Insights into mechanism of aerobic granulation in sequencing batch reactor

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Insights into Mechanism of Aerobic Granulation in Sequencing Batch Reactor

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ABSTRACT

Aerobic granulation is a novel environmental biotechnology recently developed for treating a wide spectrum of wastewater. Although extensive research effort has been dedicated to the development of aerobic granules in sequencing batch reactor (SBR), the mechanism responsible for aerobic granulation still remained unclear. Thus, this study attempted to address a basic question of what were the potential driving forces of aerobic granulation in SBR.

In the first phase of the study, four SBRs were operated at different volume exchange ratios of 20 to 80%. Results showed that a rapid aerobic granulation could be achieved in the SBR run at a high volume exchange ratio, and the characteristics of mature aerobic granules in terms of mean size, sludge volume index (SVI), granule fraction, extracellular polysacchrides (EPS) and calcium content were all closely related to the volume exchange ratio applied to SBR. To interpret the role of the volume exchange ratio in aerobic granulation, a concept of minimum settling velocity of bioparticles, which is a function of both settling time and volume exchange ratio, was proposed. It was further demonstrated that the effect of volume exchange ratio on aerobic granulation would be realized through selecting the minimum settling velocity of bioparticles. In fact, the minimum settling velocity can serve as a major selection pressure exerted on bioparticles.

In the second phase of the study, four SBRs were run at respective discharge time of 5, 10, 15 and 20 min in order to look into the effect of discharge time on aerobic granulation. It was shown that the discharge time would be a key parameter influencing aerobic granulation in SBR. A prolonged discharge time would result in a failure of aerobic granulation even though both settling time and volume exchange ratio were controlled properly. It appeared that a relatively long discharge time of 20 min could unfavour aerobic granulation, i.e. a reduced cell surface hydrophobicity and EPS production were observed.

In order to interpret the roles of various driving forces of aerobic granulation in SBR, a unified selection pressure theory was thus developed in this study. This theory showed that all driving forces of aerobic granules that have been identified so far (e.g. settling time, volume exchange ratio and discharge time) can be unified into one single parameter, i.e. minimum settling velocity of bioparticles. It was further demonstrated that aerobic granulation in SBR would be mainly determined by the magnitude of the minimum settling velocity applied, e.g. successful aerobic granulation would be achieved at a minimum settling velocity no smaller than 8 m h⁻¹. The proposed selection pressure theory indeed can provide a useful guideline for upscaling, manipulating and optimizing aerobic granular sludge in SBR.

In the third phase of the study, the roles of EPS and hydrophobicity in maintaining the structure and stability of aerobic granule were investigated. It was found that aerobic granule had a heterogeneous structure, i.e. an outer shell with high biomass density and an inner core having a low biomass density. Results further showed the outer shell of aerobic granule was composed of poorly soluble and non-easily biodegradable EPS, whereas its core part was filled with soluble and readily biodegradable EPS. Meanwhile, the shell of aerobic granule exhibited a high hydrophobicity as compared to the granule core. It is a reasonable consideration that the insoluble EPS present in the granule shell would play a protective role with respect to the structure stability and integrity of aerobic granule.

The biodegradability of EPS produced by aerobic granules was studied in the last phase of the study. Results showed that about 50% of EPS produced by aerobic granules would be readily biodegradable and could be utilized by their producers, i.e. aerobic granules under starvation condition. Kinetic analysis further revealed that the utilization rate of the biodegradable EPS was 5 times lower than that of acetate, but 50 times higher than that of those non-readily biodegradable EPS. Consequently, it seems that a large fraction of EPS produced could serve as the energy reservoir for aerobic granules which can be used under starvation condition.

Keywords: aerobic granulation, selection pressure, volume exchange ratio, discharge time, EPS, hydrophobicity, biodegradability

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LIST OF ABBREVIATIONS

APHA	American public health association
COD	chemical oxygen demand (mg L^{-1})
DO	dissolved oxygen (mg L^{-1})
ECP	extracellular polymers
EPS	extracellular polysaccharides
HRT	hydraulic retention time (hour or day)
IA	image analysis
MATH	microbial adherence to hydrocarbon
OLR	organic loading rate ($\text{kg COD m}^{-3} \text{ d}^{-1}$)
OUR	oxygen utilization rate ($\text{mg O}_2 \text{ h}^{-1}$)
SBR	sequencing batch reactor
SEM	scanning electron microscope
SLR	sludge loading rate ($\text{kg COD kg}^{-1} \text{ SS d}^{-1}$)
SOUR	specific oxygen utilization rate ($\text{mg O}_2 \text{ g}^{-1} \text{ VS h}^{-1}$)
SRT	sludge retention time (h)
SS	suspended solids (mg L^{-1})
SVI	sludge volume index (mL g^{-1})
UASB	upflow anaerobic sludge blanket
VS	volatile solid (mg L^{-1})

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Present wastewater treatment plant highly demands high performance, stable and cost-effective process, which is capable of handling not only organic loadings, but also nutrients and toxic compound. Aerobic granulation technology offers the possibility to meet all of those requirements in one process.

Aerobic granulation technology is a recently developed novel wastewater treatment process. It exhibits many advantages over conventional activated sludge systems (Lin et al. 2003; Liu et al. 2003a; Tay et al. 2003a, 2004a; Yang et al. 2003). The highly organized microbial structure of aerobic granules offers high biomass concentration to aerobic granular sludge SBR, which enables it to handle high organic or toxic loading rates (Liu et al. 2003a; Tay et al. 2003a, 2004a). The large particle size and highly compact structure of aerobic granules endow them with the characteristics of excellent settleability (Liu et al. 2005). This ensures a high solid-to-liquid separation efficiency in aerobic granular sludge SBR, which in turn minimizes the necessary reactor volume. Besides, the small foot-print of aerobic granular sludge SBR allows this technology to be even more applicable to the land-limited places, which is a common scenario in practice.

One older counterpart of aerobic granulation technology is the anaerobic granulation technology, developed more than 20 years ago (Lettinga et al. 1980). However, the anaerobic granulation reactor usually requires extremely long start-up time (normally 2-8 months); while aerobic granular sludge SBR only needs 15 to 30 days to start up. In addition, the anaerobic granulation process results in many other

drawbacks, e.g. high operation temperature and inability to handle low strength wastewater or nutrients removal (e.g. Nitrogen and Phosphate). Besides, anaerobic granulation is somehow sensitive to the characteristics of wastewater, e.g. anaerobic granules do not grow successfully on some types of wastewater, and sudden disintegration of biogranules without any apparent cause has been reported (Liu et al. 2002; Punal et al. 2003; Schmidt and Ahring 1996). In contrast, aerobic granulation technology does not have these problems (Lin et al. 2003; Liu et al. 2003a; Liu and Tay 2004; Yang et al. 2003). Hence, aerobic granulation technology appears to be a promising alternative to either the conventional activated sludge or anaerobic granulation processes for further wastewater treatment.

With the development of aerobic granulation technology, many parameters have been tested and found to affect aerobic granulation, which include substrate concentration; organic loading rate; hydrodynamic shear force; settling time; hydraulic retention time; cycle time; aerobic starvation; presence of calcium ion in feed; intermittent feeding strategy; dissolved oxygen; reactor configuration and inhibitions (de Kreuk et al. 2005; Jiang et al. 2003; Liu and Tay 2002; Liu et al. 2003d, 2003a; McSwain et al. 2004a; Pan et al. 2004; Peng et al. 1999; Qin et al. 2004a, b; Tay et al. 2001a, 2002c, 2003a; Yang et al. 2004b). The effects of these parameters on aerobic granulation are reviewed in Chapter 2. A unique feature of SBR is cycle operation. Each cycle consists of filling, aeration, settling and decanting. Selection pressure in terms of settling time has been proposed to be a driving force for aerobic granulation (Qin et al. 2004b). Previous research on anaerobic granulation showed that selection pressure in terms of upflow velocity in the upflow anaerobic sludge blanket reactor (UASB) was a crucial factor for the formation of anaerobic granules (Hulshoff Pol et al. 1988). So far, the mechanism of selection pressure in aerobic granulation is still not very clear. Besides, increasing attentions were also drawn on the stability of aerobic granules in recent years (de Kreuk and van Loosdrecht 2004; Liu et al. 2004a). Sudden disintegration of aerobic granules have been encountered both in lab and pilot scale aerobic granulation SBRs (Tay et al. 2001c, 2005). In anaerobic granules, extracellular polysaccharides (EPS) have been considered as the structure stability strengthening

material in view of their intercellular bonding ability (Forster 1991; Schmidt and Ahring 1994). However, very limited information is known about the functional roles of EPS in aerobic granules.

1.2 OBJECTIVES AND SCOPES

Selection pressure has been demonstrated to be a decisive factor in the formation of anaerobic granules (Hulshoff Pol et al. 1988); however, the mechanism of selection pressure in aerobic granulation is still not clear. Therefore, the first objective of this study was to look into the selection pressure that drives aerobic granulation in SBR. The second objective was to get a better understanding of the role of EPS with respect to aerobic granules stability. The specific objectives of this study include the following aspects:

- To identify the driving force roles of volume exchange ratio and discharge time for aerobic granulation in SBR
- To analyze the inter-relationships of volume exchange ratio, discharge time and settling time in association to the selection pressure so as to shed light on the mechanism that functions behind aerobic granulation
- To develop a selection pressure based guideline for successful aerobic granulation by means of the up-to-date understandings acquired in this study
- To give insight into the structural and metabolic roles of EPS in aerobic granules

1.3 ORGANIZATION OF THE THESIS

This thesis contains 8 chapters. Chapter 1 is a brief introduction to the research, and Chapter 2 looks into the relevant literature information. Chapters 3 and 4 investigate the key parameters in SBR that drive aerobic granulation in terms of selection pressure. Chapter 5 demonstrates that effects of those key parameters can be

Chapter 1 Introduction

actually unified into one single factor, i.e. minimum settling velocity, which can be exploited as a guideline to instruct the design and operation of up-scaled aerobic granulation reactor. EPS properties and distribution inside aerobic granules are investigated in Chapter 6. Chapter 7 particularly looks into the biodegradability of EPS produced by aerobic granules. All of the final conclusions are drawn and recommendations are proposed in Chapter 8.

CHAPTER 2

LITERATURE REVIEW

Biological wastewater treatment can be classified with respect to its metabolic function as aerobic process, anaerobic process, anoxic process, facultative process, or a combination of aerobic, anoxic or anaerobic process (Crites and Tchobanoglous 1998). Aerobic processes of wastewater treatment employ a mixed culture of aerobic organisms in the presence of oxygen to oxidize the materials in the wastewater, while anaerobic processes employ a mixed culture of anaerobic microorganisms in the absence of oxygen to hydrolyze, ferment and ultimately convert the organic solids present in the form of fats, proteins and polysaccharides to methane. The facultative process of wastewater treatment is a process in which the organisms can function in the presence or absence of oxygen, while the anoxic process utilizes microorganisms to convert the nitrate nitrogen to nitrogen gas in the absence of oxygen (Crites and Tchobanoglous 1998).

In the biological process of wastewater treatment, the biomass (mainly microbial cells) has to be separated from the liquid after the transformation of pollutants, such as organics, nitrogen and phosphorus to the microbial cells. However, dispersed individual bacteria are suspended in solution and are extremely difficult to settle due to their common features of tiny sizes (most bacteria cells are $0.5 - 3 \mu\text{m}$), slightly heavier than water specific density and negatively charged nature (Bitton 1999). The immobilization of cells provides excellent separation ability of the biomass because of its compact microbial structure. Therefore, self-immobilization of the bacterial cells is particularly important and useful for the biological wastewater treatment system. Meanwhile, a high biological conversion capacity can be attained due to high biomass concentration in the reactor systems resulting from

the cell immobilization. In this chapter, a brief review on the cell immobilization such as biofilm, anaerobic granulation and some recent research on aerobic granulation, are presented.

2.1 Biofilm

2.1.1 Introduction

Biofilm is self-immobilization of the microorganisms onto a static solid surface or suspended carriers. It can be regarded as bacteria aggregation, but onto a solid surface. An extensive use of biofilms is made widely in the field of wastewater treatment for three main reasons (Nicolella et al. 2000): (1) compared with most other industrial biological processes, large volumes of dilute aqueous solutions can be treated; (2) natural mixed populations of microorganisms, which readily form biofilms, are used; and (3) the process can be operated at high biomass concentration in the reactor, without the need of settlers for biomass recirculation. Many types of biofilm reactors have been applied for wastewater treatment, such as static biofilm which includes trickling filters, and particulate biofilm which includes biofilm upflow sludge blanket reactors, fluidized bed reactor, expanded granular sludge blanket reactor, biofilm airlift suspension reactor and internal circulation reactor.

2.1.2 Development of biofilm

For reliable operation of particulate biofilm reactors, high biomass concentrations present in stable biofilm are essential. For this reason, the carriers should be completely covered with the biofilm. However, these biofilms should be smooth; otherwise the biofilm particulates will be easily washed out of the reactor. Biofilm development is the difference between the biofilm growth and detachment. It is influenced by various processes, including adsorption and desorption of microorganisms to and from the solid surface, attachment of microorganisms to the surface, biofilm growth and detachment (Nicolella et al. 2000). The formation of

biofilm is a multiple-step process. It is the result of several physical, chemical and biological processes occurring simultaneously (Liu and Capdeville 1996; Rouxhet and Mozes 1990; Rusten et al. 1998; Selivanovskaya et al. 1997). Heijnen (1984) postulated the hypothesis that, in ideally mixed reactors, formation of biofilm covered carriers only takes place if the hydraulic retention time is less than the inverse of the maximum growth rate (dilution rate is larger than the maximum growth rate). Liu and Tay (2002) described the development of biofilm as a four-step process as follows:

Step 1: Physical movement to initiate bacterium-to-bacterium contact or bacterial attachment onto a solid surface. The forces involved in this step are hydrodynamic force, diffusion force, gravity force, thermodynamic forces such as Brownian movement, and cell mobility. Cells can move by means of flagella, cilia or pseudopods. It has been shown that cell mobility is important for both initial interaction with the surface and movement along the surface (Pratt and Kolter 1998).

Step 2: Initial attractive forces to keep stable bacteria-solid surface and multicellular contacts. Those attractive forces are (a) physical forces which include Van der Waals forces, opposite charge attraction, thermodynamic forces such as free energy of surface and surface tension, hydrophobicity, and filamentous bacteria that can link or bridge individual cells together; (b) chemical forces which include hydrogen bonding, formation of ionic pairs, formation of ionic triplets, and interparticulate bridging; and (c) biochemical forces which include cellular surface dehydration, and cellular membrane fusion.

Step 3: Microbial forces to make immobilized bacteria mature. This may include the production of ECP, such as expolysaccharides, growth of cellular cluster, metabolic change and genetic competence induced by environment, which facilitate and further strengthen the cell-cell interaction, and result in the high density of adhering cells.

Step 4: Steady state three-dimensional structure of immobilized cells shaped by hydrodynamic shear forces. The biofilm would be finally shaped by hydrodynamic shear force to form a certain structured community; however, extremely high shear force would cause serious disaggregation and inhibition of development of biofilm.

It can be seen that for cells in a culture to attach to a solid surface, a number of conditions have to be fulfilled. Among the factors involved in the development of biofilms, shear force and loading rate have been demonstrated to play a major role (Alphenaar et al. 1993; Speitel and DiGiano 1987; van Loosdrecht et al. 1995). It has been shown that the biofilm morphology is influenced to a large extent by the surface substrate loading and applied shear/detachment forces (Tijhuis et al. 1996). A moderate surface substrate loading and a high detachment force yield smooth and strong biofilm, while the combination of a high surface substrate loading rate and low detachment forces lead to rough biofilm. The strength of biofilm also appeared to be related to the shear/detachment forces applied during biofilm formation, in combination with the substrate loading.

2.1.3 Biofilm structure

Extracellular polymers

Biofilms are composed primarily of microbial cells and ECP. ECP may account for 50% to 90% of the total organic carbon of biofilm (Flemming et al. 2000) and can be considered as the primary matrix material of the biofilm. ECP may vary in chemical and physical properties, but it is primarily composed of EPS. Some of these polysaccharides are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (such as D-glucuronic, D-galacturonic, and Mannuronic acids) or ketal-linked pyruvates confers the anionic property (Sutherland 2001). This property is important because it allows association of divalent cations such as calcium and magnesium, which have been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Flemming et al. 2000). In the case of some gram-positive

bacteria, such as the staphylococci, the chemical composition of ECP may be quite different and may be primarily cationic. Hussain et al. (1993) found that the slime of coagulase-negative bacteria consists of a teichoic acid mixed with small quantities of proteins.

ECP is also highly hydrated because it can incorporate large amounts of water into its structure by hydrogen bonding. ECP may be hydrophobic, although most types of ECP are both hydrophilic and hydrophobic (Sutherland 2001). ECP may also vary in its solubility. Sutherland (2001) noted two important properties of ECP that may have a marked effect on the biofilm. First, the composition and structure of the polysaccharides determine their primary conformation. For example, many bacterial EPS possess backbone structures that contain 1,3 or 1,4 β -linked residues and tend to be more rigid, less deformable, and in certain cases poorly soluble or insoluble. Other ECP molecules may be readily soluble in water. Second, the ECP of biofilms is not generally uniform but may vary spatially and temporally. Leriche et al. (2000) used the binding specificity of lectins to simple sugars to evaluate bacterial biofilm development by different organisms. These researchers' results showed that different organisms produce differing amounts of ECP and that the amount of ECP increased with age of the biofilm. ECP may associate with metal ions, divalent cations, and other macromolecules (such as protein, DNA, lipids, and even humic substances) (Flemming et al. 2000). ECP production is known to be affected by nutrient status of the growth medium; excess available carbon and limitation of nitrogen, potassium, or phosphate promote ECP synthesis (Sutherland 2001). Slow bacterial growth will also enhance ECP production (Sutherland 2001). Because ECP is highly hydrated, it prevents desiccation in some natural biofilms. ECP may also contribute to the antimicrobial resistance of biofilm by impeding the mass transport of antibiotics through the biofilm, probably by binding directly to these agents (Donlan 2000).

Investigations on the ECP constituents reveal an evident mass difference between EPS and proteins; e.g. alginate, a kind of EPS produced by *P. aeruginosa*, has a molecular mass between 1×10^6 and 2×10^6 Dalton which correspond to 5 μm of the

thread-like molecule; nevertheless, protein only has a molecular mass of 3×10^4 Da (Grobe et al. 1995; Sutherland 2001). Whereby, it could be estimated that a volume element of $1 \mu\text{m}^2$ and 10 nm thicknesses contains around 10 molecules of EPS and 300 protein molecules. Flemming and Wingender (2001) depicted realistic size relations of EPS and proteins in Figure 2-1. They further pointed out that EPS is the relatively stable constituent in ECP but proteins always undergo a more rapid turnover. A vivid metaphor has been used by Flemming and Wingender (2001) to cover the interrelationships between ECP and its two main constituents, i.e. EPS and protein, in which the ECP matrix is compared as a house, then the EPS resemble the “wall, ceilings and floors” while proteins resemble the “furniture” of the “house” (Figure 2-1). This systematic arrangement ensures the proteins are in proximity to the cells and avoids them from being washed out of ECP, which may likely occur under conditions of convective flow. Therefore, Figure 2-1 indicates that EPS would be the main integrity-strengthening material ensuring structure and compositional stability in the ECP matrix.

Biofilm architecture

Tolker-Nielsen and Molin (2000) noted that every microbial biofilm community is unique, although some structural attributes can generally be considered universal. Biofilms are very heterogeneous, containing microcolonies of bacterial cells encased in an ECP matrix and separated from other microcolonies by interstitial voids (water channels) (Lewandowski 2000). Liquid flow occurs in these water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents. This concept of heterogeneity is descriptive not only for mixed culture biofilm (such as might be found in environmental biofilm) but also for pure culture biofilm common on medical devices and those associated with infectious diseases. Stoodley et al. (1997) defined certain criteria or characteristics that could be considered descriptive of biofilms in general, including a thin base film, ranging from a patchy monolayer of cells to a film several layers thick containing water channels. The organisms composing the biofilm may also have a marked effect on the biofilm structure. For example, James et al. (1995) showed that biofilm thickness could be

affected by the number of component organisms. Pure cultures of either

Figure 2-1 Size relationships of EPS and proteins in a volume element of an area of $1 \mu\text{m}^2$ and a thickness of 1 nm, containing 10 molecules of EPS (2×10^6 Da) and 300 protein molecules (3×10^4 Da) (Flemming and Wingender 2001).

K.pneumoniae or *P. aeruginosa* biofilms in a laboratory reactor were thinner (15 μm and 30 μm , respectively), whereas a biofilm containing both species was thicker (40 μm). Jones et al. (1969) noted that this could be because one species enhanced the stability of the other.

Biofilm architecture is heterogeneous both in space and time, constantly changing because of external and internal processes. Tolker-Nielsen et al. (2000) investigated the role of cell motility in biofilm architecture in flow cells by examining the interactions of *P. aeruginosa* and *P. putida* by confocal laser scanning microscopy. When these two organisms were added to the flow cell system, each organism initially formed small microcolonies. With time, the colonies intermixed, showing the migration of cells from one microcolony to the other. The microcolony structure changed from a compact structure to a looser structure over time, and when this

occurred the cells inside the microcolonies were observed to be motile. Motile cells ultimately dispersed from the biofilm, resulting in dissolution of the microcolony.

2.2 Anaerobic granulation

2.2.1 Introduction

A granule is a dense microbial aggregate in the form of a spherical shape. Based on physical appearance and settleability, bacterial aggregates can be classed into three types: flocs, pellets and granules (Dubourguier et al. 1988b). Flocs are conglomerates with a loose structure. After settling, a layer of flocs forms macroscopically in one fluffy layer. Pellets are conglomerates with a more dense structure than flocs. After settling they are still visible as separate entities. Granules are dense pellets with a well-defined, clear-cut appearance. They look like a granule and their shapes do not rely on the presence of water.

Anaerobic granules formed through self-immobilization of the microorganisms are the dense microbial communities that typically include millions of organisms per gram biomass. Individual species in these microecosystems are hardly able to completely degrade influent wastes. Complete degradation of the waste involves complex interactions between the resident species. Thus, granular sludge reactors are desirable in wastewater biological treatment processes because a very high number of organisms can be maintained in the bioreactors, i.e. contaminant transformation is rapid and large volumes of waste can be treated in reactors. In anaerobic granular sludge reactors, the large size and relatively high density of individual granules cause them to settle rapidly, which simplifies the separation of purified effluent from the biomass (Batstone and Keller 2001; Noyola and Moreno 1994).

2.2.2 Anaerobic granulation phenomenon

Anaerobic granulation of the sludge is commonly found in UASB or UASB-like systems. In the UASB process, wastewater enters a sludge blanket reactor at the

base and flows upwards. Within the base of the reactor, there forms a suspended blanket of anaerobic biomass which is retained there by its own density and is agitated gently by the formation of gas. At the top of the reactor, there is usually a baffle system which assists retention of the less dense fraction of the biomass and release of the gas. The biomass exists as compact anaerobic granules of up to 3 – 4 mm in diameter. The suspended solids concentration within the lower levels of the blanket or bed can be as high as 70 g L^{-1} , decreasing in the upper layers. Depending upon the type of wastewater being treated, efficient COD removals may be obtained at loadings of up to $15 \text{ kg m}^{-3} \text{ d}^{-1}$ (Sterritt and Lester 1988).

Anaerobic granulation takes place on many types of different wastewaters including industrial wastewaters such as cornstarch wastewaters (Ross 1984), sugar beet wastewater (Lettinga et al. 1980, 1984), potato starch wastewater (Paris et al. 1987), starch wastewater (Wu et al. 1985), distillery wastewater or molasses (Harada et al. 1988), apple juice wastewater (Dold et al. 1987), brewery wastewater (Fang et al. 1990), slaughterhouse wastewater (Wu et al. 1985), citrate wastewater (Wu et al. 1987), synthetic substrates such as ethanol and propionate (de Zeeuw 1984), acetate (Harada et al. 1988), mixed volatile fatty acids (de Zeeuw 1984; Harada et al. 1988), mixed sucrose and glucose (Harada et al. 1988), glucose plus peptone and meat extract (Fang and Chui 1993), as well as domestic wastewater (Vieira and Garcia 1992).

Anaerobic granulation phenomenon has also been reported in other reactor configurations treating different wastewaters such as the hybrid upflow blanket filter (MacLeod et al. 1990), anaerobic filter (Dubourguier et al. 1988a; Young and Dahab 1982), anaerobic fluidized bed reactor (Yoda et al. 1989), anaerobic baffled reactor (Bachmann et al. 1985), and in an anaerobic pond (Roam 1989). The granulation is not only restricted to methanogens in anaerobic process. It was also found in acidifying bacteria (Beefink 1987), nitrifying bacteria (de Beer et al. 1993) and denitrifying bacteria (van der Hoek 1988).

2.2.3 Advantages of anaerobic sludge granulation

There are several ideas regarding the practical benefits of the formation of anaerobic granules. McCarty and Smith (1986) calculated that at a given H_2 turnover rate (320 sec^{-1}) the distance between acetogenic and hydrogenotrophic bacteria under steady state conditions is 5 - 11 μm ; this indicates that for this high conversion rate it is favorable that the organisms are very close to each other for syntrophic relationships. This idea is supported by several researchers. Dubourguier et al. (1988a) observed a decrease in propionate degrading activities after disintegration of granular sludge. According to Fitzpatrick et al. (1989) there are several possible advantageous reasons for bacteria to form anaerobic granules:

1. Granulation of bacteria leads to heterogeneous ordered populations of syntrophic microorganisms in the form of multicellular associations under favorable physiological conditions.
2. Symbiosis, i.e. interactions between adjacent organisms and genetic exchange, is facilitated.
3. Growth within a granule, unlike freely suspended cells, may increase the nutrient uptake.
4. Granulation may protect cells from predators.
5. The diffusion distance for fermentation intermediates is minimized in granules; this is a very efficient means to conserve every fraction of energy available in a complex degradation system.
6. Under conditions where the composition of the bulk solution is unfavorable for growth (e.g. extreme pH values), a more favorable microenvironment can be created within the aggregate so that metabolism is still possible.

2.2.4 Mechanisms and models for anaerobic granulation

Microbial granulation is undoubtedly a complex process. Many factors are involved in the sludge granulation process. For anaerobic granulation, it was found that the development of granular sludge depends on the seed sludge characteristics, feed

composition, hydraulic loading rate, temperature, etc. Changes in the composition of wastewater, organic loading rate, and pH can affect the internal structure of the granules (Pavlostathis et al. 1997). Jhung and Choi (1995) suggested that the sludge granulation is more influenced by the type of wastewater rather than the type of reactor used. The presence of high energy substrates, such as glucose and sucrose, is essential for a well granulated biomass (Schmidt and Ahring 1996; Thaveesri et al. 1995). The mechanism of anaerobic granulation has been explored by a number of investigators. To date several explanations have been proposed, mostly based on qualitative observation. There is considerable controversy among these proposed mechanisms.

Thermodynamic models

The formation of anaerobic granules is a multiple-step process, and both physicochemical and biological forces are involved. It has been suggested that microbial adhesion can be defined only in terms of the energy involved in the formation of the adhesive junction. When a bacterium approaches another bacterium, the interaction between them includes repulsive electrostatic force, attractive van der Waals force, and repulsive hydration interaction (Parsegian and Rand 1991). In order to interpret the role of physicochemical forces in the anaerobic granulation process, some thermodynamic models have been developed.

Secondary minimum adhesion model

Based on theoretical calculations and experimental observations, the secondary minimum adhesion model suggests that reversible adhesion takes place in the secondary minimum of the DLVO free energy curve. The Gibbs energy of reversible adhesion is relatively small, and there is always a separation distance between two adhering bacteria. The reversible adhesion can be shifted to irreversible adhesion at the primary minimum by overcoming the energy barrier or by protruding fibrils or fimbriae, which bridge the gap between bacteria (van

Loosdrecht and Zehnder 1990). Microstructural analysis of the UASB granule supports this separation distance between bacteria at the secondary minimum.

Hydrophobic interaction and local dehydration models

Wilschut and Hoekstra (1984) proposed a local dehydration model when studying membrane fusion, and suggested that under the physiological conditions, the strong repulsive hydration interaction was the main force to keep the cells apart. The model shows that when bacterial surfaces are strongly hydrophobic, irreversible adhesion will occur. This indicates that hydrophobicity (or hydrophilicity) of the bacterial surfaces may be another important factor involved in irreversible adhesion. Hydrophobicity and hydrophilicity are usually used to describe a molecule or a structure having the feature of being rejected from an aqueous medium (i.e. hydrophobicity). Hydration interaction becomes significant at surface separations of 2 - 5 nm or less, depending on the nature of bacterial surfaces. According to surface thermodynamics theory, increasing the hydrophobicity of cell surfaces would cause a corresponding decrease in the excess Gibbs energy of the surface, which in turn promotes cell-to-cell interaction and further serves as the driving force for cell self-separation from the liquid phase. Local dehydration of the short-distance-apart surfaces has been identified as the prerequisite for bacteria adhesion. It has been shown that the hydrophobicity of bacterial surface plays a crucial role in the initiation of anaerobic granules (Tay et al. 2000).

Surface tension

In a bulk solution with a low surface tension, hydrophilic bacteria adhere to one another, while in solutions with a high surface tension hydrophobic species are likely to adhere to one another. In general, most acidogenic bacteria are hydrophilic, and methanogens appear to be hydrophobic (Daffonchio et al. 1995). The surface tension model is based on the consideration of liquid surface tension in UASB reactors (Thaveesri et al. 1995). Depending on the liquid surface tension (γ) in the UASB reactor, bacterial cells may grow in rather loose associations, in multilayered

granules ($\gamma < 50 \text{ mN m}^{-1}$) or in mixed conglomerates ($\gamma > 56 \text{ mN m}^{-1}$) (Grootaerd et al. 1997; Thaveesri et al. 1995). Thus, the adhesion of hydrophilic cells is enhanced at low liquid surface tension, while the opposite is true for hydrophobic cells.

Treating sludge granulation only as a physical or physicochemical process is not adequate. Bacterial granulation involves too many changes and unidentified components to allow one to build a purely physical model with any confidence (Parsegian and Rand 1991). The nature of bacteria makes them far from either 'dead' chemicals or 'ideal' particles. They have no rigidly definite surface boundary, simple geometry, or uniform molecular surface composition (Darnell et al. 1986; Rouxhet and Mozes 1990). It seems unlikely that an understanding of the physical forces between bacterial cells alone can fully explain bacterial granulation. The physical phenomena of microbial granulation must eventually be related to the biological triggers that control them.

Structural models

In order to understand the mechanisms behind the sludge granulation process, physical, chemical and microbiological compositions of the granules, granular microstructure analysis, and the effects of many factors on sludge granulation have been examined. Several structural models for sludge granulation arising from these observations and investigations have been proposed. These models are mainly focused on the formation of initial/embryonic granules, which are inert matters in the inert nuclei model; cation-bridged bacterial aggregates in the divalent cation-bridge model; ECP-bound bacterial cells in the ECP bonding model and Capetown's mode; filamentous bacterial aggregates in 'spaghetti' theory and the crystallized nuclei formation model; and syntrophic microcolonies in the syntrophic microcolonies model.

Inert matter

Inert matter, such as small inorganic particles and mineral precipitates, are frequently observed in anaerobic granules (de Zeeuw 1984; Dubourguier et al.

1988b; Lettinga et al. 1984). The core portion of granules often contains a much higher ash content than the exterior layer as a result of abundant crystalline precipitates of calcium carbonate and calcium bound phosphorus present in the interior core portion (Uemura and Harada 1995). The use of organic and inorganic nuclei as inert matter often yields significantly beneficial effects on anaerobic sludge granulation, and reduces the time required to form mature granules (Lettinga et al. 1980; Wirtz and Dague 1996).

Critical concentrations of phosphate, ammonium and divalent cations, such as Ca^{2+} , Mg^{2+} and Fe^{2+} , also have a stabilizing effect on anaerobic granulation (Schmidt and Ahring 1993; Shen et al. 1993). The attachment of cells to surfaces of inert matter has been proposed as the initiating step for granulation (Archer 1988). Yoda et al. (1989) reported that using 100 μm powdered zeolite as carrier, the granules developed faster. Hulshoff Pol (1989) observed that when the inert particles with size of 40 - 100 μm were removed from inoculated sewage sludge, anaerobic granulation was not observed within the period of time that was usually required for the formation of granules.

Mineral precipitates, such as calcium phosphate, calcium carbonate and ferrous sulfide, are formed as a consequence of metabolic activities and accumulated inside granules (Dubourguier et al. 1988b). Ferrous sulfide plays an important role in the anaerobic granulation process. It was attached firmly to the sheath of *Methanothrix* sp. Therefore, it was thought to stabilize bacterial aggregation within granules (Dubourguier et al. 1988b).

Divalent cations, such as calcium ion and magnesium ion, were suggested to stimulate the anaerobic granulation process by neutralizing negative charges on bacterial cell surfaces, which causes a relatively strong “van der Waals” attractive forces, or acts as cationic bridges between bacteria (Grotenhuis et al. 1991; Schmidt and Ahring 1993). Ca^{2+} , with concentrations of 100 - 200 mg L^{-1} , was found to exert a positive impact on anaerobic granulation (Hulshoff Pol et al. 1982; Mahoney

et al. 1987), which also proves the role of calcium precipitates in the granulation process.

Grotenhuis et al. (1988) found granules, after treatment with a Ca^{2+} chelating agent, EGTA (ethylene glycol-bis (β -aminowthyl ether)-N, N, -tetraacetic acid), disintegrated or became weaker. Thus, it was concluded that calcium might play an important role in anaerobic granules' structure, as calcium phosphate precipitates for adhesion of bacteria or/and a constituent of ECP.

To date, there is no conclusive evidence to show mineral precipitates are essential for anaerobic granulation. Some research results even contradict the above interpretation, e.g. the granules can be developed without the addition of any inert materials (Lettinga et al. 1985; Thiele et al. 1990).

Anaerobic granulation

Divalent cation-bridge model

Divalent cations, both Ca^{2+} at 2.0-5.0 mM and Mg^{2+} at 0.5-10.0 mM, exert a positive impact on anaerobic granulation (Grotenhuis et al. 1991; Mahoney et al. 1987; Schmidt and Ahring 1993; Teo et al. 2000). A study on membrane fusion showed that Ca^{2+} might cause conformational changes of some surface proteins or polypeptide groups that could interact with two surfaces and bridge them together (Papahadjopoulos et al. 1990). Divalent cations were suggested either to stimulate granulation by neutralizing negative charges on bacterial surfaces as a result of relatively strong van der Waals attractive forces, or to function as cationic bridges between bacteria (Grotenhuis et al. 1991; Guiot et al. 1988; Hulshoff Pol et al. 1982; Schmidt and Ahring 1993).

Extracellular polymers

ECP, mainly polysaccharides and polypeptides, may play an important role in both anaerobic granulation and stability of the granules. ECP in anaerobic granules was

first observed with a scanning electron microscope (SEM), and further studies suggested that anaerobic sludge granulation might be initiated by ECP (Ross 1984). Ross's explanation assumed that polymers were able to bond physically or electrostatically, and this bondage could form bridges which in turn attach more bacterial cells and particulate material (organic and inorganic). This agglomeration, which he called a multi-component matrix, increased to a sufficient size to settle as floc aggregates, and later on these flocs became granules. It was suggested that ECP can change the surface negative charge of the bacteria, and thereby bridge and bond two neighboring bacterial cells physically to each other as well as other inert particulate matter, and settle out as floc aggregates (Jia et al. 1996; Schmidt and Ahring 1994; Shen et al. 1993).

Zoutberg et al. (1988) reported that some hydrolytic-fermentative bacteria, such as *Selenomans ruminantium* and *Clostridium butyricum*, produced polysaccharides and formed aggregates when grown on carbohydrate-containing media in anaerobic gas-lift reactors. Approximately 30 % of the dry biomass consisted of EPS in aggregates of *Clostridium butyricum*. Harada et al. (1988) observed with SEM and transmission electron microscopy (TEM) that the ECP excreted by acidogenic bacteria appeared to assist in cell to cell attachment and the enhancement of granule strength and structure stability. These observations were also supported by the fact that anaerobic granulation in carbohydrate-containing wastewater was faster than in a volatile fatty acid (VFA) mixture (de Zeeuw 1987; Lettinga et al. 1984).

No conclusive remarks can be drawn yet that the polysaccharides produced by fermentative bacteria play an essential role in anaerobic granulation, as these bacteria do not exist in a large numbers in the granules developed on a VFA mixture or even in granules fed with carbohydrate-containing wastewater.

The Capetown model

The Capetown group proposed that, under the conditions of high hydrogen partial pressure and limited cysteine, the overproduction of amino acids (except cysteine) would induce the ECP formation to enmesh *Methanobacterium* strain AZ and other

bacteria, thus leading to the formation of granules (Palns et al. 1987; Sam-Soon et al. 1988). This model suggests that ECP is excreted by *Methanobacterium* strain AZ, a hydrogen-utilizing methanogen.

Spaghetti theory

Based on direct microscopic and SEM examinations, Wiegant (1988) proposed a “Spaghetti” theory for anaerobic sludge granulation. Filamentous *Methanothrix* are always observed as predominant bacteria in anaerobic granules from various sources. It was proposed that, in the initial development of granular sludge, *Methanothrix* attached first on precursors, and then in a branched-growth process formed a network, where other bacteria could be entrapped in its knots. These attached bacteria look like a Spaghetti structure. These aggregates will grow due to bacteria growth and attachment, and become denser and more spherical through shear stress by upflow liquid and gas during the course of growth. Finally, the aggregate develops into a compact grown granule.

Spaghetti theory supports the role of precursor aggregates in anaerobic granule initiation, which is considered a decisive step during the granulation process. There are a few ways of *Methanothrix* initially attaching to and growing on precursors. The granule initiation might be attributed to the growth of *Methanothrix sp.* on small inert particles from the inoculums (Hulshoff Pol et al. 1988), the growth of *Methanothrix sp.* on clumps of *Methanosarcina sp.* (de Zeeuw 1987), the bridging effect of *Methanothrix sp.* on microflocs originating from the inoculated digester sludge (Dubourguier et al. 1988b), or the formation of small aggregates of *Methanothrix* caused by biogas turbulence (Wiegant 1988). In this theory, precursor formation is still a decisive step in the anaerobic sludge granulation process.

Crystallized nuclei formation

Similar to spaghetti theory, the crystallized nuclei formation model suggested that the network or nucleus formation would initiate the anaerobic sludge granulation process (Zhu et al. 1997). The thermodynamic energy change of the bacterial

aggregate ecosystem would induce the growth of adhered bacteria, and then the network formation, just like the crystallization of chemicals.

Syntrophic microcolonies

It was proposed that syntrophic relationships in mixed cultures might eventually lead to the formation of stable microcolonies or consortia (Hirata 1984). Schink and Thauer (1987) calculated the diffusion distance for transfer metabolites (H_2 and acetate) and indicated aggregation of bacteria of different trophic groups was important for energetics and kinetics of substrate conversion. Dubourguier et al. (1988b) observed syntrophic microcolonies in anaerobic granules and emphasized their importance in interspecies hydrogen transfer. However, no evidence has yet been presented to prove that the formation of syntrophic microcolonies precedes the formation of anaerobic granules.

Proton translocation-dehydration theory

Based on a consideration of the proton translocating activity on bacterial membrane surfaces, Tay et al. (2000) proposed a proton translocation-dehydration theory for the molecular mechanism of anaerobic sludge granulation. The overall sludge granulation process in this theory included four stages: dehydration of bacterial surfaces, embryonic granule formation, granule maturation, and post-maturation. The proton translocating activity on bacterial membranes could cause the energization of their surfaces and the water molecules surrounding the target cell. This induced bacterial surface dehydration. By the action of external hydraulic forces, the relatively hydrophobic bacteria adhered to each other to form embryonic granules due to the weakened hydration repulsion. These granules were strengthened by further surface dehydration because of effective intermediate transference.

In these embryonic granules, which involved a series of methanogenic metabolisms, the organization sequences and distribution of each group of bacteria was

orchestrated and developed by the distribution of intermediate transference. Eventually this led to well-structured bacteria consortia, i.e. mature granules. In the post-maturation stage, the proton translocating activity kept the bacterial surfaces at a relatively hydrophobic state. The granule structure was continuously maintained by the mechanisms of proton translocating activity and ECP.

Dehydration of bacterial surfaces

During the start-up period, the organic wastewater is fed into an anaerobic reactor where the seed sludge has been inoculated. The fermentative bacteria secrete extracellular enzymes into the medium to catalyze the hydrolysis/acidification process. The organic compounds are degraded into volatile fatty acids (VFAs), coupling with the electron transport system. At the same time, the proton pumps on the membranes of these bacteria are activated. The proton translocation activity can establish a proton gradient cross the bacterial cell surface and subsequently cause surface protonation. The energized bacterial surfaces result in the breaking of hydrogen bonds between negatively charged groups and water molecules, as well as partial neutralization of the negative charges on their surfaces. This may induce the dehydration of the bacterial surfaces.

Embryonic granule formation

The fermentation of complex organic compounds supplies the substrates for acetogens and methanogens and accelerates their growth and duplication. Similarly, coupling with the electron transport on their respiration chains, the acetogenic and methanogenic bacterial surfaces are dehydrated due to the presence of high energy protons. By the action of external hydraulic forces, these relatively neutral and hydrophobic acidogens, acetogens and methanogens may adhere to each other to form embryonic granules due to the weaker hydration repulsion. These initial aggregates are strengthened by further dehydration of the bacterial surfaces, which results from the effective metabolites transference. Only those embryonic granules which can obtain energy and nutrients from the environment are selected for the

remaining biomass. Moreover, this new physiological environment begins to induce the excretion of ECP to the embryonic granule surfaces.

Granule maturation

In embryonic granules with on-going methanogenic series metabolism, the distribution of each group of bacteria depends on the orientation of intermediate metabolites transference, which is the most efficient way for anaerobes to transfer their intermediates. This will result in the formation of well-organized bacterial consortia as mature granules. Embryonic granules may also adhere to and integrate other dispersed bacteria while the original bacterial colonies continue to grow and multiply. Granule maturation resists and blocks the unrestricted multiplication of bacterial cells because of space restriction for them to grow and dispose of wastes; this space restriction and the continuous supply of substrates facilitate the production of ECP in large quantities.

Post-maturation

The proton translocating activity in mature granules keeps the bacterial surfaces at a relatively hydrophobic state. The maintenance of the structure of mature granules is achieved mainly by the mechanism of proton translocating activity.

The proton translocation-dehydration theory may be applicable not only to the sludge granulation process but also to other microbial adhesion processes. Most bacteria exhibit proton translocating activity. In methanogens, no substrate-level phosphorylation has yet been found and evidence is accumulating that *Archaeobacteria* obtain energy by means of anaerobic respiration. Some bacteria, e.g. *Streptococcus*, have no respiration chain and can only produce ATP via substrate-level phosphorylation. The proton gradients across these bacterial surfaces are often generated by proton extrusion catalyzed by membrane ATPases (H^+ /ATPases) at the expense of ATP. Moreover, the metabolic end-product efflux is an additional mechanism for proton extrusion from streptococci and other bacterial cells that

result in the generation of proton gradients. Protons are disposed as acid to regulate their cytoplasmic pH conditions. These can cause protonation and dehydration on the bacterial surfaces.

These mechanisms mentioned above are based on hypothesis or experimental observation. To date, the formation and growth of anaerobic granules are not well understood. There is still no consensus upon mechanism, and lots of controversies exist.

2.2.5 Characteristics of anaerobic granules

Microstructure

Based on the microscopic observations, a multilayer model for anaerobic granulation was initially proposed by MacLeod et al. (1990) and Guiot et al. (1992). According to this model, the microbiological composition of granules is different in each layer. The inner layer mainly consists of methanogens that may act as nucleation centers that are necessary for the initiation of granule development. H₂-producing and H₂-utilizing bacteria are dominant species in the middle layer, and a mix of species, including rods, cocci and filamentous bacteria, predominates in the outermost layer. The conversion of a target organic compound to methane depends on the spatial organization of the methanogens and other species in UASB granules. The layered structure of UASB granules is further evidenced by immunological and histological methods (Lens et al. 1995); dynamic models (Arcand et al. 1994); studies with microelectrodes (Santegoeds et al. 1999); and fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides (Sekiguchi et al. 1999; Tagawa et al. 2000).

A distinct layered structure has also been found in the methanogenic-sulfidogenic aggregates, with sulfate-reducing bacteria in the outer 50 to 100 µm and methanogens in the inner part (Santegoeds et al. 1999). Unlike the initial multilayer model proposed by MacLeod et al. (1990), recent research shows that UASB

granules have large, dark, nonstaining centers in which neither archaeal nor bacterial signals are observed (Rocheleau et al. 1999). These nonstaining centers may be formed as a result of the accumulation of metabolically inactive, decaying biomass and inorganic material (Sekiguchi et al. 1999).

Granules with a homogeneous nonlayered structure have also been reported (Fang et al. 1995; Grotenhuis et al. 1991). Filamentous microorganisms were predominant throughout such granules. Some researchers have argued that layered and nonlayered microstructures can be developed with carbohydrates and proteins as substrates, respectively (Fang et al. 1995). This phenomenon is said to originate in the different initial steps involved in the degradation of carbohydrates and proteins. The carbohydrate degradation of small molecules is faster than the subsequent degradation of the intermediates. In contrast, the initial step in the protein degradation is a rate-limiting step. Furthermore, different types of granules can also form on a give substrate (Daffonchio et al. 1995). It has been suggested that the granular microstructure is dependent on the degradation kinetics of the substrate. In other words, different dominating catabolic pathways may give rise to the different granules (Daffonchio et al. 1995; Schmidt and Ahring 1996).

Methanogenic activity

Methane-producing bacteria are one of the major species in anaerobic granules. In most research on anaerobic granules, the activity of methane-producing bacteria has been used to quantify the metabolic activity of the granules. In general, the methanogenic activity can be defined as the methane production per unit biomass per unit time or the methane production per unit reactor volume per unit time. The methanogenic activity can be used to evaluate the performance of a reactor and as an indicator of toxic or inhibitory effects on anaerobic granules.

Physicochemical properties

Research suggests that cell surface hydrophobicity plays a crucial role in anaerobic granulation (Tay et al. 2000; Teo et al. 2000). Increasing cell surface

hydrophobicity causes a decrease in the excess Gibbs energy of the surface and further facilitates cell-to-cell interactions, leading to a stable structure. Some environmental factors are known to influence cell surface hydrophobicity. These factors include starvation, oxygen level, selection pressure, and the ionic strength of the medium (Castellanos et al. 2000; Rouxhet and Mozes 1990).

Biosolids washed out from the UASB reactors have been found to be more hydrophilic than the sludge retained in the reactor (Mahoney et al. 1987). This seems to indicate that, in the presence of a selection pressure such as a high liquid upflow velocity, microorganisms having a high surface hydrophobicity can be self-immobilized to form denser aggregates that remain in the reactor. Anaerobic granules tend to become weaker as the surface negative charge increases (Quarmby and Forster 1995). In general, the surface of microorganisms is negatively charged at physiological pH values. The maximum cell immobilization strength was observed at the isoelectric point of the cells (Liu 1995; Rouxhet and Mozes 1990). Therefore, it can be concluded that anaerobic granulation is closely correlated with cell surface properties.

The size of anaerobic granules has a dual effect on the performance of a UASB system. If the granule is too small, it is likely to be easily washed out of the reactor, and this leads to operational instability. On the other hand, a large granule size reduces mass transfer within it. The large anaerobic granules in UASB reactors are usually associated with fluffy bacteria. These large granules can be easily lost with the effluent because of their low density. The size and density of anaerobic granules depend on many factors including hydrodynamic conditions, COD loading rate, and the microbial species involved. In industrial practice, a narrow size distribution of granules is preferred, and medium-sized granules with a diameter of 1.0 to 2.0 mm seem the most attractive. The density of anaerobic granules indicates their compactness. In UASB reactors, anaerobic granules can grow up to 2 to 5 mm or larger and can range in specific gravity from 1.033 to 1.065 (Pereboom and Vereijken 1994; Tay and Yan 1996). The relatively high specific gravity of

individual anaerobic granules allows them to settle rapidly. This permits good separation of solids and liquid in a compact separator.

The strength of anaerobic granules strongly influences the stability of granules. A high strength means a more compact and stable structure of the granules. Quarmby and Forster (1995) found that high COD loading rates result in low-strength anaerobic granules. This is expected. The strength of anaerobic granules depends on many factors including the microbial diversity, organics loading rate, the feed, exopolysacchride production, and hydrodynamic shear forces. High-strength anaerobic granules are desirable in industrial applications.

2.2.6 Factors influencing anaerobic granulation

A major problem associated with the UASB reactors is the long start-up period (2 - 4 months or longer) required for the development of anaerobic granules. Enhanced granulation processes are desirable to reduce the space time requirements of bioreactors. This section discussed the main factors that influence anaerobic granulation and its performance.

Upflow liquid velocity and hydraulic retention time

It has been observed that anaerobic granulation can proceed well at relatively high liquid upflow velocity, but does not occur under conditions of low hydrodynamic shear (Alves et al. 2000; Arcand et al. 1994; Arne Alphenaar et al. 1993; O'Flaherty et al. 1997). According to Alphenaar et al. (1993), granulation in UASB reactors is favored by a combination of high upflow liquid velocity and short hydraulic retention time (HRT). Usually, the effects of upflow liquid velocity on anaerobic granulation are explained by the selection pressure theory (Hulshoff Pol et al. 1988). A long HRT accompanied with a low upflow liquid velocity may allow dispersed bacterial growth and be less favorable for microbe granulation. In contrast, a short HRT combined with a high upflow liquid velocity can lead to washout of

nongranulation-competent bacteria and thus promote sludge granulation. These findings are consistent with those reported for aerobic granulation.

Evidence shows that flocculent anaerobic sludge can be converted to a relatively active anaerobic granular sludge by manipulating hydraulic stress and the settleability of the anaerobic granules. Granules with improved SVI and sludge settling velocity develop when the upflow liquid velocity is increased (Noyola and Moreno 1994). While the upflow liquid velocity has a significant positive effect on the mean granule size, its effect on the specific washout rate of the smaller particles is insignificant (Arcand et al. 1994).

Organic loading rate

The organic substrate loading rate (OLR) describes the degree of starvation of the microorganisms in a biological system. At a low OLR, microorganisms are subject to nutrient starvation, while a high OLR sustains fast microbial growth (Bitton 1999). Evidence shows that anaerobic granulation can be accomplished by gradually increasing the OLR during the start-up (Campos and Anderson 1992; Hulshoff Pol 1989; Kosaric et al. 1990; Tay and Yan 1996). It is critical to select a reasonably high OLR during start-up, to ensure rapid granulation and a stable treatment process. A simple and practical strategy for rapid start-up of anaerobic granular sludge reactors is to increase the OLR to attain only 80% reduction of biodegradable chemical oxygen demand (COD) with supplementary monitoring of effluent for washout of suspended solids (de Zeeuw 1987; Fang and Chui 1993). However, if the applied OLR is too high during start-up of UASB reactors, the biomass production rate increases and this causes hydrodynamic turbulence and can lead to washout of the seed sludge from the reactor. This often leads to unsuccessful start-up of a UASB reactor.

Characteristics of feed

Characteristics of the feed are considered a key factor influencing the formation, composition, and structure of anaerobic granules. Based on their free energy of

oxidation, organic substrates can be roughly classified into high-energy and low-energy feeds. During the UASB start-up period, high energy carbohydrate feeding can sustain the acidogens and facilitate the formation of ECP. The more readily the acidogens take up and metabolize the substrate, the more rapidly the proton pumps will be activated, and the sooner the methanogens will obtain the substrate (Tay et al. 2000). Thus, the rapid growth of acidogens due to the presence of high-energy substrate in the influent would facilitate the overall process of sludge granulation in the UASB reactors.

The granules grown on a volatile fatty acid mixture (acetate, propionate, and butyrate) under mesophilic conditions can be classified into three distinct types, according to the predominant acetate utilizing methanogens present: (1) rod-type granules, which are mainly composed of rod-shaped bacteria in fragments of about four to five cells resembling *Methanothrix*; (2) filament-type granules, which consist predominantly of long multicellular rod-shaped bacteria; and (3) sarcina-type granules, which develop when a high concentration of acetic acid is maintained in the reactor (de Zeeuw 1984; Hulshoff Pol et al. 1982). A trend has been observed towards increasing diversity of methanogenic subpopulations with an increasing complexity of the waste composition. At least four distinct microcolonies have been observed in granules treating brewery wastewater (Wu 1991). One of these microcolonies was composed of *Methanothrix*-like rods only, while the other microcolonies consisted of hydrogen-carbon dioxide utilizing *Methanobacterium*-like rods juxtaposed with three different rod-shaped syntrophs (Hickey et al. 1991). Full-scale UASB experience confirms that anaerobic sludge granulation occurs in many different types of wastewaters. Because of the extremely low growth rate of anaerobic bacteria, the energy content of the substrate is important for anaerobic granulation; however, the complexity of substrate also exerts a selection pressure on the microbial diversity in anaerobic granules. This selection pressure may in turn influence the formation and microstructure of granules through its effect on the food chain and community signaling communications.

Seed sludge

Theoretically any medium containing the proper bacterial flora can be used as seed sludge for a UASB reactor. Potential seed sludge includes manure, fresh water sediments, septic tank sludge, digested sewage sludge, and surplus sludge from anaerobic treatment plants. The start-up of UASB reactors using digested sewage sludge as seed may take several months (de Zeeuw 1984). A long start-up period is the major deterrent to the use of UASB systems. With respect to the use of digested sewage sludge as seed, de Zeeuw (1984) found that heavy, relatively inactive sludge was preferred over lighter, more active sludge because of differences in washout. Two types of sludge washouts were distinguished. These were erosion washout and sludge bed washout. Sludge bed erosion washout is a selective washout based on differences in settleability. Although digested sewage sludge is usually used for the start-up of UASB reactors, other types of seed sludge have been used successfully when granular sludge for seeding is unavailable. Wu et al. (1987) used aerobic activated sludge from a sewage treatment plant and primary sludge from an aerobic plant treating textile dyeing wastewater for start-up.

Addition of a small amount of granules to nongranular inoculums is proven to stimulate the granulation process (Hulshoff Pol et al. 1982; Xu and Tay 2002). This is probably a consequence of supplying a specific inoculum that is responsible for granulation. UASB systems can also be started up using existing granules. This lends an advantage to the UASB start-up process, although a successful start-up is not dependent on the use of precultivated granules. When feasible, inoculation with a large amount of seed granular sludge from a healthy UASB reactor is desirable. However, the availability of granular seed sludge is limited and the purchase and transport of the inoculums can be expensive. Hulshoff Pol et al. (1982) reported that the addition of crushed granular methanogenic sludge to digested sewage in a UASB reactor fed with acetate and propionate can give rise to the development of methanogenic sludge granules with a diameter of 1 to 2 mm.

Two different types of sludge may develop on the same medium, depending on the source of the inoculums. Xu and Tay (2002) used methanol-precultured anaerobic sludge to inoculate a UASB reactor. This approach accelerated the formation of embryonic granules in a laboratory-scale UASB reactor. The granulation process reached its post-maturation stage about 15 to 20 days ahead of the control reactor. Use of methanogen-enriched seed sludge for UASB inoculation can reduce the time required for start-up. It seems certain that anaerobic granulation can be expedited simply by manipulating the composition of seed sludge. This approach can greatly facilitate the start-up of UASB reactors. It is still not entirely clear which species of seed sludge contributes the most to anaerobic granulation.

Addition of polymer and cations

Synthetic and natural polymers have been widely used in coagulation/flocculation processes. These polymers are known to promote particle agglomeration and have been used to enhance the formation of anaerobic granules. El-Mamouni et al. (1998) found that supplementation with the polymer chitosan (a polymer that is similar in structure to polysaccharides) significantly enhanced the formation of anaerobic granules in the UASB-like reactors. For example, the granulation rate in the chitosan-containing reactor was 2.5-fold higher than that in the control reactor without the polymer supplementation. The polymer enhanced granules had about the same specific activity of methane production as the granules formed without the polymer. Polymeric chains enhance flocculation by bridging microbial cells. Such initial microbial nuclei are the first step in microbial granulation. In cationic polymer-assisted anaerobic granulation processes, it has been observed that the start-up period required for the development of a granular sludge blanket can be shortened significantly compared to when no polymers are used (Uyanik et al. 2002). Two mechanisms appear to be involved in polymer enhancement of anaerobic granulation. The addition of polymers to anaerobic systems likely changes the surface properties of bacteria to promote association of individual cells. Polymer may also form a relatively solid and stable three-dimensional matrix within which bacteria multiply and daughter cells are then confined. The polymer additives

appear to play a similar role as the naturally secreted ECP in aggregating anaerobic sludge.

Evidence shows that the presence of divalent and trivalent cationic ions, such as Ca^{2+} , Mg^{2+} , Fe^{2+} , and Fe^{3+} , helps bind negatively charged cells together to form microbial nuclei that promote further granulation (Mahoney et al. 1987; Schmidt and Ahring 1993; Teo et al. 2000; Yu et al. 2001). de Zeeuw (1984) reported that the rate of sludge granulation was significantly enhanced at a calcium concentration of 100 mg L^{-1} in the wastewater. Similarly, Mahoney et al. (1987) observed that granule formation was stimulated by the presence of calcium in a concentration range of 100 to 200 mg L^{-1} . Research by Teo et al. (2000) showed that an increase in Ca^{2+} concentration from 0 to 80 mg L^{-1} substantially improved the strength of anaerobic granules, as indicated by a 60% decrease in turbidity. The role of Ca^{2+} in anaerobic granulation processes is still uncertain. Calcium concentrations exceeding about 500 mg L^{-1} (Guiot et al. 1988; Thiele et al. 1990; Yu et al. 2001) are detrimental to granulation. At high calcium concentrations, problems such as the precipitation of calcium on the surface of granules and accumulation of calcium inside anaerobic granules with consequent reduced microbial activity have been reported (Yu et al. 2001).

Temperature

As a core microbial component of anaerobic granules, methanogenic bacteria grow slowly in wastewater, and their generation times range from 3 days at 35°C to as high as 50 days at 10°C (Bitton 1999). When the reactor temperature is below 30°C , the activity of methanogens is seriously reduced. This is the main reason why mesophilic UASB reactors must be operated at a temperature of 30 to 35°C for successful functioning. In addition, sludge washout and deterioration of COD removal efficiency have been reported in UASB reactors when temperature is increased in steps from 37 to 55°C (Fang and Lau 1996). Kettunen and Rintala (1998) further reported that effluent quality from a UASB reactor operated at 70°C was lower than that from reactors operated at 35 and 55°C . High temperature is

known to encourage the growth of suspended biosolids; however, extremely high temperatures inhibit bacterial growth. Extreme thermophilic UASB reactors (i.e. temperature above 55 °C) seem not to be practicable because of the additional energy that is required to maintain the high temperature and the relatively poor effluent quality. A high-temperature operation is also difficult to control.

Recently, attention has been given to the impact of low temperature on the performance of anaerobic granular sludge reactors (Angenent et al. 2001; Lettinga et al. 2001; Singh and Viraraghavan 2003; Tarre et al. 2003). Singh and Viraraghavan (2003) showed that COD removal efficiency can be as high as 70 to 90 % in a UASB reactor operated at 11 °C with a hydraulic retention time of 6 h. Similarly, the expanded granular sludge bed (EGSB) reactors have been shown to be practicable systems for anaerobic treatment of a mainly soluble and preacidified wastewater, at temperatures of 5 to 10 °C (Lettinga et al. 2001). In addition, anaerobic migrating blanket reactors (AMBRs) have also been successfully applied to treat low-strength wastewaters at low temperatures (Angenent et al. 2001). Clearly, therefore, anaerobic granular sludge systems are suitable for the treatment of municipal wastewater at low and moderate temperatures.

pH

Based on the sequence of anaerobic reaction, the microbial species involved can be roughly divided into the following three categories: (a) bacteria responsible for hydrolysis; (b) acid-producing bacteria; and (c) methane-producing bacteria. In general, the acid-producing bacteria tolerate a low pH and have an optimal pH of 5.0 to 6.0; however, most methane-producing bacteria can function optimally in a very narrow pH range of 6.7 to 7.4 (Bitton 1999). This explains why pH is more inhibitory to methane-producing bacteria than to acidogenic bacteria in UASB reactors. Once the reactor pH falls outside 6.0 to 8.0, the activity of methane-producing bacteria is reduced to a low level, and this decline in activity in turn poses serious operational problems that can lead to the failure of the reactor. Under

normal operational conditions, the pH reduction caused by acid-producing bacteria can be buffered by bicarbonate produced by the methane-producing bacteria.

2.3 Aerobic granulation

2.3.1 Introduction

Aerobic granulation is a recently exploited biotechnology for the removal of organics, nitrogen and phosphorus from wastewater; and aerobic granules as novel bio-agents have attracted great research interests because of their specific characteristics, such as excellent settling properties, compact microbial structure, high biomass retention and the ability to withstand shock and toxic loadings (Arrojo et al. 2004; Beun et al. 1999; Lin et al. 2003; Liu et al. 2003a; Morgenroth et al. 1997; Peng et al. 1999; Tay et al. 2001a, b; Yang et al. 2003). So far, most research on aerobic granulation has been conducted in SBR, while no successful aerobic granulation has been observed in continuous culture.

Compared with continuous microbial culture, the main feature of SBR is its cycle operation, each cycle consisting of filling, aeration, settling and discharging. In SBR, the settling time is likely to exert a selection pressure on the sludge particles, i.e. only particles that can settle down within a given settling time are retained in the reactor; otherwise they would be washed out of the system. Previous research showed that selection pressure in terms of upflow velocity was a driving force towards successful anaerobic granulation in UASB reactors (Alphenaar et al. 1993; Hulshoff Pol et al. 1988). Although SBR has been extensively used in research on aerobic granulation, the mechanisms of aerobic granulation in SBR are not fully understood and especially there is still no consensus on the principal driving force of aerobic granulation. Therefore, this literature review attempts to provide an overview of the various factors that could affect aerobic granulation.

2.3.2 Characteristics of aerobic granules

Compared to the loose, fluffy, and irregular conventional activated sludge flocs, the aerobic granular sludge is known to: (i) have a denser and stronger microbial structure; (ii) have a regular, smooth round shape, and a clear outer surface; (iii) be visible as separate entities in the mixed liquor during both the mixing and the settling phases; (iv) have a high biomass retention and excellent settleability; (v) be capable of withstanding high flow rates; (vi) be able to withstand high organic loading rates; and (vii) be less vulnerable than the suspended sludge to the toxicity of organic chemicals and heavy metals in the wastewater. The excellent settleability of aerobic granules simplifies the separation of treated effluent from the granular sludge.

Morphology

Microscopic examination shows that the morphology of the aerobic granular sludge is completely different from the floc-like sludge. The shape of the granules is nearly spherical with a very clear outline (Chen et al. 2001; Peng et al. 1999; Tay et al. 2001a, c). The granule size is an important parameter in the characterization of aerobic granulation. The average diameter of aerobic granules varies in the range of 0.2 to 5 mm. This is mainly due to a balance between growth and abrasive detachment as a result of the relatively strong hydrodynamic shear force in aerobic reactors (Liu and Tay 2002; Liu et al. 2003b). Hydrodynamic shear force is known to control the prevailing size of the suspended biosolids in many situations (Chisti 1999a). Methods of estimating the magnitudes of these forces under various conditions of operation have been discussed by Chisti (1999a).

Settleability

The settling properties of aerobic granules determine the efficiency of solid-liquid separation that is essential for the proper functioning of wastewater treatment systems. The sludge volume index (SVI) of aerobic granules can be lower than 50

mL g^{-1} , which is much lower than that of conventional bioflocs (Liu et al. 2003e; Qin et al. 2004a). This implies that, from an engineering perspective, the settleability of sludge can be improved significantly through the formation of aerobic granules so that it can be settled in a more compact clarifier. The settling velocity of aerobic granules is associated with granule size and structure and is as high as 30 to 70 m h^{-1} (Liu et al. 2005). This is comparable with that of the UASB granules, but is at least three times higher than that of activated sludge flocs (typical settling velocity of around 8 to 70 m h^{-1}). The high settling velocities of aerobic granules allow the use of relatively high hydraulic loads to the reactors without having to worry about washout of biomass (Beun et al. 2000; Tay et al. 2001b). Thus, aerobic granulation can lead to more biomass retention in the reactor, and this can enhance the performance and stability of the reactor. A high concentration of the retained biomass ensures a faster degradation of pollutants and relatively compact reactors.

Density and strength

The specific gravity of aerobic granules typically ranges from 1.004 to 1.065 (Etterer and Wilderer 2001; Tay et al. 2001a; Yang et al. 2005). The granules, with a high physical strength, withstand high abrasion and shear. The physical strength, expressed as the integrity coefficient (i.e., the ratio of residual granules to the total weight of the granular sludge after 5 min of shaking at 200 rpm on a platform shaker expressed as percent), is higher than 95% for the aerobic granules grown on glucose and acetate (Tay et al. 2002b). The physical strengths of aerobic and anaerobic granules are comparable. Aerobic granules with smaller sizes tend to be more compact compared to larger aerobic granules (Toh et al. 2003; Yang et al. 2004a).

Cell surface hydrophobicity

Cell surface hydrophobicity is an important affinity force in cell self-immobilization and attachment processes (Kos et al. 2003; Liu et al. 2003d; Pringle and Fletcher

1983). The role of cell surface hydrophobicity in the formation of aerobic granules has not been clear. Liu et al.(2003d) have linked the formation of heterotrophic and nitrifying granules to the cell surface hydrophobicity. The hydrophobicity of granular sludge was nearly two-fold higher than that of conventional bioflocs. A high shear force or hydraulic selection pressure imposed on microorganisms resulted in a significant increase in the cell surface hydrophobicity, while the cell surface hydrophobicity seemed not to be sensitive to the changes in the organic concentrations or loading rates in the range of 500 to 3000 mg COD L⁻¹.

Specific oxygen utilization rate

In the environmental engineering field, microbial activity of microorganisms is characterized by the specific oxygen utilization rate (SOUR). A very wide range of SOUR values for aerobic granules have been reported (Morgenroth et al. 1997; Tay et al. 2001a; Yang et al. 2003; Zhu and Wilderer 2003). The SOUR has been found to increase with the increase in shear force specified in terms of superficial air velocity. It is obvious that the increased shear force can stimulate the respiration activities of microorganisms in a very significant manner (Tay et al. 2001a). This may have to do with the effect of hydrodynamic shear on increasing the rate of oxygen transfer at the granule-liquid interface (Chisti 1999b). The biochemical reactions associated with bacterial metabolism result in an approximately liner relationship between oxygen utilization and carbon dioxide production: that is, relatively less cell mass is produced at high oxygen utilization as the metabolism is faster and more of the substrate is converted to carbon dioxide. The microbial activity represented by SOUR is inversely related to the hydraulic selection pressure in terms of the settling time (Qin et al. 2004a). The shorter settling time tends to significantly stimulate the respiratory activity of these bacteria. This implies that the microorganisms, attempt to regulate their energy metabolism in response to changes in hydraulic selection pressure.

During long storage of aerobic granules under anaerobic conditions, SOUR is used as an indicator for evaluating the metabolic activity of the granular sludge. A

decreased SOUR indicates a reduced metabolic activity of granules after storage (Tay et al. 2002b; Zhu and Wilderer 2003). Once a reactor restarts, the activity of the granules returns to normal within a few days. When treating wastewater containing toxic chemicals such as phenol, SVI and SOUR values are necessary for monitoring the physiological changes of granules. Biomass growth and substrate removal are linked to batch measurement of SOUR. From the SOUR values, we can estimate the highest permissible loading rate. Therefore, SOUR is an important characteristic for assessing the ability of aerobic granules to handle high-strength industrial wastewaters.

Storage stability

The loss of granule stability and activity during an extended idling period is related to the storage temperature. A high storage temperature accompanied with the absence of external substrate can lead to endogenous respiration and a rapid disintegration of the granules. Tay et al. (2002b) examined the effect of storage period of glucose-and acetate-fed aerobic granules on granule activity and structure and found that the aerobic granules grown on glucose only lost about 60% activity. Both granules were stored for 4 months in tap water at 4 °C. Zhu and Wilderer (2003) found that, after 7 weeks of storage of aerobic granules in ambient environment, the granules could regain microbial activity with a week.

Ng (2002) studied the effect of different storage solutions, e.g. tap water, physiological and nutrient solutions, on the stability and activity of aerobic granules stored for a period of 8 weeks at 4 °C. The granules stored in all storage media became more irregular and smaller at the end of week 8 as compared to fresh granules at week 0 and released soluble organic material with an accompanying drop in the solution pH due to cell hydrolysis. A partial disintegration of granules was observed. The decay rate of aerobic granules during the anaerobic storage appears to be correlated with the content of volatile solids (VS) in the granules. In summary, the loss of granule activity and structural integrity during storage depend

on the storage temperature, duration, the storage medium, and the characteristics of the granules.

Compared to fresh granules, the strength of the stored granules has been observed to decrease by 7-8 % for glucose- and acetate-fed aerobic granules after 4 months storage at 4 °C (Tay et al. 2002b). However, the size of aerobic granules stored in tap water and physiological solution decreased by 34 % and 22 %, respectively, at the end of 8-week storage (Ng 2002). In addition, the apparent color of aerobic granules stored in tap water and physiological solution turned from brownish-yellow (fresh granules) to gray and dark black at the end of storage. The granules that were stored in the phosphate buffered saline (PBS) experienced the least change in color. It is reasonable to expect that the change of apparent color during storage results from anaerobic metabolism of the granules.

Zhu and Wilderer (2003) reported that aerobic granular sludge could be stored for up to 7 weeks without loss of integrity and metabolic potential of the granules; that is even without the substrate and oxygen supply, and the granular sludge still remained intact. The metabolic activity of the stored aerobic granules recovered progressively as soon as the reactor was re-supplied with substrate and oxygen. This implies that aerobic granular sludge can be kept on standby in case the wastewater flow and concentration fall below the normal levels or when toxic effects occasionally occur. Clearly, the use of aerobic granulation is applicable to situations where substrate availability varies greatly with time. This is a very common scenario.

Microbial structure

Confocal laser-scanning microscopy (CLSM) has been used with different oligonucleotide probes, specific fluorochromes, and fluorescent microspheres for studying the microstructure of aerobic granules (Meyer et al. 2003; Tay et al. 2002a, 2003b; Toh et al. 2003). The obligate aerobic ammonium-oxidizing bacterium *Nitrosomonas spp.* was found mainly at a depth of 70 to 100 µm from the granules

surface, and aerobic granules contained channels and pores that penetrated to a depth of 900 μm below the granule surface. The porosity peaked at depths of 300 to 500 μm from the granule surface (Tay et al. 2002a, 2003b). These channels and pores would facilitate the transport of oxygen and nutrients into and metabolites out of the granules. Polysaccharide formations peaked at a depth of 400 μm below the granule surface. The anaerobic bacterium *Bacteroides spp.* was also detected at a depth of 800 to 900 μm from the granule surface (Tay et al. 2002d), while a layer of dead microbial cells was located at a depth of 800 to 1000 μm (Toh et al. 2003). In order to fully utilize the aerobic microorganisms in the granules, the optimal diameter for aerobic granules should be less than 1600 μm , which is twice the distance from the granule surface to the anaerobic layer (Tay et al. 2002a). Consequently, small granules will be more effective for aerobic wastewater treatment, as these granules have more live cells within a given volume of granules.

More recently, Liu et al. (2004a) observed the mushroom-like structure of aerobic granules developed at high substrate N/COD ratios. The nitrifying population was mainly located at a depth of 70 to 100 μm from the surface of the granule (Tay et al. 2002a). Research has shown that biofilm of mixed bacterial communities can form thick layers consisting of differentiated mushroom-like structures (Costerton et al. 1981) that are similar to structures observed in aerobic granules. There is strong evidence that bacteria sense and move towards nutrients (Prescott et al. 1994). It has been demonstrated that biofilm can form mushroom-like structures by simply changing the diffusion rate; that is, the biofilm structure is largely determined by nutrient concentration (Wimpenny and Colasanti 1997). Since nitrifying bacteria are slow-growing, the mushroom-like structures seem to result from the demand of the nitrifying population for nutrients. These structures improve the access of the nitrifying population to nutrients. As Watnick and Kolter (2000) noted, in mixed biofilm, bacteria distribute themselves according to who can survive best in the particular microenvironment, and the high complexity of the resulting microbial community appears to be beneficial to its stability. Consequently, the distribution of different microbial populations in a granule may have an effect on its stability.

Microbial diversity

Microbial diversity of aerobic granules has been studied by molecular biotechnology techniques (Jang et al. 2003; Meyer et al. 2003; Tay et al. 2002a; Tsuneda et al. 2003; Yi et al. 2003). Heterotrophic, nitrifying, denitrifying, P-accumulating bacteria, and glycogen-accumulating bacteria have been identified in aerobic granules developed under different conditions (Jang et al. 2003; Lin et al. 2003; Liu et al. 2003e; Meyer et al. 2003; Tsuneda et al. 2003; Yang et al. 2003). The microbial diversity of aerobic granules is closely related to the composition of culture media in which they are developed. Anaerobiosis and dead cells have been documented at the centers of aerobic granules (Tay et al. 2002d). The presence of anaerobic bacteria in aerobic granules is likely to result in the production of organic acids and gases within the granules. These end-products of anaerobic metabolism can destroy the granules or at least diminish their long-term stability.

2.3.3 Factors influencing aerobic granulation

To date, almost all aerobic granules have been cultivated only in SBR, although the reason for this is not yet understood. Evidence shows that aerobic granulation is a gradual process from seed sludge to compact aggregates, further to granular sludge and finally to mature granules (Tay et al. 2001a). Obviously, for cells in a culture to aggregate, a number of conditions have to be fulfilled. This section attempts to discuss the factors involved in aerobic granulation in order to identify the main driving forces of aerobic granulation in SBR.

Substrate composition

Aerobic granules have been successfully cultivated with a wide variety of organic substrates in SBR, including glucose, acetate, ethanol, phenol, particulate organic matter-rich wastewater and both simulated and real municipal wastewater (Arrojo et al. 2004; Beun et al. 1999; Jiang et al. 2002; Liu et al. 2003a; Morgenroth et al. 1997; Pan 2003; Peng et al. 1999; Schwarzenbeck et al. 2003; Tay et al. 2002b), while nitrifying and phosphorus-accumulating granules have also been developed

(Lin et al. 2003; Tay et al. 2002c; Tsuneda et al. 2003). These seem to indicate that the formation of aerobic granules is a process independent of or insensitive to the characteristics of the feed wastewater, while evidence shows that the microbial structure and diversity of mature aerobic granules are closely related to the type of substrates used (Liu et al. 2003a; Tay et al. 2001a, b).

Substrate loading rate

The essential role of organic loading in the formation of anaerobic granules has been recognized, i.e. a relatively high organic loading facilitates the formation of anaerobic granules in the UASB reactor (Hulshoff Pol et al. 1988; Kosaric et al. 1990). However, this is due to the high organic loading-enhanced biogas production that results in an increased upflow liquid velocity known as the major selection pressure for anaerobic granulation in the UASB reactor (Hulshoff Pol et al. 1988). In contrast to anaerobic granulation, the accumulated evidence shows that aerobic granules can form across a very wide range of organic loading rates, e.g. 2.5 – 15.0 kg COD m⁻³ d⁻¹ (Liu et al. 2003a; Moy et al. 2002), while nitrifying granules can also be developed over a very wide range of ammonia nitrogen loadings (Qin et al. 2004c; Tsuneda et al. 2003; Yang et al. 2003). As noted by Liu et al. (2003a), aerobic granulation is substrate concentration-independent, but the kinetics behavior of aerobic granules is related to the applied substrate loading (Liu et al. 2003a; Moy et al. 2002). Consequently, it seems a reasonable consideration that the effect of organic loading rate on the formation of aerobic granules is insignificant, i.e. the substrate loading in the range studied so far is not a determinant of aerobic granulation in SBR.

Hydrodynamic shear force

In a bubble column or airlift SBR, hydrodynamic shear force is mainly created by aeration that can be described by the upflow air velocity. Previous research showed that a higher shear force favored the formation of more compact and denser aerobic granules, while the stimulated production of EPS at high shear force was also

observed (Liu and Tay 2002; Tay et al. 2001b). It is well known that EPS can mediate both cohesion and adhesion of cells and play a crucial role in maintaining structural integrity in a community of immobilized cells (Liu et al. 2004c). Consequently, the enhanced production of EPS at high shear force can make granule structure more compact and stronger. Similar to the formation of a biofilm, aerobic granules can form at different levels of hydrodynamic shear forces. It appears from the current evidence that the structure of mature aerobic granules is hydrodynamic shear force-related, although hydrodynamic shear force is not a primary inducer of aerobic granulation in SBR (Liu and Tay 2002).

Feast-famine regime

SBR is operated in a sequencing cycle of feeding, aeration, settling and discharging of supernatant. In SBR, the aeration period actually consists of two phases: a degradation phase in which the substrate is depleted to a minimum, followed by an aerobic starvation phase in which the external substrate is no longer available. Thus, it is likely that micro-organisms in SBR are subjected to a periodic feast and famine regime, called periodic starvation (Tay et al. 2001a). Under the periodic feast-famine conditions, bacteria become more hydrophobic and high cell hydrophobicity in turn facilitates microbial aggregation (Bossier and Verstraete 1996; Liu et al. 2004b; Tay et al. 2001a). When bacteria are subjected to a periodic feast-famine regime, microbial aggregation could be an effective strategy for cells against starvation. In fact, the periodic feast-famine regime in SBR can be regarded as a kind of microbial selection pressure that may alter the surface properties of cells. However, more recent research showed that aerobic granules could not be successfully developed if the settling time in SBR was not properly controlled, even though a periodic feast-famine regime was present (Qin et al. 2004a, b), while negative effects of nutrient starvation on the surface properties of aerobic granules in terms of cell hydrophobicity and the content of EPS were also observed (Zhou 2004). In addition, when the starvation time in SBR was reduced from 3 h to below 30 min, no significant impact on aerobic granules was observed (unpublished data). This may imply that the periodic feast-famine regime could favor aerobic

granulation, but so far there is no solid experimental evidence to show that starvation acts as an inducing force of aerobic granulation in SBR.

Feeding strategy

McSwain et al. (2004a) recently developed an operating strategy to enhance aerobic granulation by intermittent feeding, i.e. different filling times were applied to SBR reactors, resulting in different degrees of feast-famine period for the micro-organisms. It was found that a high feast-famine ratio or pulse feeding to the SBR was favorable for the formation of compact and dense aerobic granules, i.e. the feeding strategy may influence the characteristics of aerobic granules formed in a SBR, but it is unlikely to play a role as the trigger for aerobic granulation, as discussed above.

Dissolved oxygen

Dissolved oxygen (DO) concentration is an important parameter in the operation of aerobic wastewater treatment systems. Aerobic granules can form at a DO concentration as low as $0.7 - 1.0 \text{ mg L}^{-1}$ in a SBR (Peng et al. 1999), while studies show they can also successfully develop at DO concentrations of $2 - 6 \text{ mg L}^{-1}$ (Qin et al. 2004a; Tsuneda et al. 2003; Yang et al. 2003). It appears that if aerobic conditions are maintained by sufficient aeration, the DO concentration is not a decisive parameter in aerobic granulation.

Reactor configuration

As pointed out earlier, almost all aerobic granules have so far been produced only in a column SBR. It can be understood that reactor configuration has an impact on the flow patterns of liquid and microbial aggregates in the reactor and creates a relatively homogenous circular flow and localized vortex along the reactor height; and thus microbial aggregates are constantly subjected to circular hydraulic attrition. The circular flow could force microbial aggregates to be shaped as regular granules

that have a minimum surface free energy (Liu and Tay 2002). In column SBR, a higher ratio of reactor height to diameter (H/D) can ensure a circular flowing trajectory, which in turn creates a more effective hydraulic attrition for microbial aggregates. However, a high H/D ratio may improve oxygen transfer and could also result in a reactor with a small footprint (Beun et al. 2002). Recent research showed that aerobic granules could be developed in SBRs with different H/D ratios (Pan 2003). There is no reason to believe that aerobic granulation is tightly associated with the H/D ratio, as discussed in the following sections.

Solids retention time

Solids retention time (SRT), so-called sludge age, is one of the design parameters in a continuous activated sludge process. Pan (2003) investigated the role of SRT in aerobic granulation in SBR, and found that an SRT up to 30 days had no significant influence on aerobic granulation. As noted by Liu and Tay (2002), in more than 100 years of research history of the activated sludge process, aerobic granulation has never been reported in activated sludge processes operated in an extremely wide range of SRT, from several hours to hundreds of days. It seems reasonable to think that SRT would not be a triggering or inducing force for aerobic granulation.

Cycle time

Basically, the SBR cycle time represents the duration of the substrate oxidation reaction; and it is interrelated to the hydraulic retention time. If the SBR is run at an extremely short cycle time, microbial growth can be hindered by an insufficient reaction time for micro-organisms to break-down substrates. As a result, the sludge loss due to hydraulic washout from the system cannot be compensated by the growth of bacteria. Previous research showed that when a complete washout of sludge blanket occurred, a failure of nitrifying granulation resulted at a very short cycle time (Tay et al. 2002c). In contrast, if the cycle time is kept much longer than that required for a biological reaction, hydrolysis of the biomass would eventually cause a negative effect on microbial aggregation (Chen et al. 2003; Pan et al. 2004;

Zhou 2004). The cycle time of SBR should be short to suppress biomass hydrolysis, but long enough for biomass growth and accumulation in the system. Recent research clearly demonstrated that, for SBRs operated at the optimum cycle time determined previously, aerobic granules still could not form if the settling time was kept longer than 15 min, as discussed in the next section (Qin et al. 2004a, b). This implies that cycle time is not a decisive factor for aerobic granulation in SBR.

Settling time

In a column SBR, wastewater is treated in successive cycles of a few hours each. At the end of a cycle, settling of the biomass takes place before the effluent is withdrawn. Sludge that cannot settle down within the given settling time could be washed out of the reactor through a fixed discharge port. Basically, a short settling time preferentially selects for the growth of good settling bioparticles. Thus, the settling time exerts a major hydraulic selection pressure on the microbial community. Qin et al. (2004a, b) studied the effect of settling time on aerobic granulation in SBR designed with a fixed discharge port located at a height (L) of 0.63 m and found that aerobic granules were successfully cultivated and became dominant only in SBR operating at a settling time of less than 5 min, while a mixture of aerobic granules and suspended sludge developed in SBR run at longer settling times. In aerobic granulation research, a short settling time has been commonly employed to enhance aerobic granulation in SBR (Hu et al. 2005; Jiang et al. 2002; Lin et al. 2003; Liu et al. 2003a; Yang et al. 2003). In fact, at a long settling time, poorly settling sludge flocs cannot be withdrawn effectively; and they may in turn outcompete granule-forming bioparticles. As a result, aerobic granulation could fail in SBR run at longer settling times. This seems to indicate that aerobic granules can form only at short settling times below a critical level, i.e. settling time is a decisive factor in the formation of aerobic granules in SBR.

2.4 Summary

It is known that granulation through self-immobilization of the microorganisms can increase the volumetric conversion capacity of biological wastewater treatment

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systems, and an easy separation of the sludge from the liquid can be attained. To date, successful aerobic granulations have only been achieved in SBRs. Many factors have been tested for their influences on aerobic granulation, but only the settling time appears to be decisive and to act as a driving force for aerobic granulation. However, questions still remain after those previous findings: (1) Why should settling time play such a driving force role for aerobic granules formation? (2) Are there any other factors that play the same role as settling time in aerobic granulation? and (3) What is the real driving force behind aerobic granulation? Obviously, information with respect to this key part of the aerobic granulation technology is still missing. Therefore, this study aimed to address those questions and provide a better understanding of the aerobic granulation mechanisms.

CHAPTER 3

THE ROLE OF SBR MIXED LIQUOR VOLUME EXCHANGE RATIO IN AEROBIC GRANULATION

3.1 Introduction

As reviewed in Chapter 2, aerobic granulation is newly developed technology for wastewater treatment (Liu et al. 2003a; Morgenroth et al. 1997; Peng et al. 1999; Tay et al. 2001a). In the past few years, much research has been dedicated to understanding the formation and performance of aerobic granules SBR. Qin et al. (2004a, b) proposed that the settling time of SBR could serve as a selection pressure for aerobic granulation, i.e. short settling time favors granulation. However, two basic questions remain unanswered yet: (i) in addition to settling time, is there any other selection pressure for aerobic granulation? (ii) what is the real driving force behind aerobic granulation? This and the following two chapters address these two fundamental questions. It is expected that this study will offer deeper insights into the mechanisms of aerobic granulation as well as stable operation of an aerobic granular sludge bioreactor.

3.2 Materials and Methods

Experimental set-up

Four column reactors (R1 to R4), each with an inner diameter of 5 cm and a working volume of 2.0 L, were operated in a sequencing batch mode. Figure 3-1 shows the schematic diagram of the experimental setup. R1 to R4 were operated at different volume exchange ratios of 80 to 20%, which are defined as volume of the effluent withdrawal to total working volume of the SBR (Figure 3-1). The

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sequential operations of the four reactors were kept the same at 4 h of cycle time, comprising 225 min aeration, 5 min settling, 5 min discharging and 5 min filling.

In all the four reactors, organic loading rate was maintained at $6 \text{ kg COD m}^{-3} \text{ d}^{-1}$, and air was supplied at a rate of 3 L min^{-1} . For aeration, the aerator, a type of porous stone, was installed at the bottom of each reactor. Peristaltic pumps (Masterflex, Cole-Parmer Instrument Company, USA) were used for influent filling and effluent withdrawing, while an air pump (GASR, Gast Manufacturing Inc., USA) was used for air supply.

Substrate and seed sludge

The synthetic wastewater used mainly consisted of sodium acetate, 1280 mg L^{-1} and NH_4Cl , 100 mg L^{-1} ; K_2HPO_4 , 22.5 mg L^{-1} ; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 15 mg L^{-1} ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 12.5 mg L^{-1} ; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg L^{-1} and 1.0 mL L^{-1} micronutrient solution. The microelement solution contained: H_3BO_3 , 0.05 g L^{-1} , ZnCl_2 , 0.05 g L^{-1} , CuCl_2 , 0.03 g L^{-1} , $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05 g L^{-1} , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.05 g L^{-1} , AlCl_3 , 0.05 g L^{-1} , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05 g L^{-1} , NiCl_2 , 0.05 g L^{-1} . The initial substrate concentrations to R1 and R4 were controlled at the level of $1000 \text{ mg COD L}^{-1}$. The influent pH was adjusted to 7.0 by HCl. R1 to R4 were inoculated with 2 g L^{-1} of fresh activated sludge taken from a local municipal wastewater reclamation plant.

Analytical methods

SVI, SS and COD

Sludge volume index (SVI), suspended solids (SS) and chemical oxygen demand (COD) were measured by standard methods (APHA 1998).

Size measurement

The size of bioparticles or granules was measured by a laser particle size analysis system (Malvern Mastersizer Series 2600) when the size was smaller than $1880 \mu\text{m}$.

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When the size was bigger than 1880 μm , an image analysis system (Quantimet 500 Image Analyzer, Leica Cambridge Instruments) with an Olympus SZX9 microscope was used. The laser particle size analysis system (Mastersizer), based on the principle of laser diffraction, has a measurable size range of 0.5 - 1880 μm . The image analysis (IA) system was used to measure large-size granules which are beyond the measurement limit of the laser particle size analysis system. The image analysis consists of an image processing unit and a video camera with a camera lens (Olympus SZX9).

Fraction of aerobic granules in reactor

In this study, the fraction of granules was estimated by measuring the volume of granules with a mean size above 0.4 mm in the total volume of sample. For this purpose, a bioparticle was considered as an ellipse. Thus, the fraction of granules (f) in terms of percentage was calculated as follows:

$$f = \frac{n(\pi/6)d^3}{V_t} \times 100\% \quad (3-1)$$

where V_t is the total volume of sample, and d and n are the mean diameter and number of granules in the sample, respectively.

Extracellular polysaccharides

Biomass was harvested by centrifuging a sample at 3,000 rpm for 10 min. The cold aqueous extraction technique was applied for EPS extraction (Zhang et al. 1998). Biomass was firstly suspended in 10 mL 8.5% NaCl solution containing 0.22% formaldehyde. The mixture was then chilled in ice and mixed in a homogenizer for 3 min, followed by filtration through a 0.22 μm cellulose acetate filter to remove biosolids. The filtrate was ready for EPS analysis.

5 mL filtrate was subsequently mixed with 0.5 mL of 50 mg L^{-1} phenol reagent rapidly and thoroughly, followed by adding 2.5 mL concentrated sulfuric acid and thoroughly mixing again. Let the tube stand for 10 min and then place it in a 25°C

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water bath for 15 min. Read the absorption at A_{488} for each sample against the blank. Finally determine the EPS concentration by referring to the standard curve that was developed by using glucose according to above procedures.

Calcium content

In order to analyze the calcium content of aerobic granules, granule samples were dried to a constant weight at 105 °C, and then digested with nitric acid (APHA 1998), followed by analysis with an inductively coupled plasma emission spectrometer (PerkinElmer Optima 2000).

In this study, all samples were taken at the first hour of each cycle and measurements were done in triplicates.

Figure 3-1 Schematics of R1 to R4 operated at the respective volume exchange ratio of 80, 60, 40 and 20 %

3.3 Results

3.3.1 Effect of volume exchange ratio on formation of aerobic granules

Only 2 days after startup, aerobic granules appeared in R1 operated at the highest volume exchange ratio of 80%, while aerobic granules were subsequently observed in R2 to R4 operated at a respective volume exchange ratio of 60, 40 and 20% after 6, 12 and 20 d operation. It can be seen in Figure 3-3 that larger and more spherical aerobic granules were formed at the higher volume exchange ratio. Figure 3-2 further shows that aerobic granules dominated the whole sludge blanket in the SBR run at the high volume exchange ratios, while a mixture of granules and bioflocs was actually cultivated in R4, operated at the lowest volume exchange ratio of 20%. This indicates that aerobic granulation in SBR is closely related to the applied volume exchange ratio, i.e. high volume exchange ratio would favour the formation of aerobic granules.

Figure 3-2 Fraction of aerobic granules in R1 to R4

Figure 3-3 Morphologies of aerobic granules cultivated in R1 to R4 run at respective volume exchange ratio of 80, 60, 40 and 20%, bar: 3 mm

3.3.2 Effect of volume exchange ratio on sludge settleability

In the field of biological wastewater treatment, SVI is a parameter commonly used to evaluate microbial sludge settleability. Figure 3-4 shows that large-size aerobic granules would be developed at the high volume exchange ratios, and they have low SVI values, e.g. the settleability of sludge blanket developed at the volume exchange ratio of 80% is three times superior to those found at the volume exchange ratio of 20%. In fact, the Stokes law suggests that the particle settleability is a function of the square of its diameter, i.e. larger aerobic granules would have a better settleability as shown in Figure 3-4.

Figure 3-4 SVI of sludge blanket developed at different volume exchange ratios

3.3.3 Accumulation of calcium and EPS in aerobic granules

Figure 3-5 reveals that calcium ion was accumulated significantly in aerobic granules developed at high volume exchange ratio, e.g. the calcium content in granules cultivated at the volume exchange ratio of 80% is almost 3-fold higher than those obtained at the volume exchange ratio of 20%. Previous research also

reported the excessive accumulation of calcium in attached-growth cells (Kemner et al. 2004). In this study, it was found that more EPS was produced in aerobic granules formed at high volume exchange ratios (Figure 3-6). Meanwhile, a relationship between the calcium and EPS contents in aerobic granules developed at different volume exchange ratios was shown in Figure 3-7. According to Figure 3-7, calcium content increase closely with that of EPS. In fact, the high affinity between EPS and calcium had been reported (Forster and Lewin 1972; Rudd et al. 1984). Figures 3-5 and 3-6 seems to indicate that one possible way for calcium to promote aerobic granulation is to complex with EPS, which would contribute to the three-dimensional structure and stability of the conformation of the polymer network in the aerobic granules.

Figure 3-5 Calcium content of aerobic granules developed at different volume exchange ratios

Figure 3-6 EPS content of aerobic granules cultivated at different volume exchange ratios

Figure 3-7 Granules EPS content versus calcium content

3.4 Discussion

It appears from Figures 3-3 to 3-6 that the volume exchange ratio of SBR would serve as an effective driving force for aerobic granulation. For a column SBR (Figure 3-1), the bioparticles' traveling distance above the discharging port is L (distance between water surface and discharging port), which is indeed proportionally correlated to the SBR volume exchange ratio (Figure 3-1). Thus, for a designed settling time (t_s), bioparticles with a settling velocity less than L/t_s would be washed out of the reactor, while only those with a settling velocity greater than L/t_s will be retained. These may indicate that there is a minimum settling velocity $(V_s)_{\min}$ in a SBR reactor that can be defined as follows:

$$(V_s)_{\min} = \frac{L}{t_s} \quad (3-2)$$

Equation (3-2) shows that a long L or a short settling time would result in a larger $(V_s)_{\min}$, and vice versa.

Qin et al. (2004a, b) studied aerobic granulation at different settling times, but with a fixed volume exchange ratio of 50%. The data by Qin et al. (2004a, b) as well as those obtained in this study, were thus used to calculate $(V_s)_{\min}$. Figures 3-8 to 3-11 show relationships of the fraction, mean size, SVI and calcium content of aerobic granules to the minimum settling velocity. It is obvious that both mean size and fraction of granules tend to increase with the increase in $(V_s)_{\min}$, i.e. $(V_s)_{\min}$ may serve as a selection pressure to drive aerobic granulation, and highly influence the properties of stable aerobic granules, such as SVI, mean size and the calcium content. Moreover, it appears from Figure 3-8 that, at a $(V_s)_{\min}$ less than 4 m h^{-1} , aerobic granulation is not favored in SBR; instead the growth of suspended sludge is greatly encouraged. The typical settling velocity of conventional activated sludge is generally less than 5 m h^{-1} (Giokas et al. 2003). These imply that, for a SBR operated at a $(V_s)_{\min}$ lower than the settling velocity of conventional sludge, suspended sludge could not be effectively withdrawn. As a result, suspended sludge will take over the entire reactor at low $(V_s)_{\min}$ as shown in Figure 3-8. A recent study demonstrated that suspended sludge could outcompete aerobic granules, and

such an outcompetition eventually led to instability and even failure of aerobic granulation (Liu et al. 2004d). To achieve a rapid and enhanced aerobic granulation in SBR, the minimum settling velocity $(V_s)_{\min}$ must be controlled at a level higher than the settling velocity of suspended sludge. This study provides experimental evidence that the real selection pressure towards an enhanced and stable aerobic granulation is the minimum settling velocity as defined by Equation (3-2). Any changes in the minimum settling velocity would influence the formation and stability of aerobic granules in SBR (Figures 3-8 to 3-11).

It appears from Figure 3-11 that the accumulation of calcium in aerobic granules was positively related to $(V_s)_{\min}$. Figures 3-12 and 3-13 further show that the mean size of aerobic granule increased with the calcium content of aerobic granules, while the SVI was inversely correlated with it. These results indicate that microorganisms can selectively accumulate calcium that could play a part in the

Figure 3-8 Fraction of sludge blanket aerobic granules content versus $(V_s)_{\min}$ obtained in this (●) and (○) studied by Qin et al. (2004a, b)

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Figure 3-9 Granules mean size versus $(V_s)_{\min}$ obtained in this (●) and (○) studied by Qin et al. (2004a, b)

Figure 3-10 SVI of microbial blanket versus $(V_s)_{\min}$ obtained in this (●) and (○) studied by Qin et al. (2004a, b)

Figure 3-11 Calcium content in aerobic granules versus $(V_s)_{min}$ obtained in this (●) and (○) studied by Qin et al. (2004a, b)

initiation and development of aerobic granules at high $(V_s)_{min}$. It seems that microbial aggregates can protect themselves from the hydraulic discharge from the reactor by increasing their physical size through the accumulation of calcium when the reactor is operated at high $(V_s)_{min}$. It is most likely that the selective accumulation of calcium would be a defensive strategy of microbial aggregates to resist the hydraulic discharge from the reactor through the calcium-promoted increases in their size and settleability in terms of SVI (Figure 3-13). In fact, it has been generally believed that calcium may facilitate anaerobic granulation (Schmidt and Ahring 1996; Yu et al. 2001), while evidence also shows that the removal of calcium from the anaerobic granule matrix would result in lowered strength of the UASB granules (Pereboom 1997). This and previous research seem to suggest that the accumulation of calcium in biogranules would favor the long-term stability of biogranules.

Figure 3-12 Granules mean size versus calcium content obtained in this (●) and (○) studies by Qin et al. (2004a, b)

Figure 3-13 SVI of microbial blanket versus calcium content obtained in this (●) and (○) studies by Qin et al. (2004a, b)

So far it has been well known that calcium is a constituent of EPS, which is used to adsorb and link materials in the anaerobic granulation process (Morgan et al. 1990). There is evidence that bacteria are embedded in the EPS matrix in aerobic granules, and the stability of aerobic granules is highly related to the structural strength of the EPS matrix (Liu et al. 2004c). The close association of the calcium accumulation to the granule EPS (Figure 3-7) indicates that calcium would play an essential role in binding with EPS and contribute to the stability of the conformation of the polymer network in the aerobic granules. Consequently, the formation of the EPS- Ca^{2+} -EPS bridges can substantially enhance the stability of aerobic granules.

3.5 Conclusion

This study showed that the volume exchange ratio of SBR could be manipulated as an effective selection pressure for a successful aerobic granulation in SBR, i.e. aerobic granulation would be accelerated and enhanced in SBR operated at a high volume exchange ratio. Meanwhile, the excessive production of EPS and subsequent calcium accumulation at a high volume exchange ratio was found to facilitate the formation and further improve the settleability of aerobic granules. The essential role of volume exchange ratio can be interpreted by the proposed concept of minimum settling velocity very well.

CHAPTER 4

EFFECT OF DISCHARGE TIME ON AEROBIC GRANULATION

4.1 Introduction

The combined effects of settling time and volume exchange ratio on aerobic granulation have been discussed in Chapter 3. This chapter is a follow-up of the study presented in Chapter 3, and looks into another potential selection pressure, namely discharge time of the SBR. It should be pointed out that the role of discharge time in aerobic granulation is still unknown.

4.2 Materials and Methods

Experimental set-up

Four column reactors (R1 to R4) were operated at discharge times of 5, 10, 15 and 20 min, respectively. Reactor configurations and the other operation parameters were kept exactly the same as those used in Chapter 3, except that a 50 % volume exchange ratio was equally applied in all SBRs.

Substrate and seed sludge

Media and seed sludge used can be found in Chapter 3.

Analytical methods

Cell surface hydrophobicity

Cell surface hydrophobicity was determined by the method of microbial adherence to hydrocarbons (MATH), as described by Rosenberg et al. (1980). Hexadecane was used as the hydrophobic phase. Hydrophobicity is expressed as the percentage of cells adhering to the hexadecane after 15 min of partitioning. The detailed testing procedures are as follow:

The sample was collected by centrifugation at 3000 rpm for 10 min, then it was ground and washed twice with phosphate urea magnesium (PUM) buffer (pH 7.1, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 22.2 g L⁻¹, KH_2PO_4 7.26 g L⁻¹, urea 1.8 g L⁻¹, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g L⁻¹), and resuspended in the same buffer to give an absorbance by using a spectrometer (UV-1021V, Shimadzu, 1997) at 540 nm. The 4 mL of the suspension was mixed with 1 mL of hydrophobic solvent (hexadecane), and followed by 15 min incubation at 30 °C. The mixture was vortexed for 1 min, and then maintained stagnant for 15 min for two-phase partitioning. After that, the water phase was sucked out by syringe for spectrometer measurement at 540 nm again. The hydrophobicity was calculated as the percentage of the difference compared with the initial absorbance.

Scanning electron microscope (SEM)

Microbial composition of the granules was observed qualitatively with a scanning electron microscope (SEM) (Stereosan 420, Leica Cambridge Instruments). The granules sample was gently washed with phosphate buffer solution and allowed to settle by gravity. Granules were then fixed with 4 % paraformaldehyde and left for 4 hours. The fixed granules were dehydrated by a successive gradient of 20, 40, 60, 80, 100 % ethanol solution. The dehydrated granules were finally dried by a critical point dryer (E3000, VG Mircotech, England), and were ready for observation in the SEM.

The other analytical methods have been detailed in Chapter 3.

4.3 Results and Discussion

4.3.1 Effect of discharge time on formation of aerobic granules

Figure 4-2 shows that smooth and round-shape looking aerobic granules were successfully developed at a short discharge time, and only floc-like bioparticles were cultivated in R4 operated at the longest discharge time of 20 min. Figure 4-1 shows that granule mean size appears to be inversely related to the discharge time applied in SBRs, e.g. large aerobic granules were observed at short discharge time. It seems a prolonged discharge time would be contributing to aerobic granulation, even though proper settling time and volume exchange ratios were applied in SBRs.

Figure 4-1 Granules mean size versus discharge time in stable SBRs

Figure 4-2 Morphology of the steady state sludge, bar: 3 mm

4.3.2 Effect of discharge time on fraction of aerobic granules

Figure 4-3 shows that the fraction of aerobic granules tends to decrease with the increase in discharge time of SBR, especially in R4 run at the discharge time of 20 min, where almost no aerobic granules were formed (Figure 4-3). The failure of aerobic granulation at long discharge time may imply that this parameter could serve as kind of selection pressure for aerobic granulation (Figure 4-3).

Figure 4-3 Fraction of aerobic granules in R1 to R4

4.3.3 Effect of discharge time on sludge settleability

Steady state SVIs in R1 to R4 are shown in Figure 4-4. High SVI values were found at long discharge time of SBR. This may mean that prolonged discharge time would not be favourable for producing highly compact microbial aggregates. In fact, the SVI found in R4 was similar to that of typical activated sludge flocs.

Figure 4-4 SVI versus discharge times

4.3.4 Effect of discharge time on sludge hydrophobicity

The effect of discharge time on sludge hydrophobicity is shown Figure 4-5. A low hydrophobicity is observed at a long discharge time. The hydrophobicity was increased from 26% in the seed sludge to a stable value of 71, 65, 52 and 39% in R1 to R4. These imply that the cell surface hydrophobicity would be inversely related to the discharge time, i.e. microbial community developed at short discharge time exhibits a high cell surface hydrophobicity. As shown in Figure 4-3, only partial aerobic granulation was observed in R2, R3 and R4, while aerobic granules were dominant in R1. It appears that the selection pressure induced change in cell hydrophobicity would contribute to cell-to-cell aggregation. In fact, it has been well known that cell surface hydrophobicity highly contributes to the formation of biofilm and anaerobic granules (Liu et al. 2003c, 2004b; Mahoney et al. 1987; Tay et al. 2001b).

Figure 4-5 Sludge hydrophobicity in R1 to R4 with different discharge times

4.3.5 Effect of discharge time on EPS content in aerobic granules

It has been generally believed that EPS can mediate both bacterial cohesion and adhesion and act as a decisive role in building and maintaining structural integrity of a microbial community (Liu et al. 2003c; Schmidt and Ahring 1996; Sutherland 2001). Figure 4-6 shows the effect of discharge time on the sludge EPS content. The EPS content was inversely correlated to the discharge time, i.e. a shorter discharge time would stimulate cells to produce more EPS. Figure 4-6 together with Figure 4-3, it seems to suggest that EPS would play an essential role in forming and further maintaining the structural stability of aerobic granules. A SEM view of aerobic granules in Figure 4-7 demonstrates bacteria were indeed tightly wrapped inside polymeric substances. This observation provides visual evidence of those existing EPS measured in Figure 4-6.

Figure 4-6 EPS content in R1 to R4 at different discharge times

Figure 4-7 EPS found in aerobic granules, bar: 3 μ m

4.3.6 Conclusion

It was shown in this chapter that discharge time is a key parameter that highly influences aerobic granulation in SBR. A prolonged discharge time would result in a failure of aerobic granulation, even though proper settling time and volume exchange ratios were used. According to the results obtained, a relatively long discharge time, e.g. 20 min, could worsen sludge blanket settleability and inhibit cell surface hydrophobicity and EPS production. The effects of discharge time on aerobic granulation should be taken into account in up-scaled application.

CHAPTER 5

A UNIFIED SELECTION PRESSURE THEORY FOR AEROBIC GRANULATION

5.1 Introduction

It appears from Chapters 3 and 4 that settling time, volume exchange ratio and discharge time of SBR would serve as the major selection pressures that drive aerobic granulation. In the environmental engineering literature, there is still lack of theoretical basis to unify these three selection pressures for aerobic granulation. Thus, this chapter aims to develop a unified theory for aerobic granulation in sequencing batch reactors.

5.2 A Unified Selection Pressure Theory for Aerobic Granulation

It has been proposed previously that selection pressure, in terms of minimum settling velocity $(V_s)_{\min}$, is the driving force on SBRs that acts as a selection pressure for aerobic granulation (Equation (3-2)). The development of Equation (3-2) was actually based on the assumption that all the suspended bioflocs above the effluent port were effectively discharged out of the SBR during the preset discharge time (Figure 5-1, *left*). However, in the case where the discharge action is not swift enough, i.e. a long discharge time was applied, there will be a portion of the suspension which is supposed to be washed out of reactor but then gets a chance to further settle down below the effluent port and thus be retained in the SBR (Figure 5-1, *right*). Therefore, there would exist an effective settling time instead of the preset settling time, coordinating with volume exchange ratio, to function as the minimum settling velocity, namely

$$(V_s)_{\min} = \frac{L}{\text{effective settling time}} \quad (5-1)$$

Figure 5-1 Schematics of reactor configuration and hypothetical flow during discharge in the case of $t_d \leq t_{d,\min}$ (left) and $t_d > t_{d,\min}$ (right).

Referring to Figure 4-3, there seems to exist a minimum discharge time $t_{d,\min}$ at which the fraction of aerobic granules in the SBR is close to 100 %, i.e. a full granular sludge blanket is developed at $t_{d,\min}$. If the discharge time (t_d) is set to be longer than $t_{d,\min}$, Figure 5-1 illustrates that a portion of the liquor above the discharge port would continue to settle during discharge time t_d , and this would eventually lower the effective selection pressure on microorganisms. For $t_d > t_{d,\min}$, the settling time needs to be calibrated to take account of the discharge time. Therefore, the effective settling time for use in Equation (3-2) is defined as:

Chapter 5 A Unified Selection Pressure Theory

Effective settling time = preset settling time (t_s) + relaxation of settling time due to t_d (5-2)

If the discharge flow rates at t_d and $t_{d,min}$ are Q_d and $Q_{d,max}$, respectively, they can be defined as follows:

$$Q_{d,max} = \frac{V_e}{t_{d,min}} \text{ and } Q_d = \frac{V_e}{t_d} \quad (5-3)$$

Here V_e is the exchange volume above the discharge port as shown in Figure 5-1. The hypothetical flow that can settle is $Q_{d,max} - Q_d$ (Figure 5-1). Thus, the relaxation of settling time due to t_d can be expressed as follows:

$$\text{Relaxation of settling time due to } t_d = \frac{Q_{d,max} - Q_d}{Q_{d,max}} \times (t_d - t_{d,min}) \quad (5-4)$$

Substitution of Equation (5-3) into Equation (5-4) leads to the following equation:

$$\text{Relaxation of settling time due to } t_d = \left(1 - \frac{t_{d,min}}{t_d}\right)(t_d - t_{d,min}) = \frac{(t_d - t_{d,min})^2}{t_d} \quad (5-5)$$

Thus, Equation (5-2) becomes

$$\text{Effective settling time} = t_s + \frac{(t_d - t_{d,min})^2}{t_d} \quad (5-6)$$

Combining Equation (5-6) with Equation (5-1) gives

$$(V_s)_{min} = \frac{L}{t_s + \frac{(t_d - t_{d,min})^2}{t_d}} \quad (5-7)$$

Equation (5-7) combines the three major selection pressures (i.e. t_s , t_d and L) in SBR into the minimum settling velocity required for successful aerobic granulation. Fast settling bioparticles tend to be heavy spherical aggregates while the slow settling particles tend to be small, light and of irregular shapes. Clearly, bioparticles can be selected according to their settling velocity. This has been confirmed in laboratory scale aerobic granular sludge SBRs (Liu et al. 2005). Equation (5-7) provides a plausible explanation for why t_s , t_d and L in SBRs can serve as the effective selection pressures that allow for selecting particles that settle easily.

The relationship between $(V_s)_{min}$ and the ratio of biomass of aerobic granules to the total biomass in a stable SBR operated at different selection pressures is shown in

Figure 5-2. It can be seen that the fraction of aerobic granules in the reactor increased with the increase of $(V_s)_{\min}$. Figure 5-2 shows that at $(V_s)_{\min}$ values smaller than 1.0 m h^{-1} , only suspended bioflocs are cultivated and no aerobic granules are developed. As $(V_s)_{\min}$ increased above 1.0 m h^{-1} , aerobic granular sludge blanket starts to form. At $(V_s)_{\min}$ value of 4.0 m h^{-1} , aerobic granules prevail over suspended flocs. As the typical settling velocity of suspended activated sludge is generally less than $3 \text{ to } 5 \text{ m h}^{-1}$ (Giokas et al. 2003), these results seem to indicate that if the SBR is operated at a $(V_s)_{\min}$ value below that of suspended flocs, suspended sludge would not be effectively washed out of the reactor. The specific growth rates and growth yield of aerobic granules are much lower than that of suspended activated sludge (Tay et al. 2004b; Yang et al. 2004a), i.e. suspended sludge can easily outcompete aerobic granules. Such outcompetition represses the formation and growth of aerobic granules and eventually leads to disappearance of the aerobic granular sludge blanket in the SBR if suspended sludge is not effectively withdrawn (Liu and Tay 2004; Qin et al. 2004a, b). Therefore, $(V_s)_{\min}$ must be controlled at a level higher than the settling velocity of suspended sludge, otherwise successful aerobic granulation would not be achieved and maintained stably. Equation (5-7) indicates that enhanced selection of bioparticles for rapid aerobic granulation can be realized through proper control of settling time discharge time and volume exchange ratio in SBR.

5.3 Guideline for Upscaling Aerobic Granular sludge SBR

When upscaling aerobic granular sludge SBR, settling time, discharge time and volume exchange ratio must be properly controlled and manipulated (Equation (5-7)). Compared to the volume exchange ratio and discharge time, control of the settling time is more flexible in manipulating the operation of a full-scale SBR. To avoid initial washout of biomass, settling time should be gradually shortened from 20 min to as short as 2 min (Hu et al. 2005; Lin et al. 2003; Qin et al. 2004a, b; Tay et al. 2004b). According to Figure 5-2, $(V_s)_{\min}$ for enhanced aerobic granulation should not be less than 8 m h^{-1} . Successful aerobic granulation has been obtained at settling velocities of 10 and 16.2 m h^{-1} (Beun et al. 2000, 2002).

Figure 5-2 Relationship between the fraction of aerobic granules and $(V_s)_{min}$. ●) at different settling times (Qin et al. 2004b); ○) at different volume exchange ratio (Chapter 3); ■) at different discharge time (Chapter 4).

An essential aspect of design of aerobic granular sludge SBR is the estimation of the discharge time. Discharge time greatly influences the formation of aerobic granules (Equation (5-7)) and determines the discharge pumping rate (Equation (5-3)) that relates to energy consumption. In practice, engineers and operators have limited scope for manipulating the volume exchange ratio or depth of the outlet port of the reactor. Indeed, most laboratory scale aerobic granular sludge SBRs are actually operated at a fixed volume exchange ratio (Lin et al. 2003; McSwain et al. 2004b; Morgenroth et al. 1997; Tay et al. 2001a). In practice it may be preferable to control the settling time and discharge time in order to achieve the minimum settling velocity required for aerobic granulation; hence, Equation (5-7) can be rewritten as follows:

$$t_s = \frac{L}{(V_s)_{min}} - \frac{(t_d - t_{d,min})^2}{t_d} \quad (5-8)$$

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The following example considers a full scale column-type SBR with a diameter of 4 m and reactor height of 8 m. A volume exchange ratio of 50 % is assumed. This corresponds to a discharge depth (L) of 4 m and a minimum discharge time of 5 min. The latter value is based on laboratory studies, e.g. Figure 4-3. If the minimum settling velocity is increased in steps of 2 m h^{-1} from an initial value of 8 m h^{-1} , the corresponding settling time and discharge time can be determined by Equation (9); thus,

$$\text{At } (V_s)_{\min} = 8 \text{ m h}^{-1}, t_s = 30 - \frac{(t_d - 5)^2}{t_d}$$

$$\text{At } (V_s)_{\min} = 10 \text{ m h}^{-1}, t_s = 24 - \frac{(t_d - 5)^2}{t_d}$$

$$\text{At } (V_s)_{\min} = 12 \text{ m h}^{-1}, t_s = 20 - \frac{(t_d - 5)^2}{t_d}$$

$$\text{At } (V_s)_{\min} = 16 \text{ m h}^{-1}, t_s = 15 - \frac{(t_d - 5)^2}{t_d}$$

$$\text{At } (V_s)_{\min} = 20 \text{ m h}^{-1}, t_s = 12 - \frac{(t_d - 5)^2}{t_d}$$

The above equations show relationships of the settling time to the discharge time at different desired minimum settling velocities (Figure 5-3). The salient points from Figure 5-3 are as follows: 1. any pair of t_s and t_d that satisfies the above t_s - t_d relationship would result in the desired $(V_s)_{\min}$ for aerobic granulation, i.e. a longer t_d would be compensated by a shorter t_s , and vice versa; 2. for any given settling time, we can compute the discharge time required; 3. the longest settling time for the desired $(V_s)_{\min}$ can be estimated; 4. the longest discharge time allowed for achieving desired $(V_s)_{\min}$ can be calculated. Such information is essential for rational design and operation of an aerobic granular sludge SBR.

It should be realized that the discharge time directly determines the effluent pumping rate that relates to the energy cost, i.e. a short discharge time results in a higher effluent pumping rate. From the point of view of the SBR operation, we prefer a long discharge time in order to reduce pumping power. However, a long discharge time can be obtained only by shortening the settling time as shown in

Figure 5-3. Studies in laboratory scale SBR suggest that optimal settling time for successful aerobic granulation is less than 5 min (McSwain et al. 2004b; Qin et al. 2004a, b; Tay et al. 2001a, 2004b). If a similar settling time applies to the above full scale SBR, we can compute the discharge time needed for achieving a desired $(V_s)_{\min}$ for aerobic granulation, e.g. 34.4 min for a $(V_s)_{\min}$ of 8 m h^{-1} . Consequently, Equation (5-7) offers a guide for design and operation engineers to manipulate the aerobic granulation process through adjustments of the settling time, discharge time and the volume exchange ratio.

Figure 5-3 Relationship between settling time and discharge time for a desired $(V_s)_{\min}$

5.4 Conclusion

Effects of settling velocity, volume exchange ratio and discharge time actually can be unified into one single factor, i.e. minimum settling velocity, which represents a unified selection pressure for successful aerobic granulation in SBR. By controlling minimum settling velocity within a valid range, e.g. $(V_s)_{\min} > 8 \text{ m h}^{-1}$, successful aerobic granulation would be accomplished with different combinations of setting

Chapter 5 A Unified Selection Pressure Theory

time, volume exchange ratio and discharge time values. Thus, the minimum settling velocity can be used as a guideline to upscale aerobic granular sludge SBR with various options of settling time, volume exchange ratio and discharge time.

CHAPTER 6

DISTRIBUTION OF EPS AND CELL SURFACE HYDROPHOBICITY IN AEROBIC GRANULE

6.1 Introduction

So far, most research on aerobic granulation has been focused on the cultivation and physical characteristics of aerobic granules. It is well known that the family of biogranulation has two major members, i.e. aerobic granules and anaerobic granules (Liu and Tay 2004). The structure of anaerobic granules has been studied extensively (Fang 2000; Gonzalez-Gil et al. 2001; Lanthier et al. 2002; Quarmby and Forster 1995), and EPS have been found to contribute to the build-up of the matrix structure and the stability of anaerobic granules (Liu et al. 2004c). The failure of microbial aggregation has been found to be correlated to the metabolic blocking of EPS synthesis (Cammarota and Sant'Anna 1998; Yang et al. 2004b), while an EPS deficiency would result in a weak structure of anaerobic granules (Quarmby and Forster 1995). Compared to anaerobic granules, little is currently known about the internal structure of aerobic granules as well as the EPS and cell hydrophobicity distribution in aerobic granules. Thus, this study investigated the internal structural distribution of EPS and hydrophobicity in aerobic granules as well as the essential role of EPS in aerobic granulation.

6.2 Materials and Methods

Experimental set-up

Aerobic granules were cultivated in a column reactor of 100 cm height and 5 cm diameter. The other operation parameters were the same as those used in the Chapter 3 study. Meanwhile, a 50 % volume exchange ratio was used.

Substrate and seed sludge

Synthetic wastewater used for this phase of study consisted of sodium acetate as the sole carbon source. Detailed composition is shown in Chapter 3.

The reactor was inoculated with 2 g L⁻¹ of fresh activated sludge taken from Jurong wastewater treatment plant, Singapore. The seed sludge had a SVI of 202 mL g⁻¹. The average floc size of the seed sludge was 97 µm.

Analytical procedures

EPS extraction and analysis

EPS was extracted from ground aerobic granules according to the cold aqueous technique: the ground granules were suspended in 2 mL of 8.5% NaCl and 0.22% formaldehyde solution, chilled and mixed for 3 minutes, and the sample was then centrifuged at 12000 rpm by using mini spin (Eppendorf, Germany) for 30 minutes (Jia et al. 1996). The supernatant was sampled for EPS analysis by using the method developed by Dubois et al. (1956).

EPS visualization

According to the method by deBeer et al. (1996), sliced aerobic granules were stained with 300 mg L⁻¹ calcofluor white (Fluorescent Brightener 28, Sigma) for 1 h

in 20 mL phosphate buffered saline. The samples were then rinsed with Milli-Q water to decrease the background fluorescence and were then visualized with an Epifluorescence Microscope BX-FLA-3 (Olympus, Japan). Dye fluorescence intensity was measured by using Micro Image (Olympus, version 4.5.1.22) that was coupled with the Epifluorescence Microscope.

Microscopic analysis of aerobic granule internal structure

Both intact and sectioned aerobic granules were visualized by using the Image Analyzer (IA) (Olympus Imaging Analyzing System SZX9, Japan). According to the procedure recommended by IA, the optical density (OD) of the sectioned granule sample was measured by analyzing the transmittance of the light (blue filter KB-4, Olympus) that radiated from behind specimen, and was calculated as follows:

$$OD(x, y) = -\log [(intensity(x, y) - black) / (incident - black)]$$

in which intensity(x, y) is the light intensity at pixel (x, y), black is the light intensity generated when no light goes through the sample, and incident is the intensity of the incident light. OD has been previously used to correlate with biofilm density (Bakke et al. 2001).

Sliced segment density

The weight of sliced segment was measured by using a high sensitivity balance (Perkin Elmer, AD6, USA) with precision up to 0.001 mg. IA microscopic technology coupled with the integrated Micro Image (Olympus, version 4.5.1.22) was exploited to determine the segment area so as to work out the volume by multiplying by the segment thickness. Finally, the sliced segment density was calculated by this mass weight to its volume ratio.

Hydrophobicity analysis

This method has been detailed in Chapter 3.

6.3 Results

6.3.1 Heterogeneous structure of aerobic granule

Figures 6-1 and 6-2 show that aerobic granules have an opaque outer layer with a depth of about 800 μm from the granule surface and a relatively transparent inner core. Optical density (OD) has been commonly used to quantify the biomass concentration, i.e. a high OD is correlated to a high biomass concentration or density in suspended and biofilm cultures (Gaudy and Gaudy 1980). Figure 6-3 exhibits the OD profile from the surface to the center of an aerobic granule. It was found that the OD in the granule center was close to zero, indicating a very low biomass density or a loose microbial structure. In contrast, a peak OD was observed in the outer layer of the granule, which would result from a high biomass density or a compact microbial structure. It appears from Figure 6-1 that the highly transparent center part of the granule was not void but was filled with jelly-like substances. To confirm these observations, five aerobic granules, namely No.1 to 5, were sliced and the respective mass density of the outer and the inner layer was then measured (Figure 6-4). These results indicate that the mass density of the outer layer of granule is higher than that of the core part of the aerobic granule. As can be seen in Figures 6-1 to 6-3, the core part of the granule was composed of transparent and jelly-like substances, while the outer shell of the granule mainly consists of dense materials leading to an opaque structure.

6.3.2 EPS and hydrophobicity distribution in aerobic granule

The outer shell of the aerobic granule was separated from the core part, and the corresponding EPS content as well as hydrophobicity was determined. Figure 6-5 shows that the EPS content in the core part of the granule is nearly 5 to 6 times higher than that in the shell part of aerobic granule. To localize the EPS distribution, the sliced aerobic granule was stained by calcofluor white, and was then visualized by epifluorescent microscopy. Using a fresh granule as reference (Figure 6-6), it

Figure 6-1 Cross section view of aerobic granule in dark-field visualization mode, bar: 500 μm

Figure 6-2 Cross section view of aerobic granule in bright-field visualization mode, bar: 500 μm

Figure 6-3 OD profile from aerobic granule surface into its center (*arrow*)

Figure 6-4 Mass density ratios of shell layer to core part of aerobic granules, dashed line indicates its mean value

was found that the fluorescent dye was mainly attached to the outer shell of the granule, while the fluorescence was very weak in the center of the granule (Figure 6-7). The fluorescence intensity profile in the direction of granule radius further shows that most calcofluor white stained EPS is situated in the outer shell of the granule with a depth of 400 μm below the granule surface (Figure 6-8). These may imply that the β -linked EPS would be mainly located in the outer shell of the granule. In fact, calcofluor white has been commonly used to label the β -linked EPS (deBeer et al. 1996). In addition, Figure 6-9 shows the respective hydrophobicity of the shell and core of an aerobic granule. It seems that the granule outer shell has a much high hydrophobicity than the granule core.

Figure 6-5 Ratios of EPS concentration in core part to shell layer of aerobic granules, dashed line indicates its mean value

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Figure 6-6 Microscopic view of one quarter aerobic granule cross-section before calcofluor white staining, bar: 100 μm

Figure 6-7 Microscopic view of one quarter aerobic granule cross-section after calcofluor white staining, bar: 100 μm

Figure 6-8 Distribution of fluorescence dye intensity from aerobic granule surface into its center (arrow)

Figure 6-9 Hydrophobicity ratios of shell layer to core part of aerobic granules, dashed line indicates its mean value

6.3.3 Response of aerobic granule to starvation

The fresh aerobic granules harvested from the parent reactor were starved under aeration conditions without addition of carbon and nutrient sources for 20 days. Figures 6-10 and 6-11 show changes in granule structure before and after the 20-days starvation. Compared to the fresh aerobic granule, the center part of the starved granule became very transparent. Further examination of the starved granule reveals that after 20-days starvation, the granule core part became hollow, while the outer shell still remained intact (Figure 6-12). These observations seem to suggest that the EPS present in the granule shell would not be taken up by bacteria. However, the EPS located in the core of the aerobic granule could be biodegraded under the starvation condition. In fact, many β -linked EPS, e.g. cellulose and chitin, are usually non-readily biodegradable.

Figure 6-10 IA view of aerobic granule before starvation, bar: 300 μ m

Figure 6-11 IA view of aerobic granule after starvation, bar: 300 μm

Figure 6-12 Void structure of starved aerobic granule, bar: 300 μm

6.4 Discussion

Figures 6-1 to 6-4 show that aerobic granules have a heterogeneous structure consisting of a dense outer shell and a loose-structure core, while the outer shell of the aerobic granule is mainly composed of β -linked EPS which is more hydrophobic and non-readily biodegradable under the starvation condition (Figures 6-5 to 6-12). These may imply that the EPS detected in the core of the aerobic granule would be easily biodegradable. According to their physico-chemical properties, EPS can be classified into bound EPS and soluble EPS, while only soluble EPS would be biodegradable (Laspidou and Rittmann 2002).

Previous research showed that substrate could only penetrate to a depth of 800 μm below the aerobic granule surface (Tay et al. 2003b). This implies that a nutrient deficient situation would be encountered in the core part of aerobic granules as shown in Figures 6-1 and 6-2. There is evidence that the nutrient deficiency condition would induce the production of soluble EPS. It has been reported that compared to a balanced culture, 10 times more soluble EPS was secreted in nutrient deficiency culture (Aquino and Stuckey 2003). This provides a plausible explanation for the enhanced production of soluble EPS in the granule core (Figure 6-5). It is a reasonable consideration that soluble EPS would be less important in constructing and maintaining the structural stability of the aerobic granule, i.e. EPS property and distribution in aerobic granules instead of their quantity would play a crucial role in improving the stability of aerobic granules. In addition, it appears from Figures 6-5 to 6-9 that the high hydrophobicity of the outer shell of an aerobic granule would result from the insoluble β -linked polysaccharides accumulated in the shell. In fact, insoluble β -linked EPS has been found to serve as the backbone of the biofilm structure (Sutherland 2001).

The long-term starvation test clearly shows that the β -linked polysaccharides in the outer shell of granule are not biodegradable (Figures 6-10 to 6-12). The non-biodegradable outer shell of aerobic granule may serve as a protective barrier

responsible for biological stability and ecological integrity of the granule. It is obvious that hydrolysis or disappearance of this insoluble EPS would result in disintegration of the granule. In fact, the destruction of biofilm structure was observed after a key biofilm EPS was hydrolyzed (Skillman et al. 1999).

6.5 Conclusion

This study showed that aerobic granules had a heterogeneous structure comprising a dense and hydrophobic outer shell and a less hydrophobic and loose core. The outer shell of the aerobic granule was composed of poorly soluble and non-biodegradable EPS that was responsible for the physico-chemical and biological stability and integrity of the aerobic granule matrix, while the core part of the granule was filled with readily soluble and biodegradable EPS.

CHAPTER 7

BIODEGRADABILITY OF EXTRACELLULAR

POLYSACCHARIDES PRODUCED BY AEROBIC GRANULES

7.1 Introduction

In the past decades, intensive research effort has been dedicated to a sound understanding of the role of EPS in the formation of biofilm, and anaerobic and aerobic granules (Danese et al. 2000; Liu et al. 2004c; Schmidt and Ahring 1994; Tay et al. 2001c). It has been thought that EPS should not be biodegraded by their own producer; i.e. EPS-producing organisms are unable to utilize their own EPS as carbon source (Dudman 1977; Obayashi and Gaudy 1973; Pirog et al. 1977; Sutherland 1999). On the contrary, increasing evidence shows that EPS could be utilized by their own producers (Boyd and Chakrabarty 1994; Patel and Gerson 1974; Ruijsenaars et al. 2000; Zhang and Bishop 2003).

Aerobic granules' core part, where enormous amounts of hydrophilic EPS are distributed, has been found to become void as a result of starvation, as described in Chapter 6. Compared to suspended bioflocs, high EPS content in aerobic granules was also measured (Figures 3-6 and 4-6). However, little is known about the biodegradability of EPS produced by aerobic granules as well as their contribution to the stability of aerobic granules. Therefore, this study attempted to investigate the biodegradability of EPS produced by aerobic granules. It is expected that such results would offer in-depth insights into the role of EPS in constructing and maintaining the matrix structure of aerobic granules, and also provide additional experimental evidence for arguments about EPS biodegradability.

7.2 Materials and Methods

Cultivation of aerobic granules

Aerobic granules were cultivated in a lab-scale SBR with a diameter of 5 cm and a working height of 100 cm. The SBR was inoculated with activated sludge taken from a local municipal wastewater treatment plant, and was operated in a time sequence of 5 min settling, 5 min discharging, 5 min filling and 225 min aeration at 25 °C. Effluent was withdrawn from an outlet located at 50 cm height from the bottom of the reactor. Air was introduced from the bottom of the reactor through a fine bubble aerator at a rate of 3 L min⁻¹. The synthetic wastewater used mainly consisted of sodium acetate, 2560 mg L⁻¹ and NH₄Cl, 200 mg L⁻¹; K₂HPO₄, 45 mg L⁻¹; CaCl₂·H₂O, 30 mg L⁻¹; MgSO₄·7H₂O, 25 mg L⁻¹; FeSO₄·7H₂O, 20 mg L⁻¹. This gave a loading rate of 6 kg COD m⁻³ d⁻¹.

Starvation test of aerobic granules

The starvation tests of aerobic granules were carried out in a 1 liter beaker at 25 °C for 20 days. In these tests, 4 g suspended solids of aerobic granules were added to 1 liter of the above synthetic wastewater, but free of sodium acetate. During the tests, changes in the EPS content of the aerobic granules were determined.

Biodegradability test of EPS

EPS were first extracted from fresh and starved aerobic granules using the methods described below, respectively. In the batch tests of EPS biodegradability conducted at 25 °C, initial EPS concentration was adjusted to 100 mg COD L⁻¹, while initial granules concentration was kept at 1 g SS L⁻¹. Each test lasted for 30 hours. The EPS concentrations in terms of polysaccharides, protein and COD were determined during the test period. A control test with sodium acetate as the sole substrate was also done for comparison purposes.

Analytical methods

Fresh aerobic granules taken from the SBR were gently rinsed with deionized (DI) water, and were then ground with a mortar. The ground granules were further homogenized with DI water by using a vortexer (Vision Scientific) for 1 min. The EPS of the granules were extracted according to the cationic exchange resin method developed by Frolund et al. (1996). In this method, the homogenized granules in DI water were mixed with cationic exchange resin (DOWEX Marathon C sodium form with a size of 20 to 50 mesh) at a ratio of 70 g resin to 1 g dry biomass of granules. The granules-resin mixture was stirred at 4 °C and 750 rpm for 4 hours, and pH was automatically controlled at 6.9 to 7.2 by adding 0.1 M HCl solution. After the 4 hours extraction, the supernatant was filtered through 0.45 µm cellulose-acetate filters and the filtrate was used for EPS determination.

EPS were determined by the method developed by Dubois et al. (1956). In this study, a fluorescence dye, namely calcofluor white (Fluorescent Brightener 28, Sigma), was used to visualize the distribution of the beta-linked EPS in aerobic granules according to the method by deBeer et al. (1996). In this method, a sliced aerobic granule was stained with 300 mg L⁻¹ calcofluor white for 1 h in 20 mL Phosphate Buffered Saline. The stained granules were rinsed with Milli-Q water to reduce the background fluorescence, and were then visualized with an Epifluorescence Microscope BX-FLA-3 (Olympus, Japan).

The oxygen utilization rate of the aerobic granules was studied using a respirometer (Columbus Instruments Micro-Oxymax). An Image Analyzer (Quantimet 500 Image Analyzer, Leica Cambridge instruments) was employed for the observation of granule morphology, and the integrated Micro Image software (Olympus, version 4.5.1.22) was used to determine the mean size of granules. In this study, suspended solids (SS), volatile solids (VS), sludge volume index (SVI) of granules, and the chemical oxygen demand (COD) of samples were determined according to standard methods (APHA 1998). All the measurements were done in triplicates.

7.3 Results

7.3.1 Change of EPS in aerobic granules under starvation condition

Fresh aerobic granules taken from the SBR had an initial SOUR of $62 \text{ mg O}_2 \text{ g}^{-1} \text{ VS h}^{-1}$; SVI of 55 mL g^{-1} and a mean diameter of 1.6 mm. They were then subjected to aerobic starvation without addition of external carbon source for 20 days. Results showed that the EPS in aerobic granules decreased after aerobic starvation, e.g. about 75% of the EPS of aerobic granules were utilized at the end of the 20-day starvation (Figure 7-1). This may imply that part of the EPS produced by aerobic granules would be biodegradable by their own producers under the studied condition. Image analysis further revealed that even after the 20-day starvation, the structure of aerobic granules still remained intact, but they became much more transparent compared to the fresh granules (Figure 7-2). It should be realized that the EPS remaining after the 20-day starvation period would represent the fraction of non-biodegradable EPS in the total EPS produced, which accounted for about 25% of the total EPS in the granule (Figure 7-1). This small portion of non-biodegradable EPS in the granule would be responsible for the structural integrity of the aerobic granule.

7.3.2 Biodegradability of extracted EPS from aerobic granules

To further look into the biodegradability of granule EPS, the EPS extracted from fresh aerobic granules were fed, as sole carbon source, to the pre-starved aerobic granules in order to avoid the interference of their own energy storage on the batch experiments.

Biodegradability of EPS extracted from fresh aerobic granules.

Figure 7-3 shows the biodegradation profiles of EPS obtained from the batch experiments. A rapid biodegradation of EPS in the first 10 hours was observed and

Figure 7-1 Change of EPS content in aerobic granules in the course of 20 days starvation

Figure 7-2 Morphology of aerobic granules before (a) and after (b) 20 days starvation, bar: 1 mm

was followed by a stationary phase, i.e. about 50% of EPS could be utilized by their own producers as the external carbon source. According to Figure 7-3, the average linear biodegradation rate of EPS was estimated as $15.2 \text{ mg EPS g}^{-1} \text{ SS d}^{-1}$.

Figure 7-3 Biodegradability of EPS extracted from fresh aerobic granules

Biodegradability of EPS extracted from starved aerobic granules.

It appears from Figure 7-3 that more than 50% of the EPS extracted from fresh aerobic granules were non-biodegradable under the studied conditions. To confirm such an observation, the EPS extracted from the starved aerobic granules were fed to aerobic granules as the sole external carbon source. Figure 7-4 shows no significant biodegradation of the EPS extracted from the starved granule, indicated by a very slow average biodegradation rate of $0.38 \text{ mg g}^{-1} \text{ SS d}^{-1}$ for EPS.

Figure 7-4 Biodegradability of EPS extracted from starved aerobic granules

7.3.3 Distribution of non-biodegradable EPS in aerobic granule

As shown in Figures 7-1, 7-3 and 7-4, the EPS produced by aerobic granules can be classified into two major groups under the studied conditions, i.e. biodegradable EPS and non-biodegradable EPS. Previous research revealed that the non-biodegradable EPS would mainly belong to the family of beta-linked EPS (Chapter 6). To further look into the distribution of the beta-linked EPS in aerobic granules, a sectioned starved-aerobic granule was stained with calcofluor white. Figure 7-5 shows that the most stained EPS after the 20-day starvation were situated at the outer-shell of the aerobic granule, while a significant void space was observed in the core-part of the starved aerobic granule.

7.3.4 Comparison of biodegradability of acetate and extracted EPS

For comparison purposes, batch experiments fed with sodium acetate and the EPS extracted from the fresh and starved aerobic granules were conducted, respectively.

Figure 7-5 Visualization of beta-linked EPS in aerobic-granule after 20 days starvation by the epifluorescence microscopy, (a): unstained granule; (b): stained granule, bar: 200 μm

The initial acetate and extracted EPS concentrations were kept at the same level of 100 mg COD L^{-1} . Figure 7-6 shows that the average biodegradation rate of acetate, EPS extracted from fresh and starved aerobic granules was 5.4 mg COD mg^{-1} SS d^{-1} , 1.1 mg COD mg^{-1} SS h^{-1} , and 0.018 mg COD mg^{-1} SS h^{-1} , respectively. Such results are in a good agreement with those presented in Figures 7-3 and 7-4. In this study, the respirometric test using fresh aerobic granules in the presence of external acetate was also carried out (Figure 7-7). These results indicate that the OUR was closely related to the availability of the external and internal energy sources.

7.4 Discussion

The results in Figures 7-1 and 7-2 seem to indicate that the EPS produced by aerobic granules were mainly composed of biodegradable and non-biodegradable EPS under the studied conditions. Figures 7-3 and 7-4 further revealed that at least 50% of EPS produced by aerobic granules would be biodegradable. In fact, previous research also showed that EPS extracted from biofilms could be effectively degraded by their own producers (Zhang and Bishop 2003).

Figure 7-6 Biodegradability of acetate and EPS extracted from aerobic granules. 1: acetate (+); 2: EPS extracted from fresh aerobic granules (○); 3: EPS extracted from starved aerobic granules (●)

Figure 7-7 The OUR profile in response to substrate consumption

The utilization rate of the biodegradable EPS by their own producers was 5 times slower than that of acetate, while it was almost 50 times faster than that of the non-biodegradable EPS (Figure 7-6). Theoretically, the biodegradable EPS would not contribute to the structural stability of granules because of their relatively high biodegradability, but they can indeed serve as energy source during the starvation of granules. And only the non-biodegradable EPS would be responsible for the spatial integrity of aerobic granules because of their extremely slow biodegradation rate or high biological stability (Figure 7-6). It should be noted that three regimes can be seen in Figure 7-7, i.e. (i) the regime of acetate utilization indicated by the highest OUR; (ii) the regime of the EPS biodegradation after the complete depletion of acetate; and (iii) the regime of endogenous metabolism with the lowest OUR. It seems the OURs of these three regimes would be well correlated to the biodegradation rates of acetate, and of EPS extracted from fresh and starved aerobic granules.

In fact, the role of EPS in maintaining the spatial structures of biofilms, and of aerobic and anaerobic granules have been reported, but with no differentiation of biodegradable and non-biodegradable EPS (Flemming and Wingender 2001; Liu et al. 2004c). As discussed in Chapter 6, most of the biodegradable EPS was located in the core part of the aerobic granules, and this part of EPS could be depleted after biodegradation during the long-term starvation, and subsequently result in a void structure in the starved granules. This and previous research clearly indicate that only non-biodegradable EPS would be responsible for the structural integrity and stability of aerobic granules, and biodegradable EPS with a large fraction in the total EPS produced by aerobic granules would mainly serve as spare energy pool during aerobic starvation.

7.5 Conclusion

This study, probably for the first time, showed that the EPS produced by aerobic granules could be divided into two major groups, namely biodegradable and non-readily biodegradable EPS. Nearly 50% of the total EPS produced can be re-utilized

Chapter 7 Biodegradability of EPS

by their own producers under the starvation conditions, and this part of the biodegradable EPS would not make any contribution to the structural stability of aerobic granules. Meanwhile, it was found that the structural integrity of aerobic granule was tightly related to the non-readily biodegradable EPS which was mainly located on the outer-shell of the aerobic granule. The kinetic studies further revealed that the specific utilization rate of the biodegradable EPS was 5 times slower than that of acetate, but 50 times faster than the non-readily biodegradable EPS. The extremely low biodegradation rate of the non-readily biodegradable EPS in turn supports the point of view that they are the main contributor to the matrix stability of aerobic granules.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Volume exchange ratio could be an effective driving force for aerobic granulation in SBR

The volume exchange ratio could be exploited as an effective driving force of aerobic granulation in SBR, i.e. a small volume exchange ratio would hinder aerobic granulation, while a high exchange ratio would favor and enhance aerobic granulation in SBR. It was also found that high volume exchange ratio would stimulate the excessive production of EPS and subsequent calcium accumulation that would in turn facilitate the formation and subsequent stability of aerobic granules.

Discharge time could be an additional driving force of aerobic granulation in SBR

Discharge time was found to be an additional driving force of aerobic granulation besides the volume exchange ratio in SBR. A prolonged discharge time could result in a failure of aerobic granulation even though both settling time and volume exchange ratio were properly controlled. According to the present study, a discharge time longer than 20 min would be unfavorable for aerobic granulation. It appears that the role of discharge time should be seriously taken into account in the design and operation of a full-scale aerobic granular sludge SBR.

A unified theory is developed for aerobic granulation in SBR

To interpret the effects of settling time, volume exchange ratio and discharge time on aerobic granulation in SBR, a unified theory was further developed. This theory showed that the above three driving forces indeed could be unified into a single one, i.e. minimum settling velocity that is a function of the settling time, volume exchange ratio and discharge time. The data analysis indicated that the minimum settling velocity for aerobic granulation should not be smaller than 8 m h^{-1} . It became clearer that the minimum settling velocity can be used as a guideline to upscale aerobic granular sludge SBR with various options of settling time, volume exchange ratio and discharge time.

Aerobic granule had a heterogeneous structure with non-uniform distribution of EPS and cell surface hydrophobicity

Aerobic granule had a heterogeneous structure consisting of a dense and hydrophobic outer shell and a loose and less hydrophobic core. The outer shell of aerobic granules was composed of the poorly soluble and non-readily biodegradable EPS that was responsible for the physico-chemical and biological stability and integrity of the aerobic granule, while the core part of the granule was filled with soluble and readily biodegradable EPS, which would be used as an energy reservoir under carbon starvation conditions.

EPS could be partially biodegraded by their own producers

It was shown that the EPS produced by aerobic granules could be divided into two major groups, namely biodegradable and non-readily biodegradable EPS. Nearly 50% of the total EPS produced can be re-utilized by their own producers under the starvation conditions, and this part of the biodegradable EPS would not make any significant contribution to the structural stability of aerobic granules. Meanwhile, it was found that the structural integrity of aerobic granules was closely related to the

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non-readily biodegradable EPS which was mainly located on the outer-shell of the aerobic granule. The kinetic studies further revealed that the specific utilization rate of the biodegradable EPS was 5 times slower than that of acetate, but 50 times faster than the non-readily biodegradable EPS. The extremely low biodegradation rate of the non-readily biodegradable EPS in turn provides a plausible explanation for the fact that they would be the main contributor to the stability of aerobic granules.

8.2 Recommendations for future study

This study offered deeper insights into the mechanism of aerobic granulation in SBR. Further study is desirable to apply this selection pressure theory in the design and operation of a full-scale aerobic granular sludge SBR. Moreover, the potential role of the newly found biodegradable EPS in nitrogen and phosphate removal by aerobic granules deserves further investigation.

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