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Controlling factors and involved mechanisms on forming alginate like extracellular polymers in flocculent sludge

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ABSTRACT

Alginate like extracellular polymers (ALE) recovered from excess sludge have been evaluated as an eco-friendly, cost effective and sustainable alternative to highly valued materials. However, the ALE extraction from flocculent sludge ranges normally from 90 to 190 mg/g VSS, which is only equivalent to the lowest edge of the ALE production from aerobic granular sludge (AGS). But flocculent sludge is much higher in production than AGS and thus a further investigation was expected on key factors and associated mechanisms controlling ALE formation of flocculent sludge. The investigation was conducted by lab-scale sequencing batch bioreactors. The experiments revealed that flocculent sludge with starch used as an influent substrate contained the highest ALE production (220.3 ± 8.0 mg/g VSS). Low temperature was favorable to enriching ALE, up to 303.3 ± 21.5 mg/g VSS at 12°C . Moreover, ALE reached up to 137.8 ± 13.2 mg/g VSS at C:N = 5:1 and slightly declined with increased or decreased the C/N ratio. The specific ALE yield was 63.7 mg ALE/(g BOD_5) at a low organic load, which was twice as high as that with high organic loads. However, SRT had a minor effect on ALE formation. Obviously, such scenarios as starch-rich and low temperature could promote the ALE production. Furthermore, the characteristic analysis including alginate equivalent, different fractions and hydrogel forming property among different ALE, confirmed that the ALE extracted from flocculent sludge had a potential in substituting for commercial alginates. However, different working conditions would exert a significant influence on the composition and chemical properties of ALE, which implies that the controlling some parameters could be an approach to directionally cultivating ALE for their unique structures and potential applications.

1. Introduction

As a complex high-molecular-weight mixture of biopolymers in aggregates in biological wastewater treatment systems, such biopolymers as alginate like extracellular polymers (ALE) and sulfated polysaccharides have been identified as highly valuable raw biomaterials [1–5]. For examples, many researches [6–8] reported the potential applicability of the biopolymers as bio-sorbents for heavy metal and toxic organic compounds removal. In addition, the combination of the biopolymers with $\text{Fe}_2(\text{SO}_4)_3$ is promising to be applied in raw drinking water treatment [9]. For the reason, the biopolymers are being considered as a potential alternative to conventional chemical polymers because of their biodegradability, non-toxicity and high efficiency.

The evaluation of ALE properties and structures in the previous studies indicated that ALE could be applied in the industries of food,

paper, textile, medical, construction and even in agriculture and horticulture [1–5]. Lin et al. [1] and Kim et al. [5] concluded that ALE extracted from aerobic granular sludge (AGS) was suitable as a substitute for the biomaterials for non-flammable surface coating. Much attention to recovering the biopolymers has focussed on ALE from AGS. Biopolymers extraction from AGS has been scaled-up to a demonstration-scale plant in Zutphen, the Netherlands [10]. The recent study [10] also revealed that there was a similarity of the biopolymers extracted from flocculent sludge, up to 60% with respect to chemical structures compared to the commercial alginate; the alginate equivalent of the extracted ALE was also over 400 mg/g ALE (40%–60% of the mannuronic acid (M) and guluronic acid (G) units); moreover, ALE had a good capacity of the ionic hydrogel formation. These results implies that the ALE extracted from flocculent sludge was also promising as a potential substitute for commercial alginates [10]. However, a lower ALE

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level was normally associated with flocculent sludge (ranging from 90 to 190 mg/g VSS) [10], which is only at the lowest edge of ALE from AGS (200 to 350 mg/g VSS [11–14]). In practice, there are a larger amount of excess flocculent sludge produced in wastewater treatment plants (WWTPs) comparing with AGS. There were relatively few studies on recovering extracellular biopolymers from CAS and moreover the existing studies on EPS from CAS mostly focused on identifying and evaluating their roles in biological nutrient removal and sludge disposal processes. The economic value of EPS from CAS seems largely ignored.

Under the circumstance, improving the ALE formation in flocculent sludge for recovering the biopolymers is significant and expected for a highly valuable biomaterials application. Among them, the biopolymers/ALE are usually associated with the substrate concentration and operational conditions, such as influent substrate, organic load, dissolved oxygen (DO), temperature, pH, sludge retention time (SRT), etc., which governs the substrate utilization rate and accordingly the formation rate of the biopolymers in aggregates [1,15–21]. For example, a high DO concentration could promoted microbial metabolisms and thus result in the high production of the biopolymers [19,20]. Also, temperature and pH directly affected the metabolic process and thus changed the production and compositions of the biopolymers [18,22]. Furthermore, the biopolymers would be consumed or converted by the hydrolysis and fermentation processes as a result of the disintegration of sludge flocs under limited oxygen or depletion conditions [19]. Finally, the changes of dominant microbes in the system under different SRTs would also affect the biopolymers formation [18,20]. Based on the findings in our pervious study [10], increasing soluble organics (SCOD) content and lowering nutrient content (C/N and C/P ratios) in the influent could be favorable to the ALE formation [10]. And different bacteria in biological nutrient removal (BNR) processes had either a positive or a negative effect on the ALE formation [10]. However, it is still unknown that the deeply associated mechanisms and the optimum working conditions for the ALE formation in flocculent sludge. For the purpose of recovering highly valued biomaterials, further ascertaining the associated mechanisms and the key factors controlling the ALE formation in flocculent sludge should be necessarily effective to improve the potentials of biomaterials' recovery.

For the above reason, flocculent sludge in the study was cultured in the lab-scale sequencing batch bioreactors (SBRs) with different working conditions, including different carbon sources, organic load, substrate (C/N), temperature and solid retention times (SRTs). The study focused on the nature, production and recovery of ALE associated with their characteristics and properties. The involved mechanisms of different parameters affecting the ALE formation were at a top priority. Principal component analyses (PCA) were also applied to evaluate and figure out the significant variables controlling ALE formation of flocculent sludge. Thus, the effective measurement and/or optimization for the ALE improvement were finally summarized for the sustainable operation of WWTPs.

2. Materials and methods

2.1. Cultivation and collection of flocculent sludge

Flocculent sludge was cultured in lab-scale aerobic/anoxic sequencing batch reactors (SBRs) with a working volume = 5.0 L. The bioreactors were conducted daily at three consecutive cycles. As shown in the [supplementary materials](#) (Fig. S1), each anaerobic/aerobic cycle comprised of an 8.0 h period including 4.5 h aerobic, 2.5 h aerobic, and 1.0 h settling. After settling, one-thirds of the supernatant liquid was discharged (about 10 min) and fed with fresh influent (about 8 min in aerobic phase). Finally, five minutes prior to the end of the aerobic phase, the designed volume of mixed liquor was withdrawn to maintain a SRT of approximately 15 days. DO was kept at 3.0–4.0 mg/L in the aerobic phase. Stirring speed was at 150 rpm/min in the aerobic and anoxic phase.

2.2. Experimental set-up

As shown in [Table 1](#), five groups (I–V) were conducted with different influent parameters and operational conditions for flocculent sludge cultivation. Each group was controlled with carbon sources (glucose, starch and sodium acetate), organic load ($L = 0.20, 0.36$ and 0.50 kg $\text{BOD}_5/\text{kg MLSS}\cdot\text{d}$), influent substrate (C/N = 3.0, 5.0 and 7.0), temperature ($T = 12, 18$ and 24 °C) and solid retention time (SRT = 10, 15 and 20 d), respectively, to figure out the key factors on ALE formation. The other designed substrates of the latter group were followed with the results from the former group as shown in [Table 1](#). Other substrate and trace elements are shown in [Table S2](#). Moreover, two parallel bioreactors were set up for each different operational parameter.

In the study, the performance COD, nitrogen and phosphate removal for different SBRs were measured and the results were listed in [Table S3](#). The average removal efficiency of COD, TN, $\text{NH}_4^+\text{-N}$, TP and $\text{PO}_4^{3-}\text{-P}$ were stable at the high levels of around 88.6%–93.8%, 70.4%–84.4%, 96.0%–99.8%, 79.2%–83.2% and 93.4%–97.6%, respectively. After lasting for at least 15 days with stable nutrient removal, sludge samples were collected from the different SBRs every day and concentrated by a 0.15-mm filter, and then stored in a refrigerator (4 °C) for ALE extraction.

2.3. ALE extraction protocols

ALE was extracted by the method of heating + Na_2CO_3 , as described in Felz et al. [12,22]. The mixture contained dry sludge (g), demineralized water (mL) and Na_2CO_3 (g) with the ratio of 1:50:0.25 and then was heated at 80 °C for 35 min in the water bath. Hence, ALE was thus released from sludge agglomerates and then dissolved in the supernatant. By adjusting pH of the supernatant to 2.2, the biopolymers could gel for ALE further purification. Other detailed procedures are described in the [supplementary materials](#) (Text S4). Extraction of ALE from every different flocculent sludge were conducted in duplicate.

2.4. Analytical methods of characterizations

Colorimetric assays were chosen to provide the relevant insights about the composition contents of ALE. Polysaccharide (PS) and protein (PN) analyses were detected by a phenol-sulfuric acid assay with D-glucose and the Lorry assay with bovine serum albumin respectively, based on the conceptual framework proposed by Dubois et al. [23] and Lowry et al. [24]. A commercial alginate (extracted from brown-algae, viscosity 4–12 cP, 1% in H_2O) was chosen as the standard with the phenol-sulfuric acid assay to evaluate the amount of alginate equivalents, which represents the alginate purity of the ALE extraction [10,22,25].

The chemical functional groups of the biopolymers were analyzed by a Thermo Fisher Fourier transform infrared spectrometer (FT-IR). The FT-IR spectra in KBr pellets (98 mg KBr + 2 mg sample) were recorded at the wavenumber of 4000–400 cm^{-1} . The spectra of commercial sodium alginate was also recorded as the standard to estimate of the similarity of different ALE from the commercial alginate by the FT-IR software [26]. Moreover, three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy was also applied to identify fluorescent compounds contained in biopolymers [21]. 3D-EEM spectra were collected with subsequent scanning emission (Em) spectra from 220 to 550 nm at 1 nm increment by varying the excitation (Ex) wavelength from 200 to 500 nm at 5 nm increments [10]. UV-visible absorbance measurements were also used for detecting other characterizations such as the humification and aromaticity degree of the biopolymers from 800 to 200 nm using an Agilent Cary 5000 UV-Vis-NIR spectrophotometer (resolution 1.0 nm) [10,27].

The fractions of mannuronic acid (M) and guluronic acid (G) polyunits in the biopolymers were tested to analyze the gel-forming capacity of the biopolymers [12,28]. These were detected by the methods of

Table 1

Influent parameters and operational conditions in different bioreactors for cultivating flocculent sludge.

| Groups | COD (mg/L) | NH ₄ Cl (mg N/L) | KH ₂ PO ₄ (mg P/L) | Carbon source | Organic load ^a | C/N ^b | T (°C) ^c | SRT (d) ^d |
|--------|----------------------------|-----------------------------|--|-------------------------------------|---------------------------|-------------------|---------------------|----------------------|
| I | 500 | 25 | 5.0 | Glucose Starch Sodium acetate | 0.36 | 20 | 24 | 15 |
| II | 400 700 1 000 150 | 20 35 50 | 4.0 7.0 10 | Sodium acetate | 0.20 0.36 0.50 | 20 | 24 | 15 |
| III | 250 350 | 50 | 5.0 | Sodium acetate | 0.36 | 3.0 5.0 7.0 | 24 | 15 |
| IV | 500 | 25 | 5.0 | Sodium acetate | 0.36 | 20 | 12 18 24 | 15 |
| V | 500 | 25 | 5.0 | Sodium acetate | 0.36 | 20 | 24 | 10 15 20 |

^a Organic load (L), kg BOD₅/(kg MLSS·d).^b C/N, BOD₅/TN.^c T, temperature.^d SRT, solid retention time.

partial acid hydrolysis, as described in [supplementary materials](#) Text S5 [28]. The fraction of G or M were the sum of the half of GM blocks and the total of GG blocks or MM blocks, respectively. the monomer ratios of G:M was the ratio of the average fraction of G to M, which directly reflected the capacity of forming hydrogel. Ionic hydrogel formation was also tested with 2.5% (w/v) CaCl₂ solution to evaluate the ionic hydrogel properties of ALE according to the procedures described by Lin et al. [28] and Felz et al. [22]. The ALE was cross-linked in the CaCl₂ solution at room temperature for about 16–18 h.

The other parameters including COD, BOD₅, TN, NH₄⁺-N, TP, PO₄³⁻-P, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and pH were detected according to the standard methods [29].

2.5. Statistical analysis

In the Group II with different organic load, it is more meaningful to evaluate the specific ALE yield as calculated by Eq. (1).

$$Y_{ALE/BOD_5} = \frac{E_{ALE}}{BOD_{5,Inf} - BOD_{5,Eff}} \quad (1)$$

Where, Y_{ALE/BOD_5} , the specific ALE yields, which means the ALE production with per gram organics (BOD₅) consumption; E_{ALE} , the ALE extraction; $BOD_{5,Inf}$ and $BOD_{5,Eff}$, the BOD₅ concentration in influent and effluent, respectively.

Analysis of variance (ANOVA) was also used to discuss the difference of ALE extracted from flocculent sludge with different working conditions based on the data obtained from the duplicated bioreactors. For each solution and for each method, samples were taken in duplicate to ensure the accuracy of experimental data. Furthermore, PCA could reduce the dimensionality of the data-set and thus transform the results into useful knowledge that could be easily interpreted. Therefore, it was applied to evaluate the cause-effect relationship correlations between ALE production and operational factors and thus to identify the decisive factors affecting ALE formation. In this study, relationships were observed by retaining the first two principal components (PC1 and PC2) and plotting in two dimensions where the cases and loading were set in the biplot.

3. DecimalComplex, results and discussion

3.1. ALE extraction

The amount of ALE extracted from different flocculent sludge are shown in Fig. 1. As shown in Fig. 1, the estimated extractions were at the range of 120–300 mg/g VSS, which were comparable to those ALE extracted from practical flocculent sludge (90–190 mg/g VSS) [10] and were also similar to those results reported in the previous studies [30,31]. To an extent, therefore, flocculent sludge cultured by synthetic wastewater under different operational conditions in lab-scale SBRs was presentative of excess sludge from practical WWTPs. Moreover, Fig. 1 also indicated that some factors significantly affected the ALE formation in flocculent sludge ($p < 0.05$). Among others, the ALE production in the sludge with starch being a carbon source reached to 220.3 ± 7.9 mg/g VSS, which was much higher than those fed with glucose and sodium acetate. Also, low temperature (12 °C) could be favorable to the ALE formation, up to 303.3 ± 12.5 mg/g VSS. By contrast, different organic load and SRT had minor effects on the ALE production ($p > 0.05$).

3.2. Alginate equivalent

ALE are the main structural components in extracellular biopolymers, and has been identified as the promising substitute for commercial alginates [1,5]. The alginate equivalent is the important parameter for evaluating the purity of alginate, which represents the highly valuable products in the biopolymers [10,25] and as depicted in Fig. 2. It indicates that the average alginate equivalent of different ALE was as high as 550 mg/g ALE. Moreover, with starch as carbon source, or at C/N = 5.0, or temperature ≥ 18 °C, the alginate equivalent of ALE considerably reached to the highest level, up to 650 mg/g ALE.

3.3. Composition content

Different compositions were measured to analyze the constituents in ALE and the results are shown in Fig. 3. As shown in Fig. 3, PN and PS were the major constituents in ALE and the corresponding amounts ranged of 300–470 mg/g ALE and 100–230 mg/g ALE, respectively. PN were significantly enriched in the biopolymers, characterized by the high PN/PS ratios of 2.2–4.1 except for the flocculent sludge fed with starch as carbon source (PN/PS = 1.5) and temperature at 24 °C (PN/PS = 1.8). Moreover, the DNA content was at the similar range of 10–23 mg/g ALE in different ALE, which indicated that the release of intracellular DNA from the cell lysis and extracellular DNA from extracellular

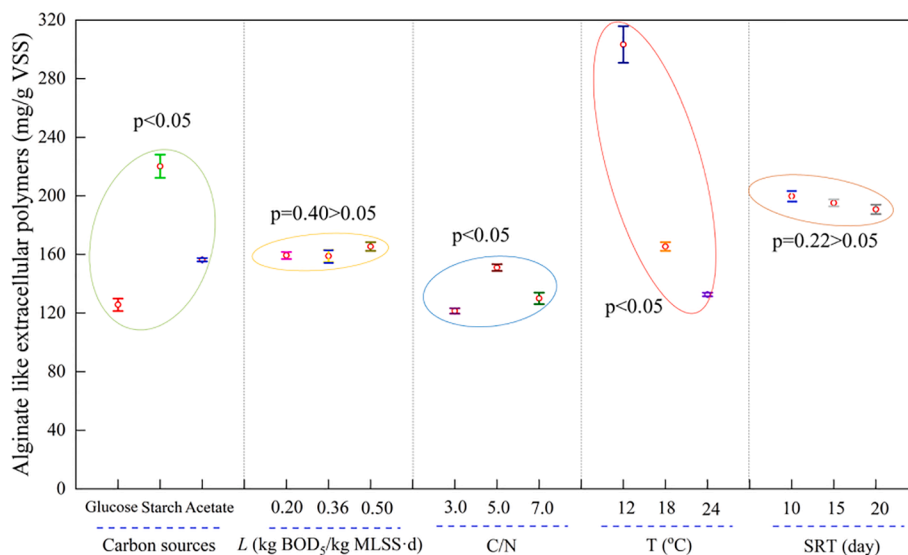


Fig. 1. Alginate like extracellular polymers (ALE) extracted from flocculent sludge cultured with different operational parameters.

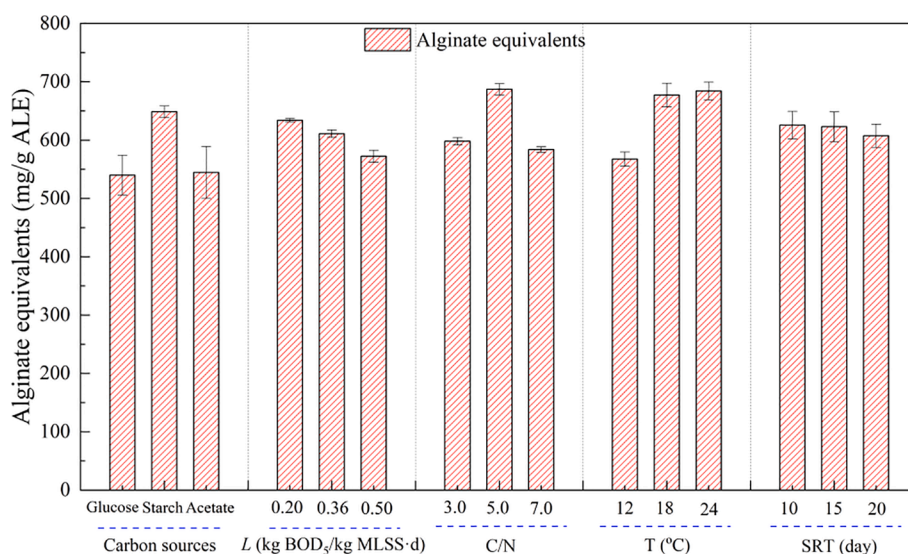


Fig. 2. Alginate equivalents in the alginate like extracellular polymers (ALE).

biopolymers could be at the same level.

3.4. Gel-forming capacity

The fractions of the isolated biopolymers including a family of copolymers comprising of mannuronic acid (M) and guluronic acid (G) units were in an irregular block pattern of varying proportions of GG, MG and MM blocks. Their proportion, distribution and length determined the chemical and physical properties of alginate molecules [28]. The fractions of mannuronic acid (M) and guluronic acid (G) poly-units in the biopolymers were applied to evaluate the gel-forming capacity of the biopolymers [22,28]. As shown in Fig. 4, the recovery yields of different blocks were at 40%–65%, with about 20%–35% GG blocks and 12%–28% MG blocks in the chemical structure of all the biopolymers. Moreover, the monomer ratios of G:M of the biopolymers from different flocculent sludge was at the range of 2.0–3.0. The GG-rich ALE flocs benefited the flocculent sludge that formed a compact gel structure due to its higher affinity towards divalent cations than the other two blocks [28,32]. These results were consistent with the above analysis of alginate equivalent and also demonstrated that ALE was the important

structure in the flocculent sludge biopolymers.

According to Lee and Mooney [33] and Hay et al. [34], furthermore, the ionic hydrogel properties of ALE were also tested by dripping in Ca^{2+} solution to evaluate the capacity of forming hydrogel. The results revealed that Ca^{2+} -ALE beads (cross-linked through Ca^{2+} ions) displayed the great ionic hydrogel forming property among different ALE, which demonstrated that there was a hydrogel being stabilized by divalent ions and represented the similar gel formation to commercial alginate.

In summary, the above experimental results indicated that the compositions and characteristics of ALE were reformed by different operational parameters, but the high purity and the special physical and chemical properties indicated that ALE in flocculent sludge were the promising substitutes to commercial alginate. On the other hands, the ALE yield was relatively low in flocculent sludge, but the results demonstrated that adjusting or optimizing some parameters in biological systems might be favorable to the ALE formation. Therefore, the associated mechanisms of different factors involving in the ALE formation were analyzed in the following sections to conclude the optimal strategies for the high production of ALE.

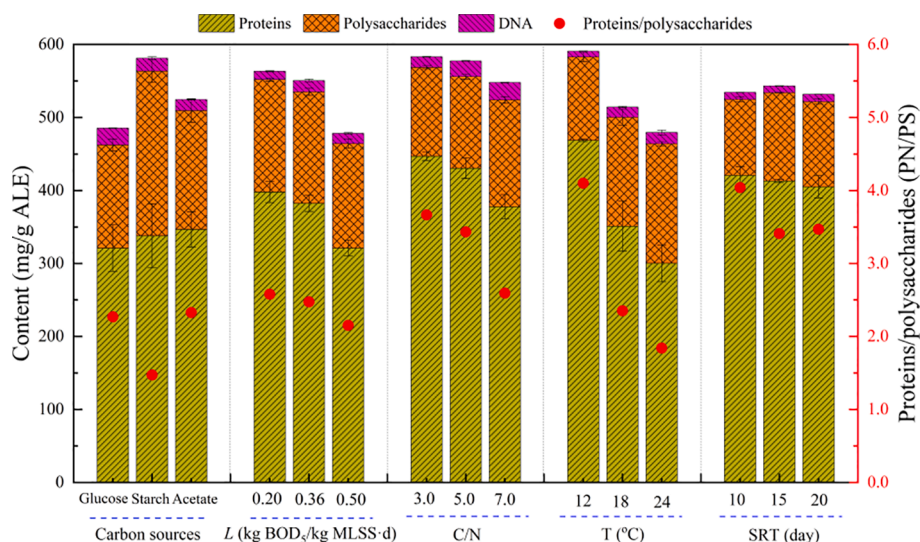


Fig. 3. Protein (PN) and polysaccharide (PS) contents in the alginate like extracellular polymers (ALE) with different operational parameters.

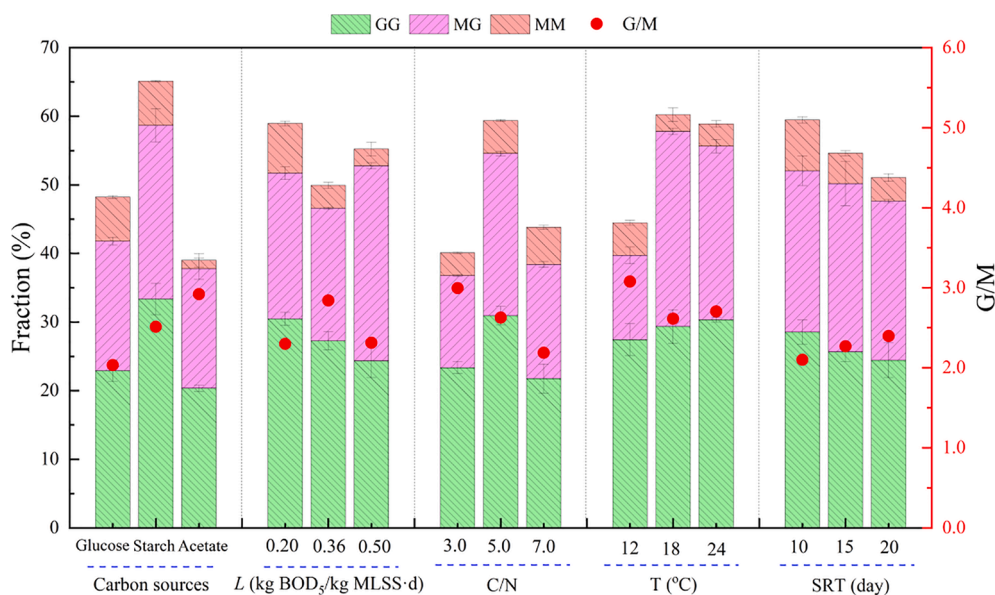


Fig. 4. Fractions of different structural blocks in the alginate like extracellular polymers (ALE).

4. Controlling factors and involved mechanisms

4.1. Carbon sources

As shown in Fig. 1, the ALE extraction revealed that the influent with starch as carbon source had a noticeable effect on forming ALE in flocculent sludge and the production reached up to the highest level of 220.3 ± 8.0 mg/g VSS, followed by sodium acetate (156.4 ± 1.2 mg/g VSS) and glucose (125.7 ± 4.3 mg/g VSS). Similarly, González-García et al. [35] also found that starch as carbon source contributed to biosynthesizing more extracellular polymers than glucose, galactose and xylose for the marine bacterium. Moreover, Wang et al. [36] demonstrated that the biopolymers were slightly higher in sludge samples cultivated with starch than that of glucose.

These results indicated that large molecule organic (such as starch) was more favorable for biopolymers/ALE synthesis than small molecules organics (like glucose and sodium acetate). It might be due to that starch could not only serve as a carbon source but also play a key role in adsorption bridging for bacterial proliferation [37]. According to the

extended derjaguin-laundau-verwey-overbeek theory (XDLVO), an aggregation matrix forms when the distance between bacteria is shortened enough to overcome the maximum repulsive force [38]. Macromolecular organic matters could directly shorten the energy potential barrier and rapidly form stable sludge flocs [38], which was proved by better sludge flocculation performance of starch-rich sludge than glucose and sodium acetate in the cultivation process. Moreover, the UV-Visible spectra are depicted in Fig. S6 and the evaluated parameters are listed in Table 2. The E_2/E_3 , $SUVA_{254}$ and E_2/E_4 value of the starch-rich ALE were the lowest, at 4.30, 2.75 and 5.00, respectively, which means the low content of aromaticity and aliphatic compounds. Also, E_4/E_6 of starch-rich ALE was the highest, at 2.38, which indicated that it was at the low degree of humification and aromaticity. In all, the starch-rich ALE performed the higher adsorption bridging capacities and had more large molecular organics in the extracellular biopolymers than others ALE [27,39,40].

As for small molecular organics such as sodium acetate and glucose, the sludge fed with sodium acetate was more favorable than glucose for the formation of biopolymers [16]. More easily biodegradable organics

Table 2
Results of UV–Visible spectra [27,39,40].

| Parameters | Glucose | Starch | Sodium acetate | Functions |
|---------------------------|---------|--------|----------------|---|
| E_2/E_3 ^a | 5.05 | 4.30 | 5.12 | Molecular weight of dissolved organic materials (DOM) |
| $SUVA_{254}$ ^b | 4.73 | 2.75 | 3.34 | Unsaturated structural components |
| E_2/E_4 ^c | 5.89 | 5.00 | 5.27 | Condensation degree of humic substances |
| E_4/E_6 ^d | 2.25 | 2.38 | 2.32 | Humification and aromaticity degree |

^a E_2/E_3 , the UV–Visible absorbance ratio at 250 and 365 nm, which reflects the molecular weight of dissolved organic materials (DOM) contained in ALE extraction.

^b $SUVA_{254}$, the value of the UV–Vis absorbance at 254 nm normalized by per mg TOC of ALE extraction, which indicates that the absorbance of unit TOC (mg/L) at 254 nm and reflects the unsaturated structural components in ALE extraction.

^c E_2/E_4 , the ratio of absorbance at wavelengths of 240 and 420 nm, which is inversely proportional to the condensation degree of humic substances.

^d E_4/E_6 , the absorbance ratio at 465 and 665 nm, which represents the information of humification and aromaticity degree.

such as sodium acetate were involved in the electron transfer and energy metabolism processes and could promote a higher level of extracellular enzymes in the extracellular matrix [41]. Moreover, readily biodegradable organic substrates like sodium acetate could directly cross the cell membrane and enter cell interior to be easily converted into acetyl Co-A and thus participate in the tricarboxylic acid (TCA) cycle. However, complex organic substances (starch, sucrose, etc.) need to be hydrolyzed and broken into small molecules by a multi-step bioreactions [16]. Therefore, a high level of enzymes including extracellular enzymes were likely involved in the complex organic metabolism [42], which was also confirmed by the higher proteins content in ALE, as shown in Fig. 3.

4.2. Organic load (L)

With different organic load (L), ALE production was about 160 mg/g VSS, which implied that the influent organic load seemed nothing to do with ALE formation. Indeed, there was also no obvious difference on biomass produced between different influent organics by measuring the observed biomass yield (Y_{obs}) in Corsino et al. [43]. Moreover, the specific ALE yields (Y_{ALE/BOD_5}) were calculated to evaluated the ALE production for per gram organics (BOD_5) consumption and the results are shown in Table 3. Table 3 showed that $Y_{ALE/BOD_5} = 63.7$ mg ALE/(g BOD_5) at $L = 0.20$ kg BOD_5 /kg VSS-d was twice as high as at $L = 0.36$

Table 3
Specific alginate like extracellular polymers (ALE) yields (Y_{ALE/BOD_5}) and fluorescence spectroscopy analysis [45].

| Organic load (kg BOD_5 /kg VSS-d) | ALE extraction (mg/g VSS) | Y_{ALE/BOD_5} (mg ALE/g BOD_5) | Different regions by three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy ^a | | | | |
|-------------------------------------|---------------------------|-------------------------------------|---|--------|---------|--------|-------|
| | | | Flu I | Flu II | Flu III | Flu IV | Flu V |
| 0.20 | 159.3 ± 2.4 | 63.7 | 0.9% | 2.7% | 2.4% | 55.7% | 38.4% |
| 0.36 | 158.9 ± 4.2 | 36.3 | 1.1% | 3.3% | 1.5% | 62.8% | 31.3% |
| 0.50 | 165.5 ± 3.0 | 26.5 | 1.2% | 3.5% | 2.1% | 62.1% | 31.1% |

^a Flu I and Flu II indicate aromatic protein-like substances (aromatic protein I and II), respectively. Flu IV represents soluble microbial by-product-like substances (SMPs) and protein-derived compounds. Flu III and Flu V include fulvic acid-like and humic acid-like organics.

and 0.50 kg BOD_5 /(kg VSS-d), 36.3 and 26.5 mg ALE/(g BOD_5), respectively. The possible mechanism might be attributed to that a large part of organic matters would be oxidized and decomposed to carbon dioxide under a high organic load to provide the energy for cellular metabolism and microbial proliferation rather than biosynthesize biopolymers. On the other hand, the utilized efficiency of organics at low organic load was higher and promoted extracellular structural biopolymers formation [44]. In this study, 3D-EEM fluorescence spectroscopy results of ALE are shown in Fig. 5. It depicted that the fraction of soluble microbial products (SMPs) in ALE (Region IV as shown in Table 3) increased obviously along with the increase of organic load, which proved the higher organic oxidation rather than the biosynthesis for the biopolymers formation.

Moreover, the content of PN decreased from approximately 400 mg/g ALE to 320 mg/g ALE with the increase of organic load, while PS was almost identical, at about 150 mg/g ALE. It was due to that the microorganisms would produce more exoenzymes or exoproteins outside the cells to maintain the stable metabolism under the conditions of lower organic deprivation and the chronic starvation. Furthermore, low organic load could promote the high content of PN in biopolymers matrix [44]. Also, the alginate equivalent of ALE also increased from 572.4 ± 10.3 mg/g ALE to 634.2 ± 3.0 mg/g ALE with decreasing organic load, which implied that more alginate formed under low organic load.

4.3. Substrate (carbon over nitrogen, C/N)

Fig. 1 showed that the ALE formation in flocculent sludge was also influenced by the C/N ratio. The ALE extraction reached to a peak of 137.8 ± 13.2 mg/g VSS at C/N = 5.0. On the contrary, the production declined slightly with increasing or decreasing C/N. A profound emphasis in several reports were given that C/N was associated with ALE production [15,17,18]. However, there was no a fixed favorable C/N ratio, for example, an optimum C/N ratio was at 0.5 mentioned by Liu et al. [15], but it was at 20 suggested by Ye et al. [17] and even at 40 reported by Durmaz and Sanin [46].

The results of PN and PS showed that C/N ratio had significant effects on the components of ALE. With decreasing C/N, the PN content increased, whereas the PS content decreased, which resulted in a rise on the PN/PS ratio. The functional groups of ALE, commercial alginate and three different polysaccharides (sodium acetate, glucose and starch) were detected by FT-IR and the results are illustrated in Fig. 6. Fig. 6 illustrates that the functional groups of ALE were significant different from the completely polysaccharides, but they were much similar to the commercial alginate, which indicates that ALE extracted from the flocculent sludge seem to have the similar characters as compared to the commercial alginate.

The different corresponding band assignments are also listed in Table 4. Among them, Amide I region ($1700\text{--}1600\text{ cm}^{-1}$) of ALE suggested their PN compositions were markedly different, which indicated that ALE formation at a low C/N ratio had more protein-like substances. Moreover, the band near 1136 cm^{-1} (C–O–C stretching vibrations belonging to carbohydrate) and 1072 cm^{-1} (C–H in-plane bending belonging to polysaccharides) also represented more polysaccharide dominated structures in the biopolymers at high ratio of C/N. Moreover, there are also significant differences in the bands, like C–H stretching at 2930 cm^{-1} (CH_2 groups) and 2960 cm^{-1} (CH_3 groups). Increasing nitrogen concentration (a low C/N ratio) favored protein synthesis in sludge matrix and decreased the extracellular polysaccharide accumulation due to that almost all carbon sources would be consumed for microbial proliferation and metabolism [17,47,48]. Moreover, shortage of carbon source could result in cellular autolysis and endogenous respiration and thus promote more exoenzyme protein [20,50]. Also, a high C/N ratio would be favorable for microbial synthesis of polysaccharide due to the increase of ATP content [50].

The quantitative similarity of chemical functional groups between

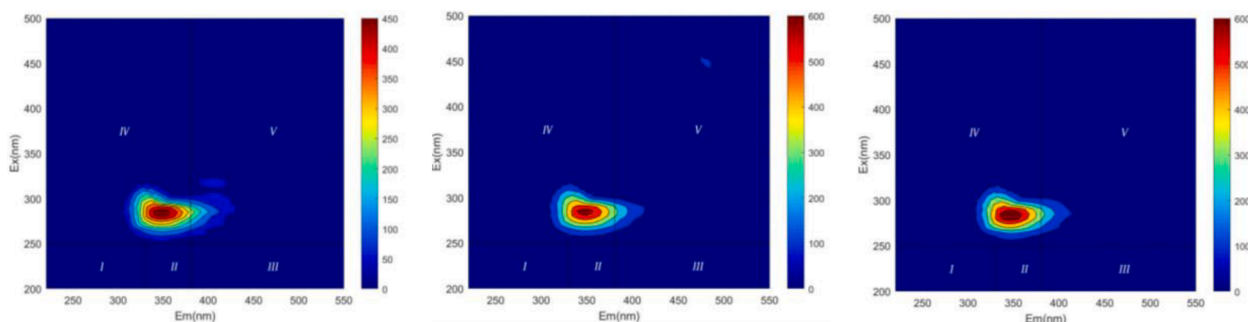


Fig. 5. Three-dimensional excitation–emission matrix (3D-EEM) fluorescence spectroscopy of the alginate like extracellular polymers (ALE).

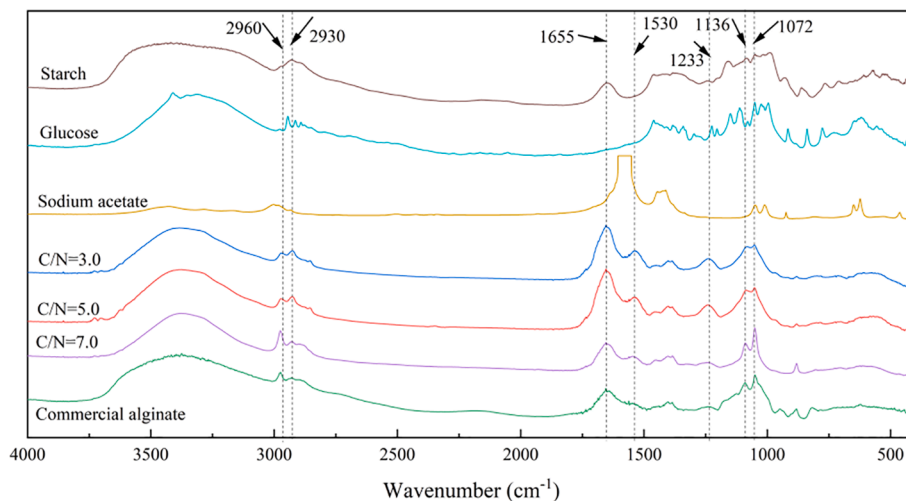


Fig. 6. Fourier transform infrared (FT-IR) spectra of different alginate like extracellular polymers (ALE), commercial alginate and other three polysaccharides (sodium acetate, glucose and starch).

Table 4

Different band assignments for the FT-IR spectral features (cm^{-1}) of alginate like extracellular polymers (ALE) [51–54].

| Wavenumber (cm^{-1}) | Vibration types | Corresponding groups |
|---------------------------------|---|----------------------------|
| 950 | O–P–O stretching | Nucleic acids |
| 1072 | C–H in-plane bending | Polysaccharides |
| 1233 | C–N stretching; C–OH stretching | Amide III; Polysaccharides |
| 1393 | C–H stretching in $-\text{CH}_3$ | Amines and Lipids |
| 1450 | CH_3 bending, CH_2 stretching | Methyl, Methylene |
| 1530 | C–H stretching, N–H bending | Amide II |
| 1655 | C = O stretching | Amide I |
| 2930 | C–H stretching (CH_2 groups) | Carbohydrates and Lipids |
| 2960 | C–H stretching (CH_3 groups) | Carbohydrates and Lipids |

ALE in different flocculent sludge and commercial alginate, reached up to 58.5% for $\text{C}/\text{N} = 3.0$ and 63.2% for $\text{C}/\text{N} = 7.0$, while it reached to highest level at 69.2% for $\text{C}/\text{N} = 5.0$. Moreover, at $\text{C}/\text{N} = 5.0$, the corresponding alginate equivalent (as shown in Fig. 2) reached to a maximum of 687.1 ± 9.6 mg/g ALE, while it was only at 583.9 ± 4.7 mg/g ALE and 598.2 ± 6.5 mg/g ALE for $\text{C}/\text{N} = 3.0$ and 7.0, respectively. Clearly, the optimal C/N had a noticeably positive effect on promoting the production of the alginate in extracted ALE.

4.4. Temperature

The previous studies have revealed that the temperature was one of the most important parameters influencing ALE formation in sludge

[18,21]. The experimental results in this study also showed that there was a sharp decline of ALE production along with the increasing temperature. At $T = 12^\circ\text{C}$, ALE extraction was at 303.3 ± 21.5 mg/g VSS, and rapidly decreased to 165.5 ± 3.0 mg/g VSS at 18°C and 132.7 ± 1.2 mg/g VSS at 24°C , respectively. However, Sutherland [49] and Nichols et al. [55] reported that the decrease of temperature could cause a decline on growth rate and the synthesis of cell wall polymer, which made more precursors available for biopolymers synthesis. Similarly, Gao et al. [56] also supported that the biopolymers in flocs gradually decreased with increasing temperature, while the soluble biopolymers in supernatant gradually increased. It might be due to that the transferring capacities of nutrient and oxygen were significantly low and the viscosity of sludge also increased at low temperature, which caused the flocs gathering in clusters tightly and extracellular substances were wrapped to form a dense colloidal network. Thus, enzymatic activities, electron transfer capacities as well as substance conversion capacities would be hindered seriously, resulting in low metabolites including structural extracellular biopolymers [49]. Meanwhile, 3D-EEM fluorescence spectroscopy are depicted in Fig. S7 and the fractions analysis is described in Fig. 7. It showed the obviously higher fluorescence intensity of solubility-like microbial metabolites and protein-derived compounds fractions (Region Flu IV) at 24°C than that of at 12°C , while fulvic acid-like and humic acid-like substances (Region Flu V) were lower at high temperature. It implies that bacterial metabolism was indeed disrupted at low temperature, as described above.

However, the alginate equivalent at low temperature (12°C) was only at 567.6 ± 12.3 mg/g ALE, which was much lower than 677.3 ± 20.5 mg/g ALE at 24°C . These results indicated that the increase of ALE in flocculent sludge matrix at low temperature was other content such as

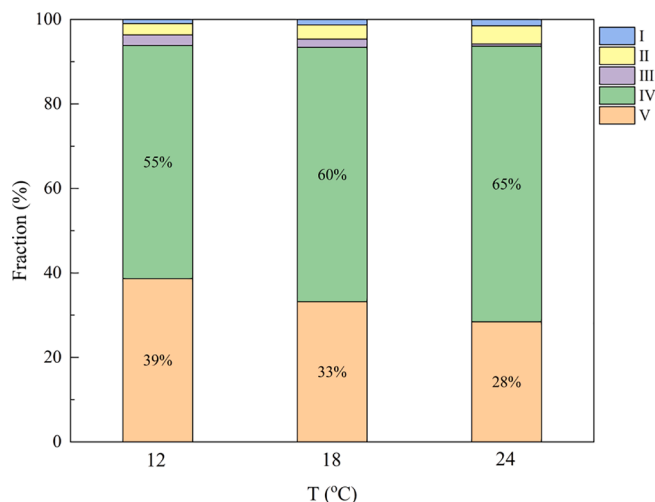


Fig. 7. Fractions of the alginate like extracellular polymers (ALE) by three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy.

humic substances rather than the structural alginate polymers. This was also consistent with the G and M fractions, as shown in Fig. 4.

Moreover, 1.0 mL per drop of extracted ALE was also dripped into a 2.5% (w/v) CaCl_2 solution to evaluate the formation of drop-shaped Ca^{2+} -ALE beads (cross-linked through Ca^{2+} ions), which demonstrated the ionic hydrogel forming property [22,28]. The ALE was cross-linked in the CaCl_2 solution at room temperature for about 16–18 h and the average diameter of hydrogel was measured and evaluated. The results were depicted in Fig. 8, which reveals that Ca^{2+} -ALE beads displayed different hydrogel forming properties between different ALE at different temperature. As also shown in Fig. 8, the ionic hydrogel properties of ALE at 12 °C were also of poor performance (loosely hydrogel at about 24 mm) than ALE at 18 °C (about 13 mm) and 24 °C (about 11 mm), which implies that the temperature would affect the hydrogel abilities of ALE, i.e., low temperature was not favorable for the high purification of alginate (main hydrogel forming structure) of the ALE production. There were also significant changes of PN and PS content. PN decreased from 468.6 ± 1.6 mg/g ALE to 351.0 ± 34.1 mg/g ALE with temperature increased, while PS showed an increase from 114.3 ± 6.3 mg/g ALE to 149.3 ± 11.0 mg/g ALE. Similar results were also observed by Neyens et al. [57].

4.5. Solid retention times (SRT)

The results at different SRT showed that ALE extraction was all at the range of 190–200 mg/g VSS. PN and PS were at the range of 400–420 mg/g ALE and 100–120 mg/g ALE, respectively, which indicated that SRT had minor effect on ALE formation and their compositions. By contrast, Duan et al. [58] gave a totally different idea; they concluded that a short SRT implied a relatively faster and higher active microbial

metabolism and a shift of dominant bacterial populations, resulting in more extracellular carbohydrate and protein accumulated in the biopolymers. The settleability and compressibility of the sludge with different SRT were associated with the extracellular biopolymers and the flocculation performance. A long SRT apparently realized a better sludge flocculation and a better settling capacity [18,21,59,60]. In this study, however, there was no difference between sludge flocculation and the settling capacity at different SRT. Anyway, different SRT still needs to be further investigated.

5. Further analysis and outlook

5.1. Principal component analyses (PCA)

Since the PCA is based on the correlation matrix, the results can be interpreted as the correlations of the original correlated environmental/operational factors with each uncorrelated variables (principal components, PCs) and the behaviors of each variable is ascribed to each loading value [61]. The results of loadings for the different cultivation parameters in the five PCs are given in Table S8. Moreover, Fig. 9 showed the biplot of the first 2 PCs to visualize the combined behaviors of significant parameters that affect ALE formation, which revealed that PCA reduced the dimensionality into two significant principal components (PCs) that represented around 80.2% of the total variance ($\text{PC1} = 42.7\%$, $\text{PC2} = 37.5\%$). Based on the highest total variance of 42.7% in PC1, carbon sources and C/N presented the high negative loading values, which indicated that PC1 decreased with increasing carbon sources and C/N. Moreover, temperature had a relatively highly positive loading in PC1

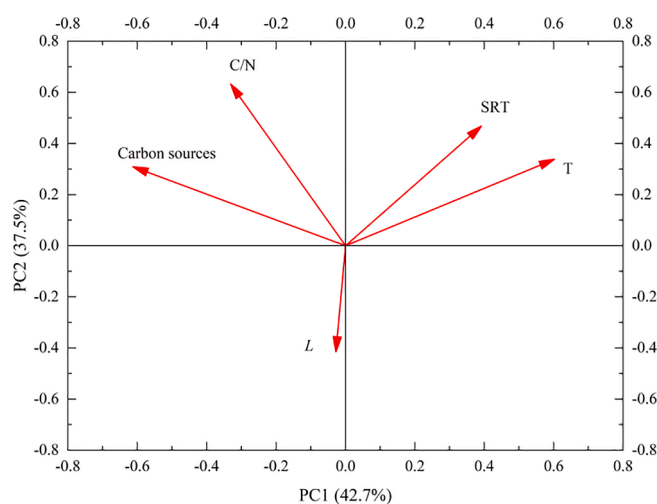


Fig. 9. Loading biplot for principal component analysis (PCA) for the correlations of operational parameters with the alginate like extracellular polymers (ALE) formation.

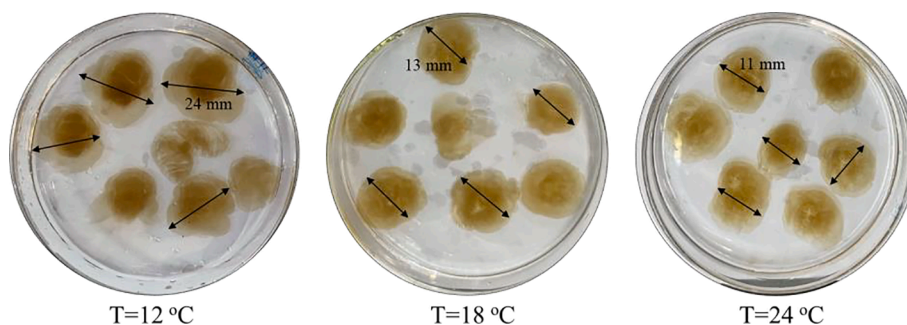


Fig. 8. Ionic hydrogel properties of the alginate like extracellular polymers (ALE).

(0.60). Therefore, the parameters in PC1 could be effective to describe the behaviors of carbon/nitrogen sources and temperature performance. PC2 explained about 37.5% of the total variance, and the effects of carbon sources on PC2 were diminished since it had very low absolute loadings (0.31). The PC3 was largely correlated to organic load rate (0.83) and SRT (0.51) although presenting only 19.5% of the explained variance.

The PCA results revealed that biopolymers content correlated with carbon sources, substrate and temperature. Among them, temperature was a key factor that affected ALE formation, followed by carbon sources and substrate (C/N). By contrast, organic load had little effect on ALE production.

5.2. Highly valuable biopolymers recovery

In summary, it is important to figure out the optimal conditions for the biopolymers/ALE formation for the perspective of highly valuable biopolymers recovery from flocculent sludge. In practice, it should also be considered that the environmental/operational conditions are suitable for a high efficiency of BNR process. Starch and temperature are two significant contributory factors to improving ALE production. However, some scenarios with starch-rich could only enhance the ALE production. Moreover, low temperature only occurs in the winter but is not favorable to BNR process. Adjusting the influent characteristics, including C/N and organic load in the BNR processes without affecting the effluent quality of WWTPs, would be also some approaches to maximizing the ALE production. Moreover, the results also imply that it would be possible to achieve a directional cultivation of the biopolymers/ALE with different proportions of polysaccharide or protein and even with some special chemical/physical properties. Therefore, the feasible applications or economic benefits for highly valuable biomaterials recovery could be improved.

On the other hand, all of these parameters are working together on forming ALE in practice, thus the multi factors instead of independent variables should be paid more attention for ascertaining the optimal strategies on ALE formation and characteristics in flocculent sludge in future study.

6. Conclusions

Alginate like extracellular polymers (ALE) recovered from excess flocculent sludge has been identified as a kind of highly valuable biomaterials for potential applications. However, ALE extraction from flocculent sludge is often limited by the low level of ALE formation in flocs matrixes. With the study, the key factors and associated mechanisms controlling the ALE formation were experimentally identified for flocculent sludge to improve the ALE production from flocculent sludge. Based on the production and recovery of the biopolymers from flocculent sludge, the influences of carbon sources, organic load, substrate (C/N), temperature and solid retention times (SRT) on alginate like extracellular polymers (ALE) formation were basically identified and evaluated with this study. The experimental results indicate that carbon sources and temperature had the major effects on the ALE formation. The ALE production could reach to 220.3 ± 8.0 mg/g VSS with starch being carbon source, which was higher than sodium acetate and glucose. In addition, low temperature was more favorable to enriching extracellular biopolymers, and the ALE production could reach to 303.3 ± 21.5 mg/g VSS at 12°C . Moreover, a higher organic load could decrease the specific ALE yields ($Y_{\text{ALE}/\text{BOD}_5}$). C:N = 5:1 in the influent could contribute a highest ALE production among different C/N ratios, up to 137.8 ± 13.2 mg/g VSS. Interestingly, SRT had a minor effect on the ALE production, always ranging from 190 to 200 mg/g VSS under different SRTs. The PCA analysis confirmed that temperature was a key factor affecting the ALE formation, followed by carbon sources and substrate (C/N). On the other hand, there were the noticeable differences on the fractions and chemical properties of the ALE under different

working conditions. In summary, adjusting or optimizing operational parameters could improve the ALE formation in flocculent sludge and thus promote the highly valuable materials' recovery from conventional WWTPs. Moreover, the study also concludes that the directional cultivation of the biopolymers/ALE with some special chemical/physical properties could be an approach to improving the value of recovered biomaterials and expend the potential applications.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2022.135792>.

References

- [1] Y.M. Lin, K.G.J. Nierop, E. Girbal-neuhauser, M. Adriaanse, M.C.M. Van Loosdrecht, Sustainable Materials and Technologies Sustainable polysaccharide-based biomaterial recovered from waste aerobic granular sludge as a surface coating material, *SUSMAT*. 4 (2015) 24–29, <https://doi.org/10.1016/j.susmat.2015.06.002>.
- [2] Y. Lin, C. Reino, J. Carrera, J. Pérez, M.C.M. van Loosdrecht, Glycosylated amyloid-like proteins in the structural extracellular polymers of aerobic granular sludge enriched with ammonium-oxidizing bacteria, *MicrobiologyOpen* 7 (6) (2018) e00616.
- [3] W. Xue, Q. Zeng, S. Lin, F. Zan, T. Hao, Y. Lin, M.C.M. Van Loosdrecht, G. Chen, Recovery of high-value and scarce resources from biological wastewater treatment: Sulfated polysaccharides, *Water Res.* 163 (2019), 114889, <https://doi.org/10.1016/j.watres.2019.114889>.
- [4] S. Felz, T.R. Neu, M.C.M. van Loosdrecht, Y. Lin, Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers, *Water Res.* 169 (2020), 115291, <https://doi.org/10.1016/j.watres.2019.115291>.
- [5] N.K. Kim, N. Mao, R. Lin, D. Bhattacharyya, M.C.M. van Loosdrecht, Y. Lin, Flame retardant property of flax fabrics coated by extracellular polymeric substances recovered from both activated sludge and aerobic granular sludge, *Water Res.* 170 (2020), 115344, <https://doi.org/10.1016/j.watres.2019.115344>.
- [6] B.S. Inbaraj, C.P. Chiu, G.H. Ho, J. Yang, B.H. Chen, Effects of temperature and pH on adsorption of basic brown 1 by the bacterial biopolymer poly(γ -glutamic acid), *Bioresour. Technol.* 99 (2008) 1026–1035, <https://doi.org/10.1016/j.biortech.2007.03.008>.
- [7] G. Guibaud, N. Tixier, A. Bouju, M. Baudu, Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb, *Chemosphere*. 52 (2003) 1701–1710, [https://doi.org/10.1016/S0045-6535\(03\)00355-2](https://doi.org/10.1016/S0045-6535(03)00355-2).
- [8] D.Q. Cao, W.Y. Yang, Z. Wang, X. Di Hao, Role of extracellular polymeric substance in adsorption of quinolone antibiotics by microbial cells in excess sludge, *Chem. Eng. J.* 370 (2019) 684–694, <https://doi.org/10.1016/j.cej.2019.03.230>.
- [9] F. Ma, L.N. Zheng, Y. Chi, Applications of biological flocculants (BFs) for coagulation treatment in water purification: Turbidity elimination, *Chem. Biochem. Eng. Q.* 22 (2008) 321–326.
- [10] J. Li, X. Hao, W. Gan, M.C.M. van Loosdrecht, Y. Wu, Recovery of extracellular biopolymers from conventional activated sludge: potential, characteristics and limitation, *Water Res.* 205 (2021), 117706, <https://doi.org/10.1016/j.watres.2021.117706>.
- [11] S.S. Adav, D.J. Lee, Extraction of extracellular polymeric substances from aerobic granule with compact interior structure, *J. Hazard. Mater.* 154 (2008) 1120–1126, <https://doi.org/10.1016/j.jhazmat.2007.11.058>.
- [12] S. Felz, S. Al-zuhairi, O.A. Aarstad, M.C.M. Van Loosdrecht, Y.M. Lin, Extraction of Structural Extracellular Polymeric Substances from Aerobic Granular Sludge (2016) 1–8, <https://doi.org/10.3791/54534>.
- [13] M. Boleij, T. Seviour, L.L. Wong, M.C.M. van Loosdrecht, Y. Lin, Solubilization and characterization of extracellular proteins from anammox granular sludge, *Water Res.* 164 (2019), 114952, <https://doi.org/10.1016/j.watres.2019.114952>.
- [14] L. Zhu, J. Zhou, M. Lv, H. Yu, H. Zhao, X. Xu, Specific component comparison of extracellular polymeric substances (EPS) in flocs and granular sludge using EEM and SDS-PAGE, *Chemosphere*. 121 (2015) 26–32, <https://doi.org/10.1016/j.chemosphere.2014.10.053>.

- [15] W.J. Liu, K. Wang, B.Z. Li, H.L. Yuan, J.S. Yang, Production and characterization of an intracellular biofloculant by *Chryseobacterium daeguense* W6 cultured in low nutrition medium, *Bioresour. Technol.* 101 (2010) 1044–1048, <https://doi.org/10.1016/j.biortech.2009.08.108>.
- [16] F.X. Ye, G. Peng, Y. Li, Influences of influent carbon source on extracellular polymeric substances (EPS) and physicochemical properties of activated sludge, *Chemosphere* 84 (2011) 1250–1255, <https://doi.org/10.1016/j.chemosphere.2011.05.004>.
- [17] F. Ye, Y. Ye, Y. Li, Effect of C/N ratio on extracellular polymeric substances (EPS) and physicochemical properties of activated sludge flocs, *J. Hazard. Mater.* 188 (2011) 37–43, <https://doi.org/10.1016/j.jhazmat.2011.01.043>.
- [18] T.T. More, J.S.S. Yadav, S. Yan, R.D. Tyagi, R.Y. Surampalli, Extracellular polymeric substances of bacteria and their potential environmental applications, *J. Environ. Manage.* 144 (2014) 1–25, <https://doi.org/10.1016/j.jenvman.2014.05.010>.
- [19] H.S. Shin, S.T. Kang, S.Y. Nam, Effect of carbohydrates to protein ratio in EPS on sludge settling characteristics, *Biotechnol. Bioprocess Eng.* 5 (2000) 460–464, <https://doi.org/10.1007/BF02931948>.
- [20] G.P. Sheng, H.Q. Yu, X.Y. Li, Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review, *Biotechnol. Adv.* 28 (2010) 882–894, <https://doi.org/10.1016/j.biotechadv.2010.08.001>.
- [21] G.P. Sheng, H.Q. Yu, Characterization of extracellular polymeric substances of aerobic and anaerobic sludge using three-dimensional excitation and emission matrix fluorescence spectroscopy, *Water Res.* 40 (2006) 1233–1239, <https://doi.org/10.1016/j.watres.2006.01.023>.
- [22] S. Felz, P. Vermeulen, M.C.M. van Loosdrecht, Y.M. Lin, Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS), *Water Res.* 157 (2019) 201–208, <https://doi.org/10.1016/j.watres.2019.03.068>.
- [23] M. Dubois, K. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, A colorimetric method for the determination of sugars, *Nature* 168 (4265) (1951) 167–168.
- [24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275, [https://doi.org/10.1016/s0021-9258\(19\)52451-6](https://doi.org/10.1016/s0021-9258(19)52451-6).
- [25] Y. Lin, M. de Kreuk, M.C.M. van Loosdrecht, A. Adin, Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant, *Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in pilot-plant* 44 (11) (2010) 3355–3364.
- [26] J. Li, X. Hao, M.C.M. Van Loosdrecht, Y. Luo, D. Cao, Effect of humic acids on batch anaerobic digestion of excess sludge, *Water Res.* 155 (2019) 431–443, <https://doi.org/10.1016/j.watres.2018.12.009>.
- [27] R. Liu, X. Hao, M.C.M. van Loosdrecht, P. Zhou, J.I. Li, Dynamics of humic substance composition during anaerobic digestion of excess activated sludge, *Int. Biodeterior. Biodegradation* 145 (2019) 104771.
- [28] Y.M. Lin, P.K. Sharma, M.C.M. van Loosdrecht, The chemical and mechanical differences between alginate-like exopolysaccharides isolated from aerobic flocculent sludge and aerobic granular sludge, *Water Res.* 47 (2013) 57–65, <https://doi.org/10.1016/j.watres.2012.09.017>.
- [29] G.Q. Isolation, Standard methods for the examination of water and wastewater treatment methods for the examination of water and wastewater, *Public Health* (1999).
- [30] C.M. Schambeck, B.S. Magnus, L.C.R. de Souza, W.R.M. Leite, N. Derlon, L. B. Guimarães, R.H.R. da Costa, Biopolymers recovery: dynamics and characterization of alginate-like exopolymers in an aerobic granular sludge system treating municipal wastewater without sludge inoculum, *J. Environ. Manage.* 263 (2020) 110394.
- [31] Y.C. Yang, X. Liu, C. Wan, S. Sun, D.J. Lee, Accelerated aerobic granulation using alternating feed loadings: Alginate-like exopolysaccharides, *Bioresour. Technol.* 171 (2014) 360–366, <https://doi.org/10.1016/j.biortech.2014.08.092>.
- [32] T. Seviour, M. Pijuan, T. Nicholson, J. Keller, Z. Yuan, Understanding the properties of aerobic sludge granules as hydrogels, *Biotechnol. Bioeng.* 102 (2009) 1483–1493, <https://doi.org/10.1002/bit.22164>.
- [33] K.Y. Lee, D.J. Mooney, Alginate: Properties and biomedical applications, *Prog. Polym. Sci.* 37 (2012) 106–126, <https://doi.org/10.1016/j.progpolymsci.2011.06.003>.
- [34] I.D. Hay, Z.U. Rehman, M.F. Moradali, Y. Wang, B.H.A. Rehm, Microbial alginate production, modification and its applications, *Microb. Biotechnol.* 6 (2013) 637–650, <https://doi.org/10.1111/1751-7915.12076>.
- [35] Y. González-García, A. Heredia, J.C. Meza-Contreras, F.M.E. Escalante, R. M. Camacho-Ruiz, J. Córdova, Biosynthesis of extracellular polymeric substances by the marine bacterium *saccharophagus* degradans under different nutritional conditions, *Int. J. Polym. Sci.* 2015 (2015) 1–7.
- [36] H. Wang, H. Deng, L. Ma, L. Ge, The effect of carbon source on extracellular polymeric substances production and its influence on sludge floc properties, *J. Chem. Technol. Biotechnol.* 89 (2014) 516–521, <https://doi.org/10.1002/jctb.4147>.
- [37] Y. Liu, H.H.P. Fang, Influences of extracellular polymeric substances (eps) on flocculation, settling, and dewatering of activated sludge, *Crit. Rev. Environ. Sci. Technol.* 33 (2003) 237–273, <https://doi.org/10.1080/10643380390814479>.
- [38] J. Xu, H.-Q. Yu, X.-Y. Li, Probing the contribution of extracellular polymeric substance fractions to activated-sludge bioflocculation using particle image velocimetry in combination with extended DLVO analysis, *Chem. Eng. J.* 303 (2016) 627–635.
- [39] J.L. Weishaar, G.R. Aiken, B.A. Bergamaschi, M.S. Fram, R. Fujii, K. Mopper, Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon, *Environ. Sci. Technol.* 37 (2003) 4702–4708, <https://doi.org/10.1021/es030360x>.
- [40] J. Polak, W.W. Sułkowski, M. Bartoszek, A. Luty, D. Pentak, A. Sułkowska, Spectroscopic study of the effect of biological treatment on the humification process of sewage sludge, *J. Mol. Struct.* 834–836 (2007) 229–235, <https://doi.org/10.1016/j.molstruc.2006.12.065>.
- [41] C.S. Laspidou, B.E. Rittmann, Non-steady state modeling of extracellular polymeric substances, soluble microbial products, and active and inert biomass, *Water Res.* 36 (2002) 1983–1992, [https://doi.org/10.1016/S0043-1354\(01\)00414-6](https://doi.org/10.1016/S0043-1354(01)00414-6).
- [42] X.Y. Li, S.F. Yang, Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge, *Water Res.* 41 (2007) 1022–1030, <https://doi.org/10.1016/j.watres.2006.06.037>.
- [43] S.F. Corsino, M. Capodici, M. Torregrossa, G. Viviani, Physical properties and Extracellular Polymeric Substances pattern of aerobic granular sludge treating hypersaline wastewater, *Bioresour. Technol.* 229 (2017) 152–159, <https://doi.org/10.1016/j.biortech.2017.01.024>.
- [44] P. Rusanowska, A. Cydzik-Kwiatkowska, P. Świączak, I. Wojnowska-Baryla, Changes in extracellular polymeric substances (EPS) content and composition in aerobic granule size-fractions during reactor cycles at different organic loads, *Bioresour. Technol.* 272 (2019) 188–193, <https://doi.org/10.1016/j.biortech.2018.10.022>.
- [45] Q. Dai, L. Ma, N. Ren, P. Ning, Z. Guo, L. Xie, H. Gao, Investigation on extracellular polymeric substances, sludge flocs morphology, bound water release and dewatering performance of sewage sludge under pretreatment with modified phosphogypsum, *Water Res.* 142 (2018) 337–346, <https://doi.org/10.1016/j.watres.2018.06.009>.
- [46] B. Durmaz, F.D. Sanin, Effect of carbon to nitrogen ratio on the composition of microbial extracellular polymers in activated sludge, *Water Sci. Technol.* 44 (2001) 221–229, <https://doi.org/10.2166/wst.2001.0626>.
- [47] Z. Wang, M. Gao, Y. Xin, D. Ma, Z. She, Z. Wang, C. Sun, Y. Ren, Effect of C/N ratio on extracellular polymeric substances of activated sludge from an anoxic-aerobic sequencing batch reactor treating saline wastewater, *Environ. Technol. (United Kingdom)* 35 (2014) 2821–2828, <https://doi.org/10.1080/09593330.2014.924563>.
- [48] A.G. Geyik, F. Çeçen, Production of protein- and carbohydrate-EPS in activated sludge reactors operated at different carbon to nitrogen ratios, *J. Chem. Technol. Biotechnol.* 91 (2016) 522–531, <https://doi.org/10.1002/jctb.4608>.
- [49] I.W. Sutherland, Biofilm exopolysaccharides: A strong and sticky framework, *Microbiology* 147 (2001) 3–9, <https://doi.org/10.1099/00221287-147-1-3>.
- [50] Y. Mengistu, C. Edwards, J.R. Saunders, Continuous culture studies on the synthesis of capsular polysaccharide by *Klebsiella pneumoniae* K1, *J. Appl. Bacteriol.* 76 (1994) 424–430, <https://doi.org/10.1111/j.1365-2672.1994.tb01098.x>.
- [51] X. Yin, S. Qiao, J. Zhou, X. Quan, Using three-bio-electrode reactor to enhance the activity of anammox biomass, *Bioresour. Technol.* 196 (2015) 376–382, <https://doi.org/10.1016/j.biortech.2015.07.096>.
- [52] Z. Zhang, Y. Ji, R. Cao, Z. Yu, X. Xu, L. Zhu, A novel mode of air recycling favored stable operation of the aerobic granular sludge process via calcium accumulation, *Chem. Eng. J.* 371 (2019) 600–608, <https://doi.org/10.1016/j.cej.2019.04.083>.
- [53] Z. Niu, Z. Zhang, S. Liu, T. Miyoshi, H. Matsuyama, J. Ni, Discrepant membrane fouling of partial nitrification and anammox membrane bioreactor operated at the same nitrogen loading rate, *Bioresour. Technol.* 214 (2016) 729–736, <https://doi.org/10.1016/j.biortech.2016.05.022>.
- [54] F. Jia, Q. Yang, X. Liu, X. Li, B. Li, L. Zhang, Y. Peng, Stratification of Extracellular Polymeric Substances (EPS) for Aggregated Anammox Microorganisms, *Environ. Sci. Technol.* 51 (2017) 3260–3268, <https://doi.org/10.1021/acs.est.6b05761>.
- [55] C.M. Nichols, J.P. Bowman, J. Guezennec, Effects of incubation temperature on growth and production of exopolysaccharides by an antarctic sea ice bacterium grown in batch culture, *Appl. Environ. Microbiol.* 71 (2005) 3519–3523, <https://doi.org/10.1128/AEM.71.7.3519-3523.2005>.
- [56] J. Gao, Q. Zhang, K. Su, R. Chen, Y. Peng, Biosorption of Acid Yellow 17 from aqueous solution by non-living aerobic granular sludge, *J. Hazard. Mater.* 174 (2010) 215–225, <https://doi.org/10.1016/j.jhazmat.2009.09.039>.
- [57] E. Neyens, J. Baeyens, R. Dewil, B. De Heyder, Advanced sludge treatment affects extracellular polymeric substances to improve activated sludge dewatering, *J. Hazard. Mater.* 106 (2004) 83–92, <https://doi.org/10.1016/j.jhazmat.2003.11.014>.
- [58] L. Duan, Y. Song, H. Yu, S. Xia, S.W. Hermanowicz, The effect of solids retention times on the characterization of extracellular polymeric substances and soluble microbial products in a submerged membrane bioreactor, *Bioresour. Technol.* 163 (2014) 395–398, <https://doi.org/10.1016/j.biortech.2014.04.112>.
- [59] B.Q. Liao, D.G. Allen, I.G. Droppo, G.G. Leppard, S.N. Liss, Surface properties of sludge and their role in bioflocculation and settleability, *Water Res.* 35 (2001) 339–350, [https://doi.org/10.1016/S0043-1354\(00\)00277-3](https://doi.org/10.1016/S0043-1354(00)00277-3).
- [60] J. Li, K. Kisara, S. Danielsson, M.E. Lindström, G. Gellerstedt, An improved methodology for the quantification of uronic acid units in xylans and other polysaccharides, *Carbohydr. Res.* 342 (2007) 1442–1449, <https://doi.org/10.1016/j.carres.2007.03.031>.
- [61] V. Vasilaki, E.I.P. Volcke, A.K. Nandi, M.C.M. van Loosdrecht, E. Katsou, Relating N₂O emissions during biological nitrogen removal with operating conditions using multivariate statistical techniques, *Water Res.* 140 (2018) 387–402, <https://doi.org/10.1016/j.watres.2018.04.052>.