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Aerobic Granulation: Advances and Challenges

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Abstract Aerobic granulation was developed in overcoming the problem of biomass washout often encountered in activated sludge processes. The novel approach to developing fluffy biosolids into dense and compact granules offers a new dimension for wastewater treatment. Compared with conventional biological flocs, aerobic granules are characterized by well-defined shape and compact buildup, superior biomass retention, enhanced microbial functions, and resilient to toxicity and shock loading. This review provides an up-to-date account on development in aerobic granulation and its applications. Granule characterization, factors affecting granulation, and response of granules to various environmental and operating conditions are discussed. Maintaining granule of adequate structural stability is one of the main challenges for practical applications of aerobic granulation. This paper also reviews recent advances in addressing granule stability and storage for use as inoculums, and as biomass supplement to enhance treatment efficiency. Challenges and future work of aerobic granulation are also outlined.

Keywords Aerobic granulation · Stability · Formation · Review

Introduction

Ever since the initial reports on aerobic granules in a continuous upflow aerobic sludge blanket bioreactor [1] and in a sequencing batch reactor [2], extensive research work on

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aerobic granulation has been investigated by many researchers [2–8]. Granulation is a self-immobilization phenomenon in which fluffy biological solids assemble and agglomerate as dense and compact granular sludge under controlled loading and operating conditions. Just like anaerobic granular sludge, aerobic granules exhibit superior characteristics over normal biological flocs, such as well-defined shape and structure, high settling velocities, and biomass retention, plus ability to withstand high organic loadings [3, 6, 9]. Because of its unique features, the application of aerobic granular sludge is perceived as one of the promising wastewater treatment technologies.

Aerobic granules have been examined in various laboratory-scale bioreactors treating a variety of high-strength substrates containing biodegradable as well as toxic substances [9–12]. The formation of nitrifying granules and phosphorus accumulating granules was also successfully demonstrated [11, 13, 14]. Extensive work on granule characterization, factors affecting granulation, response of granules to various environmental and operating conditions, and granulation mechanisms has been documented.

There is, however, limited study of pilot- or full-scale aerobic granular sludge systems. For practical large-scale industrial operation, continuous-flow reactors are preferred over batch-operated SBRs for lower plant installation cost and easy reactor operation, maintenance, and control. A study had revealed that aerobic granules developed in a continuous-flow reactor would become unstable faster than those in a sequencing batch reactor (SBR) [15]. On the other hand, it appeared from other studies that stable granule structure can only be sustained for a limited time in an SBR [16–18]. It appears that maintaining granule of adequate structural stability poses a major challenge for large-scale operation. The current bottleneck underscores the need for further research on granule stability for practical applications.

This paper presents a review on recent development in aerobic granulation encompassing granule formation and characterization as well as response of granules to various environmental and operating conditions. As maintaining granule of adequate structural stability is a major challenge for practical applications, this paper also reviews recent advances addressing granule stability and storage for use as inoculums, and as biomass supplement to enhance treatment efficiency of bioreactors. Challenges and future work of aerobic granulation are also outlined.

Characterization, Granulation, and Treatment Performance

Granule stability may associate with one or more of the attributes of granules. A good understanding on granule characterization and formation would help in a better understanding of granule stability. Granule characteristics can be broadly categorized as physical, chemical, and biological attributes as discussed in the following sections.

Physical Attributes

One important physical attribute of aerobic granules is their settling velocity. Granules could settle with a speed ranging from 25 to 70 $m h^{-1}$, which are significantly higher than that of flocs with 7 to 10 $m h^{-1}$ [19, 20]. This means settleability of sludge can be improved significantly through the formation of granules and it would enable desirable biomass retention in the reactor resulting in good organic degradation efficiency and reactor stability. Moreover, high granule settling velocity would allow reactor be operated with high hydraulic flow, which could translate into a relatively compact reactor design.

Granule with a high physical strength would withstand high abrasion and shear. The physical strength, expressed as integrity coefficient, is higher than 95% for the aerobic granules grown on glucose and acetate [21]. Smaller size granules tend to be denser than larger aerobic granules [22, 23]. Adav et al. [8] evaluated the hydraulic characteristics of aerobic granules using size exclusion chromatography. It was reported that intra-granular transport for molecular compounds larger than 140,000 Da can be severely hampered by diffusion resistance.

Chemical Attributes

The hydrophobicity of aerobic granules was reported twofold higher than normal bioflocs [13]. While cell surface hydrophobicity showed insignificant effects on organic loading rates, increase in shear force would increase cell surface hydrophobicity [13]. EPS are metabolic products accumulating on the surface of bacterial cells, which could alter the physico-chemical characteristics of cellular surface such as charge, hydrophobicity, and other properties. Using ultrasound followed by chemical reagents formamide and NaOH extraction method, Adav and Lee [24] reported that total polysaccharides/total proteins (PS/PN) ratios ranged between 3.4 and 6.2 for granules, which were much higher than that for sludge flocs (approximately 0.9).

Microbial activity of microorganisms is often measured with the specific oxygen utilization rate (SOUR). A wide range of SOUR values (34–168 mg O_2 g^{-1} VSS h^{-1}) for aerobic granules have been reported [2, 25–27]. The SOUR has been found to increase with the increase in superficial air velocity which is normally associated with liquid shear force. It has been shown that the increased shear force can significantly stimulate the respiration activities of aerobic microorganisms [25] at granule–liquid interface [28]. Liu and Tay [7] emphasized that high shear force assisted formation of compact and denser aerobic granules by stimulating production of extracellular polysaccharides. The density, settling velocity, and strength of the granules were proportionally related to the applied shear force [29]. High aeration rates (2.0 to 6.0 l air min^{-1}) were proposed to be essential for maintaining granule stability [30]. High aeration rate is considered to provide high shear force for eroding surface of granules to yield smooth surface, to simulate bacterial strains to secret more extracellular polymeric substances (EPS) for enhancing structural integrity, to reduce substrate transfer resistance from external pool to granule surface, and to provide sufficient oxygen for substrate degradation.

The microbial activity represented by SOUR is inversely proportional to the settling time [20], viz., the shorter settling time tends to stimulate respiratory activity of these microorganisms. Aerobic microorganisms may attempt to regulate their energy metabolism in response to changes in hydraulic selection pressure associated with settling time. SOUR is a good indicator for monitoring the physiology changes of granule especially when treating wastewater containing toxic substances. Biomass growth and substrate removal can be linked to batch measurement of SOUR, and estimation of permissible organic loading rate. Hence, SOUR is an important characteristic for assessing the ability of aerobic granules to handle high-strength industrial wastewaters [31].

Biological Attributes

Use of scanning electron microscope (SEM), light microscopy, and confocal laser scanning microscopy (CLSM) together with fluorescence in situ hybridization (FISH) enables microscopic view into the microbial structure of aerobic granules [32–35]. The taxonomic microbial

diversity in wastewater treatment plant and phenol-fed aerobic granules showed the dominance of proteobacteria [36–38]. Jiang et al. [38] identified ten isolates from matured phenol-fed granules of which six had taxonomic affiliations with β-proteobacteria, three with Actinobacteria, and one with γ-proteobacteria. Whiteley and Bailey [37] studied the bacterial populations in the specific compartments of an operational industrial phenol remediation system using proteobacterium group specific probes and found the majority to be β- and γ-proteobacterium. Gram and Neisser stains and FISH analyses showed that most of the filamentous bacteria in aerobic granules cultivated in brewery wastewater belonged to the genus *Thiothrix* or to *Sphaerotilus natans* [39]. FISH–CLSM technique identified that obligate aerobic ammonium-oxidizing bacterium *Nitrosomonas* spp. was mainly at a depth of 70 to 100 μm from the granule surface, while anaerobic bacterium *Bacteroides* spp. was detected at a depth of 800 to 900 μm from the granule surface [21, 40–44].

Microbial diversity was closely related to the structure of granules and the composition of culture media in which they were developed. Anaerobiosis and dead cells occurring at the centers of aerobic granules was reported [35]. Presence of anaerobic microorganisms in aerobic granules may result in organic acids and gases produced within the granules. These end products of anaerobic metabolism could eventually lead to disintegration of aerobic granules.

Using CLSM coupled with different specific fluorochromes, fluorescent microspheres, and oligonucleotide probes, the interior structure of bioaggregates developed during aerobic granulation process were examined [42, 43, 45]. The fluorescent staining and CLSM experiments demonstrated that microbial aggregation has a loose structure, with protein and β-polysaccharides distributing over the granule interior, and with lipids and α-polysaccharide at the rim regime. In particular, a core of dead cells was surrounded by live cells. The mature granule has a non-cellular protein core with networks by β-polysaccharides. Cells were accumulated at outer regimes. Based on CLSM images, the granulation process is speculated to be composed of (1) cell–cell aggregation to form granule seed; (2) cells grow and develop to a size that limits substrate transport, hence leading to cell lysis at inner core; (3) the granule structure is compacted by shear force and leaves a non-cellular protein-β-D-glucopyranose polysaccharide core as the lysed products. The mature granule has a smooth outer surface to minimize damage by granule–granule collision, while the protein–polysaccharide core provides mechanical strength for granule to resist shear stress. The functional strains principally distributed at the rim regime to minimize substrate transport resistance [35].

Granulation

Aerobic granulation is a progressive evolution process of biological solids evolving from fluffy seed flocs to dense granules. Unlike anaerobic granulation which appears to be spontaneous, aerobic granules can be cultivated with controlled loading and operating strategy. It has been reported that granulation is influenced by a variety of factors, including reactor startup and operating conditions such as seed sludge, substrate composition, organic loading rate, feeding strategy, reactor design and hydrodynamics, settling time, exchange ratio, and aeration intensity [2, 3, 6, 9, 11, 21, 26, 31, 40–44, 46, 47]. The following sections discuss the influence of these environmental and operating conditions and the response of granules to these factors.

Seeding and Cultivation of Inoculums

Different microbial community in seed sludge exhibited different agglomeration abilities arising from variation of physico-chemical characteristics [48] which can be associated with

microbial strain, hydrophilic or hydrophobic nature, or it can be a result of certain EPS activities in which case intra-species differences can be found [49, 50]. The greater number of hydrophobic microbial community in the seed could result in faster aerobic granulation with excellent settleability [51]. Activated sludge has been used as seed in most aerobic granulation studies. Aerobic granules, however, can be cultivated by seeding completely autotrophic nitrifying microorganisms in SBR [52]. The autotroph-cultivated granules matured after 43 days of cultivation.

Adav and co-workers [53] successfully cultivated single culture aerobic granules using three separate isolates from a phenol-fed aerobic granule. The isolates presented high phenol-degrading (66.1±11.8 mg phenol g VSS^{-1} h^{-1}) and high auto-aggregation capabilities (autoaggregation index of 78.5%). Conversely, isolates with low (autoaggregation index of 4.4%) or no auto-aggregation capability failed to develop into single-culture granules. It was noted that lectin–saccharide interactions correspond to the cell–cell aggregation arising from specific pairing of the isolates. Jiang et al. [54] also cultivated phenol-degrading granules with two strains—one strain with high phenol degrading capability (1.4 g phenol g VSS^{-1} day^{-1}) but low flocculating potential (autoaggregation index of 2.3%), and the other strain with (0.5 g phenol g VSS^{-1} day^{-1}). Aerobic granules were cultivated using single culture *Corynebacterium* sp. DJ1 with high auto-flocculating (autoaggregation index of 74.8%) and high phenol-degrading capabilities (162 mg phenol g^{-1} VSS h^{-1}) [12]. The cultivated granules were subsequently used to degrade phenol at concentrations greater than 2,500 mg l^{-1} with good efficiency. On the other hand, suspended form of DJ1 strain experienced severe inhibition at 2,000 mg l^{-1} phenol, indicating the ability of granulation in protecting microbial community from toxicity.

Bacterial and fungal granules were cultivated from $CaCO_3$ alkalinity medium of pH 8.1 and pH 3, respectively [55]. Both bacterial and fungal granules were able to degrade waste effectively, with the former exhibited dense structure while the latter showed a fluffy and loose interior structure. Fungal granules can form more rapidly than bacterial granules, but with weaker interior strength. It is clear that functional bacterial strains must exist in the seed sludge to induce cell aggregation leading to granulation.

Type and Loading of Substrate

Various types of substrate were used to cultivate aerobic granules. These included glucose, acetate, phenol, starch, ethanol, molasses, sucrose, and other synthetic wastewaters [2–4, 6, 9, 40–44, 46, 56–58]. Inorganic carbon source dominated by nitrifying bacteria was used to cultivate aerobic granules [33, 59]. Literature on granulation with actual wastewaters was also reported [60–64]. Granules were cultivated from activated sludge using nitrobenzene as sole carbon and nitrogen sources [65]. A degradation rate of nitrobenzene of 28.8 mg l^{-1} h^{-1} at 600 mg l^{-1} nitrobenzene was reported. Aniline concentration of up to 6,000 mg l^{-1} was used as the sole carbon and nitrogen sources to cultivate granules [66]. Two pure strains, *Pseudomonas* sp. adx1 and *Achromobacter* sp. adx3, were isolated from the granules, which can degrade aniline at 0.924 g g^{-1} h^{-1} and 0.645 g g^{-1} h^{-1}, respectively. Certainly, the type of substrate dictates the diversity and dominancy of the bacterial species, granule surface, and structure [31, 67].

It has been reported that substrate organic loading rate (OLR) posed insignificant effect on the formation of aerobic granules. Aerobic granules have been cultivated with OLRs ranging from 2.5 to 15.0 kg chemical oxygen demand (COD) m^{-3} day^{-1} [9, 19]. Beun et al. [3] also suggested that OLRs from 2.5 to 7.5 kg COD m^{-3} day^{-1} did not appear to have a direct impact on granule formation. Most studies postulated that formation of aerobic granule in SBR does not depend on substrate concentration. It had been reported, however,

that kinetics behavior and morphology of granules were related to substrate loading [9, 19]. High organic loading enhanced biogas production causing higher upflow liquid velocity serving as a selection pressure for granulation [68]. At an OLR of 6.0 kg COD m^{-3} day^{-1}, the dense aerobic granules grew larger but gradually lost their stability corresponding to notable growth filamentous microbial community [18].

It was reported that the optimal OLR for granulation was 2.52 kg COD m^{-3} day^{-1} [69]. While it had been found that granulation cannot be accomplished at OLRs less than 2 kg m^{-3} day^{-1} [30], granules were successfully cultivated at OLRs of 1.05–1.68 kg COD m^{-3} day^{-1} [70] over a cultivation period of a year. A long granule cultivation time of over 400 days using SBR were recorded with municipal wastewater as the feed with low OLR [71]. Also using SBR, aerobic granules were cultivated by increasing OLR with acetate medium from 2.7 to 22.5 kg COD m^{-3} day^{-1} [72]. Suppression of filamentous bacterial growth was noted with increasing OLR. After cultivation for 120 days, the aerobic granules successfully treated real winery wastewater at an OLR of 6 kg m^{-3} day^{-1}.

Reactor Operating Mode

It appears that aerobic granules had been successfully cultivated only in sequencing batch reactor (SBR). The sequential operation of SBR consists of feed filling, aeration, settling, and effluent decanting. The settling time and exchange ratio of liquid volumes at the end of each cycle serve as a selection pressure to remove non-granular biomass from the reactor. Short cycle time results in short hydraulic retention time (HRT) favoring rapid granulation. With cycle time increased from 1.5 to 8 h, the specific biomass growth rate of granular sludge decreased from 0.266 to 0.031 day^{-1}, while the corresponding biomass growth yield (Y_{obs}) decreased from 0.316 to 0.063 g VSS g^{-1} COD [73]. Granules cultivated at 1.5-h cycle time showed the largest size, while those cultivated at 4-h cycle exhibited the densest structure.

Short settling time washes away poorly settling solids and retains only well settling granules [19, 20, 26, 57, 74, 75]. Granule surface characteristics may change with the settling time. Granulation in three identical columns fed with acetate-containing wastewaters at different settling times of 10, 7, and 5 min was investigated [76]. A shift in microbial community for granules cultivated at different settling times was noted. Short settling time leads to washout of non-flocculated strains, and the flocculated strains can be enriched in granules without competition from non-flocculated strains being washout.

The aeration period in SBR operation consists of two stages: a degradation stage in which substrate is consumed followed by aerobic starvation stage in which substrate is no longer available. Tay et al. [6] hypothesized that the periodic starvation increases cell surface hydrophobicity, which in turn facilitates aerobic granulation in the SBR. There has been a split in the opinions on the effect of starvation on microbial adhesion and granulation. While induction of the favorable cell surface hydrophobicity due to starvation has been claimed [6, 13, 77], detrimental impact of starvation on surface hydrophobicity has been reported [78]. It is interesting to note that the starvation cycle in the SBR operation is not a prerequisite for aerobic granulation [73, 79]. In some cases, starvation enhances stability of aerobic granules, while prolonged starvation weakens the granule stability [80, 81] cultivated aerobic granules in SBR by alternating the OLR (0.96, 1.92, and 3.84 kg COD m^{-3} day^{-1}). It was reported that by alternating the feed, granules can be cultivated readily with ammonium-oxidizing bacteria present in the outer granule layers and nitrite-oxidizing bacteria existing in both outer and inner layers.

Environmental Factors

Most research findings suggested that dissolved oxygen (DO) in the reactor liquid is not a controlling factor for aerobic granulation. Granules can be developed at DO levels as low as 0.7–1.0 mg l^{-1} [4] to as high as 2–6 mg l^{-1} [26, 33, 82]. Most aerobic granulation work was carried out at room temperatures (20–25 °C). Granules were reported, however, in a SBR operated at 8 °C. The granules cultivated were in irregular shape with excessive growth of filamentous microbes, causing severe biomass washout and unstable operation [83]. Aerobic granulation at low temperatures is not feasible at the current knowledge.

The pH of reactor content has a profound impact on the microbial growth. Fungi would become prolific at low pH and may play a role in the initial granulation [3, 74, 84]. Fungi could release protons in exchange for NH_4^+, which would further reduce the pH. It has been observed that granulation at a pH of 4.0 was dominated by fungus with granule size approaching as large as 7 mm. At a higher pH of 8.0, granulation was dominated by bacteria with granule size reduced to 4.8 mm [55].

Treatment Performance

Treatment performance of a biological system for wastewater treatment can be improved by using aerobic granular sludge in ways that allow high conversion rates and efficient biomass separation to minimize the reactor volume. The merits of aerobic granulation over other biological treatment systems are attributed to the fact that biomass is grown in a compact form as granules. This eliminates the use of the large settling tanks and allows much higher biomass concentrations grown in a granular structure in the reactors. This granular structure has many advantages. Due to diffusion gradients, the various process conditions usually accommodated in various tanks are now accommodated inside the granular sludge. Thus, effectively only one tank is needed without the need for large recycle flows. Much higher biomass concentration leads to an equivalent decreased reaction time, and thereby reactor volume. Furthermore, due to the compact morphology of the granules, a much shorter settling time can be included in the process cycle of an aerobic granulation system in order to keep the biomass in the system.

Most aerobic biological systems are used to treat low strength wastewaters associated with organic loading rates of up to 1 kg COD m^{-3} day^{-1}. Feasibility of applying aerobic granulation technology for treatment of high-strength organic wastewaters was demonstrated [9] in which aerobic granules were able to sustain the maximum organic loading rate of up to 15.0 kg COD m^{-3} day^{-1} while removing more than 92% of the COD without compromising granule integrity. Aerobic granules have been applied to degrade phenol and pyridine [38, 41, 57] which appear to be detrimental to aquatic organisms. Yang et al. [26] investigated the simultaneous removal of organics and nitrogen by aerobic granules. Heterotrophic nitrifying microbial populations in aerobic granules and alternate nitrification and denitrification with nitrification rate up to 97% and COD removal efficiency of 95% were reported.

Most biological phosphorus removal (BPR) processes are based on suspended biomass cultures and require large reactor volumes. Although full-scale experience shows a strong potential of the BPR, difficulties in assuring stable and reliable operation have also been recognized. In attempts to overcome the problems associated with the conventional bio-removal of P, Lin et al. [11] successfully developed phosphorus-accumulating microbial granules in SBRs operated at substrate P/COD ratios ranging from 1/100 to 10/100 by weight. The soluble COD and PO_4–P profiles showed that the granules had typical P-accumulating characteristics, with concomitant uptake of soluble organic carbon and the

release of phosphate in the anaerobic stage, followed by rapid phosphate uptake in the aerobic stage.

Granule Disintegration and Stability

Deterioration in granule stability over time is a major issue of aerobic granulation for full-scale operation. Studies have shown that aerobic granules would disintegrate after prolonged operation [6, 8, 9, 31, 35, 40, 57, 59]. Hypotheses to account for stability deterioration over a long-term operation are discussed in the following sections.

Growth of Filamentous Population

High growth rate of bacterial strains encourages proliferation of filamentous microbes causing a rapid increase in granule with larger size, loose structure, and low density. Overgrowth of filamentous bacteria can lead to fluffy and loose granules that can be easily washed out of the reactor. Liu and Liu [17] showed that overgrowth of filamentous microorganisms can lead to reactor failure when the DO was low. Excessive growth of filamentous microbes causing severe biomass washout and unsteady operation was also noted in a separate study [85]. The filaments tend to jam up the pipelines causing operational problems of the reactor system. To screen out loose biosolids from the SBR, a large height-to-diameter ratio of reactor column was suggested. The suggested configuration would also serve as a selection pressure for granules cultivation and to maintain granule stability [3].

Zheng et al. [18] noted that microbial community in granules was dominated by filamentous microbes and inferior degrading efficiency at high OLRs. Operated at 6.0 kg COD m^{-3} day^{-1}, the dense granules grew larger with remarkable filamentous growth but gradually deteriorate in their stability [18]. It had also been reported that granules can become unstable at high OLRs of up to 8 kg m^{-3} day^{-1} [2, 3, 30]. The filament-dominating granules developed to large sizes that restrict oxygen transfer, which leads to development of anaerobic conditions within the core of the granules. The inherent biochemical reactions of anaerobes such as biogas and fatty acids formation would weaken the internal structure of the granule.

Granule development with phenol-containing wastewater aerated at different intensities was studied [42]. No granules had been formed under low aeration intensity (1 $lmin^{-1}$). At high aeration rate (3 $lmin^{-1}$), stable granules (1–1.5 mm) with compact interior were cultivated. At intermediate intensity (2 $lmin^{-1}$), large granules (3–3.5 mm) with overgrown filaments were noted. It was hypothesized that intermediate aeration neither yields sufficient oxygen supply nor breaks down overgrown filaments, and this leads to reactor failure. In addition to promoting granule development, strong shear force caused by intense aeration would also provide sufficient oxygen to suppress filament growth.

Anaerobic Degradation within Granules

When granules grow oversize to such an extent that its radius is larger than the mass transfer limit, anaerobic conditions could develop within the granule. Subsequent anaerobic metabolites such as fatty acids and biogas can weaken the granule internal structure resulting in disintegration of granules [18]. Obligate anaerobic *Bacteroides* sp. over the entire interior of the granules stored at 4 °C for 60 days were spotted [24]. Endogenous respiration might occur within the granule core due to anaerobic activities. Separate studies also revealed that

the cell core was free of oxygen since the active cell layer accumulated at outer regime consumed most of the oxygen intake [86, 87].

Proteolytic bacteria belonging to genera *Pseudomonas*, *Raoultella*, *Acinetobacter*, *Pandoraea*, *Klebsiella*, and *Bacillus* inside the aerobic granules had been identified [88]. These bacterial strains were thought to be responsible for granule deterioration within the core. Granules tend to disintegrate when EPS productivity becomes low, for example, under high OLRs condition [89]. Lopez et al. [72] cultivated aerobic granules using SBR by increasing OLR for acetate medium from 2.7 to 22.5 kg COD m^{-3} day^{-1}. These authors noted that the granule size with a particle diameter between 3.0 and 4.0 mm was reduced to below 2.0 mm when treating real winery wastewaters at lower OLR (6 compared with 22.4 kg COD m^{-3} day^{-1}). Possible problem for oxygen supply limit at high OLRs was also addressed.

It was reported that rupture of mature granules is attributable to clogging of pores and channels in granules, hence hindering nutrients intake by the microbes [90]. Moreover, mineral complexes associated to granule EPS matrix would dissolve at low pHs causing damages to the granule.

Granule becomes weaker structurally under an extended starvation. It was observed that granule became unstable during an extended idling period under high storage temperature [31]. A high storage temperature in the absence of substrate supply can lead to endogenous respiration and a rapid disintegration of the granules. After an examination of the effect of storage time of granules on microbial activity and structure, it was found that the activity of the granules can be reduced by as much as 90% after being stored in tap water for 4 months at 4 °C. In a separate study, it was revealed that after 7 weeks of storage under ambient environment, the granules regained microbial activity within a week [27].

In another study, it was observed that granules stored in all storage media for 8 weeks shrank in size and became irregular in shape [91]. Cell lysis took place releasing soluble cellular substances accompanying with a sharp pH drop. Disintegration of granules was observed. The decay rate of disintegrating aerobic granules stored under anaerobic condition appeared to correlate with the content of volatile solids in the granules. In essence, deterioration in activity and structural integrity of stored granules depended on the storage temperature, duration, storage medium, and characteristics of the granules.

Aerobic granular sludge could be stored for up to 7 weeks at room temperatures without deterioration in integrity and metabolic potential of the granules [27]. The granule structure and characteristics remained intact even without the substrate and oxygen supply, viz., they did not significantly change in size, color, or settleability. The metabolic activity of the stored granules recovered progressively as soon as they were rejuvenated with substrate and oxygen. This interesting finding implies that aerobic granular sludge is resilient to certain level of shock in case the substrate supply falls below the normal levels or when toxic spikes occur. In another study, it was noted that aerobic granules can maintain structural integrity when stored at high phenol concentrations [40]. The stability was deemed to be induced by phenol toxicity inhibiting anaerobic proteolytic strains at granule core, hence enhancing granule stability. The activity of aerobic granules stored in a refrigerator at 4 °C for 7 months can be recovered in COD removal capability after 2 weeks of recuperation [92]. It was also demonstrated that storing at sub-freezing temperature (−20 °C) best preserved activity and structural integrity for aerobic granules [40]. Meanwhile, Zhu [93] claimed that their granules remained stable even after storage for 2 years in tap water at an ambient temperature (16–26 °C).

It was postulated that stored granules become unstable when hydrolysis of intra-granular proteins takes place [88]. Hydrolysis of protein core gradually deteriorates granule stability, eventually leading to granule breakup. From an examination on a hydrolyzed core using fluorescent staining and CLSM experiments, it was shown that large void spaces appeared

within the aged granule. Once the interior structure becomes too weak to withstand the hydraulic shear, the granule will be disintegrated.

Role of Extracellular Polymeric Substances

Extracellular polymeric substances (EPS) are metabolic products accumulating on the surface of bacterial cells, which alter the physico-chemical characteristics of cellular surface such as charge, hydrophobicity, and other properties. EPS constituting proteins, polysaccharides, humic acids, and lipids secreted by bacteria help to initiate aerobic granulation process [13, 67, 94]. It was hypothesized that EPS bridge bacterial cells and other particulates into an aggregate forming the precursor of a granule [13].

Non-soluble β-polysaccharides form the outer shell of aerobic granules to withstand shear [80]. On the contrary, a non-cellular protein core in aerobic granules provides mechanical stability to the granule [45, 95]. Selective removal of proteins exhibited minimal impacts on structural stability of granules [95]. Conversely, hydrolysis of β-polysaccharides caused disintegration. Granule structure is supported by a network composed principally of β-polysaccharides as the backbone for embedment of proteins, lipids, α-polysaccharides, and cells [96]. In essence, enrichment of certain EPS enhanced microbial granulation and granule stability.

Proteins rather than polysaccharides were enriched in the sheared granules [42]. It was found that total polysaccharides/total proteins (PN/PS) ratios ranged between 3.4 and 6.2 for granules, which was much higher than that for sludge flocs (approximately 0.9). The enriched protein contents present an essential feature for aerobic granules [45]. Storage modulus of granular EPS was contributed by polysaccharide rather by protein contents [97]. It was concluded that the exopolysaccharides contribute to sludge granulation. Li et al. [98] attempted to correlate the EPS quantity and the aerobic biogranulation in a membrane bioreactor (MBR). Under appropriate polysaccharides/proteins ratio of 0.6, stable granules can be maintained in the MBR.

Augmentation of Granule Stability

A few strategies were proposed to enhance granule stability for practical operation. This includes:

1. applying appropriate OLR and DO,
2. promoting slow-growing organisms,
3. strengthening granule core, and
4. inhibiting anaerobic growth.

Applying Appropriate OLR and DO

It has been established that applying an appropriate OLR is essential to control granule stability [60, 81, 85, 99]. Li et al. [81] proposed that operating at high OLRs (>2.0 kg COD m^{-3} day^{-1}) is an effective strategy to control fungal bloom and maintain granule stability. Unsuccessful cultivation of granules at <4 kg COD m^{-3} day^{-1} using glucose and peptone as substrate was reported [30]. Conversely, granules subject to high OLRs may disintegrate [9, 17, 18]. It was noted that acetate-fed granules were unable to withstand an OLR >9 kg COD m^{-3} day^{-1} [9]. In another study, it was observed that the dominant microbial population in the sucrose-based granules fed with an OLR of 6 kg COD m^{-3} day^{-1} shifted from bacterial to filamentous growth prior to disintegration [18]. It appears that optimum OLRs for

granulation processes are depending on a variety of factors, and they have to be determined from experimental study.

Much research has asserted that high shear forces are necessary for the formation of aerobic granular sludge in SBRs. In order to distinguish the role of shear force and dissolved oxygen on granule formation, a study found that even when subjected to a high shear force, aerobic granules could not form at a dissolved oxygen less than 5 mg l^{-1} under a static fill condition [100]. In contrast to most research findings that DO is not a controlling factor for aerobic granulation [4, 26, 33, 82] as discussed in the "Environmental Factors" section, these results indicated that the substrate removal kinetics and dissolved oxygen are more significant to granule formation than shear force.

In another study, it was found that granules would disintegrate as DO decreased to <40% saturation [101]. On the other hand, anoxic respiration could improve granule formation if nitrate or nitrite was used as electron acceptor [102]; granules were successfully developed at a DO of 2 mg l^{-1} supplemented with nitrate. It was hypothesized that occurrence of denitrification heterotrophic growth within the core assisted sludge granulation. Without nitrate or nitrite serving as electron acceptors, heterotrophic growth can occur only at a thin layer of granule outer surface. At low DO concentrations, fast-growing microorganisms such as flocculated filamentous microbes proliferate and subsequently are washed out of the system [101]. High mechanical mixing did not induce sludge granulation, and that DO instead of shear stress would promote granulation [103].

Overgrowth of filamentous microorganisms could result in granule disintegration when the DO is inadequate especially operating at high OLRs [17]. Granules may grow oversize at high OLRs, and DO may not be diffused into the internal matrix due to limitation of mass transfer in oversized granules. This would create an anaerobic environment beyond the DO diffusion limit extending into the granule core [18]. The subsequent anaerobic metabolites such as volatile fatty acids and biogas including the toxic hydrogen sulfide may damage the granule structure leading to disintegration. Existence of proteolytic bacteria of genera *Pseudomonas*, *Raoultella*, *Acinetobacter*, *Pandoraea*, *Klebsiella*, *Bacillus*, and uncultured bacterium inside aerobic granules has been reported [104]. These anaerobic bacteria are likely to play a role in granule deterioration resulting in granule disintegration.

Promoting Slow-growing Microorganisms

As discussed above, instead of allowing fast-growing filamentous microbes to thrive in the culture, promoting slow-growing organisms such as phosphate or glycogen accumulating bacteria could enhance granule stability at low oxygen saturation (20%) [105]. Other slow-growing microbes such as the denitrifiers would thrive with nitrate or nitrite supplement, which could penetrate into the granule core. In essence, granule structure can be strengthened by promoting heterotrophic growth within the granule.

Application of a pre-anoxic feast period would enhance granule formation at reduced aeration rates [106]. Nitrification can be initiated by alternating anoxic feast/aerobic famine operation with nitrate supplement. On the contrary, nitrification became unstable at high OLRs as heterotrophic outcompetes autotrophic growth. Oxygen transfer was proposed as the limiting factor for granulation. Use of nitrate/nitrite as electron acceptors during the anoxic feast period is vital in reducing energy demand of granular sludge systems.

With both autotrophic nitrifiers and heterotrophic denitrifiers thriving at the outer layers, the granules convert ammonium to nitrogen gas by alternating oxic and anoxic sequence at OLRs [88]. The adverse effect from this hypothesis is the formation of nitrogen gas from denitrification. Nitrogen gas would accumulate within the granule interior, thereby increasing

its buoyancy causing granules to be floated up and washed out from the top weirs. Floating granules had been observed in a separate study [10].

A so-called concentration-to-extinction approach to isolating functional consortium from aerobic granules cultivated at OLR up to 21.3 kg m^{-3} day^{-1} was adopted [88]. The functional consortium was identified as strain *Zoogloea resiniphila* and two uncultured strains, *Acinetobacter* sp. clone JT2 and bacterium clone P1D1-516, with the latter uncultured strains corresponding to intra-granular proteins binding.

Strengthening Granule Core

It was reported that phenol-fed granule exhibited denser β-polysaccharides network in the granule interior and showed better stability than the acetate-fed granules [88]. It was hypothesized that positive divalent and trivalent ions could bind with negatively charged cells to form microbial nuclei [107]. Studies revealed that addition of Ca^{2+} ions accelerated aerobic granulation [108], and bacterial alginate reacted with calcium ions principally contributed to stability of aerobic granules [109]. It was believed that Ca^{2+} enhances the polysaccharide contents in the aerobic granules [108].

Accelerated formation of granules with larger size and denser interior was reported in reactor dosed with 10 mg l^{-1} Mg^{2+} [110]. Granules were formed in 16 days with 100 mg Ca^{2+} l^{-1} in the feed, while a longer period of 32 days was noted for granules to form in the feed without Ca^{2+}. The granules formed with Ca^{2+} ions showed superior settling and strength properties. Granulation can be accomplished when free ammonia was kept below 23.5 mg l^{-1} [14, 23, 111]. Increase in free ammonia concentration would decrease cell hydrophobicity which affected EPS production and granulation. The ratio of extracellular polysaccharides (PS) to proteins (PN) declined from about 2.50 to 0.58 with the increase of the free ammonia concentration from 2.5 to 39.6 mg N l^{-1}, i.e., high free ammonia concentration can repress the production of cell polysaccharides [111]. Autotrophic nitrifying granules were cultivated after 120 days using NH_4^+–N laden inorganic wastewaters [112]. The presence of ammonium-oxidizing bacteria and nitrite-oxidizing bacteria in the granules was reported.

Compressive strength in the range between 0.16 and 0.42 N/mm^2 of a calcium-enriched aerobic granule (with a Ca^{2+} content of 86.8–151.1 mg g^{-1} SS) was reported [113]. The granules had a significantly higher compressive strength compared with other counterparts. This is mainly attributed to the Ca precipitation in the granules.

Inhibiting Anaerobic Growth

It has been known that existence of anaerobic conditions within the granule could weaken the granule internal structure resulting in disintegration of granules. Inhibiting the growth of anaerobic microbes will help to maintain stable granules in the reactor. Removal of too large a granule or applying toxic substances like phenol could also minimize the adverse effects brought about by anaerobic strains at the core. However, this strategy was not feasible when treating non-toxic influents or when a conventional SBR was used with only light and small granules removed in the decanting stage [35].

Adav et al. [24] concluded that phenol-fed aerobic granules could be better preserved than acetate-fed granules at low temperatures up to sub-freezing temperature (−20 °C). The granules retained 80–99% of the original level of activity after 48 h of reactivation. It was postulated that addition of phenol in the storing solution significantly preserved aerobic bioactivity at all temperatures while suppressing growth of anaerobes. Too high a concentration of phenol, however, inhibited activity of the granules [114].

Challenges and Future Work

Maintaining granule with adequate structural integrity is one major challenge that hinders practical application of aerobic granulation. Current bottleneck of aerobic granulation development highlights the need for further research in granule stability for full-scale operation. There is a need to explore ways or techniques in developing granules with sustainable integrity.

A technological know-how for cultivating granules of adequate structural stability for storage has been described for the first time [35]. Storing granules in a completely dried condition for recultivation was explored by the authors. Aerobic granules with an average size of 5.4 mm cultivated with synthetic wastewater containing propionic acid as the sole carbon source were subject to drying at 25 °C for 24 h and were kept dried at room temperature (24–29 °C). The granules shrank to an average size of 2.6 mm upon drying. The granules were recuperated with the same synthetic wastewater after being dried for 21 days. The granules resumed to their original appearance with a mean diameter of 5.3 mm upon recultivation. In addition, it was reported that COD removal of the dried granules upon recuperation was not affected by the drying. The removal efficiency was comparable with active fresh granules without subjecting to drying. It appears that the drying did not cause notable impact on the granules in terms of morphology and functionality.

The interesting finding may suggest the use of drying as a novel treatment of granule for storage purpose. Dried granules can be stored and handled with ease comparing with that of wet granules. The novel drying technique of aerobic granules would allow convenient storage and handling of granules for use as inoculums for rapid startup, and as granule supplement to enhance treatment of bioreactor systems. Further research is needed to ascertain other impact of the drying such as settleability, density, surface hydrophobicity, specific oxygen uptake, strength, and ECP content of granule.

A finding accounting on cultivated granules with durable stability in continuous-flow reactors has been reported recently [115]. A column-shaped vessel was operated as the continuous-flow reactor, with effluent drained over a top weir at a hydraulic retention of 19 h. Coarse bubbles were produced below the membrane module at a flow rate of 3 $\mathrm{l min^{-1}}$. Seeded with municipal wastewater activated sludge, granules were formed upon cultivation of 60 days. Subsequently, acetate substrate was fed and its concentration maintained at 4,800 mg $\mathrm{l^{-1}}$ at an OLR of 7.0 kg COD $\mathrm{m^{-3}\ day^{-1}}$ throughout the continuous-flow reactor operation. The granules remained intact during the operation. Enriched with heterotrophic community and high concentrations of phosphate salts, the granules remained structurally stable and maintained a satisfactory organic degradation of around 85% COD removal over a 216-day test.

A mathematical model to describe the storage and growth activities of denitrifiers in aerobic granules under anoxic conditions was developed [116, 117]. In a separate study, a mathematical model describing simultaneous autotrophic and heterotrophic growth for aerobic granules in SBR was proposed [118]. Experimental tests were conducted to calibrate the coefficients and validate the model. The simulation output reveals that influent substrate and ammonium nitrogen dictate the composition of heterotrophic and autotrophic biomass. The autotrophs were mainly distributed at the outer layer of granules, while the heterotrophs thrived in the entire granule interior.

To further explore the potential of aerobic granulation technology, the following future studies are proposed:

1. Enrichment and distribution of certain EPS components in promoting granulation and maintaining granule stability during reactor operation and storage. Using CLSM coupled with different specific fluorochromes, the spatial distribution of proteins, α-, β-

polysaccharides, and lipids within the granule can be examined for a better understanding on granule internal structure and stability.

2. As most research work on aerobic granulation has been based on sequencing operating mode using SBR systems, operating and loading parameters of reactor treating various substrates in continuous operating mode need to be established. Continuous operation of reactor is advantageous over batch or sequencing mode for efficient full-scale operation.
3. Use of drying as a treatment of granule for storage purpose seems feasible. Other methods such as encapsulation treatment and further research are to be explored to ascertain stability of aerobic granules and other impact of the treatment. Novel treatment of granules would allow convenient storage and handling for use as inoculums and as granule supplement to enhance treatment of existing systems.
4. Coupling granulation technology with other treatment systems, for example MBR to complement benefits from both processes. A study [119] indicated that aerobic granulation has the potential of reducing irreversible fouling on MBR membranes by mitigating development of internal biofilm.
5. Cultivation of aerobic granules with genetically engineered microbial species with multiple targeted genes for removing multiple toxicants by single transformed bacterium. Treatment capacities of aerobic granulation system can be easily adjusted to accommodate varying loading rates, wastewater composition, and treatment goals by bioaugmentation with genetically engineered granules.

Conclusions

Aerobic granulation evolves as a promising technique for high strength and/or toxic wastewater treatment. Stability of aerobic granules for practical applications remains a major challenge that has yet to be resolved. There is a need to explore ways or techniques in developing granules with sustainable integrity. Growth of filamentous microorganisms, the role played by extrapolymeric substances, has been challenged with respect to granule stability. Strategies for generating a more stable granule were discussed, which include selection of slow-growing microorganisms, inhibiting the activity of anaerobic bacteria, along with strengthening the core of the granule. Findings of granule cultivated with durable stability in a continuous-flow reactor and granule storage with drying technique are encouraging. The novel drying technique of aerobic granules would allow convenient storage and handling of granules for use as inoculums and as granule supplement to enhance treatment of bioreactor systems. Further research is needed to ascertain other impacts of the drying, and to ascertain stability of granules in pilot- and full-scale long-term operation and storage.

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