated from acetate-fed granules

Strains	Coaggregating partner (coaggregation score)	Autoaggregating score	Number of coaggregating partners
a, strong autoaggregators (7 or 23.3%)			
S08	None	3	0
S10	None	3	0
S13	None	3	0
S15	None	3	0
S21	None	3	0
S26	None	3	0
S28	None	4	0
b, strains with mild autoaggregating(<3) ability but cannot coaggregate (1 or 3.3%)			
S19	None	2	0
c, strains with both autoaggregating & coaggregating ability (8 or 27%)			
S02	S03 (1), 23 (1)	2	2
S23	S17 (1), S20 (2)	1	2
S24	S20 (1)	2	1
S25	S03 (1), S20 (1)	2	2
S29	S03 (1), S06 (1)	2	2
S30	S17 (1)	1	1
S32	S07 (1), S 20 (1)	2	2
S35	S01 (1), S04 (1), S06 (1), S09 (1), S16 (1), S20 (1),	2	6
d, non-aggregating strains with coaggregating ability (13 or 43%)			
S01	S09 (3), S35 (1)	0	2
S03	S09 (1), S12 (3), S14 (2), S16 (1), S25 (1), S29 (1)	0	6
S04	S05 (1), S35 (1)	0	2

Strains	Coaggregating partner (coaggregation score)	Autoaggregating score	Number of coaggregating partners
S05	S04 (1)	0	1
S06	S29 (1), S35(1)	0	2
S07	S20 (2), S32 (1)	0	2
S09	S01 (3), S03 (1), S35 (1)	0	3
S12	S03 (3), S20 (1)	2	2
S14	S03 (2)	0	1
S16	S03 (1), S35(1)	0	2
S17	S23 (2), S27 (1), S30 (1)	0	3
S20	S07 (2), S12 (1), S23 (2), S24 (1), S 25 (1), S32 (1), S35 (1)	0	7
S27	S17 (1)	0	1
e, non-autoaggregating strains without coaggregating ablity (1 or 3.3%)			
S18	None	0	0

Table 4.4 Aggregating interactions among isolates from phenol-degrading granules

Strains	Coaggregating partner (coaggregation score)	Autoaggregating score	Number of coaggregating partners
a, strong autoaggregators (1 or 2.4%)			
F23	None	4	0
b, strains with mild autoaggregating(<3) ability but cannot coaggregate (0 or 0%)			
c, strains with both autoaggregating & coaggregating ability (10 or 23.8%)			
F4	F9 (2), F28 (2), F29 (2), F31 (2), F39 (2), F41 (3)	1	6
F11	F6 (3), F15 (3)	2	2
F12	F1 (2), F7 (2), F21 (2), F30 (2), F39 (2), F41 (3)	1	6
F13	F9 (2), F18 (2), F30 (2), F42 (3)	1	4
F17	F15 (2), F18 (2), F21 (2), F39 (2), F40 (2), F41 (2)	1	6
F25	F6 (3), F15 (2), F18 (3), F30 (2)	1	4
F27	F9 (2)	1	1
F28	F4 (2)	1	1
F41	F3 (2), F4 (3), F7 (3), F12 (3), F14 (2), F17 (2), F26 (2), F33 (2), F35 (2)	1	9
F42	F2 (2), F3 (2), F13 (3), F19 (2), F24 (2),	1	5
d, non-autoaggregating strains with coaggregating ability (30 or 71.4%)			
F1	F9 (1), F12 (2), F27 (2)	0	3
F2	F42 (2)	0	1
F3	F8 (1), F40 (1), F41 (2), F42 (2)	0	4
F5	F9 (1), F21 (1)	0	2
F6	F9 (3), F11 (3), F20 (1), F25 (3)	0	4
F7	F8 (2), F12 (2), F14 (2), F18 (2), F38 (1), F41 (3)	0	6
F8	F3 (1), F7 (2)	0	2

Strains	Coaggregation partner (coaggregation score)	Autoaggregating score	No. of coaggregating partners
F9	F1 (1), F4 (2), F5 (1), F6 (3), F13(2), F22 (1), F33 (1), F34 (1),	0	8
F10	F21 (1)	0	1
F14	F7 (2), F41 (2)	0	2
F15	F11(3), F17 (2), F25 (2)	0	3
F16	F42 (2)	0	1
F18	F7 (2), F13 (2), F17 (2), F25 (3)	0	4
F19	F42 (2)	0	1
F20	F6 (1)	0	1
F21	F5(1), F10(1), F12 (2), F17 (2), F33 (1), F39 (1)	0	6
F22	F9 (1)	0	1
F24	F42 (2)	0	1
F26	F29 (1), F41 (2)	0	2
F29	F4 (2), F26 (1),	0	2
F30	F25 (2)	0	1
F31	F4 (2), F35 (1), F37 (1)	0	3
F33	F9 (1), F21 (1), F37 (1), F41 (2)	0	4
F34	F9 (1)	0	1
F35	F31 (1), F41 (2)	0	2
F36	F38 (10), F40 (1)	0	2
F37	F31 (1), F33 (1)	0	2
F38	F7 (1), F36 (1),	0	2
F39	F4(2), F12 (2), F17 (2), F21 (1)	0	4
F40	F3 (1), F17 (2), F36 (1)	0	3
e, non-autoaggregating strains without coaggregating ability (1 or 2.4%)			
F32	None	0	0

Table 4.5 Comparison of aggregating populations in activated sludge, acetate-fed granule and phenol-degrading granule

Aggregating ability	Proportion (%)		
	Activated sludge	*Acetate-fed granule*	*Phenol-fed granule*
Strong autoaggregator (autoaggregating score >2), and unable to coaggregate (number of coaggregating partner =0)	32.4	23.3	2.4
Mild autoaggregator (autoaggregating score =1 or 2), and able to coaggregate (number of coaggregating partner >0)	27	27	23.8
Non-autoaggregating bacteria (autoaggregating score =0), but able to coaggregate (number of coaggregating partner >0)	18.9	43	73.8

4.3.5 Sequence Analysis of Aggregating Strains from Acetate-fed Aerobic Granules and Phenol-degrading Granules

Several isolates with outstanding aggregating ability were selected to evaluate the phylogenetic diversity. The 16S rRNA genes were partially sequenced. The nearest relatives of the chosen aggregating strains were compared by BLAST and the results were presented in Table 4.6. Polygenetic information of S15, S35 and F41 were analyzed as they were identified to be strain1, strain 2 and strain 3 respectively.

Amplification of 16S rRNA and partial gene sequencing revealed that strain 1 had a 99% identity to an uncultured bacterium clone PL-34B4 (as shown in Table I). Strain 2 matched sequence of *Leadbetterella byssophila* species with a sequence identity of 94%. Strain 3 matched the strain PG-04 with 98% identity which was isolated in phenol-degrading granules previously (Jiang et al. 2004). This culture was closely related (at identity value of 87.7%) to *Propoinibacterium cyclohexanicum* strain IAM

14535. The sequences of strain 2 and strain 3 were found to belong to *Cytophaga-Flavobacteria-Bacteriodes* (CFB), and *Actinobacteria*, respectively.

Table 4.7 shows a comparison of the aggregating capability of strain1, strain 2 and strain 3 in aerobic granule microbial community. The microscopic pictures of Gram stained cells were shown in Figure 4.11. Strain 1 in acetate-fed granule was an autoaggregating culture, and can mildly coaggregate with 6 other community members, resulting in mild aggregating index (visual score=1 or 2). Strain 2 presenting in both acetate-fed and phenol-degrading granule bacterial community was able to autoaggregate but cannot coaggregate with other strains. Strain 3 was able to coaggregate with other strains with a low autoaggregating score. It was reported previously to be slow-growing bacteria with the phenol-degrading ability (Jiang et al. 2004). This strain was found to coaggregate with 9 strains in phenol-degrading granule community.

Table 4.6 Sequence analysis of selected strains isolated from acetate-fed aerobic granules and phenol-degrading granules

Strains	Sequence length (bases)	Closest relatives (accession number)	Identity (%)	Phylogenetic Division
S03	1128	*Xanthomonas sp.* 3C_3 (AY689031)	98%	*γ-proteobacterium*
S15 (strain 2)	1452	*Leadbetterella byssophila* strain 4M15 (AY854022)	94%	*CFB group*
S35 (strain 1)	858	Uncultured bacterium clone PL-34B4 16S ribosomal RNA gene (AY570623) Uncultured bacterium clone CJRA66 16S ribosomal RNA gene (DQ202150)	99%	*Unclassified*
F7	850	*Comamonas testosteroni* 16S ribosomal RNA gene (AF532871)	98%	*β-proteobacterium*
F9	1036	*Methylobacterium sp.* (D25306)	98%	*α-proteobacterium*
F18	1157	*Dokdonella koreensis* strain DS-140 16S ribosomal RNA gene (AY987369) *Pseudoxanthomonas daejeonensis* strain TR6-08 16S ribosomal RNA Gene (AY550264)	93%	*γ-proteobacterium*
F21	960	*Afipia genosp.* 8 (U87774) *Bosea thiooxidans* (AJ250800)	99%	*α-proteobacterium*
F39	616	*Mesorhizobium sp.* ST-2 16S ribosomal RNA gene (AY225401)	97%	*α-proteobacterium*
F40	832	*Acidovorax avenae* (AY512827)	99%	*β-proteobacterium*
F41 (strain 3)	824	*Eubacterium sp.* PG-04 16S ribosomal RNA gene (AY566577)	98%	*HGC Gram positive*

Table 4.7 Aggregating capability of strain 1, 2, and 3

Name	Aggregating capability	Gram staining	Morphological type
Strain 1 (S15)	Auto-aggregating strain	negative	Rod
Strain 2 (S35)	Auto-aggregating and co-aggregating strain	negative	Cocci
Strain 3 (F41)	Co-aggregating strain, with mild auto-aggregating ability	positive	Rod

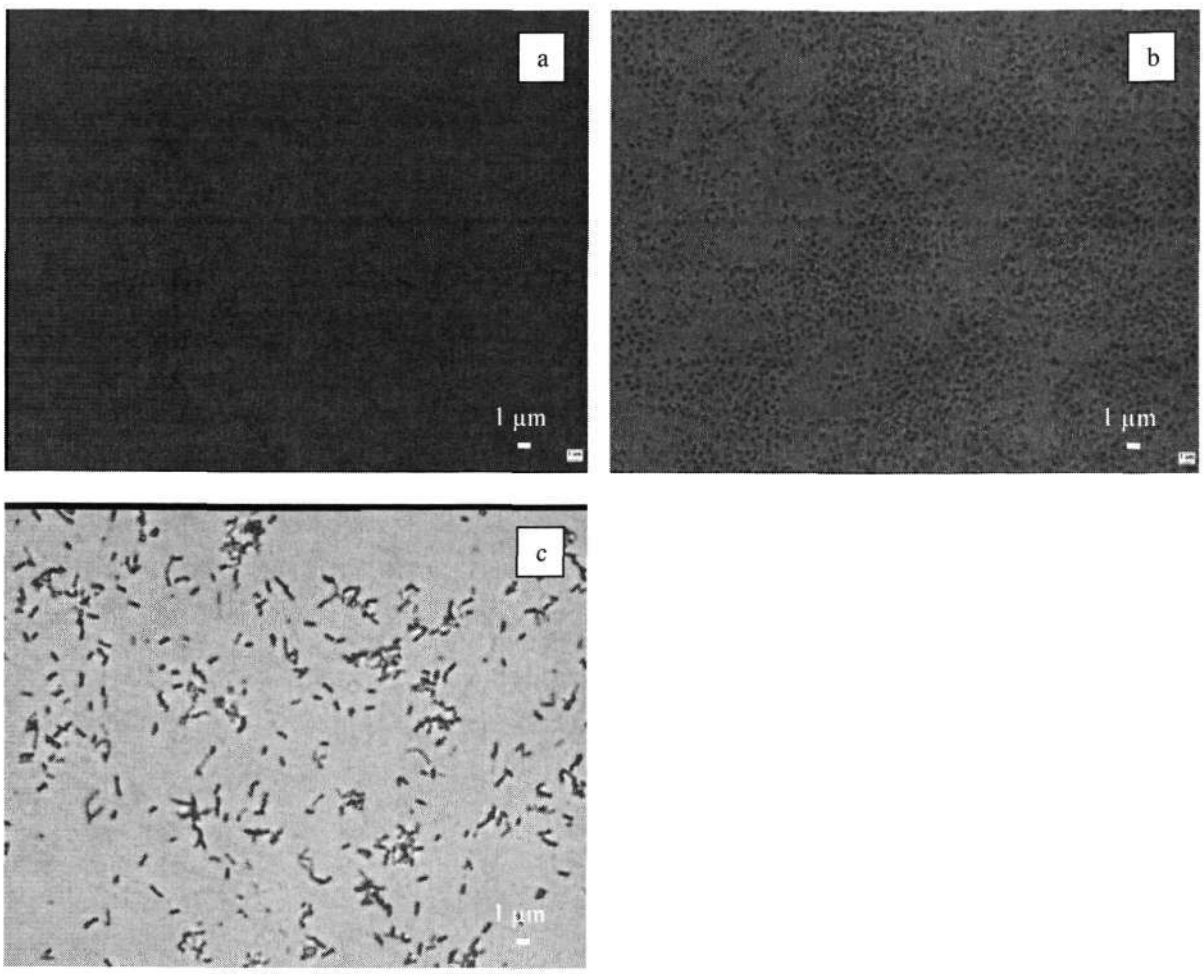

Figure 4.11 Gram-staining picture of: (a) strain 1 (S15); (b) strain 2 (S35); and (c) strain 3 (F41)

4.4 DISCUSSION

4.4.1 The Reactor Performances

In the present study, synthetic wastewater consisting of phenol as sole carbon source was applied for the cultivation of phenol-degrading granule by using acetate-fed granule as seed. Sodium acetate was not provided as buffering substance to facilitate the growth of microbes which cannot resist or survive under phenol toxicity. The results show that the acetate-fed aerobic granules were able to adapt to phenol in less than 10 days. Biomass concentration and settling ability were improved visibly, and a more clear-cut shape and compact structure was formed. The aerobic granule presented outstanding capability of resistance and rapid adaptation to high phenol loading at 2.4 kg m^{-3} day^{-1}.

Phenol-degrading granules size were stabilized in a relatively small range around 800-1,400 μm with excellent settling ability and high biomass retention after 11 days. It was reported that the optimal diameter of aerobic granule was less than 1,600 μm (Tay et al. 2002). Aerobic granules in small size could favor the acquisition of preferable kinetic behaviors, possibly due to the small mass transfer limitation (Liu et al. 2005) and also the feasibility of oxygen inside aerobic granule, which is crucial for conversion rate of various components (De Kreuk et al. 2007; Ni et al. 2008b). This can explain the stability of the present SBR system for more than one year of operation.

4.4.2 Microscopy of Acetate-fed and Phenol-degrading Granules

SEM observation showed that the surface of R1 granule in early stage exposure to phenol was predominantly composed of cocci and short rods as in control reactor R2. After operation for 11 days, the surface of granule in R1 contained more morphological types of microorganisms in cocci, short rods, long rods and filamentous form, which

aggregated to form an intricate structure embedded in EPS matrix, as the result of microbial response to chemical and toxic shock.

There were mainly two types of distinctly morphological econiches on mature phenol-degrading granule after 31 days: fungus and filamentous forms combined with large-sized microorganisms, and microaggregates composed of small-sized microorganisms in isotype. Sometimes the occurrence of overgrowth of filamentous microorganisms in aerobic granule system indicated the deterioration of system performance, accompanied with the increase of SVI and wash out of poor settled granules (Arrojo et al. 2004). However, filamentous microorganisms seemed to serve as backbone for stabilization of the entire granule structure and act as the scaffold for growth of small-sized microorganisms in this study. The lysis of wrapped fungi would further result in aggregates or clusters of residual bacteria colonized on the mature phenol-degrading granules.

In this study, four fungi possessing autoaggregating and coaggregating ability were isolated from phenol-degrading granules. Bacteria and eukaryotes were recently reported to interact with each other via small molecules (Dudlera et al. 2006). Thus these fungi were supposed to mediate the cell-cell interactions among bacteria in phenol-degrading granule community. Microbes were thought to be distributed according to the capability to survive best in the particular microenvironment, while the high complexity of microbial community would benefit the stability in a multi-species biofilm community (Watnick et al. 2000). Therefore the presence and dominance of filamentous microorganisms can be regarded as one of the response of microbial community to the changes of external environment. Furthermore, the coaggregating and autoaggregating interactions among fungi and bacteria showed to be important in the development of phenol-degrading granules. This distribution of microorganisms facilitated the bacteria to retain on the extended structure of phenol-degrading granules where mass transfer limitation were minimized (Chen et al. 2007), which differentiated with cell distribution in acetate-fed granules. Besides this, the coaggregation among

fungi and non-phenol-degrading or non-phenol-resistant bacteria provided a close proximity of coaggregating partners, and further facilitated the substrate exchange, metabolic production distribution, and synthesis of enzymes (Grenier 1992; Ishii et al. 2005). Fungi were reported to be capable of transforming polycyclic aromatic hydrocarbons (PAHs) co-metabolically to detoxified metabolites, which can be utilized by other microorganisms that cannot intake phenol as carbon source (Juhasz and Naidu 2000).

By tracking the change of morphological traits of granules in both R1 and R2, the ruffled components on granule surface were found to easily develop into secondary aggregates, and subsequently detached from original coagulation of granules to develop into big granules with regular shape and compact structure. As in this study, small granule in compact structure and smooth surface displaced the irregular big granules in system gradually after detaching from irregular and elongated granules. It was demonstrated that multi-species biofilm can form thick layers consisting of mushroom-like structures, which was similar to the observation of this study (Costerton et al. 1994). An aerobic granulation pathway was proposed that granules could replicate by divisions and budding (Vlaeminck et al. 2009). In the current study, the secondary structures of aerobic granule suggested a strategy for granule development without negative influences on other granule properties. With this strategy, granules could avoid the rapid growth in size caused by intense increase of organic loading, which improved the granule stability consequently.

4.4.3 Shift of Aggregating Populations

Population shifts of bacterial community were observed after 6 days of the exposure of acetate-fed granule to phenol. Dominant bands in acetate-fed granule community disappeared, along with the increased abundance of microbial diversity. The community diversity increased in a complex microbial community of aerobic granule as a response to phenol shock. This finding was different from the report that phenol

loading rate had strong negative effect on the microbial diversity in activated sludge (Watanabe et al. 1999).

During the adaptation process, the fast decay and loss of predominance of strain 1 in DGGE pattern can be correlated to its reliance on acetate utilization and failure in resistance or tolerance to phenol. In contrast the weakly autoaggregating strain 3 which was a phenol-degrader and can coaggregate with nine community members promptly predominated in phenol-degrading granule. This implied the favor of coaggregating interactions under the chemical and toxic shock. The aerobic granule community was believed to supply excellent protection for species against the phenol toxicity through the compact structure and active cell-cell interactions among microbes (Di Gioia et al. 2004; Jiang et al. 2006a; Tay et al. 2005a). Econiches would be supplied by the microbial aggregates formed by aggregating interactions. They would facilitate the gene transfer, exchange of interspecies substrate, distribution of metabolic products, and enhanced cell respiratory activities among the participating partners, which were located in close proximity as the result of microbial aggregation (Ishii et al. 2005).

The presence of phenol shock influenced the proportion of autoaggregating and coaggregating microorganisms dramatically. All isolates from phenol-degrading granule were able to either autoaggregate or coaggregate. The proportion of coaggregating microorganisms was as high as 98%, while only 26% of phenol-degrading granule cultures were able to autoaggregate. In acetate-fed granule cultures, the proportion of coaggregating bacteria was comparably low, and proportion of autoaggregator was relatively high when compared to phenol-degrading granule. In activated sludge, however, the proportion of coaggregating bacteria was minimal, along with highest portion of bacteria which cannot participate in the aggregating interactions.

Aggregating population dynamics presented here suggest that high participating level of microbial populations into coaggregating interactions contribute to the well-structured microbial community of high diversity, and the presence of toxic compound

stimulates the emergence of high portion of coaggregating microorganisms. It also suggests that both autoaggregating and coaggregating interactions benefit the formation and development of aerobic granules, while autoaggregating interactions were more important in sludge floc formation. Furthermore, the ability to coaggregate of microorganisms could be a crucial determinant for the aerobic granulation in phenol degradation.

The bacterial populations in aerobic phenol-degrading granule intended to possess more coaggregating abilities and a more complex multi-species community within a sheltered structure. Thus, the shift of aggregating populations encourages more microbial community members to participate in coaggregating interactions. This was used as one of the strategies to increase community tolerance against toxicity. It was reported that the environmental signals produced by toxicity, physical stress, and predation would lead to gene activation of cells or genetic rearrangements, therefore resulting in facilitating coaggregation (Bossier et al. 1996). The favor of coaggregating population in phenol-degrading granule could be supported by emergence and propagation of weakly autoaggregating bacteria *Eubacterium sp.* PG-04 (strain 3) which can coaggregate with 9 other isolates. *Eubacterium sp.* PG-04 was reported to be slow-growing bacteria (Jiang et al. 2004). Its existence and predominance in phenol-degrading aerobic granules implied the significance of slow growing bacteria with coaggregating ability to tolerate and survive in adverse environment. Slow-growing bacteria were selected to decrease the oxygen requirement by microorganisms in aerobic granules, in order to overcome the mass transfer resistance, and subsequently facilitate the stability of aerobic granular sludge (De Kreuk et al. 2004; Liu et al. 2004b). The coaggregating ability and phenol degrading compatibility assisted the predominance of this strain, which further contributed to the maintenance of population abundance of bacterial community and the long-term stability of aerobic granules under inhibition of phenol.

The predominance of *Eubacterium sp.* PG-04 in phenol-degrading granule was also

reported previously (Jiang et al. 2004). The microbial community structure of aerobic granule was probably dependent on the initial inocula. However, natural occurrence and predominance of some functionally important bacteria was thought as response of microbial community to the similar hydraulic selection pressure and inhibition induced by phenol. Therefore, this strain could possibly be one of the important populations in phenol-degrading aerobic granules. The coaggregating ability of *Eubacterium sp.* PG-04 with 9 strains in the bacterial community suggests that it can provide a core for aggregates to bridge the interactions among strains which may not coaggregate with each other directly. Also, the phenol degradation for the whole aggregate would be possibly enhanced when phenol-degrading strains and non-phenol-degrading strains were mixed (Prpich et al. 2005). Furthermore, the existing compact structure and diverse distribution of bacteria in acetate-fed granule community can provide the shield to the coaggregating partners and the proximity of taxonomically differentiated bacteria. This could explain the fast adaptation of acetate-fed granules to the phenol shock and the transition into phenol-degrading granules.

4.5 SUMMARY

This chapter demonstrated the fast adaptation (10 days) of acetate-fed granule to phenol-degrading granule without addition of acetate as co-substrate. The phenol-degrading granule was stabilized in long term operation. In phenol-degrading granule, 97.6% of the populations were able to participate in the coaggregating interactions and 2.4% of isolates possessed strong autoaggregating ability. In acetate-fed granule, the proportion of isolates with coaggregating ability was 70%, while the proportion of strong autoggregating isolates was 23.3%. In contrast, in the activated sludge populations, comparably low portion of 45.9% was able to coaggregate, and a relatively high proportion of 32.4% was strong autoaggregator, while the rest 21.7% did not show any aggregating ability. Coaggregating bacteria *Eubacterium sp.* PG-04 emerged and dominated in phenol-degrading granule community, and persisted in the community for more than 400 days. The dynamics of aggregating population in

activated sludge, acetate-fed granule and phenol-degrading granule suggested that aggregating ability was important in formation of aerobic granules, and coaggregating ability favored the adaptation process and development of phenol-degrading granule from acetate-fed granule.

CHAPTER FIVE
ACCELERATED AEROBIC GRANULATION WITH AUGMENTED AGGREGATING MONO-ISOLATE

5.1 INRODUCTION

Aerobic granules are initiated and formed under several selecting pressures. In most studies, aerobic granules were seeded from acclimated municipal activated sludge (Jiang and Liu 2009; Liu and Tay 2006; Liu et al. 2008b). Both aerobic and anaerobic granules were reported to seed aerobic granulation (Fang et al. 2009; Tay et al. 2005a; Wang et al. 2005). Stable aerobic granules were also formed after five weeks cultivation with pure bacteria strain *Bacillus thuringiensis* as inocula, which was both phenol reducer and autoaggergator (Adav and Lee 2008a, c, d).

Several factors are known to affect aerobic granulation, including seeded biomass, bioreactor configuration, organic loading rate, substrate composition, hydrodynamic shear force and so on. (Liu et al. 2004a). In conjunction with the selection pressures manipulated and regulated by operational parameters, bioaugmentation is regarded as a strategy to reach accelerated granulation and elevated performance. Some attempts have been made to form aerobic granules through bioaugmentation. Either co-strains were augmented into acclimated activated sludge, in order to reinforce the competition of augmented strains in activated sludge community (Ivanov et al. 2006; Jiang et al. 2006a); or mono strain was seeded to form pure-cultured granules (Adav et al. 2008b). However, little has been reported on the feasibility of bioaugmentation with single aggregating strain in non-acclimated activated sludge. Compared to augmentations of co-strains or pure-culture, mono-strain is easier to manipulate in bioaugmentation process with less economic cost.

Therefore, this chapter focused on bioaugmentation by using comparatively small quantity of aggregating strain to accelerate granule formation and enhance system performance under the common operational parameters comparable to other studies,

such as Tay et.al (2002a, b; 2005a, b). This study aims to provide information on the bioaugmentation strategy adopted in aerobic granulation by inoculating mono-aggregating-culture, and to contribute to the understanding of the role aggregating interactions could take in aerobic granulation.

5.2 METHODS AND MATERIALS

5.2.1 Experimental Design and Set-up

Experiments were performed in two column-type sequencing batch reactors (SBR) R1 and R2 designed and operated as described in Chapter Three. Both reactors were fed with acetate as sole carbon and energy source by using synthetic wastewater solution with the following composition: CH_3COONa 1538 mg l^{-1}; NH_4CL 175 mg l^{-1}; K_2HPO_4 15 mg l^{-1}; KH_2PO_4 12.5 mg l^{-1}; $CaCl_2.H_2O$ 15 mg l^{-1}; $MgSO_4 \cdot 7H_2O$ 12.5 mg l^{-1}; and $FeSO_4 \cdot 7H_2O$ 10 mg l^{-1}. This synthetic wastewater had a substrate concentration equivalent to 1,200 mg l^{-1} of chemical oxygen demand (COD) concentration, resulting in COD loading of both reactors at 3.6 kg COD m^{-3} d^{-1}. Experiment was carried out under step-wise increased selective pressure imposed by the gradually reduced settling period in sequentially operational cycle time of 4 h. With the influent filling time and effluent withdrawal time fixed during the whole experimental operation, in the beginning two days of cultivation, reactors were operated sequentially in a cycle of 4 hours with 5 min of influent filling, 200 min of aeration, 30 min of settling and 5 min of effluent withdrawal. In the second phase of day 3 to day 5, the settling time was reduced to 10 min with aeration and mixing time of 220 min correspondingly. Since day six, the biomass in reactors was allowed to settle for 2 min and aeration time was 228 min sequentially.

5.2.2 Seeding Biomass

Activated sludge was collected from an aeration tank in a local municipal wastewater plant (Ulu Pandang) and aerated overnight before inoculated into

bioaugmented reactor R1 and control reactor R2. The control process in R2 without bioaugmentation was started with 600 ml of activated sludge topped up to 800 ml mixed liquor to achieve initial biomass concentration of 3.17 g SS l^{-1}. The experimental process in R1 was seeded with 300 ml of seed culture S35 and 500 ml of activated sludge (total volume of 800 ml equivalent to R2). The initial biomass concentration in R1 was 3.09 g SS l^{-1} with the amount of S35 bioaugmented at approximately 11% of total biomass in dry weight. S35 was previously isolated from acetate-fed aerobic granules as described in Chapter Four. It was cultivated in Nutrient Broth (Difco, Detroit, USA) for 48 h at 25°C using sterile flask on a shaker at 150 rpm before experiment. Then cell of S35 was harvested by centrifuge at 3000×g for 20 min and resuspended in PBS buffer (pH 7.2) after washing twice with the same solution.

5.2.3 Selection of Aerobic Granule Seeds

The strain augmented into reactor R1 was gram-negative bacteria S35, which was isolated previously from the acetate-fed aerobic granule as described in Chapter Four. It had a 99% identity to Uncultured bacterium clone PL-34B4 (AY570623). Strain S35 was detected to dominate in acetate-fed granule which was used for the seed of phenol-degrading granules. This strain was able to coaggregate with 6 other microbial community members in acetate-fed granules, with an autoaggregating score of 2, as described in previous study of Chapter Four (as shown in Figure 5.1)

Figure 5.1 Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA fragments from aerobic granules cultivated in previous experiment as described in Chapter Four. Lane 1: phenol-degrading granule in experimental reactor R1 on day 41; Lane 2: strain S35, and Lane 3: seeded acetate-fed granule

5.2.4 Analytical Methods

Wastewater samples in both R1 and R2 were periodically measured for pH value, total organic carbon (TOC), mixed liquor volatile suspended solid (MLSS), volatile suspended solids (VSS) and sludge volume index (SVI) using standard methods (APHA 1998). Granule size was determined either by a laser particle size analysis

system (MasterSizer Series 2000, Malvern, U.K.) or an image analysis system (Quantimet 200 Image Analyser, Leica Cambridge Instruments, Cambridge, U.K.).

Extracellular Polymeric Substances (EPS) in the aerobic granules was extracted as described previously (Tay et al. 2005a). Approximately 0.5 g wet weight of fresh granules was suspended in a 2-ml tube with 1.5 ml of extraction solution containing 8.5% NaCl and 0.22% formaldehyde. The mixture was dispersed by a Mini-Beater (Biospec, Bartlesville, OK, USA) at 480 strokes min^{-1} for 5 minutes without glass beads, and then centrifuged at 12,000 rpm for 30 min in succession. The clear upper supernatant was then extracted for the measurement of carbohydrate content using phenol-sulfuric method (Dubois et al. 1956) and for the analysis of protein by the bicinchoninic acid protein assay (Stoscheck 1990).

5.2.5 DNA Extraction

Approximately 200-300 mg (wet weight) of aerobic granule was harvested in duplicate in each SBR column and used immediately for DNA extraction as previously described in Chapter Four. The extracted DNA was precipitated overnight with sodium acetate - ethanol mix (2 volumes:0.1 volume) at −20 °C and dissolved in 100ul of sterile MilliQ water. Extracted DNA samples were stored in a −20°C freezer before further use.

5.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE-PCR amplification was performed as described in Chapter Four. Touchdown PCR was conducted (Watanabe et al. 1998) with a mastercycler gradient thermal cycler (Eppendorf AG, Hamburg, Germany) using PCR primers P2 and P3.

5.3 RESULTS

5.3.1 Reactor Performance

Figure 5.2 shows the biomass MLSS accumulation in augmented reactor R1 and control reactor R2. During the beginning three days of operation, no severe washout of biomass in R1 was observed, however the retainable biomass in R2 was sharply reduced from 5.28 g l^{-1} to below 4.7 g l^{-1}. Throughout the entire experiment duration, biomass concentration in R1 remained higher than that in R2. These observations indicated a more stable operation system in R1 with a better acclimatization. It was found that granular sludge floc gradually appeared in the bioaugmented reactor R1 (Figure 5.3a), in which more compact and denser sludge aggregates can be visually observed. In contrast, the fluffy structure of activated sludge still prevailed in the control reactor R2 (Figure 5.3b).

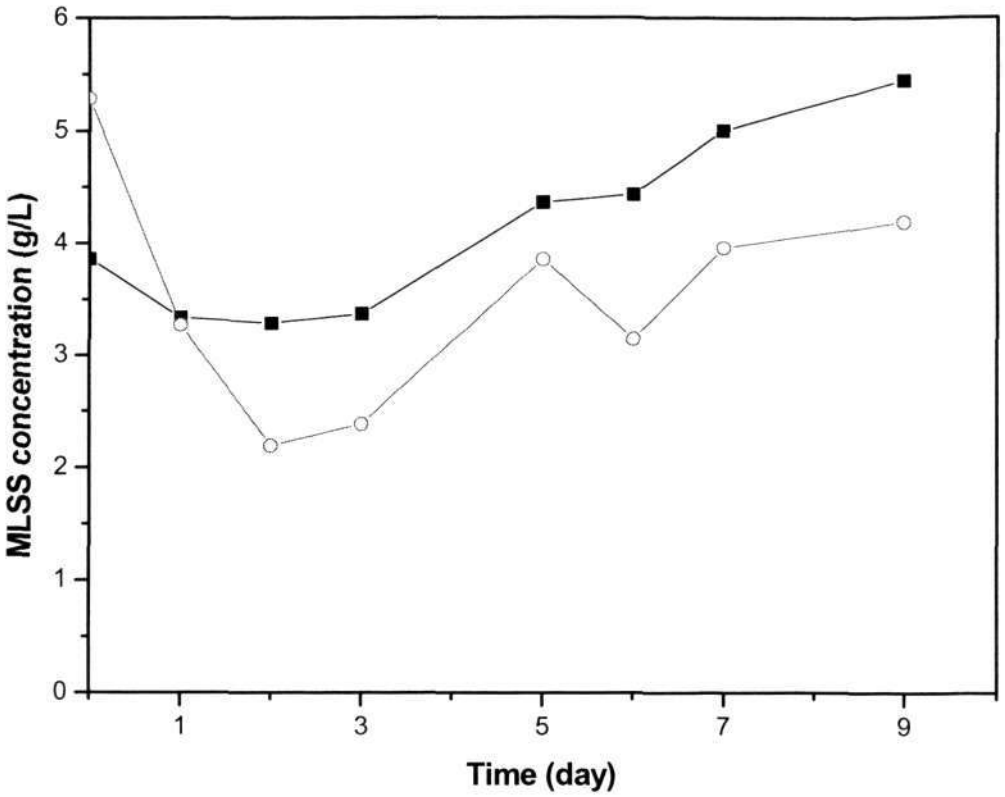

Figure 5.2 Time profiles of biomass concentration in experimental reactor R1 (■) and control reactor R2 (○)

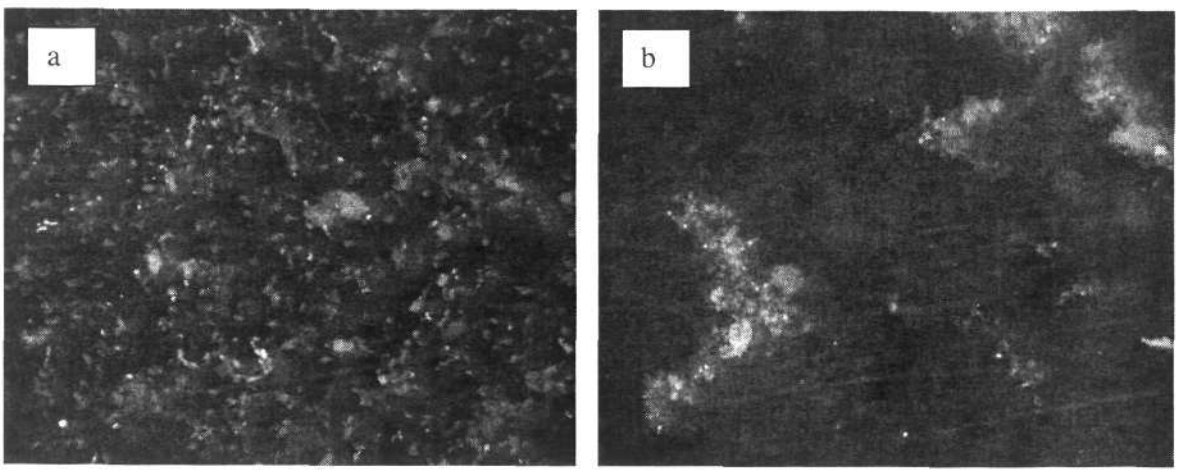

Figure 5.3 Image analysis of microbial aggregates formed after operation for 3 days: (a) experiment reactor R1; (b) control reactor R2.

Reactors R1 and R2 were both operated by feeding with sodium acetate at the same loading rate as the sole carbon and energy source. At beginning of the experimental operation, settling ability of augmented biomass in experimental reactor R1 was worse than control reactor R2, due to the addition of seed bacteria S35 that dispersed in the mixed slurry, resulting in comparably higher sludge volume index (SVI) value of augmented biomass in R1 at approximately 230 ml g^{-1} SS, when compared to initial SVI value at 179 ml g^{-1} SS of activated sludge in R2 (Figure 5.4). However, sharp decrease of SVI value of biomass in R1 to less than 100 ml g^{-1} SS was observed after only one day of reactor operation. On day 2 the SVI value in R1 dropped to 83 ml g^{-1} SS, which was close to the typical SVI of mature granules. The aerobic granules in R1 continued to possess better settling characteristics than R2 from day 1. SVI values in R1 persisted in 35 to 41 ml g^{-1} SS from day 5, while a high SVI value of 93 to 110 ml g^{-1} SS was recorded in R2 at the same time. It seems certain that the bioaugmentation of aggregating bacteria S35 into activated sludge would be responsible for the observed sludge settling as shown in Figure 5.4.

At first day after bioaugmentation, no significant difference on TOC removal efficiency was observed for experimental reactor R1 and control reactor R2, which were both around 74% (as shown in Figure 5.5). Thereafter, significantly enhanced TOC removal efficiency was found in augmented biomass for R1 since day 2, and subsequently stabilized above 90% since day 3. Meanwhile, the deteriorated removal efficiency appeared for R2 on day 2 and day3. Since day 3 the TOC removal efficiency for R2 was maintained around 70% to 80%.

After one day cultivation, augmented biomass in R1 had a mean diameter of 213 μm, while the average biomass size in R2 was 102 μm. During the initial five day cultivation, granular sludge with mean size ranged from 213 to 436 μm was observed in R1, obviously bigger than the biomass in R2 (Figure 5.6).

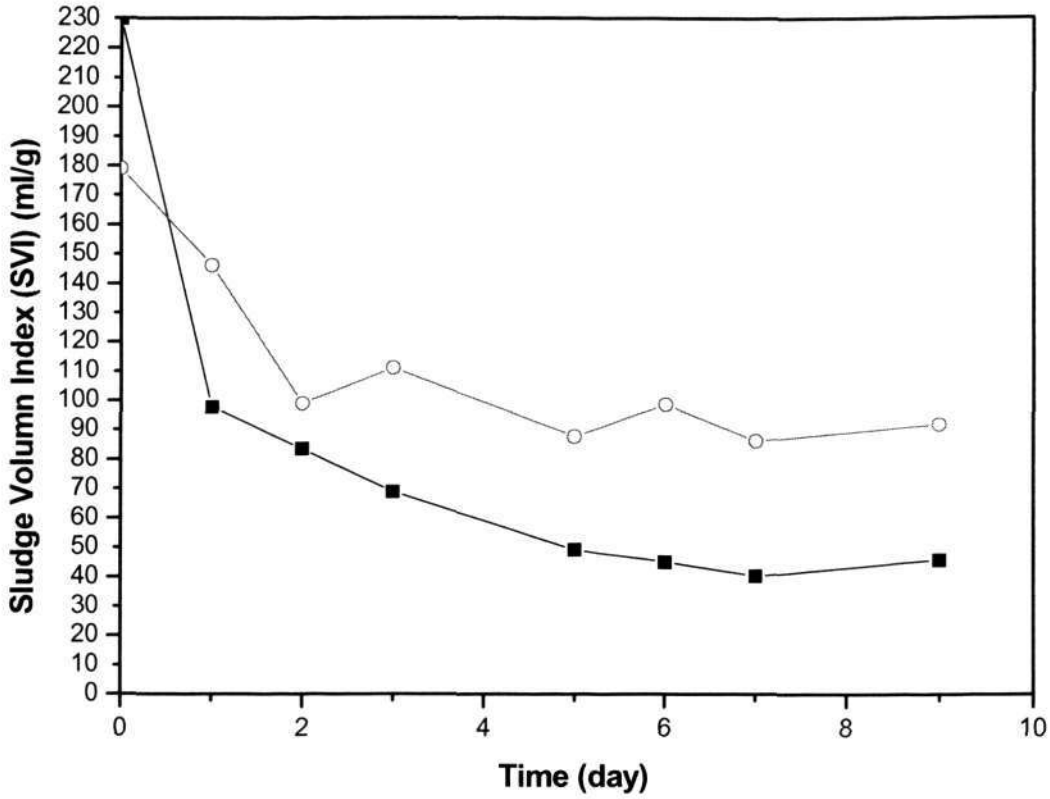

Figure 5.4 Time profiles of sludge volume index (SVI) in experimental reactor R1 (■) and control reactor R2 (○)

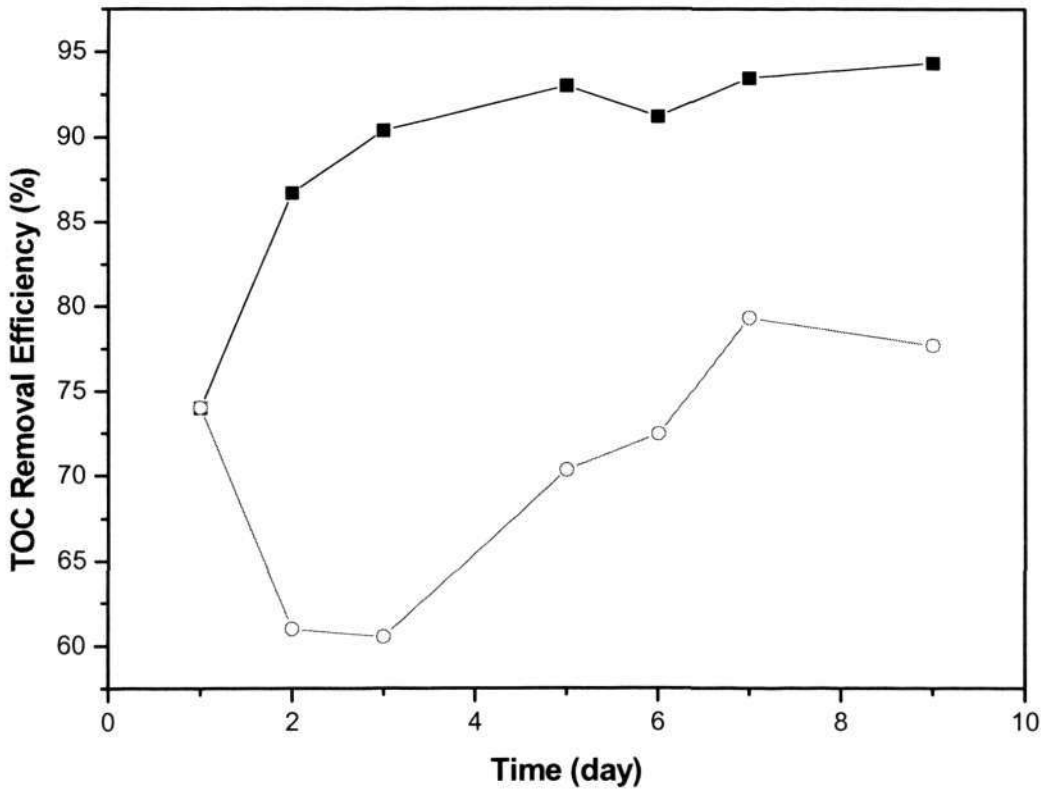

Figure 5.5 Time profiles of TOC removal efficiency in experimental reactor R1 (■) and control reactor R2 (○)

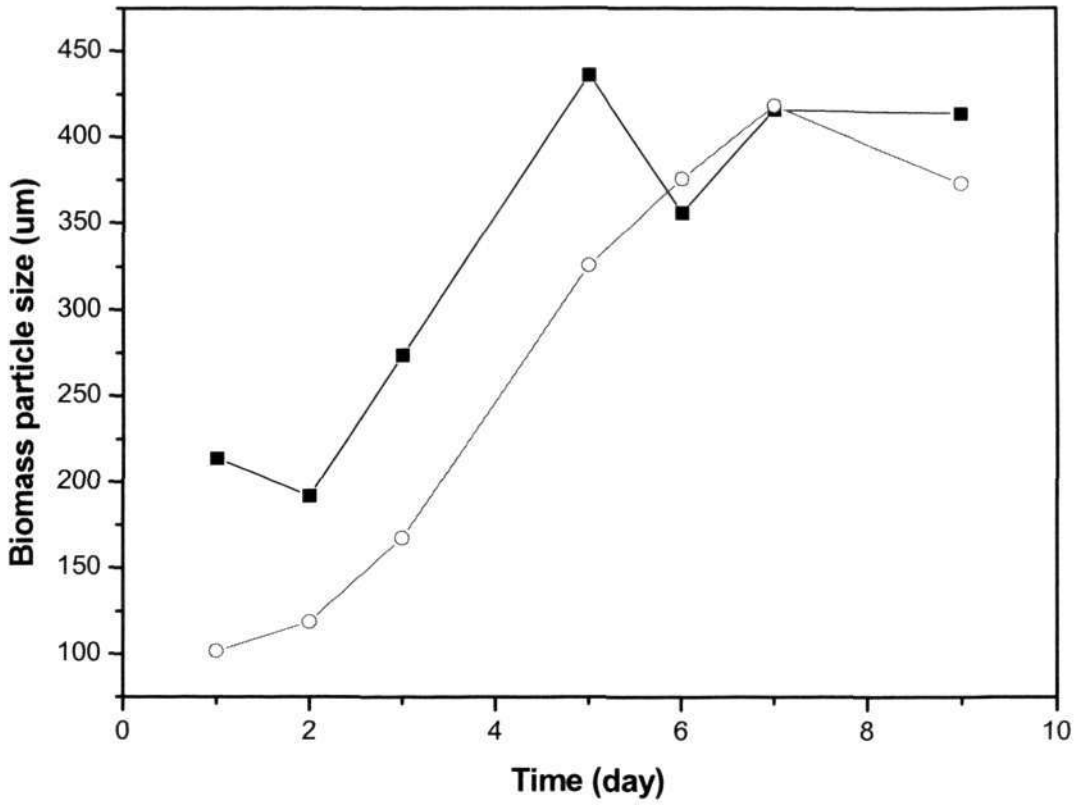

Figure 5.6 Time profiles of mean biomass particle size in experimental reactor R1 (■) and control reactor R2 (○)

5.3.2 Morphological Changes

Image analysis of the time dependent development of biomass in experimental reactor R1 and control reactor R2 is shown in Figure 5.7. After three days of cultivation, dense microbial aggregates appeared in R1, co-existed with flocculent sludge (Figure 5.7a). By that time, the mean biomass size of total biomass in R1 had exceeded 0.27 mm and the SVI value had decreased to 70 ml g^{-1} SS. In the control reactor R2, loose and fluffy sludge was present with mean biomass size at 0.17 mm and SVI value as high as 111 ml g^{-1} SS (Figure 5.7b). By day 5, more compact

granular sludge appeared in R1, and SVI value declined greatly to 41 ml g^{-1} SS simultaneously, indicating the replacement of loose and irregular activated sludge floc by good settling granular aggregates eventually in R1.

Figure 5.8 shows the morphologies of biomass in experimental reactor R1 and control reactor R2 at day 5 of cultivation. The granular sludge formed in R1 by using augmented activated sludge with strain S35 as seed consisted in clumps of microorganisms congregated together (Figure 5.8 a). However, the SEM examination of biomass in R2 demonstrated loose and irregular morphology of typical activated sludge structure (Figure 5.8b).

The settling performances of biomass in R1 and R2 were also different. It was observed from Figure 5.9 that, most portion of the granular aggregates in R1 tended to settle well within 2 min, leaving a clear supernatant in upper level of reactor with little significant washout of suspended biomass. Whereas in control reactor R2 seeded with activated sludge, biomass took more time for settlement.

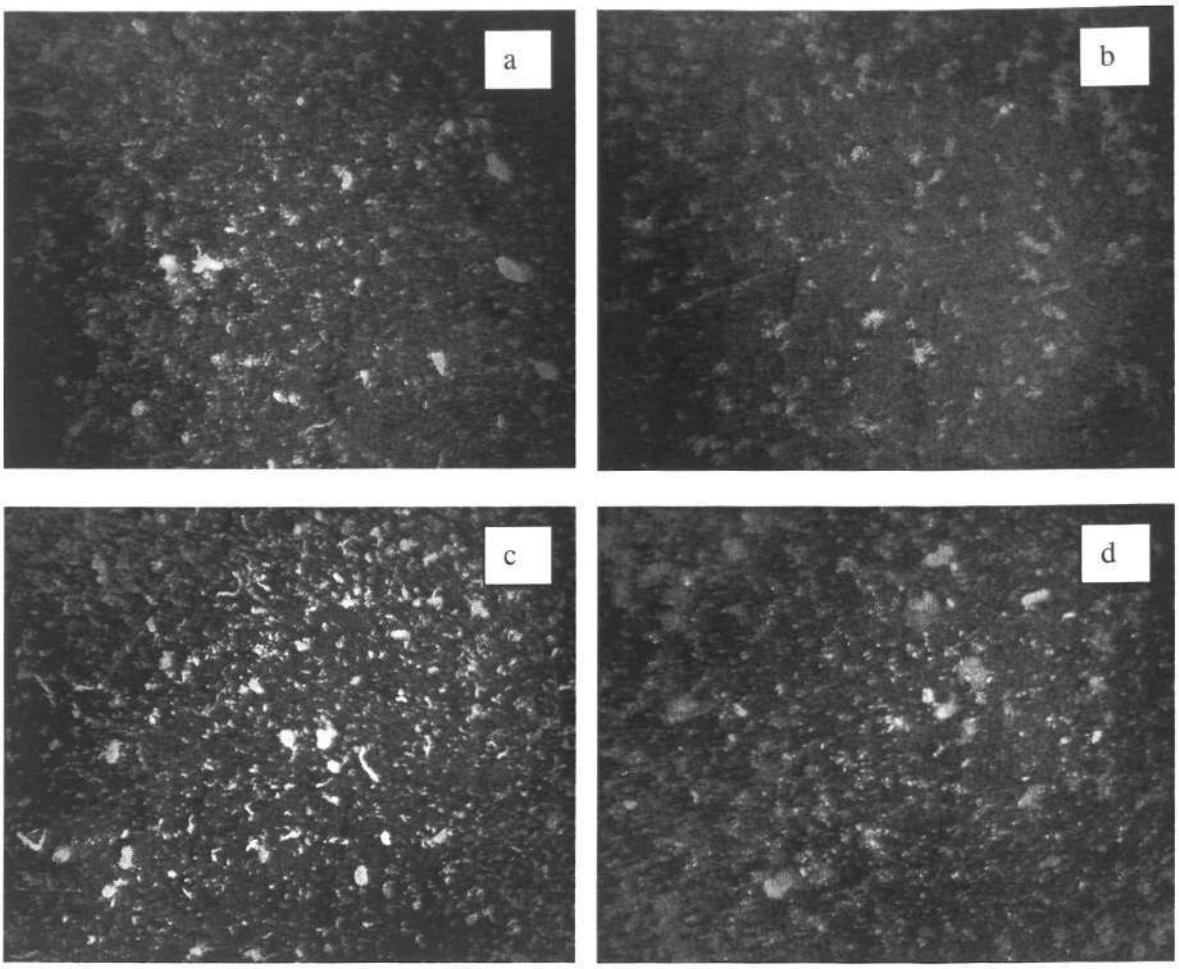

Figure 5.7 Morphological changes during cultivation: (a) in the experimental reactor R1 on day 3; (b) in the control reactor R2 on day 3; (c) in the experimental reactor R1 on day 5; and (d) in the control reactor R2 on day 5

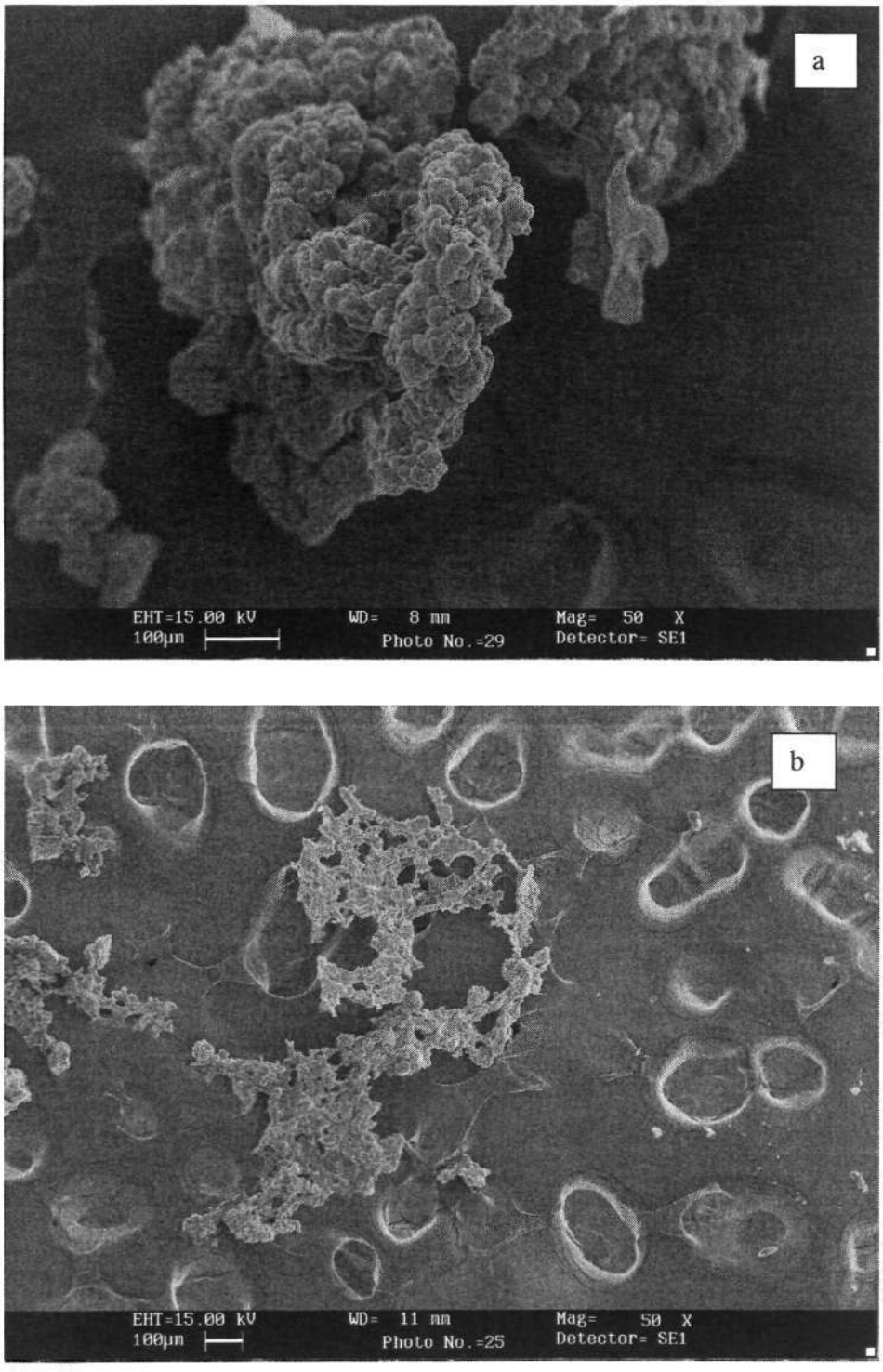

Figure 5.8 SEM picture of biomass cultivated for 5 days in: (a) granular aggregates emerged in experimental reactor R1; (b) loose structure of sludge in control reactor R2

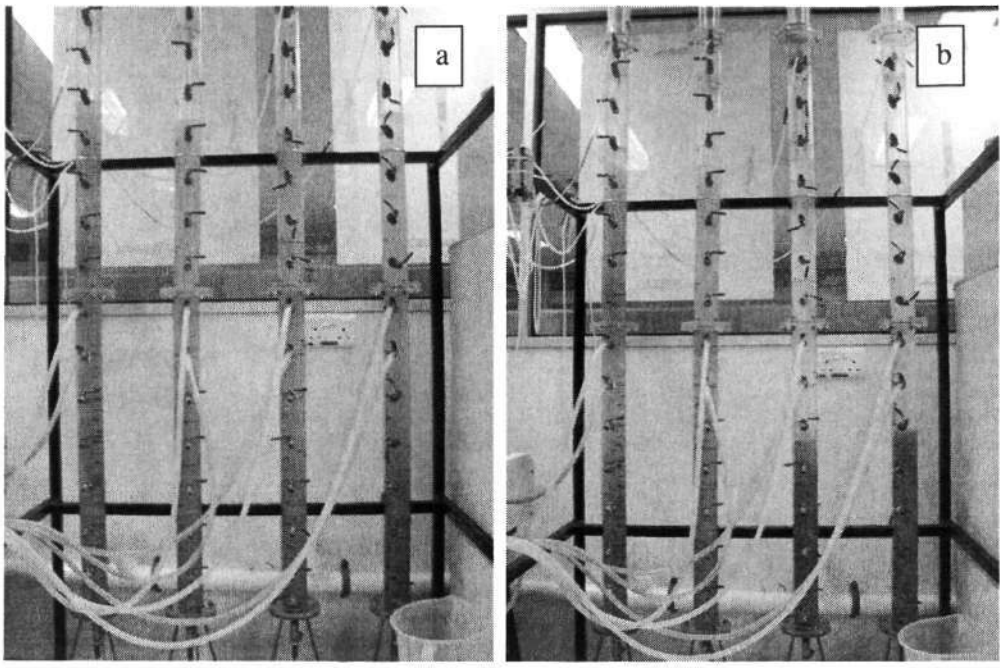

Figure 5.9 Photograph of SBR columns in operation cycle: (a) columns at start of settling phase; (b) columns after settlement for 2 minutes. Column 1 and 2: control reactor R2 (in duplicate); Column 3 and 4: experimental reactor R1 (in duplicate).

5.3.3 Detection of Dominant Populations by DGGE

To overcome the shorcomings related to the conventional plating method, and to take account to the unculturable strains, DGGE was used for the generation of genetic fingerprints to provide information on the total community composition and diversity of aerobic granules and microorganisms. Figure 5.10 shows the representative DGGE profiles of amplified 16S rRNA genes from municipal activated sludge used for granule seed, bioaugmented biomass in R1, non-bioaugmented biomass in R2, and pure strain S35. Bacterial community diversity of biomass from both reactors was observed to be high when compared with the seed activated sludge, after cultivation with acetate for three days in SBRs. The result showed that aerobic granule can maintain a complex community diversity and structure under harsh environment. Furthermore, the composition of bacterial communities in both reactors after cultivation for 12 days was conserved to some extent, as revealed by similar DGGE band patterns.

However, two distinct bands were identified in the DGGE profile of experimental reactor R1 biomass. The band associated with strain S35 was observed to co-migrate with band in R1 biomass on day 3 and day 12, while no such observation was made in R2 biomass. It probably indicates that strain S35 embedded in local microbial community of activated sludge and persisted as a dominant strain during the cultivation of augmented granular sludge in R1.

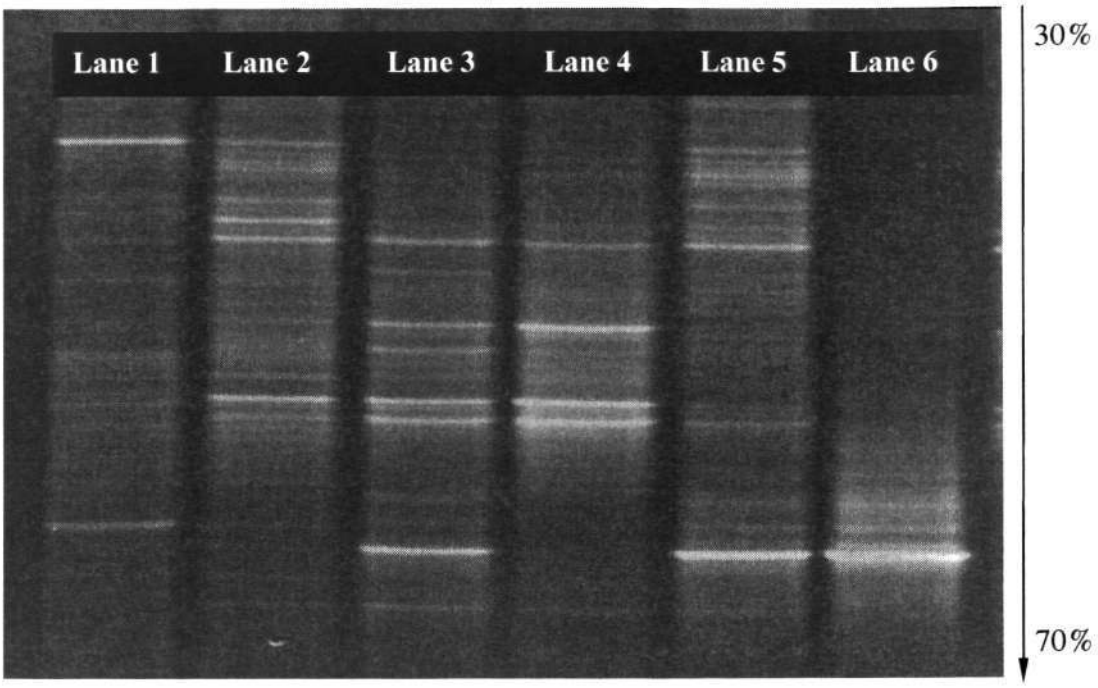

Figure 5.10 Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA fragments from experimental reactor R1 and control reactor R2. Lane 1: municipal activated sludge for granule seed; Lane 2: control reactor R2 on day 3; Lane 3: experimental reactor R1 on day 12; Lane 4: control reactor R2 on day 12; Lane 5: experimental reactor R1 on day 3; and Lane 6: strain S35.

5.4 DISCUSSIONS

5.4.1 Bioreactor Performance and Formation of Acetate-fed Aerobic Granules

After cultivation for two days with activated sludge augmented with 10% dry weight of pure culture S35, the SVI for R1 dropped dramatically from 230 to 83 ml g^{-1} SS, which was close to the typical SVI of mature granules. The SVI for R1 was further stabilized around 40 to 50 ml g^{-1} SS since day 5. In the meantime, the SVI

for R2 was around 150 ml g^{-1} SS at day one without bioaugmentation, and ranged in 86 to 111 ml g^{-1} SS from day 5 to day 9, which were higher than the SVI for R1 through the entire experiment since the first day of operation. SVI of sludge inoculated in R2 was around 150 ml g^{-1} SS. The biomass concentration in R1 initiated at 3.9 g l^{-1} and increased to approximately 5.4 g l^{-1} at day 9 after the bioaugmentation, while the biomass concentration in R2 decreased from 5.3 g l^{-1} at initial to 4.2 g l^{-1} after operation of nine days without augmentation of S35.

In comparison, the augmented reactor R1 possessed better settling ability, higher biomass retention and larger mean biomass size than those for control reactor R2, indicating the enhancement of granular aggregation with addition of S35. The microbial aggregates in wastewater treatment system can be classified as either flocs or granules differentiated by the morphologies and other properties. Flocs are in irregular shape with size normally smaller than 0.5 μm, while granules have compact and dense structure in certain defined shape (McSwain et al. 2005; Toh et al. 2003). The sludge volume index (SVI) of floc is normally above 120 ml g^{-1}, whereas the granules have better settling ability with SVI less than 50 to 80 ml g^{-1}. In this study, after five days of bioaugmentation, aerobic granules appeared in R1 based on the lower SVI of 41 ml g^{-1}, relatively higher biomass size of 436 μm and the morphological observation.

The operating strategies of step-wise increased hydraulic selection (i.e. incremental adjustments in settling time for biomass in both reactors) according to the biomass settling performance were applied to this study. Step-wise increment of either hydraulic selection or microbial selection was proved to improve the biomass settleability, enrich the slow-going microorganisms, as well as washing out non-aggregating bacteria, all of which will stimulate the early granulation and benefit stability of aerobic granules (Wang et al. 2007; Yi et al. 2006, Adav et al. 2009a). However, the step-wise increased hydraulic selection pressure is not capable of inducing the appearance of aerobic granules in five days of cultivation. Together with the augmentation of strain S35, which possessed autoaggregating and coaggregating abilities, the rapid transition and evolution of amorphous activated

sludge to compact structure was able to be seen in R1. As the result of the compact structure and good settling ability, more biomass could be retained to withstand the adverse environment, such as high shearing force or organic load fluctuations.

The results of this study showed that augmentation with a relatively small amount of S35 (initially 11% in dry weight) into municipal activated sludge can accelerate the formation of aerobic granules to less than five days, without a period of acclimatization before cultivation. No deterioration in aerobic granule properties or reactor performance was observed through augmentation of mono-strain when compared with augmentation with two taxonomically distinct strains (Ivanov et al. 2006). Therefore, bioaugmentation with mono-culture could be more economic and applicable in microbial manipulation for pilot-scale or full-scale wastewater treatment systems, though the optimized amount of bioaugmented S35 were unknown yet.

5.4.2 Selection of Augmented Culture

In the present study, the selection of augmented strains was judged on the basis of numerical abundance in aerobic granule community and its corresponding aggregating properties. S35 showed high numerical abundance, as well as relatively high autoaggregating and coaggregating ability in acetate-fed granules, therefore was selected for the bioaugmentation to accelerate and enhance development of aerobic granules fed by acetate.

The aggregates emerged rapidly in the activated sludge after addition of S35 for three days were probably due to it aggregating ability, especially the ability to autoaggregate. These microaggregates could be capable of providing a nucleus for attachment and embedment of other types of cells through coaggregation interactions among the microbial community. In this way, several strains that cannot coaggregate with each other would participate to form a multigeneric aggregate with the mediation of S35. Therefore the fast formation of microbial aggregates (Figure 5.3a) would subsequently facilitate both the initial aerobic

granulation process and the eventual appearance and further dominance of aerobic granules after five days.

In addition, it was also possible that the occurrence of S35 could improve the TOC removal efficiency through metabolic cooperation among S35 and some bacteria in activated sludge, as implied by significantly enhanced TOC removal efficiency for R1 after two days of bioaugmentation (Figure 5.5). Coaggregating interactions could facilitate the transfer of metabolic products by providing close proximity of coaggregating partners, as reported previously (Ishii et al. 2005). As a result, the development of the multi-species aerobic granule community was gradually altered by integral aggregation processes triggered by the presence of S35.

5.4.3 DGGE Detection and Microbial Diversity

From the comparison of denaturing gradient gel electrophoresis (DGGE) patterns of 16S rRNA genes of municipal activated sludge as bioseeds and SBRs biomass (both R1 and R2), a drastic increase of bacterial population abundance and rapid shift of community structure were observed in SBR community. The enhanced microbial diversity and bacterial abundance in granular aggregates are possibly associated with the compact structure and other properties of aerobic granules. The compact granule structures would facilitate the chemical gradients within the biomass due to mass transfer limitation, hence resulted in different chemical stratification and niches as the basis of complex spatial distribution of different bacterial populations (Meyer et al. 2003).

In this study, DGGE patterns revealed a dynamic change in the R1 microbial community structure from R2 since the bioaugmentation for three days. However, the DGGE patterns of the two reactors R1 and R2 evolved eventually to similar patterns, only differentiated by two extra distinct DGGE bands in R1 after bioaugmentation for 12 days. This suggests that the presence of several numerically significant bacterial populations and possible cell-cell interactions among them could evidently affect the integrated performance of the microbial community. The

augmentation of small amount of strain S35 into seed sludge was shown to be correlated to the alternation of community diversity, and possibly lead to changes of physiological traits of the R1 biomass and reactor performances.

5.5 SUMMARY

Effects of bioaugmentation of mono-culture S35 isolated from acetate-fed aerobic granules to accelerate aerobic granulation and enhance reactor performance were investigated in present study. The results showed that the bioaugmentation strategy succeeded in speeding up the aerobic granule formation and enhancing the reactor performance, evidenced by better granular sludge profile for bioaugmented biomass. Aerobic granules appeared in five days with augmentation of S35 (10% in dry weight) into activated sludge. Compared with previous studies on application of two strains in bioaugmentation, mono-culture S35 showed the similar effects on fast start-up of aerobic granules fed with benign substrate with comparably small amount of augmented strains. The co-culture bioaugmentation might be preferred to resistant toxic inhibition, such as in cultivation of phenol-degrading aerobic granules.

The strategy to select numerically abundant bacteria with relatively high autoaggregating and coaggregating abilities from pre-cultivated acetate-fed granules for the bioaugmentation of fast formed aerobic granules fed with the same substrate was investigated in this study. This appeared to be successful in the present experiment. The present study could help to broaden the knowledge of aerobic granulation technology by manipulation of microbial selection.

The monitoring of the microbial community adaptation by DGGE profile during experimental operation indicated that the augmented strain S35 was immobilized in the granules. The presence of S35 was related to the alternation of microbial diversity in aerobic granules, accompanied by different properties and better performance of augmentation biomass.

CHAPTER SIX
REAGGREGATION OF DISINTEGRATED AEROBIC GRANULES BY USING NOVEL AEROBIC GRANULAR ISOLATE

6.1 INTRODUCTION

The industrial feasibility of aerobic granulation for wastewater treatment gains increasing research interests and pilot reactors are currently designed and operated by using this environmental biotechnology (Inizan et al. 2005). As one of the major technical bottlenecks, the instability of aerobic granulation in industrial wastewater ambient demands special attention. Unstable aerobic granules are more susceptible to shear force, accompanied with deteriorated granule strength. Then disintegration of dense granule structure may subsequently occur, resulting in significant washout of biomass, serious impairment in biodegradation of target compounds, and eventual failure of granulation system. Therefore, the instability of aerobic granulation and the limitation on long term operation will significantly diminish the economic competitiveness of this technology in comparison of conventional activated sludge system (Inizan et al. 2005).

Some research attempts were made to improve aerobic granule stabilities and physical strength, e.g., optimization of the environmental conditions and the manipulation of hydraulic and microbial selection pressure (Liu et al. 2004b, 2006; Tay et al. 2001a; Wang et al. 2007). However, despite the control of operational parameters and nutrient supply, destabilization and loss of the aerobic granules still occurred after a period of operation (Inizan et al. 2005; Liu et al. 2006). The destabilization is thought to be resulting from fast-growth of aerobic granules. The consequent mass transfer limitation and the anaerobic fermentation products from anaerobic core inside the large-sized granules can explain the weak strength of aerobic granules (Liu et al. 2005b; Su et al. 2005). Furthermore, the strategy to

enhance aerobic granule stability by selecting the slow-going bacteria requires long cultivation time accordingly.

Severe economic loss will arise if the aerobic granulation fails suddenly during the consecutive wastewater treatment, especially in the industrial applications. Therefore, the recovery of disintegrated aerobic granules in terms of compact structure, good settling ability and high removal efficiency is crucial to aerobic granulation, when granule destabilization or lysis occur while other attempts fail. However, to date, the studies on reaggregation of disintegrated aerobic granules in SBRs still remain untouched and not well known yet.

Therefore, the present study aimed to investigate the feasibility to recover the destroyed aerobic granules by bioaugmentation of aggregating strain S15 that was isolated from aerobic granules as described in Chapter Four. Strain S15 was found to be dominant in both aerobic acetate-fed and phenol-degrading granules. The SBR process performance and microbial community dynamics were tracked.

The results demonstrated the feasibility to recover disintegrated granular sludge. It was shown that the augmented aggregating culture can enhance the reaggregation extent of disintegrated granular sludge. The accelerated reaggregation using bioaugmentation of aggregating bacteria as demonstrated in this study has important implications for the recovery of aerobic granulation system by the manipulation of microbial community. The works of this Chapter are expected to be useful for the deeper understanding of the role that aggregation can play in aerobic granulation process.

6.2 METHODS AND MATERIALS

6.2.1 Experimental Design and Set-up

Experiments were performed in two column-type sequencing batch reactors (SBR) R1 and R2, which were designed and operated as described in Chapter Three. Both

reactors were fed with acetate as sole carbon source by using synthetic wastewater solution as described in Chapter Five.

6.2.2 Seeding Biomass

Deteriorated and disintegrated aerobic granules precultured with acetate as sole carbon and energy source were collected from a 20 cm internal diameter SBR and stored in 4°C for 72 h before filling in experimental reactor R1 and control reactor R2. The non-bioaugmented reactor R2 was started with 500 ml of disintegrated granular sludge topped up to 800 ml mixed liquor with initiate biomass concentration of 5.49 g SS l^{-1}. The experimental process in R1 was seeded with 100 ml of seed culture S15 and 450 ml of disintegrated granular sludge to reach the mixed biomass concentration of 6.49 g SS l^{-1} (S15 was weighted 23.9% dry weight (w/w) of the mixed biomass). Culture S15 was previously isolated from acetate-fed aerobic granules as described in Chapter Four. S15 was cultivated in Nutrient Broth (Difco, Detroit, USA) at 25 °C for 96 h, and then harvested and washed twice in PBS buffer (pH=7.2), followed by resuspending in PBS buffer (pH=7.2). The final concentration of S15 suspension was measured to be 15.52 g SS l^{-1} before augmentation into experimental reactor R1.

6.2.3 Selection of Aerobic Granule Seeds

The autoaggregating strain S15 was gram-positive bacteria (Accession No. AY854022) isolated from acetate-fed granule, as described in Chapter Four. It was found to be dominant in both acetate-fed granule and phenol-degrading granule, as shown in Figure 6.1.

Figure 6.1 Denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA fragments from aerobic granules cultivated in previous experiment as described in Chapter Four. Lane 1: phenol-degrading granule seeded by acetate-fed granule; Lane 2: strain S15; and Lane 3: acetate-fed granule as bioseed for the cultivation of phenol-degrading granule

6.2.4 Analytical Methods

Measurements of pH value, total organic carbon (TOC), suspended solid (SS), volatile suspended solids (VSS), sludge volume index (SVI), granule size was as described in Chapter Five.

Extracellular polymeric substances (EPS) in the aerobic granules were extracted as described previously (Tay et al. 2005a). Approximately 0.5 g wet weight of fresh granules was suspended in a 2-ml tube with 1.5 ml of extraction solution containing 8.5% NaCl and 0.22% formaldehyde. The mixture was dispersed by a Mini-Beater (Biospec, Bartlesville, OK, USA) at 480 strokes min^{-1} for 5 minutes without glass beads, and then centrifuged at 12,000 rpm for 30 min in succession. The clear upper supernatant was then extracted for the measurement of carbohydrate content using phenol-sulfuric method (Dubois et al. 1956) and for the analysis of protein by the bicinchoninic acid protein assay (Stoscheck 1990).

6.2.5 Whole Cell Hydrophobicity

The cell hydrophobicity of granules was measured using microbial adhesion to hydrocarbon (MATH) method as described previously by Rosenberg et al. (Rosenberg 1980, 2006). The relative hydrophobicity of granules was expressed as the percentage migration of dispersed granule cells from an aqueous phase to a hydrocarbon phase after 15 min of partitioning. Hexadecane was used as the hydrophobic phase. Freshly collected aerobic granules were dispersed by using a mini-Beadbeater (Biospec, Bartlesville, OK, USA) without glass beads for 5 min at 420 stroke min^{-1}, and then washed twice with PBS. The dispersed granule cells were resuspended in the same solution and optical density at 600 nm (OD_{600}) was adjusted to 1.5-2.5. Four milliliters of dispersed granule suspension was mixed with 2 ml hexadecane. As the blank, 4 ml of PBS was added in place of the granule suspension to 4 ml hexadecane in a test tube simultaneously. The mixture was vortexed for 15 sec before incubation for 15 min without shaking, and the OD_{600} value was measured after incubation.

6.2.6 DNA Extraction and Denaturing Gradient Gel Electrophoresis (DGGE)

Approximately 200–300 mg (wet weight) of aerobic granule was harvested in duplicate in each reactor column, and used immediately for DNA extraction as described in Chapter Four. The DGGE-PCR amplification was performed as described in Chapter Four.

6.3 RESULTS

6.3.1 Selection of Bioaugmented Strain

S15 had a 94% identity to *Leadbetterella byssophila* strain 4M15 (accession No.: AY854022), which belonged to the *Cytophaga-Flavobacteria-Bacteroides* (CFB) group. S15 matched the closest identified strain with less than 97% identity, suggesting it to represent an unknown bacterial strain. Through the DGGE profiles, this strain was found to be dominant in both granule communities across the entire conversion process from acetate-fed granule to phenol-degrading granules. It showed good adaptation to chemical and toxic shock imposed by phenol and the COD loading fluctuation of nearly two fold increments. S15 also possessed capability of moderate autoaggregating in microbial community of acetate-fed granules.

Therefore, S15 was eligible for bioaugmentation to recover the disintegrated aerobic granular sludge, due to the aggregating ability it showed and the distinguished acclimatization to diverse operational conditions and chemical composition.

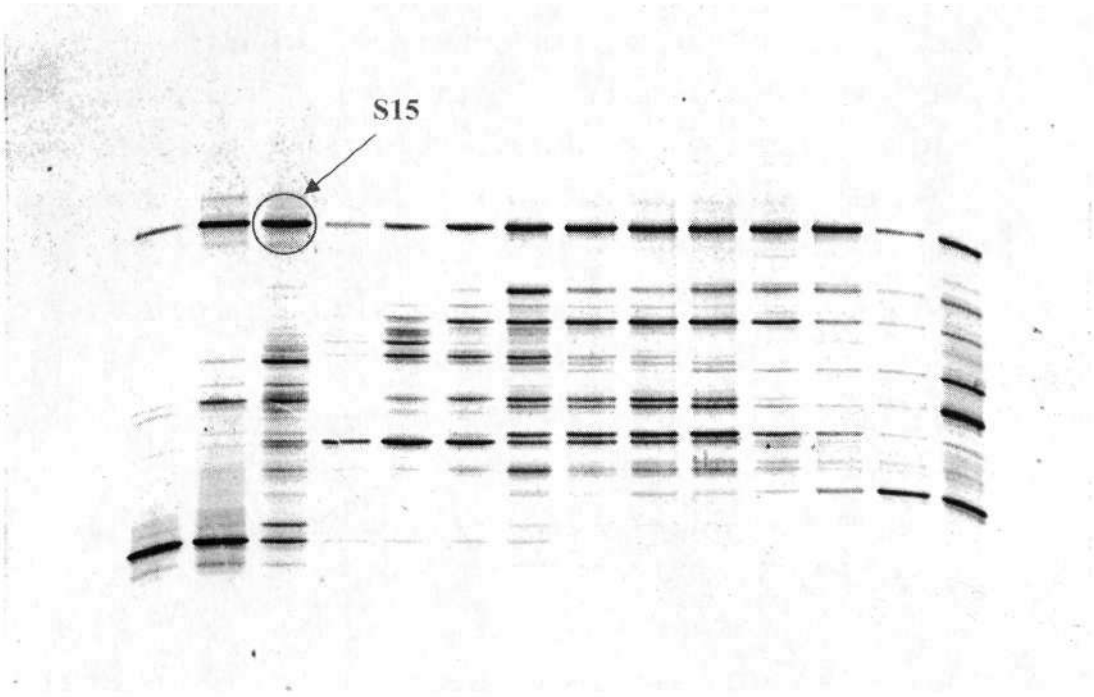

Figure 6.2 DGGE profiles of aerobic phenol-degrading granules with acetate-fed granules as seeds. Red circle: S15 as one of the dominant bacterial strains through the course of cultivation

6.3.2 Reactor Performance

The sludge volume index (SVI) of experimental reactor R1 was always lower than in control reactor R2 during the entire operation period since day 1, due to the addition of suspended strain S15 of 24% in dry weight. The SVI in the experimental reactor R1 experienced fast decrease and reached 77 ml g^{-1} after 7 days of cultivation, which was close to the typical SVI of mature granules (Figure 6.3). By the end of experiment at day 27, the SVI value had decreased to 32 ml g^{-1} SS in R1, indicating the predominance of fast-settling biomass, whereas SVI of the biomass in R2 stabilized around 54 - 69 ml g^{-1} since day 19.

The biomass retention in R1 and R2 are shown in Figure 6.4. Both reactors initiated with the same biomass concentration at 3.2 g l^{-1}. Thereafter, the rapid growth of

biomass was found in experimental reactor R1, together with the fast decrease of SVI. These evidences suggest the good adaptation of disintegrated granular sludge to the new operation environment in R1, with the presence of augmented strain S15. Throughout the whole experiment period, the R1 biomass concentration was continuously higher than that in R2, and reached 12.8 ± 1.6 g SS^{-1} at the end of the experiment, while the R2 biomass concentration maximized at 8.26 ± 0.1 g SS^{-1}.

The biomass size in R1 was noticed to be much bigger than the biomass size in R2 throughout the entire experiment. The average biomass size in R1 increased dramatically to 438 μm after 1 day of bioaugmentation, when the particle size averaged at 278 μm in control reactor R2. In R1, the average particle size ranged from 386 μm to 465 μm during the entire experiment, while the particle size fluctuated around 210 μm to 278 μm. Together with the observation that biomass concentration increased continuously and SVI decreased rapidly, the small fluctuation in biomass size in R1 indicated that the morphological characterization of R1 biomass changed from the granular sludge in form of bioflocci into the dense and compact structure without long period of transition. The similar tendency of biomass morphology from loosen biomss to compact aggregates could also be deduced from the comparable stabilization in biomass size and the improvement on biomass concentration and decrease in SVI. However, this morphological change on R1 biomass was expected to be more notable, as more significant change on SVI and biomass concentration, along with bigger biomass size was found in R1.

The COD removal efficiency of granular sludge in R1 was stable at 91-99% after 9 days of cultivation (Figure 6.6). The biomass in R2 was not able to degrade COD as efficiently as that in R1, with COD removal efficiency fluctuating in the first 19 days and stabilized around 90% since then.

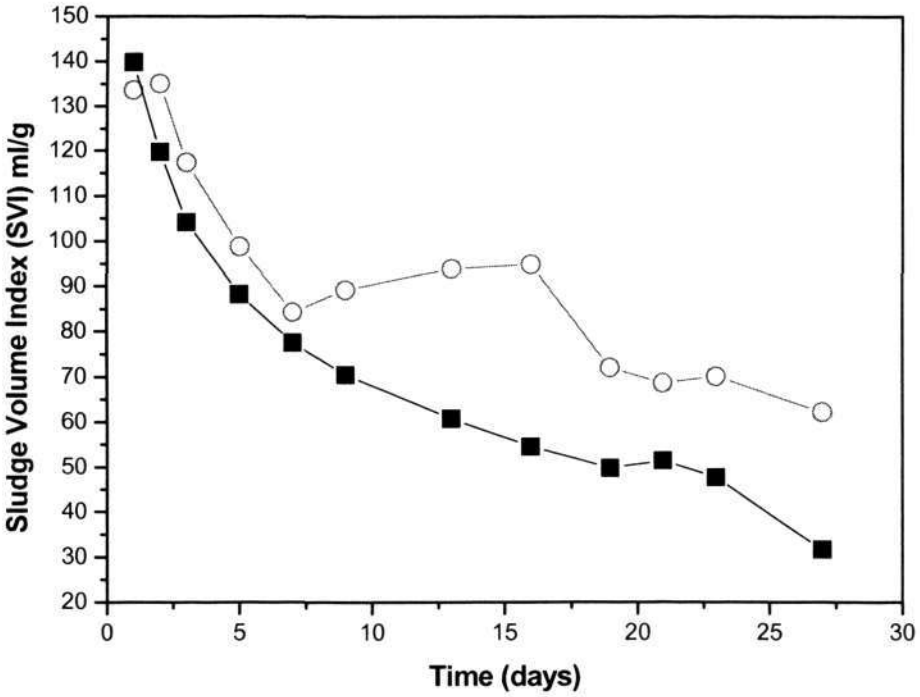

Figure 6.3 Time profiles of sludge volume index (SVI) in experimental reactor R1 (■) and control reactor R2 (○)

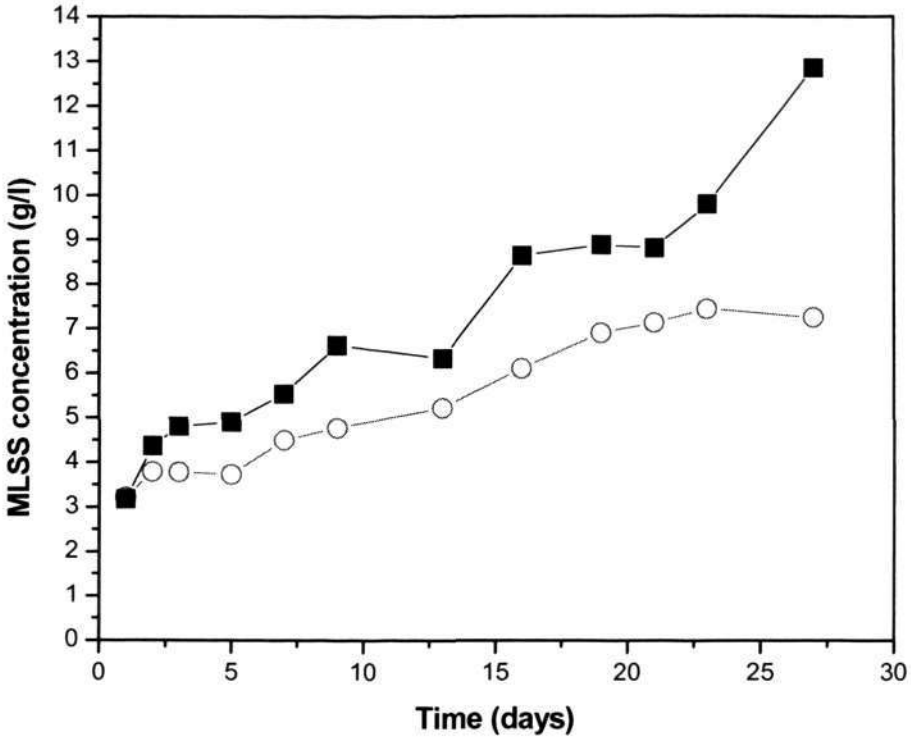

Figure 6.4 Time profiles of Biomass (MLSS) concentration in experimental reactor R1 (■) and control reactor R2 (○)

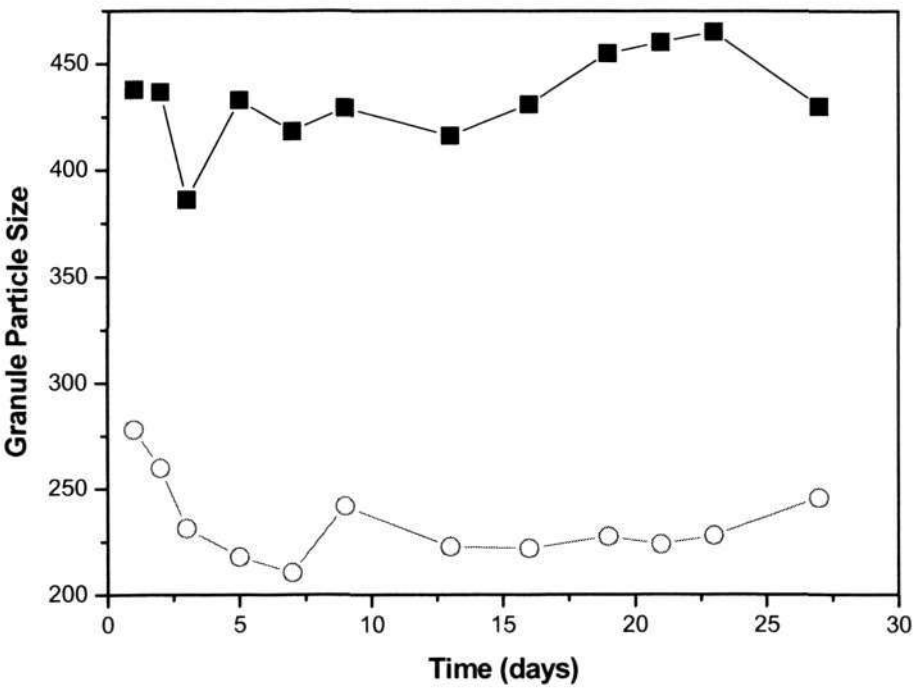

Figure 6.5 Time profiles of granule particle size in experimental reactor R1 (■) and control reactor R2 (○)

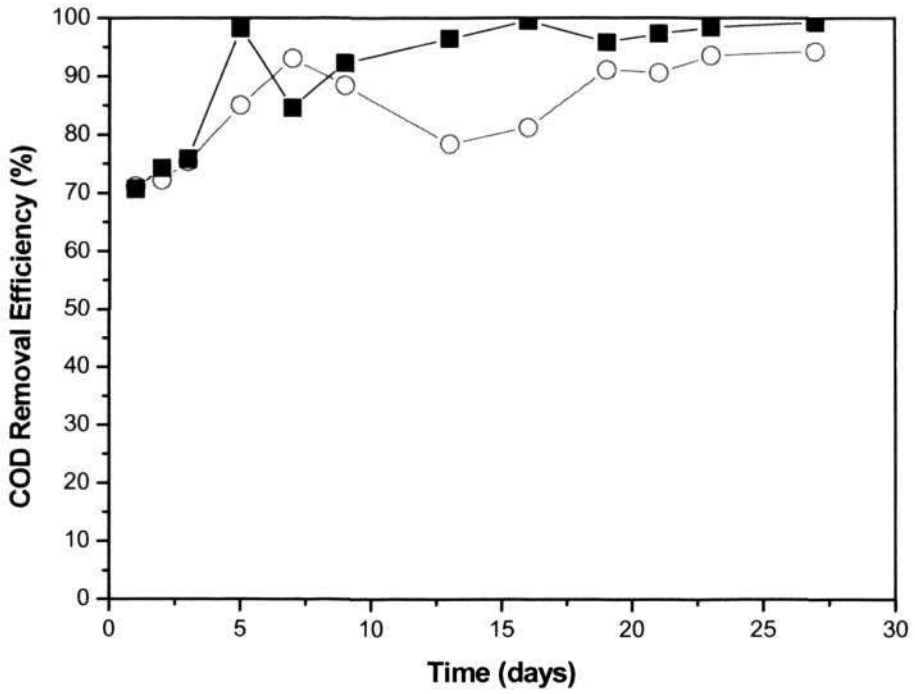

Figure 6.6 Time profiles of COD removal efficiency in experimental reactor R1 (■) and control reactor R2 (○)

6.3.3 Granule Characteristics

Figures 6.7 and 6.8 show the time profiles of extracellular polymeric substances (EPS) contents and compositions of the biomass in R1 and R2. In this study, EPS was examined with respect to the sum of polysaccharide (PS) and protein (PN). The amount of PS contents in both reactors R1 and R2 continued increasing and reached 3 to 7-fold higher after two weeks of cultivation. PS in R1 biomass ranged between 0.7 to 4.8 mg g^{-1} SS throughout the entire operation period, higher than the PS content in R2 between 0.4 to 2.7 mg g^{-1} SS. Compared with PS in both R1 and R2, PN contents were relatively higher. PN of R1 biomass ranged from 4.4 to 9.2 mg g^{-1} SS, slightly lower than PN in R2 that ranged from 4.5 to 15.5 mg g^{-1} SS.

The changes of PN and PS contents in both reactors resulted in sharp decrease of PN/PS ratio, despite the increase of total EPS contents (equals to PS+PN) in both reactors (Figure 6.8). The EPS for R1 was continuously lower than that in R2. The initial PN/PS ratios in R1 and R2 on day 1 were approximately 10 and 20 respectively. The PN/PS ratios for R1 were relatively lower when compared with that for R2. These ratios were stabilized at approximately 2 to 4 for R1 and 4 to 5 for R2, respectively, from day 13 to the end of the reactors operation.

As seen in Figure 6.9, the initial hydrophobicity in both reactors was around 38%. Then the cell hydrophobicity in R1 increased to 74% at day 13 and finally stabilized at around 64% after the formation of aerobic granules. The hydrophobicity in R2 varied between 38% and 55% during the experiment period. In comparison, the experimental reactor R1 had relatively lower cell hydrophobicity than that for R2 till day 13, then exceeded the hydrophobicity for R2 after the reformation of granules.

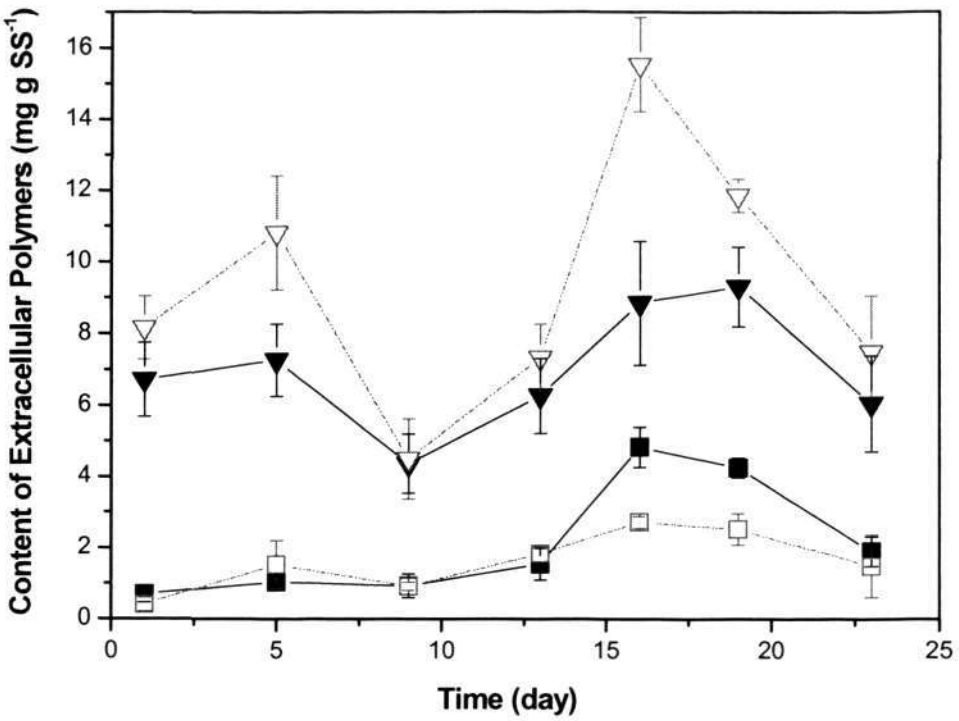

Figure 6.7 Time profiles of EPS contents in experimental reactor R1 and control reactor R2. PN content in R1 (▾), PN content in R2 (▽), PS content in R1 (■), PS content in R2 (□)

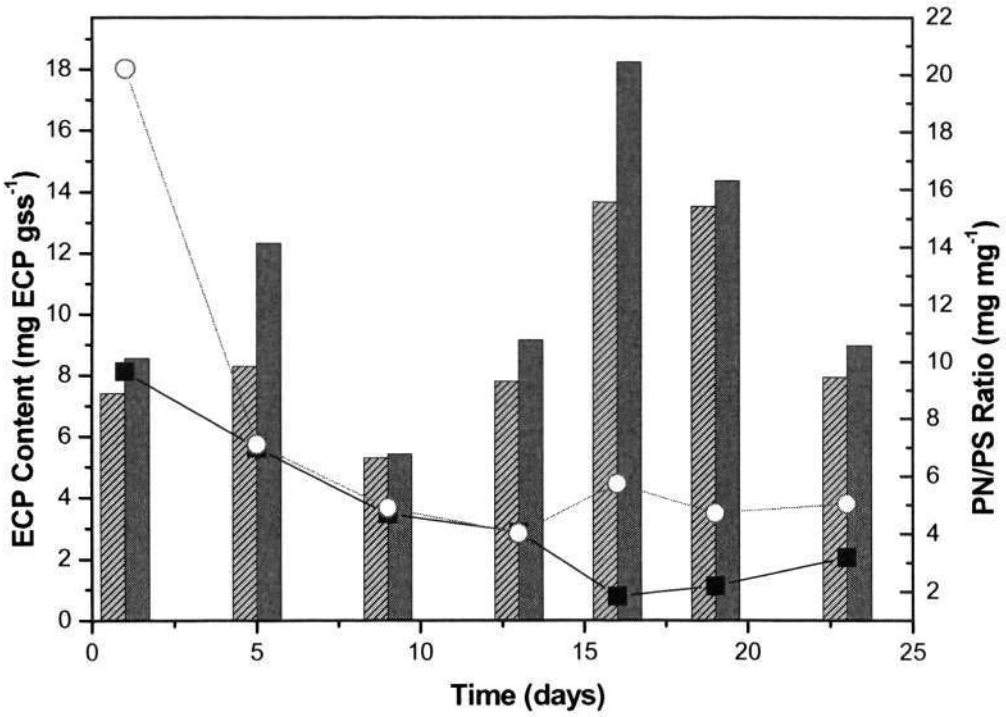

Figure 6.8 Time profiles of EPS compositions and contents in experimental reactor R1 and control reactor R2: PN/PS ratio in R1 (■), PN/PS ratio in R2 (○), EPS (PN+PS) content in R1 (□), and EPS (PN+PS) content in R2 (□)

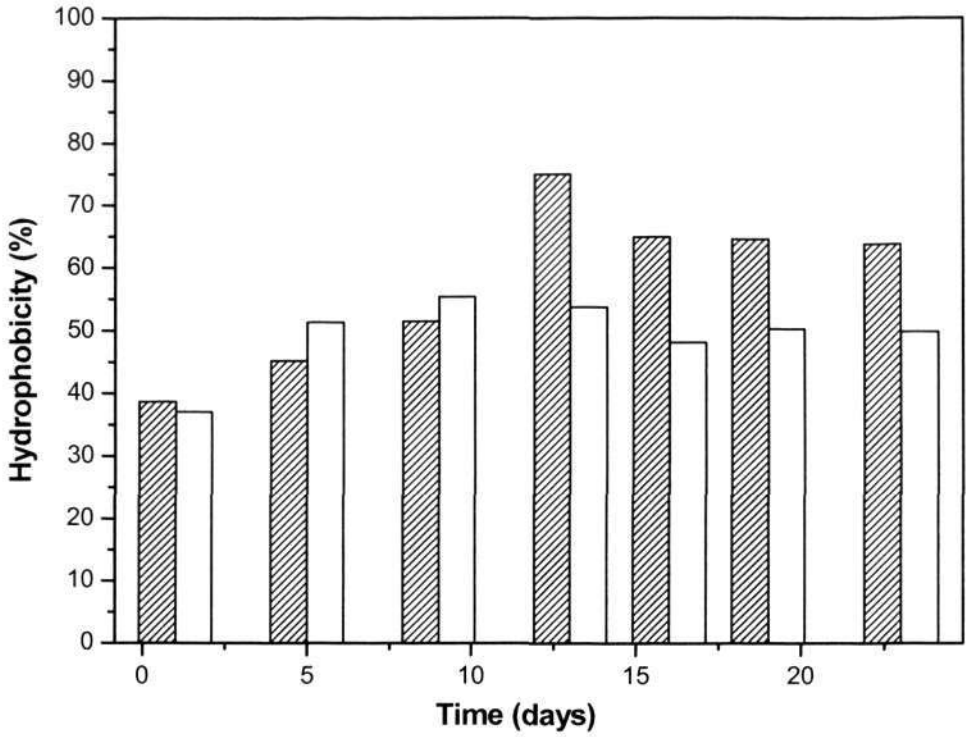

Figure 6.9 Time profiles of hydrophobicity in experimental reactor R1 (□) and control reactor R2 (□)

6.3.4 Microbiology Observation of Granules

Image analysis (IA) of the time dependant development of the reaggregated granules from disintegrated granular sludge is shown in Figure 6.10. Biomass in both reactors underwent different processes of development and formed into granular aggregates with different characterization. Granular flocs first appeared in augmented reactor R1 on day 1 of reactor operation, when irregular and loose form of sludge was observed in control reactor R2. On day 5, integrated granules and granular sludge started to coexist in reactor R1, when the mean biomass size exceeded 0.4 mm and the SVI value decreased to 88 ml g^{-1}. At the same time, biomass in R2 still appeared in form of loose sludge.

Granular aggregates arising in R1 on day 5 accompanied with flocculent bioflocs could also be confirmed by SEM observation (Fig 6.11), whereas only sludge could be found in R2. Dense and compact granules were recovered in experimental reactor R1 after two weeks. Finally, compact granules with regular spherical and clear-cut shape replaced the bioflocci and dominated in R1 on day 19, with average biomass size of 450 μm as observed by IA (Figure 6.9). Meanwhile, small granular aggregates co-existed with flocci in control reactor R2, reaching mean biomass size of approximately 270 μm.

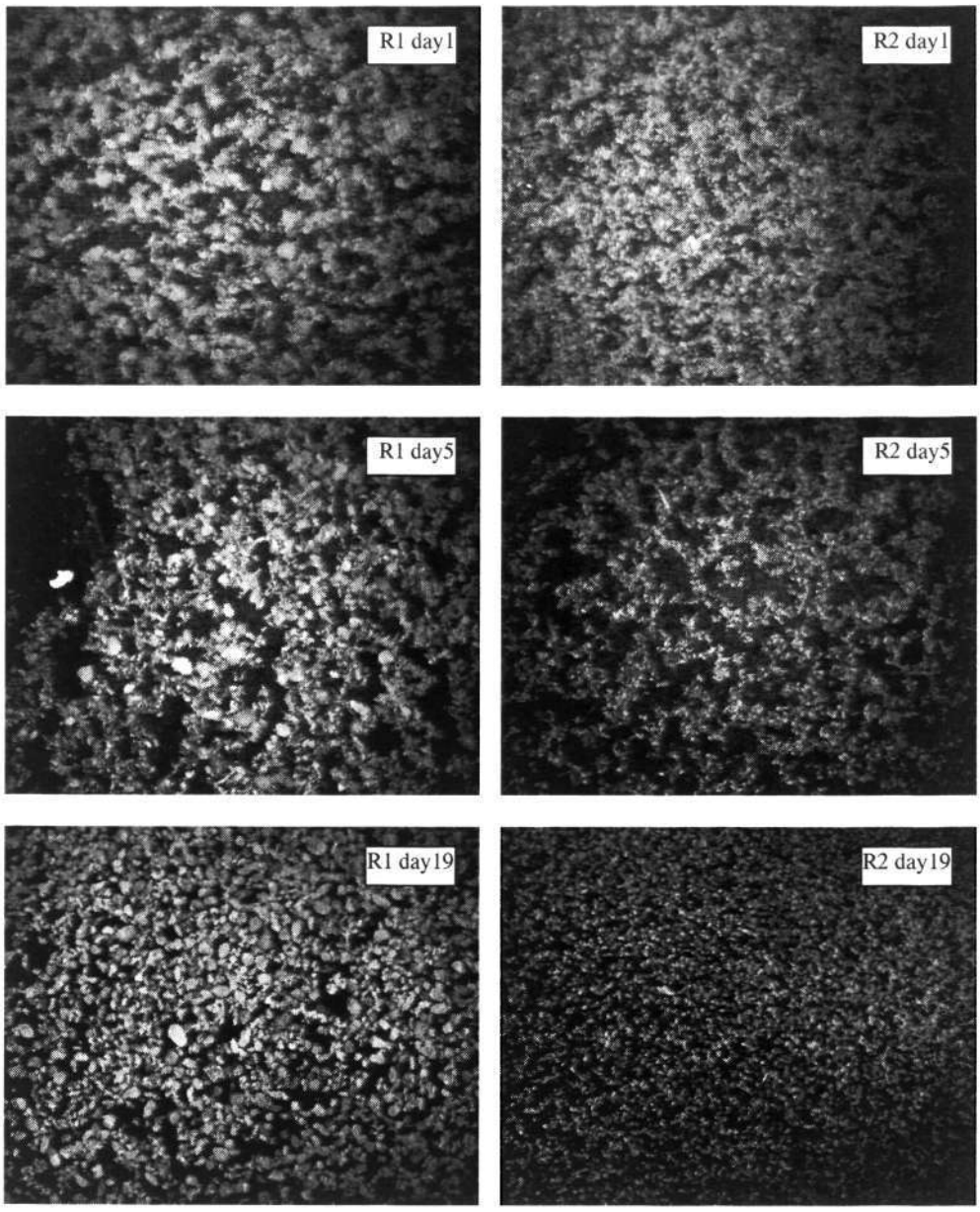

Figure 6.10 Morphological changes of aerobic granules in control reactor R1 and experimental reactor R2

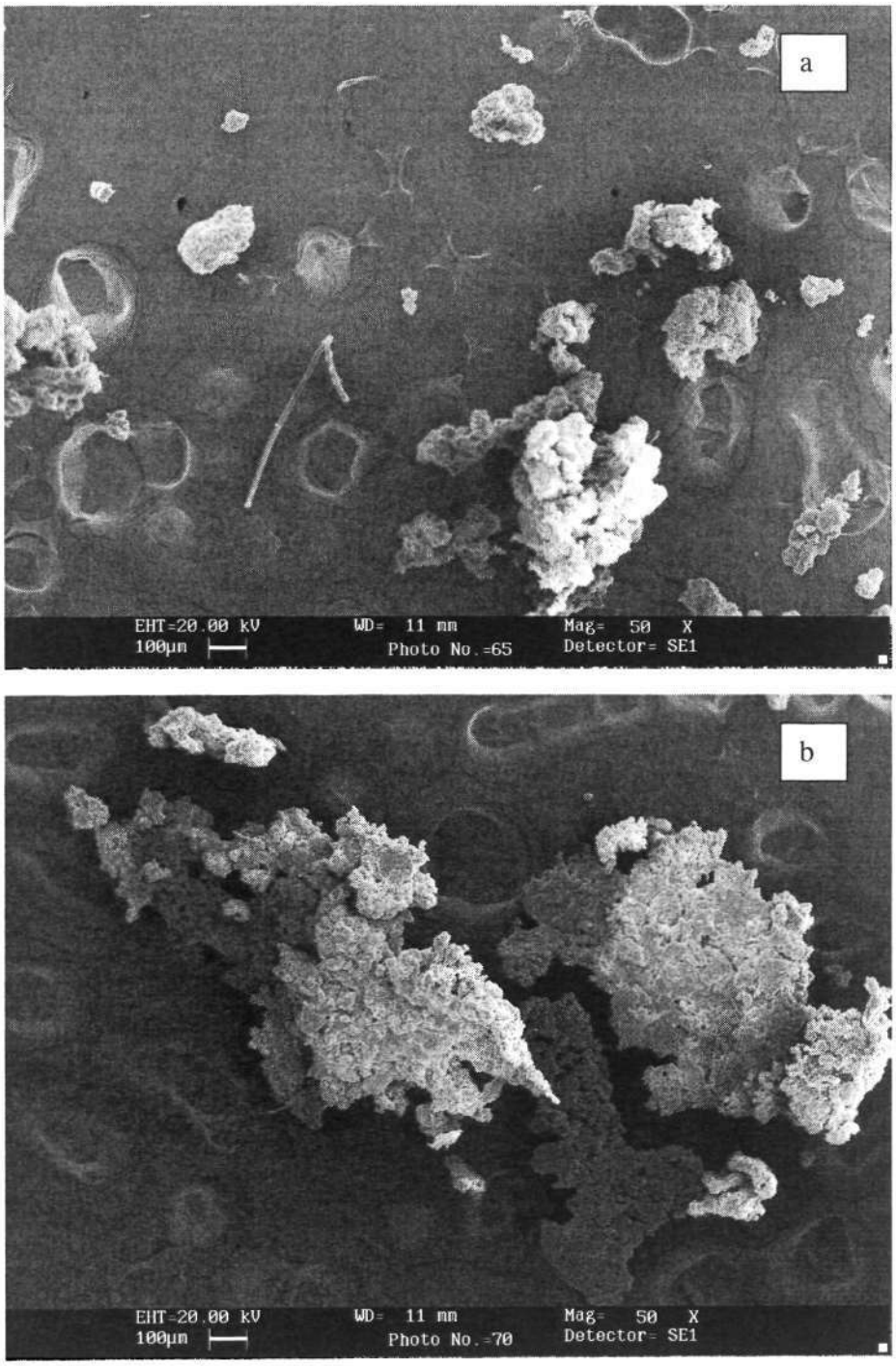

Figure 6.11 Scanning electron micrographs of biomass on day 5 in: (a) experimental reactor R1; and (b) control reactor R2

6.3.5 Detection of Dominant Populations by DGGE

Figure 6.12 shows the denaturing gradient gel electrophoresis (DGGE) fingerprints of PCR-amplified 16S rRNA fragments from biomass in R1 and R2 through the entire experiment period. The DGGE band patterns showed to be slightly different in R1 and R2 since the first day of bioaugmentation. The presence of band associated with S15 in the bacterial community of experimental reactor R1 was concurrent with alternative density of other bands, when compared with the DGGE fingerprints of R2. Although the strength of band associated with S15 declined to under the detection limit after five days of bioaugmentation, the R1 DGGE bands pattern continued to be diverse from the R2. This implies that the existence of S15 had long-term effect on the composition of microbial community, thus further affecting the development of bacterial community structure.

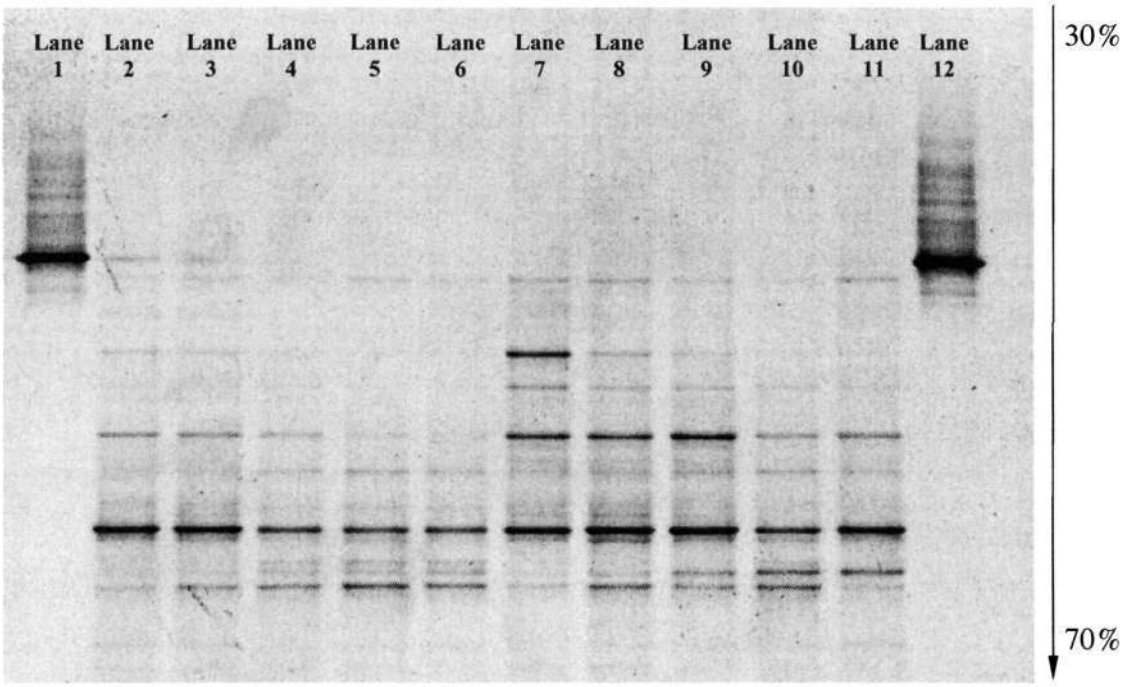

Figure 6.12 An ethidium bromide-stained 10% polyacrylamide denaturing gradient gel (30% to 70%) with DGGE fingerprints of PCR-amplified 16S rRNA fragments after PCR amplification of nucleic acids from inoculated strain S15, biomass taken from experimental reactor R1 and control reactor R2. Lane 1 and Lane 12: isolate S15; Lane 2, 3, 4, 5, 6: experimental reactor R1 on day 1, day 5, day 13, day 19, day23; Lane 7, 8, 9, 10, 11: control reactor R2 on day 1, day 5, day 13, day 19, day23

6.4 DISCUSSION

6.4.1 Reactor Performance

Microbial aggregates were reported to break up and reaggregate in an almost reversible manner under cycled-shear conditions (Chaignona et al. 2002), and the aggregates formation rate and corresponding biomass size are the result of equilibrium between aggregation and disaggregation (Jarvis et al. 2005). Though the formation and stability of aerobic granules are also regarded as the result of dynamic growth and decay equilibrium of biomass in the reactor, the destruction of aerobic granules could occur after a period of operation time and poor-settling biomass was severely washed out resulting in eventual failure. It seems that the reaggregation of biomass and achievement of a new equilibrium before the complete washing out of biomass are not naturally occurring in reactor without triggers, such as hydraulic selection pressure, or microbial selection. In Chapter Five, the fast formation and enhanced performance of aerobic granules was investigated by augmentation of an aggregating strain S35, which was isolated previously from granules. In this study, the efforts to accelerate the reaggregation of disintegrated biogranular sludge and to improve the aerobic granulation with addition of S15 were studied.

The characteristics of granular sludge in both reactors (R1 and R2) improved after cultivation in laboratory scale SBR reactor with altered operational factors of aeration rate, organic loading rate, hydraulic retention time (HRT), and settling time that were comparable to other SBRs as reported previously by Tay et al. (Tay et al. 2002b, 2003). In the present study, the reaggregation of disintegrated granular sludge in both reactors responded in similar way, however, to different extents. The reaggregation of biomass taking place in bioaugmented reactor R1 overcame control reactor R2, with evidence in granular sludge size, settling ability, COD removal efficiency, and morphological observations. This accelerated reaggregation of aerobic granular sludge with bioaugmentation of a granule aggregating bacteria has not been reported elsewhere.

In this study, the granular sludge properties in both R1 and R2 were recovered, although to different extents, along with boosted hydrodynamic turbulence and aeration transfer in SBRs of 5-cm internal diameter, when compared with cultivation in a 20-cm internal diameter reactor. This phenomenon was possibly associated with the enhanced supply of aeration, which was found to promote the reflocculation of activated sludge (Zhu 2003). However, the disintegrated granular sludge resulted from oxygen depletion was only reaggregated to a certain extent after introduction of strong aeration in R2, evidenced by relatively poor characteristics in term of biomass size, settling ability and so on. The result implied an irreversible damage occurred on granular sludge once disintegrated, which was also observed in activated sludge previously (Wilen et al. 2000).

Meanwhile, the better recovery of disintegrated granule was found in R1 with the presence of augmented S15. The dramatic increase of R1 biomass size within one day after bioaugmentation was probably attributed to the bioflocs formed through the facilitation of autoaggregating strain S15, as observed by IA. The autoaggregation of S15 and interactions with other community microbial members are likely to have important ecological role for the integral development and maintenance for granular communities, considering the fast formation of big sized bioflocs and the persistence of S15 in both acetate-fed granules and phenol-degrading granules (as discussed in Chapter Four). The microbial aggregates formed after addition of S15 were capable of providing precursors for attachment of other cells, which might explain the fast transmission of loose flocs into dense granule structures, as little fluctuation on the particle size was found since day 1 while SVI and other properties improved gradually through the entire experiment period.

6.4.2 Biomass Characteristics

EPS compositions and contents varied in R1 and R2 biomass along with the different reactor performances. EPS was believed to facilitate the cell-to-cell adhesion or adherence of bacteria to microbial aggregates surface and serve as

diffusion limitation barrier between toxicants and microorganisms in activated sludge and aerobic granules (Wingender et al. 1999a, 1999b), thus contributing to the aggregate stability (Liu et al. 2002a). It was observed that deflocculation caused by oxygen limitation in activated sludge was in association with inhibition of EPS production and possible EPS hydrolysis and degradation (Nielsen et al. 1996). However, no reverse relationships of EPS contents and reaggregation of granular sludge could be found in both R1 and R2, based on the experimental results in this study. This suggests that the total content of EPS has no direct correlation with the reaggregation of granular sludge. In the present study, both R1 and R2 demonstrated much higher amount of PN than PS, which was consistent with the results of Adav et al. (2007b), McSwain et al. (2005), and Chen et al. (2007). The results of EPS compositions in present study and above reports differed from previous investigations on aerobic granules and biofilms, which described the production of PS as a main component in EPS (Tsuneda et al. 2003).

In this study, the composition of EPS with respect to PN/PS ratio was found to be associated with the reaggregation extent of disintegrated granular sludge and reactor performance. On the first day after bioaugmentation with S15, the PN/PS ratio in bioaugmented reactor R1 was more than 2-fold lower than that in R2. Throughout the entire experiment operation, R1 was found with lower amount of PN, and comparably lower PN/PS ratio than those in R2, except for day 5 and day 13, when the comparable PN/PS ratios were found along with the observation of transition process occurred from mixture of biofloc and sludge to granule. Furthermore, PN/PS ratio in both R1 and R2 decreased sharply, accompanied with improvement of sludge properties, when the disintegrated sludge were transferred to small-column SBRs with higher agitation.

These collective results suggested that the low PN/PS ratio and lower PN content favored the reaggregation of aerobic granular sludge with good properties. The interaction and proportion between PS, PN and other macromolecules were revealed to determine the stability of the matrix (Sutherland 2001). The low PN/PS ratios were found to benefit the sludge floc stability (Sheng et al. 2006), and be

detected in aerobic granules in comparison with the loose and amorphour bioflocs (Tay et al. 2004b).

The content of EPS was observed to differ in quantity and character as a result of numerous environmental factors (Di Iaconi et al. 2005; McSwain et al. 2005; Sheng et al. 2006). As the operational parameters were uniform in R1 and R2, the presence of S15 was the only explanation for the difference of EPS composition. Microorganisms are found to regulate EPS synthesis and the matrix composition, therefore to alter their aggregating ability as a microbial response to the environment stress (Ahimou et al. 2007). The finding in this study that addition of coaggregator S15 associated with the increased portion of polysaccharide in total EPS suggests an alternative method to enhance the stability of aerobic granules through the manipulation of microbial community composition.

In this study, comparably high cell hydrophobicity was observed in R1 to be correlated to the dominance of well settled granules since bioaugmentation for thirteen days. Though the detailed role of cell hydrophobicity in formation of aerobic granules was still under investigation, increasing evidences show positive role of high hydrophobicity in improvement of aerobic granule settleability and formation (Liu et al. 2003a, 2004c). Cell hydrophobicity was found of a prime responsibility for cell attachment. A high hydrophobicity of cells would result in strong cell-to-cell interactions and further favor the formation of dense structure of aggregates. Cell hydrophobicity was reported previously to be induced by high shear force or periodical starvation (Tay et al. 2001a).

6.4.3 DGGE Detection and Community Diversity

The formation of aerobic granules is a gradual process through acclimation of bacterial populations, rather than instant change from loosen sludge into compact and dense aerobic granular structures (Liu et al. 2004b), despite the time for aerobic granulation as short as several days (Ivanov et al. 2006). In this study, however, instant increase of granular aggregates size was observed within one day after the

addition of S15. The size was maintained at the comparable level during the experiment period, though the co-existence of granule aggregates and porous granular sludge was replaced by the clear-cutting and compact granules eventually, resulting in an even size distribution.

The fast conglutination of microaggregates among disintegrated granular sludge after addition of S15 suggests the immediate cell-cell interactions or other mechanisms such as the change of EPS compositions triggered by augmentation of S15. The conglutination could establish the proximity of participant partners for aggregating interactions, and result in higher chance for exchange of nutrients and removal or distributions of metabolic products in existing granular sludge (Ishii et al. 2005), which further benefit the reaggregation of disintegrated granular sludge. The microniches formed through the cell-cell interactions could serve the habitation, protection and proliferation of other functional important bacteria.

The microbial community in disintegrated granules seemed to have relatively high potential for microbial aggregation when compared with common activated sludge. The fast elimination of band associated with S15 in the DGGE profile was observed, indicating that S15 was not detected as a dominant strain by DGGE profile after augmentation for five days. However, the trend of granulation in R1 was not obstructed or reversed by the decline of augmented S15, implying the strong recovery ability of disintegrated granular community with the trigger of S15.

The long-term survival of augmented strains was previously regarded as one of the bottlenecks in bioaugmentation processes (Akkermans et al. 1994; Fantroussi 1999, Fantroussi et al. 1999). However, in this study, the whole system performance showed to be independent on the long-term survival of the augmented strain S15. This implies that the elimination and eventually loss of abundant dominance for augmented strains in indigenous bacterial community does not necessarily indicate the failure of bioaugmentation. The similar phenomena were also reported in the formation of phenol-degrading granules (Jiang et al. 2006). This might be due to the different function of augmented strains in microbial communities, such as acting as

the primary colonizer or as physiological bridge to promote formation of microniches which facilitate the development of other bacteria (Rickard et al. 2003). The DGGE results in current study and findings from Jiang's research may suggest that the enhanced performance is not necessarily due to the shifts in community composition, but in the existing population metabolism after the augmentation. In this experiment, operational parameters and external conditions in both R1 and R2 were comparable to most of other aerobic granulation systems in SBRs (Liu et al. 2004a; Moy et al. 2002; Tay et al. 2002a). The two reactors only differentiated in the presence of augmented strain S15 with moderate autoaggregating and coaggregating ability in disintegrated granular sludge. The addition of this strain S15 was demonstrated to significantly accelerate and enhance the reaggregation of disintegrated granular sludge to form dense and compact structure with excellent settling ability within 13 days. The study of bioaugmentation with autoaggregating granule isolate in the role of recovery of disintegrated granular sludge may provide critical information for the understanding of reaggregation of granular sludge.

6.5 SUMMARY

This study showed that the reaggregation of disintegrated granular sludge could be accelerated and significantly enhanced through the addition of strain S15 which was dominated in both acetate-fed granules and phenol-degrading granule as described in Chapter Four. The strengthen aeration supply and shear force in 5-cm inner diameter SBRs can initiate the reaggregation of granular sludge in both R1 (with augmentation of strain S15) and R2 (without augmentation of strain S15). However, the extent of reaggregation of granules was enhanced significantly in R1 with evidence of better granule characterizations and system performance.

The EPS contents were found to be not directly correlated to the evolution of disintegrated sludge into compact granules. Meanwhile, the low PN content and low PN/PS ratio was shown to favor the granule reaggregation. The higher level of whole cell hydrophobicity was also observed to accompany elevated reaggregation of granular sludge in R1. The observation of this study was in line with the previous findings that bacteria could respond to sophisticated integrated intercellular signals

by altering the aggregating performance and result in changes of cell morphology, surface hydrophobicity or EPS production (Gurdon et al. 2001; Wirgant 1988).

Although the elimination of augmented inocula S15 in microbial community of R1 was observed though the monitoring on time-dependent DGGE profile, the better reactor performance and higher reaggregation extent of biomass in R1 was independent on the long-term survival of S15. It implied that the microbial community in disintegrated granules has relatively high potential for microbial aggregations when compared with common activated sludge as the bioseed for granulation.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

Aerobic granulation is a relatively new form of cell immobilization technology that is attracting considerable research attention. Aerobic granules are self-immobilized aggregates of microorganisms formed in sequencing batch reactors (SBRs). Unlike activated sludge flocs, microbial granules have a well defined appearance and are still visible as separate entities after settling. Extensive studies have been done on aerobic granulation. However, the underlying molecular mechanisms are still unclear, such as the role of cell-cell aggregating interactions (including autoaggregation and coaggregation) in aerobic granulation.

The results in this study demonstrated that the aggregating interactions are important in the formation and integration of aerobic granules. Strong autoaggregating and coaggregating bacteria inhabit mature aerobic granules. However, for the formation of microaggregates in flow environment developed from dispersed aerobic granules, coaggregation among large fraction of free cells with mild autoaggregating ability was preferred.

From the inhibition testings EDTA, electrolytes, simple sugar, heat treatment and pH value on two selected coaggregating pairs partnered by granule isolates B6-25 with B7-8 and B6-25 with B6-2, it was found that both pairs were sensitive to electrolyte concentration and extreme pH, and were slightly inhibited by the presence of EDTA. The inhibition of glucose on coaggregating pair between B6-25 and B7-8

implicated the occurrence of lectin-saccharide interactions for coaggregation in aerobic granule bacterial community.

The SBR performance of phenol-degrading granules by using pre-cultured acetate-fed granules as seed was studied. The acetate-fed granule community adapted to phenol as sole carbon source in four days, implied that the strategy to add benign substrates as co-substrate was not necessary for the adaptation of mature aerobic granule to high phenol loading.

The aggregating populations in activated sludge, acetate-fed granules and phenol-degrading granules were screened. The aerobic granules appeared to own much more microbial community members involved in cell-cell aggregations, possibly due to the high hydraulic selective pressures in SBRs, when compared with activated sludge. Among these three microbial communities, phenol-degrading granules had the highest percentage of coaggregating populations. It was demonstrated that coaggregating interactions were boosted as the community response to the combined effect of high hydraulic pressure and phenol toxicity. As a result, a more complex and integral multi-species community with high taxonomical diversity were developed in phenol-degrading granules, in comparison to acetate-fed granules.

Granule isolate S35 possessing both autoaggregating and coaggregating capability was successfully bioaugmented into activated sludge to accelerate the formation of aerobic granules in three days with enhanced reactor performance. The bioaugmentation with monoculture was demonstrated to be feasible for aerobic granulation. The results of this study deepen the understanding on the role of aggregating interaction during the initial development stage of aerobic granules, and

provide information on manipulation of the aerobic granule community with the aid of aggregating bacteria.

Augmentation of autoaggregating granule isolate S15 was shown to facilitate and hasten the reaggregation of disintegrated aerobic granules with improved reactor performance. The disintegrated granular sludge community demonstrated high potential to recover with the trigger provided by S15 presence. Though the elimination of S15 abundance in the community occurred after five days of bioaugmentation, the reaggregating process of disintegrated granular sludge continued while aerobic granules appeared in day 5. Therefore aggregating interactions seemed to be an integral part of aerobic granulation. With the facilitation of exotic aggregating bacteria, the microbial community of disintegrated granular sludge would recover quickly.

7.2 RECOMMENDATIONS FOR FUTURE WORK

This study demonstrated that the higher percentage of microbial populations was involved into aggregating interactions among cells in aerobic granules compared with activated sludge, implemented with the finding that the microbial community presented more complicated cell-cell interactions when exposed to a more adverse environment. Application of these aggregating bacteria isolated from aerobic granule into fast formation of benign substrate fed granules and accelerated reformation of disintegrated granular sludge were also studied. The reactor performance was demonstrated to be enhanced by the bioaugmentation strategy.

Based on the current findings of this study, the following aspects would be recommended to be further conducted in the following aspects:

- the physiology and kinetic characteristics of these two isolates needs to be

further studied to better understand these strains' roles in aerobic granulations. Moreover, the mechanisms behind bioaugmentation of S15 and S35 require further investigation.

- The effect of the amount and the inoculation manner of S15 and S35 are to be established for the better manipulation of aerobic granulation. The optimization of the operational parameters is also desired, for the improvement of system operations and more economical cost.

- The detailed study of behavior of these augmented strains as a member of multi-species community can be further determined by *in situ* studies.

- The impact of augmented strains on the formation and reaggregation of aerobic granules fed with toxic and recalcitrant substrate deserve further investigation.

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