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Alginate-like exopolysaccharides in aerobic granular sludge: A review

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ABSTRACT

Granular instability and a long granulation start-up period are significant challenges in implementing a full-scale aerobic granular sludge system. Identifying extracellular polymeric substances (EPS) components and their interactions is the key determinant to overcoming these challenges. Because of the high complexity of EPS, alginate-like exopolysaccharides (ALE) can be used as a model component as it is identified as the principal constituent of EPS. The feasibility of extracting ALE in aerobic granular sludge has been intensively studied; however, ALE characterization is still ongoing, and its role in EPS are still overlooked up until now. Moreover, research on its characteristics has demonstrated ALE to be a potential resource recovery. This review highlighted ALE's physical and chemical characteristics, factors influencing ALE composition and content in aerobic granular sludge, and ALE as possible resource recovery. This review also discusses the correlation between ALE, EPS, and granulation processes in aerobic granular sludge systems.

1. Introduction

Aerobic granular sludge is a dense and self-immobilized aggregate consisting of microbial consortiums that concurrently remove carbon, nitrogen, phosphorus, and other contaminants only in a single reactor [1]. It has been shown that aerobic granular sludge is a more efficient and novel method of treating wastewater than conventional activated sludge [2,3]. Aerobic granular sludge system demonstrated several advantages such as high rates of organic matter and nutrient removal, lower land footprint, lesser investment cost, lower energy, and better effluent quality [4,5]. In addition, aerobic granular sludge has demonstrated to be a potential source for resource recovery. However, a long period of granulation start-up and granular stability remains the key challenge for this approach.

Extracellular polymeric substances (EPS) is recognized as the key determinant in the granulation process to sustain aerobic granular sludge's structure stability [6]. However, EPS is a very complex biofilm, and its component's characterization is still far from complete. Alginate-like exopolysaccharides (ALE) is found to be the major constituent of EPS [7]. Therefore, ALE can be used as a model component for EPS characterization. ALE was successfully extracted in both floccular and granular sludge and is the key factor aiding in granulation as the concentration of ALE increases along with granulation [8]. On the other hand, a high concentration of ALE was also found in the floccular sludge [9,10]. Nevertheless, there are limited studies that have been done to understand the relationship between ALE and EPS. The understanding of EPS and ALE content dynamics is crucial for biofilm controlling and to further understand the granulation process.

ALE is heavily associated with alginate or algin, a naturally occurring anionic polymer typically found in the cell walls of brown seaweed. Seasonal changes heavily influence alginate production from brown seaweed, and a significant quantity of wastewater is produced during the extraction process. Some bacteria can also produce alginate, but the operation cost is expensive. ALE recovery also sparks interest as it is shown to represent more than 50% of the recovery of valuable materials that WWTP can generate, compared to biogas, cellulose, bioplastics, and phosphate [11,12]. Moreover, several studies have highlighted the extraction of polyhydroxyalkanoates (PHA), tryptophan, and phosphorus in aerobic granular sludge, but the yield of extractable ALE from aerobic granular sludge is the most abundant at about 15–25% [8,13]. Additionally, recent research indicates that ALE may be employed in a variety of industrial applications, such as a coating material

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and as a biosorbent for dyes and phosphate [14-16]. As a result, market circumstances are particularly favorable for the production of ALE from aerobic granular sludge at the moment. For example, a total of 85-kilo tons of ALE is projected to be recovered in the Netherlands from ten separate WWTPs and is predicted to generate 170 million euros by 2030 [12]. However, a complete chemical analysis of ALE is still forthcoming.

Therefore, the study of ALE is beneficial in improving strategies for biofilm control in wastewater treatment plants (WWTP) and may help model future aerobic granular sludge systems. In addition, ALE may be a relevant approach to direct the operation of WWTPs towards resource recovery. However, several key aspects such as ALE content dynamics and characterization need to be understood. ALE needs to attain some particular characteristics to be able to compete with commercial alginate. ALE characterization has not been completed yet, and the causes and processes governing the composition and content of ALE have not been thoroughly explained. Moreover, the relationship between ALE, extracellular polymeric substances (EPS), and granulation has not been fully understood. This paper reviews previous studies on ALE characterization, factors that affect the production of ALE, as well as the relationship between ALE and EPS along with granulation.

2. The roles of ALE in aerobic granulation

Polysaccharides that are secreted outside the cell (extracellular environment) in form of capsules or biofilm are known as EPS. It works as a bond between bacteria, promoting the creation of biofilms and contributing to the production of aerobic granules. [17]. It protects granules against environmental pressures, giving them mechanical stability. Additionally, it provides cell adherence to surfaces and serves as storage for carbon and water [18]. The increasing attention in the research on aerobic granular sludge system sparks interest in evaluating the recovery potential of EPS [18]. EPS is highly complex; therefore, ALE can be used as a model component for biofilm EPS as it was found that ALE is the major constituent in EPS. ALE is classified as a hydrogel and is said to provide toughness, flexibility, hydrophobicity, and a compact structure that shields microorganisms on the granules [19-21]. Additionally, ALE contributes to the robustness of AGS aggregation by embedding components such as proteins and lipids [20,22].

Several studies have reported that ALE composition in mature granules is higher than in flocs. Schambeck et al. [10] reported an increase of 29% of ALE extracted from dense and round-shaped granules. Yang et al. [9] found comparable findings, in which ALE content was more than tripled after granulation. Microbial compression and collision may be enhanced during granule maturation, resulting in the stimulation of ALE synthesis [24]. Additionally, alginate secretion through the development of blebs on the bacterial cell surface has been recently observed [25]. These theories may support the increase of ALE during the maturation phase of aerobic granules. In contrast, Huang et al. [23] found ALE in mature granules but not in flocs and showed a comparison between granules with and without ALE. Fig. 1 shows granule without ALE on day 98 (a) and granule with ALE on day 118 (b), in which granule with ALE exhibit a more compact and elastic structure. Similar results were demonstrated by Lin et al. [20] in which the presence of ALE may provide granules with aggregate compaction, surface toughness, and hydrophobicity.

Alginate's gelation process is unique from the gelation process of other polysaccharides or proteins. Polysaccharides such as gelatin, glucuronan, and pectin gel only at a specified temperature, while proteins need denaturation prior to gelation by heating, high pressure, or chemical treatment [26,27]. ALE demonstrates alginate's remarkable gelation behavior with a variety of divalent cations across a broad temperature and pH range. ALE can cross-link with multiple cations and form ionic hydrogels, providing sorption sites for different compounds [19,28,29]. To study the hydrogel properties of ALE, ALE was dropped into $CaCl_2$ solution, and brownish Ca^{2+}-ALE beads were observed (Fig. 2) [8,10,30]. The ability of ALE to form hydrogel is essential for aerobic granule production and mechanical stability. Therefore, the capacity of aerobic granule-derived polymers to form hydrogels is crucial for granule development and its mechanical stability [20]. Moreover, a high percentage of GG blocks in ALE extracted from aerobic granular sludge may suggest their significant role in the granule strength [8,9,31]. The gel-forming capabilities of ALE blocks are in the following order: MM blocks $\leq$ MG blocks < GG blocks, while the flexibility order of ALE blocks is as follows: GG blocks < MM blocks < MG blocks [8].

Compared to flocs, ALE concentration after granulation was higher, more stable, and steadily increasing [10]. ALE content increases as granulation happen, and gelation is crucial for aerobic granules' stability and formation [20]. This result implies that ALE may be required for mature granule formation. However, a high ALE concentration does not always imply granulation, since studies also found a high ALE content in floccular sludge [9,10]. Moreover, it was revealed that EPS content dynamics do not follow the same pattern as ALE [10]. This means that even though ALE is a portion of EPS, different factors may contribute to EPS production in the aerobic granular sludge. Therefore, granulation is a process that enriches ALE content in the aerobic granular sludge but ALE content does not influence the EPS content in the aerobic granular sludge as EPS dynamics appear to be a much more complex phenomenon [10].

3. ALE characterization

Alginate or algin is a naturally occurring anionic polymer that occurs naturally in the cell walls of brown seaweed. It acts as the main skeletal compound of the brown seaweed, responsible for the mechanical strength and flexibility of the seaweed in order to withstand the force of the water in which it grows [32]. Alginates building blocks are unbranched polysaccharides composed of two uronate sugars, β-D-mannuronate (M) and C-5 epimer α-L-guluronate (G). The uronate sugars are linked by 1–4 glycosidic bonds [33]. The monomers of the alginate can be arranged in homopolymeric (MM and GG) or heteropolymeric block structures (MG). Different grouping, proportions, and distribution, as well as the length of these monomers blocks, determine the chemical and physical properties of the alginate molecules [34].

Alginates form a gel when they come into contact with divalent cations such as calcium ions (Ca^{2+}). The gel-forming characteristics are highly correlated with G-blocks content and G-blocks bind to cations more efficiently than M-blocks. Thus, the greater the proportion of G-blocks in the gel, the stiffer it is [34]. Additionally, G-blocks enable inter-chain ion binding in an 'egg-box'-like structure, as seen in Fig. 3, which is required for hydrogel formation. At the moment, all commercially available alginates are derived from brown seaweeds, including *Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum nodosum*, and *Macrocystis pyrifera* [34]. Alginate is utilized in a wide variety of sectors, most notably food and cosmetics. It has been widely employed in the medical, pharmaceutical, and biotechnology sectors in recent years.

ALE is predominantly comprised of both mannuronic and guluronic sugars, just like commercial alginate. However, studies showed that ALE is comprised of complex biopolymers; therefore, its composition is far more complicated than that of commercial alginate. However, the origins of the biopolymers remain

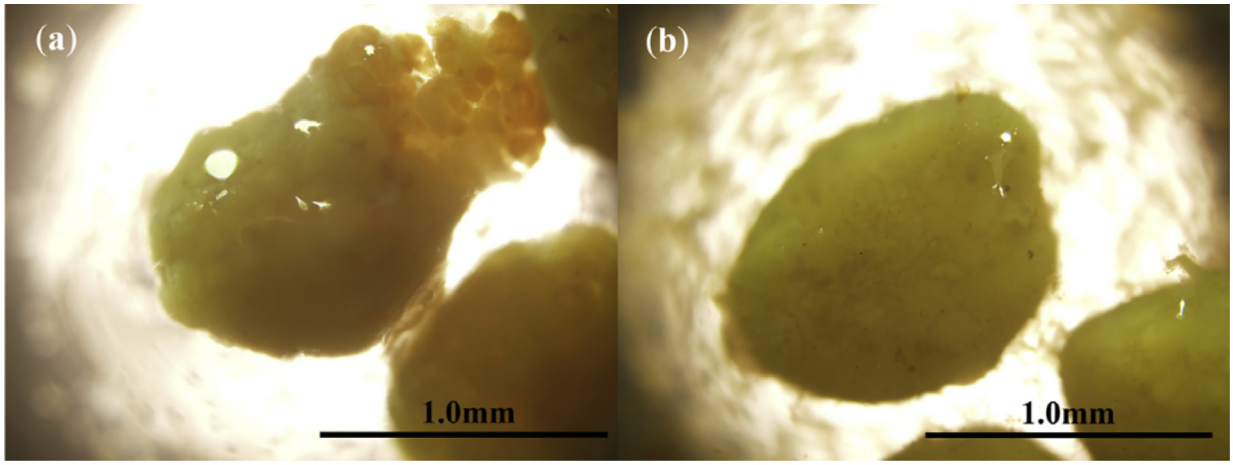

Fig. 1. (a) Granules without ALE and (b) granules with ALE [23].

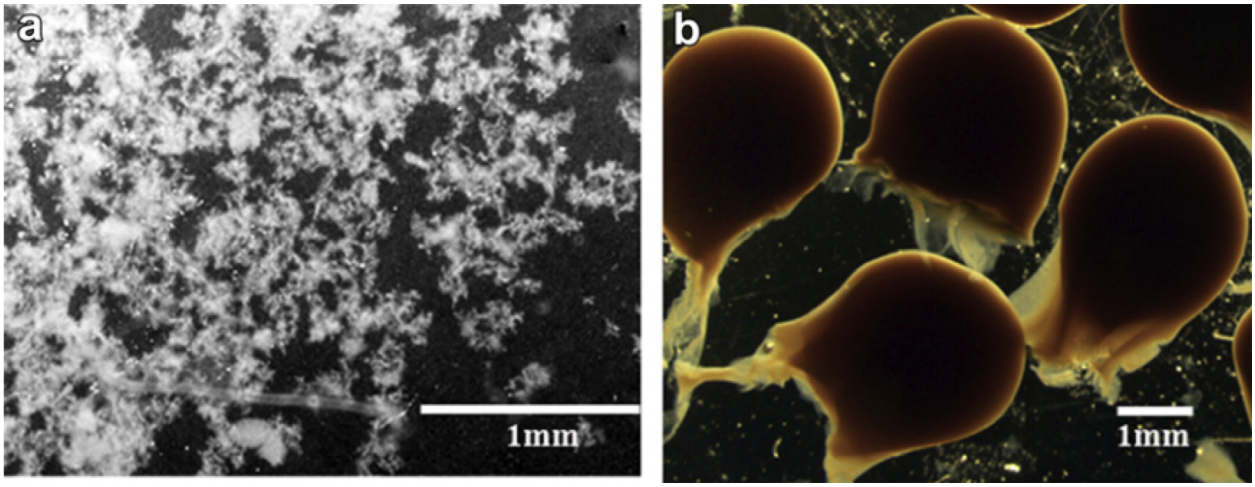

Fig. 2. ALE's morphology under the microscope after cross-linking with $CaCl_2$: (a) Na^{2+}-ALE from flocs (b) Na^{2+}-ALE from aerobic granular sludge [10].

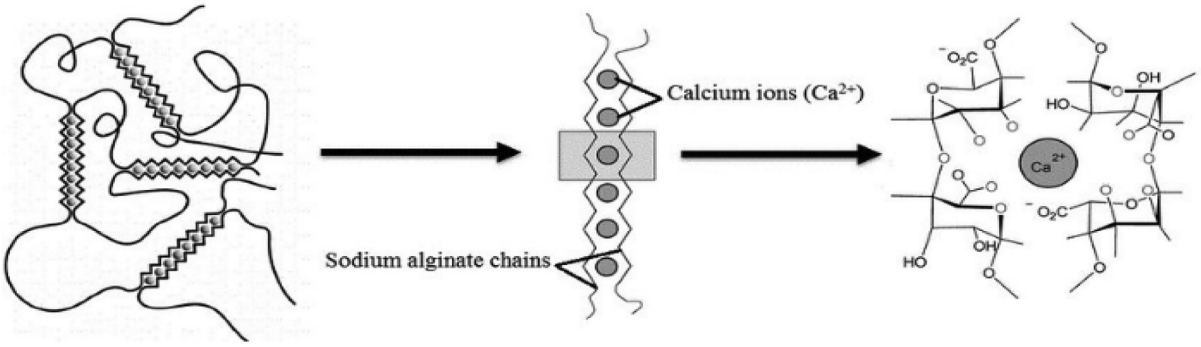

Fig. 3. Alginate gel formation and 'egg-box' structure model in the presence of calcium [32].

unknown. According to fourier-transform infrared spectroscopy (FTIR) studies, ALE is composed of proteins, humic acids, sugars (neutral, uronic, alcoholic, and amino), glycoconjugates, and lipids [29,30,35]. The complex structure of ALE confers to its adsorbent capabilities for a variety of chemicals. For instance, the humic acids included in ALE are high in carboxylic acids and phenols, implying that they might be used as chelating agents [28]. Additionally, humic acids and proteins may form complexes with cationic metals that are beneficial for a variety of adsorbent applications [28].

Meng et al. [35] investigated the composition of ALE using gas chromatography-mass spectrometry (GC–MS). Galactocide was found to be the primary compound found in ALE, followed by glucosamine, lipid content, including esters (L-alaninate, phosphoric acid propyl ester) and fatty acids (palmitic acid, hexadecanoate).

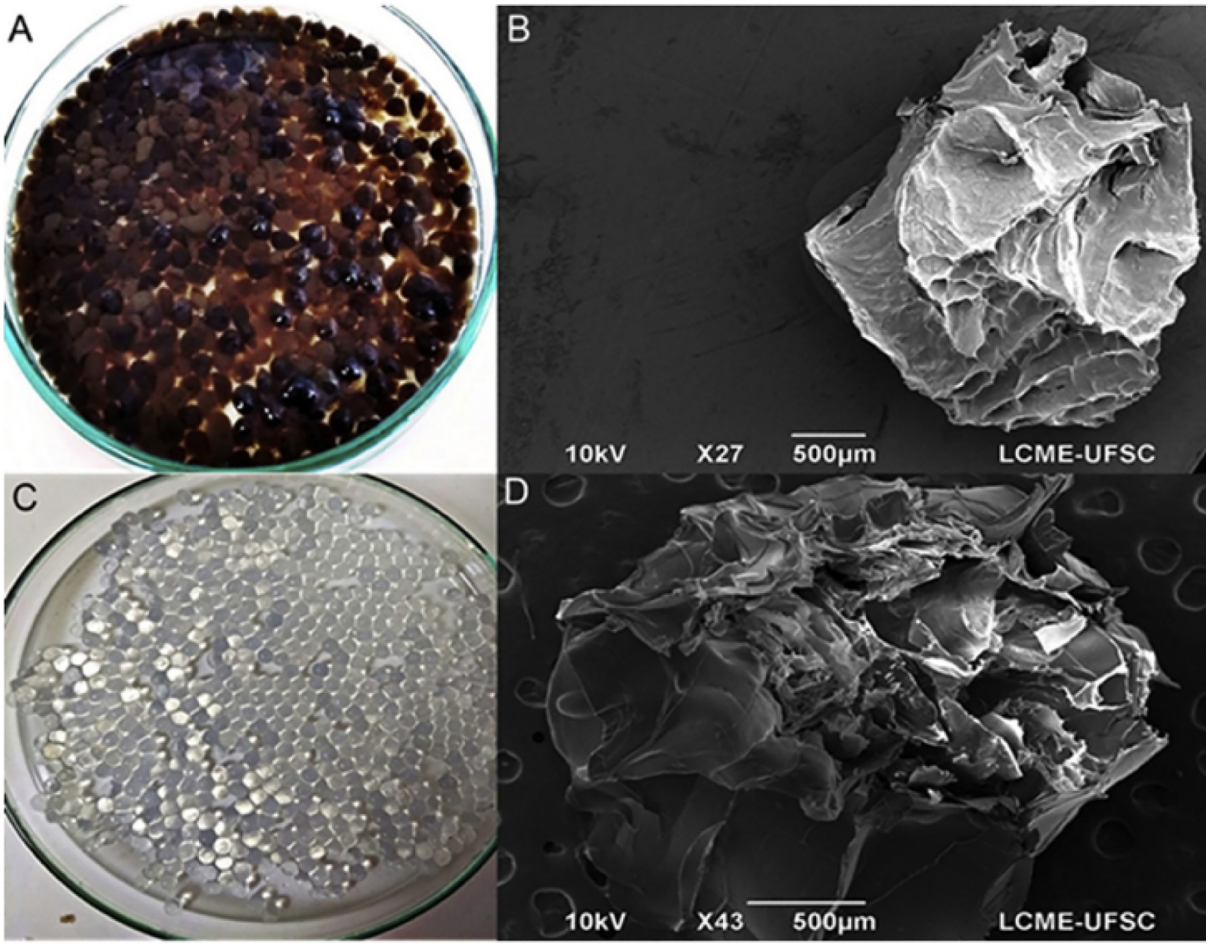

Fig. 4. (a) ALE (b) SEM images of ALE (c) Commercial alginate beads (d) SEM images of commercial alginate beads [12].

In addition, octadecanamide derivative such as octadecanamide and N-nitroso was also detected in ALE. Glucosamine is a commonly used dietary supplement for people with osteoarthritis, and it was found recently to help in cancer treatment [36,37]. Lipid content and octadecanamide derivative present ALE have the potential to be a water-barrier property and a friction coefficients reduction in products.

Schambeck et al. [10] performed Scanning Electron Microscope (SEM) measurements to the Ca^{2+}-ALE beads, and they showed similar physical structures before and after granulation. The Ca^{2+}-ALE beads were distinguished by the presence of different sized pores, rough and irregular pores, suggesting a solid cross-link with Ca^{2+}. EDX analyses were also performed to determine the Ca^{2+}-ALE beads' elemental composition. The beads were mostly composed of carbon, chlorine, calcium, and oxygen, with traces of sodium [10]. However, EDX's limitation in identifying hydrogen, which is undoubtedly available in the beads, should not be left unnoticed. Ladnorg et al. [15] addressed some structural differences between ALE and commercial alginate beads (Fig. 4). When analyzed using SEM, the rough surface and shrinkage of the spheres were observed due to the lyophilization process [38]. In addition, ALE was detected to be brownish as a result of the humic acids present in the aerobic granular sludge [29].

4. Operational conditions associated with the production of ALE

Up until now, there are still few published studies on how operational conditions in the aerobic granular sludge system can affect ALE production (Table 1). Sections 4.1 to 4.6 highlight the major operating conditions that affect ALE production in aerobic granular sludge system.

4.1. Operational cycle

The operational cycle configuration in the aerobic granular sludge system may affect the production of ALE in which adding a short anoxic phase was reported to increase the ALE content [39]. This is most likely due to the brief starvation period, which may have prompted bacteria to use EPS as an electron donor. While a prolonged anoxic phase may allow for more EPS accumulation, unstable granules will develop, resulting in low settleability and biomass loss owing to the low shear stress [40,41].

4.2. Granule size

Rollemberg et al. [39] reported a higher ALE content in mature granules with 1.0 and 1.5 mm in diameter. However, low ALE content was observed when the granules were too large (diameter greater than 2.0 mm). Reduced EPS production due to carbon diffusion limits may result in a reduction of the ALE content of extremely big granules [42]. Ali et al. [43] demonstrated that smaller-diameter granules were mostly found in the reactor's blanket, while the larger ones were at the bottom of the reactor. Therefore, it is possible to take the sludge from the blanket as it may possess a greater ALE content. However, further study is required to identify the granule composition in full-scale reactors.

Table 1
Summary of research regarding ALE in aerobic granular sludge.

System Configuration	COD and carbon source	Operational Cycle	Operational Conditions	ALE yields (mg/g-VSS)	Source
SBR, Pilot-scale 110 L	COD: 513 mg/L Municipal wastewater	Feeding: 60 min, Anoxic condition: 15 min Aerobic condition: 234 min Settling: 30 min Decanting: 6 min	Operational period: 308 days Volume exchange ratio (VER): 65% Normal seasonal temperatureNo pH and dissolved oxygen (DO) control	354–457	[10]
SBR, Lab-scale 3 L	COD: 1,350 mg/L Synthetic saline wastewater (1–85 g NaCl/L)	Anaerobic feeding: 30 min Aerating: 315 min Settling: 10 min Decanting: 5 min	Aeration: Superficial upflow air velocity of 1.1 cm/s VER: 50%Sludge retention time (SRT) : 28–30 days Temperature: 21 ± 1 °C pH: 7.2 ± 0.1	116–171	[50]
SBR, Lab scale 7.6 L	COD: 500 mg/L Synthetic wastewater	Feeding: 60 min Anaerobic condition: 30–60 min Aerobic condition: 205–265 min Anoxic condition: 0–36 min Settling: 5–20 min Decanting: 1 min	Aeration: Superficial upflow air velocity of 1.2 cm/s VER: 50% SRT: 10–20 days Hydraulic retention time (HRT): 12 h	≈226-289.7	[39]
CFR, Lab-scale 20 L	COD: 600 mg/L Synthetic saline wastewater (10–40 g NaCl/L)	–	Aeration: 1.9 cm/s in sludge return zone and 0.6 cm/s in aeration zone Light intensity: 300 μmol/m^2s (12 h light period) SRT: 19 ± 9 days HRT: 9 h Temperature: 23 ± 2 °C DO: 7–9 mg/L	17.3–28.5	[51]
SBR, Lab scale 5.5 L	COD: 400–800 Synthetic saline wastewater (30 g NaCl/L)	Feeding: 10 min Aerating: 240 min Settling: 3–10 min Decanting: 3 min Idling: 60 min	Aeration: 2.0 L/min at the bottom of the reactor and 1.2 cm/s of surface rising speed VER: 50% SRT: 20 days HRT: 8 h	14.3–45.3	[23]
SBR, Lab Scale 2 L	COD: 600 mg/L Synthetic saline wastewater (0–30 g NaCl/L)	Feeding: 2 min Non-aerating: 30 Aerating: 199 min Settling: 4 min Decanting: 2 min Idling: 3 min	Aeration: 3.0 L/min through air bubble diffuser at the bottom Temperature: 23 ± 2 °C DO: 7–9 mg/L	26.8–49.8	[35]
SBR, Pilot Scale 110 L	COD: - Municipal wastewater	6 h cycle	Operational period: 120 days SRT: 14 days	174.57	[15]
SBAR, Lab Scale 1.1 L	COD: COD: 400 mg/LSynthetic wastewater added with seawater (0–100%)	Feeding: 6 min Aerating: 120 min Settling: 5 min Decanting: 5 min Idling: 5 min	Operational period: 140 days Aeration: superficial upflow air velocity of 1.2 cm/s VER: 50% HRT: 4.8 h pH: 7.2 Temperature: 23 ± 1 °C.	53	[22]
SBR, Lab Scale 2 L	COD: 300 mg/L Synthetic wastewater	Anaerobic condition: 120 min Aerobic condition: 180 min Settling: 60 min	VER: 50% HRT: 12 hr SRT: 32 days Temperature: 10 ± 0.5 °C pH: 7.0 ± 0.1 Constant mixing speed of 500 rpm	52	[49]
SBR, Lab Scale 2.6 L	COD: - Synthetic wastewater	Anaerobic feeding: 60 min Aerating: 112 min Settling: 3 min Decanting: 5 min	Aeration: 4 L/min from porous diffuser placed at the bottom of the reactor VER: 57% HRT: 5.2 hr Temperature: 20 °C DO: 20% air saturation	125	[53]

Table 1 (*continued*)

System Configuration	COD and carbon source	Operational Cycle	Operational Conditions	ALE yields (mg/g-VSS)	Source
SBR, Pilot-scale 980 L	COD: - Domestic wastewater	Feeding and decanting: 90 min Aeration: 240 min Settling: 30 min	Operational period: 342 days Aeration: Bottom airflow of 234 L/min and an up-flow velocity of 0.55 cm/s VER: 50% SRT: 19 ± 9 days Room temperature No pH control	183	[14]
–	COD: - Simple synthetic wastewater, complex synthetic wastewater, and real wastewater	–	–	333–449	[30]
SBR, Pilot-scale	COD: 585 mg/L Municipal wastewater	Anaerobic feeding and simultaneous effluent extraction: 60–120 min Reaction period: 225–285 min Settling and decanting: 15 min	Hydraulic capacity: 5 m^3/day Biomass concentration maintained at 8–10 g TSS/L Oxygen controlled at 2 and 3 mg/L during aeration No pH and temperature control	160	[8,20]
SBR, Lab scale 4.78 L	COD:- OLR: 4.4–17.4 kg COD/m^3h Synthetic wastewater	Feeding: 6 min Aerating: 223–208 min Settling: 5–20 min Decanting: 6 min	Operational period: 342 days Aeration: Superficial up-flow air velocity of 2.65 cm/s VER: 57.9% Temperature: 28–30 °C	100	[9]

4.3. Sludge retention time (SRT)

Sludge discharge is a crucial operational procedure in the aerobic granular sludge system as it aids in the removal of phosphorus, maintaining granule stability, and lowering the suspended solids content of treated effluent [44]. A long SRT was reported to decrease ALE content, possibly due to increased endogenous respiration, which results in the utilization of EPS as a carbon source [39,42]. Polyphosphate accumulating organisms (PAOs) were associated as microorganisms responsible for the production of ALE. The reduction of SRT was reported to be advantageous for the growth of PAOs, thus causing a higher ALE production.

4.4. COD:N ratio and organic loading rate (OLR)

The COD:N ratio is also a critical parameter that affects the granules' stability. Literature reports that a high ALE production is associated with an increasing COD: N ratio value due to a high EPS production in the granules [1]. However, an extreme COD: N ratio value can cause nitrogen deficiency and the outgrowth of filaments, subsequently causing granule disintegration. Another problem linked with a very high COD: N ratio value is the increase of granules diameter (greater than 2.0 mm), which may cause difficulties for carbon diffusion to occur. Franca et al. [45] reported nitrogen deficiency and granular instability when the COD: N value is greater than 20. Another result reported by Rollemberg et al. [39] when the COD: N value is around 30, a decrease in ALE content was observed. On the contrary, the microbial community will remarkably change when the value COD: N ratio is close to one. EPS content can be significantly reduced, and therefore nitrification and granule stability will be affected as well as ALE content [9,46,47]. Thus, ALE production is influenced by substrate concentration and subsequently by the organic loading rate (OLR). Increased feed OLR induces the release of extracellular c-di-GMP by functioning strains, resulting in the production of excess ALE [9]. Therefore, research on optimization should be done to ensure operational stability while also contributing to resource recovery.

4.5. Microbial communities

The presence of high levels of ALE during granulation is associated with stable microbial community composition. A robust microbial population facilitates the formation of metabolic pathways for carbon and nutrient absorption during granulation. Hence, together with simultaneous carbon, nitrogen, and phosphorus removal, the stable microbial community generated a higher and stable ALE. The microbial communities' composition in aerobic granular sludge systems can provide knowledge regarding the microorganisms associated with ALE production. Genus *Pseudomonas* and *Azotobacter*, and phylum Proteobacteria were reported to be linked with ALE production [31,48]. Families belonging to Proteobacteria such as Xanthomonadaceae, Caulobacteraceae, Sphingomonadaceae, and Alcaligenaceae were reported to be present in high ALE production of aerobic granules.

The formation of ALE is also strongly associated with the presence of PAOs and glycogen-accumulating organisms (GAOs) in the aerobic granular sludge system's microbial populations. Rollemberg et al. [39] reported that PAOs and GAOs (Rhodobacteraceae and Competibacteraceae) dominated the microbial communities in the aerobic granular sludge system that produces the highest ALE. Similar results were observed by Schambeck et al. [10] that the production of ALE is associated with the presence of *Defluvicoccus* (GAOs) and *Tetrasphaera* (PAOs). A dense granular sludge caused by the presence of *Accumulibacter* (PAO) may contribute to the high production of ALE at low temperatures [49]. This finding can be used in future research measuring the ALE con-

tent in PAO-enriched granules in a process known as enhanced biological phosphorus removal (EBPR).

4.6. Salinity

Osmotic stress is one of the important environmental factors influencing ALE production. ALE production was found to be increasing as the salinity increased at the range of 40–80 g NaCl/L and stopped increasing when salinity was more than 80 g NaCL/L [50]. The same results were also reported by Meng et al. [48] in which ALE production was increased in 1% salinity but not in 3% salinity. Another study by Meng et al. [51] reported a significant decrease in ALE production and low granular stability in high salinity conditions. The reason behind this is that moderate salinity may motivate the expression of algC in *Pseudomonas*, thus activating the phosphomannomutase enzyme. Phosphomanomutase enzyme is part of the alginate biosynthesis mechanism [52]. However, under excessive salinity, this enzyme can be hindered. ALE's gel-forming capacity and chain flexibility may also be heavily suppressed in high salinity conditions [48].

The presence of moderate salinity could also cause the loss of Mg^{2+}, resulting in the increase of GG blocks of ALE. Similar results were reported by Li et al. [22] in which with increasing amounts of saltwater, ALE content and the fraction of GG blocks in ALE rose. The increase of GG blocks content in ALE is beneficial as it has an affinity for divalent cations. In contrast, excessive salinity can inhibit GG-blocks production due to the toxicity of sodium ions to alginate relative genes or enzymes [48].

5. Perspectives and further research

Gas chromatography-mass spectrometry (GC–MS) analysis of ALE extracted from various substrates, as well as other complementary spectroscopic analyses like Nuclear Magnetic Resonance (NMR), Raman Spectroscopy, and X-ray Photoelectron Spectroscopy (XPS), are necessary for a more complete understanding of the ALE structure and composition. Additionally, operational parameters and conditions must be optimized in order to ensure the ALE's operational stability and resource recovery. It is noted that the study of ALE is mostly focused on using synthetic wastewater. Therefore, real wastewater must be utilized in the study of ALE as synthetic wastewater cannot thoroughly represent the applicability of the system in real time. Finally, more studies need to be conducted on investigating the relationship between ALE and EPS.

CRediT authorship contribution statement

Sasmitha Aulia Zahra: Conceptualization, Writing – original draft. **Norhayati Abdullah:** Writing – review & editing, Supervision. **Koji Iwamoto:** Writing – review & editing, Supervision. **Ali Yuzir:** Project administration, Funding acquisition. **Shaza Eva Mohamad:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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