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How to Form Aggregates of Free Anammox Bacteria to Improve Sedimentation Performance

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Abstract: Free anammox bacteria are superior in growth rate, but poor sedimentation performance limits their application in sewage treatment. In this study, we investigated how to form aggregates of free anammox bacteria to improve sedimentation performance. Calcium addition tests proved that free anammox bacteria could aggregate and form a larger entirety with better sedimentation performance through calcium cross-linking with alginate-like exopolysaccharides (ALEs). This was indicated by the particle size increasing by 411.45% and sedimentation performance (measured with supernatant transmittance) increasing by 195.35% after adding calcium for 12 h. Soluble extracellular polymeric substance (S-EPS) extraction and freeze–thaw testing elucidated that providing more cross-linking sites can strengthen the cross-linking, as indicated by the sedimentation performance increasing by 158.57% and 394.80%, respectively. Static experiments showed that cross-linking time was equally important. The sedimentation performance improved with longer static times under no severe external disturbances, with a 324.61% improvement after 84 h. However, the bacteria burst and the anammox activity disappeared after freeze–thaw treatment. Based on the above test results, a potential method for forming aggregates of free anammox bacteria to improve sedimentation performance was proposed: extract S-EPS with centrifugation first, add calcium, and keep the sludge free from external hydraulic interference.

Keywords: free anammox bacteria; sedimentation performance; microcolonies interactions

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1. Introduction

Anaerobic ammonium oxidation (anammox) has become increasingly significant in carbon-neutral processes due to its reduced greenhouse gases, lack of organic carbon requirements, and considerable energy conservation [1,2]. As the main functional bacteria in the system, the anammox bacteria have a wide range of doubling times (2.1–25 d) [3–5]. Most anammox cultivation systems adopt aggregate-based technology, and the larger size restricts mass transfer, resulting in a long doubling time (10–25 d) [3,6]. The prolonged start-up time limits the widespread application of anammox technology. However, when anammox bacteria grow as free or suspended cells, their maximum growth rate can be increased to 0.33 d^{-1} (corresponding to a doubling time of 2.1 d) [4]. Unfortunately, free anammox bacteria do not perform well in sedimentation performance and are easily lost with effluent. Consequently, rapidly enriching large numbers of free anammox bacteria and forming aggregates of free anammox bacteria to improve their sedimentation performance before use may reduce start-up times. Previous research has achieved the enrichment of free anammox bacteria [7,8], but limited studies have focused on how to improve the settling performance of free anammox bacteria simply and quickly.

For anammox granules, the formation mechanism has been proposed [9]. The primary basis is made up of interactions between individual anammox bacteria. A second arrange-

ment level is made up of anammox bacterial cells encapsulated in a thin extra-cellular polymeric substance (EPS) layer. Finally, combining these microcolonies with other bacteria and polymers results in a larger entity. Accordingly, microcolonies in free anammox bacteria may aggregate to form a larger entirety with better sedimentation performance by aggregation. In fact, in our previous study, it has been observed that the sedimentation performance of free anammox bacteria picked out from the reactor improved after being stored at room temperature for a few days. As previously mentioned, EPS is crucial for the adhesion and aggregation of microorganisms, which contribute to construction and maintenance of the structure of the microbial community [10,11]. Alginate-like exopolysaccharide (ALE), a typical component of EPS, has highly similar structure and properties to alginates extracted from macroalgae [12]. The ALE gel plays a significant role in preserving the gel-like structure of the granules [13,14]. Hydrogen bonding and Ca^{2+} help it build stable interchain cross-links and bridges, creating "egg-box models" that keep cells together [15–17]. Alginate has been used to produce artificial hydrogels by cross-linking with Ca^{2+} [18], and a longer cross-linking time proved advantageous for increasing the structure's hardness [19]. ALE has also been detected in anammox sludge [20]. Therefore, we hypothesize that aggregates of free anammox bacteria could be formed, with ALE within EPS cross-linking with Ca^{2+} in water as a glue adhering and enveloping these microcolonies.

The cross-linking time and sites are crucial in determining the strength of the structure. Previous studies have demonstrated that extended cross-linking time was beneficial for enhancing the hardness of gel beads, and low cross-linking agent concentration would cause the gel beads to be soft and easily broken [19]. Consequently, providing sufficient cross-linking time through static without external hydraulic interference, and providing more sites through concentration or increased solute, may strengthen cross-linking. EPS is categorized into soluble EPS (S-EPS), loosely bound EPS (LB-EPS), and tightly bound EPS (TB-EPS) [11]. LB-EPS and TB-EPS have a higher concentration [21], which would contribute more ALE. S-EPS is weakly bound to cells or dissolved into a solution, which can be easily separated through centrifugation [22]. Removing S-EPS using centrifugation would expose LB-EPS containing more ALE to increase the cross-linking sites. Freeze–thaw is a widely adopted method in the food industry to achieve concentration. The formation of ice crystals during freezing reduces the content of free water in a polysaccharide solution, resulting in a local concentration increase. These methods may enhance cross-linking and form aggregates of free anammox bacteria to improve sedimentation performance, but this has not been experimentally proven.

This study aimed to investigate a highly efficient approach to form aggregates of free anammox bacteria with better sedimentation performance, which was characterized by the supernatant transmittance and sedimentation layer height. A calcium addition experiment was conducted to assess the possibility of ALE cross-linking with Ca^{2+} to enlarge the size of free anammox bacteria. Furthermore, we analyzed how cross-linking sites affect the cross-linking effect through S-EPS extraction and freeze–thaw, and determined the effect of cross-linking time through static experiments. Combining these findings, a practical method was proposed to improve sedimentation performance through forming aggregates of free anammox bacteria.

2. Materials and Methods

2.1. Biomass Origin

Free anammox bacteria were collected from a laboratory-scale membrane bioreactor (MBR) (Binhaiyibo, Yancheng, China) equipped with a hollow fiber membrane unit (absolute pore size: 0.1 μm). The MBR was fed with synthetic medium as previously described [7]. The mixed liquid suspended solids (MLSS) were approximately 1300 mg/L. The free anammox bacteria were kept in free planktonic with a wave-making pump. The particle size of the free anammox bacteria was below 50 μm.

2.2. Static Culture Experiment

The samples (100 mL suspension) were harvested from the MBR and transferred to two 50 mL centrifuge tubes. The synthetic wastewater contained 10 mg N/L NH_4^+ and 10 mg N/L NO_2^-. The synthetic wastewater included the same components as the original anammox reactor. The initial pH was maintained at around 8.0, and oxygen concentration was regulated by injecting N_2 into the influent. The oxygen concentration was regulated by removing N_2 from the influent. The tubes were neither shaken nor vibrated throughout the experiment. The experiment was conducted in duplicate.

The sedimentation performance of the samples was evaluated using supernatant transmittance and sedimentation layer height. The microcolonies interacted with each other to form a larger entity, leading to increased sedimentation performance. Fewer microcolonies were in the supernatant for the same static time, resulting in higher light transmission of the supernatant. Simply, the higher the supernatant transmittance, the better the sedimentation performance of the samples. The same applies to the sedimentation layer height. The specifics of the operation are as follows: shake the sample upside down repeatedly and place it into a 100 mL graduated cylinder. After 30 min, measure the height of the sedimentation layer, and take the supernatant 2 cm below the liquid level, measuring the light transmission at 550 nm using a UV spectrophotometer UV-4800 (UNICO, Shanghai, China).

2.3. Calcium Addition

The reactor setup for the calcium addition experiment was the same as the static experiment. The only difference was that the experimental group received an additional 18 mg $CaCl_2 \cdot 2H_2O$ per 100 mL, and no additional calcium was added in the control group. As previously described, the sedimentation performance and particle size were measured after 0.5 h and 12 h of calcium additions. To explore the effect of adding calcium on EPS content, the EPS content before and after adding calcium was determined. A modified heat method extracted S-EPS, LB-EPS, and TB-EPS from the samples [23]. The centrifuge model in this experiment is TDZ5-WS (Cence, Changsha, China). The samples were centrifuged for five minutes at 4000 rpm. S-EPS was collected after the supernatant was filtered using a 0.45 μm membrane. The remaining sludge pellet was resuspended with NaCl solution (0.05%, preheated to 70 °C), vortexed for 1 min, and immediately centrifuged at 4000 rpm for 10 min. The supernatant was collected as LB-EPS after filtering through a 0.45 μm membrane. The leftover sludge pellets were resuspended in a 0.05% NaCl solution and heated for 30 min in a water bath at 60 °C. The suspension was vortexed for one minute again before being centrifuged at 4000 rpm for 15 min. The supernatant was collected as TB-EPS after filtering through a 0.45 μm membrane. The PS constituent of EPS was analyzed using the anthrone method with glucose as the standard reference [24]. Samples and glucose solutions with different concentrations were prepared. Subsequently, 1 mL of each solution needed to be added to respective tubes and mixed with 5 mL of anthrone reagent. We rapidly cooled the solution by immersing it in an ice bath and shaking to mix. We sealed the tubes before heating to prevent evaporation then heated the tubes in a boiling water bath at 95 °C for 10 min, and swiftly transferred the container to a cold bath to bring the solution back to room temperature. Polysaccharide content was evaluated via spectrophotometer at 680 nm, using the sample's absorption value and standard curve for calculation. The PN constituent was analyzed using the Lowry method with bovine albumin serum as the standard reference [25]. Solution A was obtained by mixing an equal volume of 40 g/L Na_2CO_3 solution with 0.2 mol/L NaOH solution, while solution B was obtained by mixing an equal volume of 10 g/L $CuSO_4 \bullet 5H_2O$ solution with 20 g/L potassium sodium tartrate solution. Solution A and solution B were evenly mixed in a 50:1 ratio to produce solution C. For protein content determination, we took samples and different concentrations of bovine serum protein solution 1 mL, followed by the addition of 5 mL of C solution and thorough shaking. The mixture was left for 10 min before adding 0.5 mL of 1 mol/L Folin-phenol, followed by thorough mixing. After incubating at room

temperature for 30 min, the mixture was analyzed for absorbance at 680 nm. Utilizing the standard curve of the sample, the protein concentration was determined from the absorbance value. Both PN and PS results were the average value of two parallel tests.

2.4. S-EPS Extraction

The samples were centrifuged at 4000 rpm for five minutes and the supernatant was removed, which could remove S-EPS. The leftover pellet was transferred to 250 mL centrifuge tubes. Separately, we added distilled water to reach a liquid volume of 50 mL. The supernatant transmittance and sedimentation layer height was measured as previously described. An aggregation experiment was used to evaluate the role of S-EPS in the adherence of anammox bacteria. The aggregation tests were conducted following the methodology described in a prior study [21]. The samples were resuspended in 0.9% NaCl (pH 7.0, 25 °C) before and after S-EPS extraction. Adjusting the $OD_{600,0}$ of the suspension to 0.6, 3 mL of suspension was then put into the cuvette. The anammox microcolonies accumulated and sank to the bottom of the cuvette as time passed, and the $OD_{600,t}$ at the upper part of the cuvette was measured after 1, 2, 3, 4, 6, 7 and 8 h, respectively. Anammox consortia aggregate percentages (A_t) were calculated based on the following equation:

$$Aggregation\ percentage(A_t, \%) = \frac{OD_{600,0} - OD_{600,t}}{OD_0} \times 100 \quad (1)$$

The aggregation process can be regarded as a reversible chemical reaction. The kinetics of aggregation were described using a pseudo-first-order Equation (2) [26].

$$A_t = A_e(1 - e^{-tk}) \quad (2)$$

where the k (h^{-1}) is the fitted pseudo-first-order rate constant, A_e (%) is the percentage aggregation at equilibrium, and A_t (%) is the percentage aggregation at a certain time.

2.5. Freeze–Thaw Treatment

A 400 mL sample was extracted from the MBR and divided into eight centrifuge tubes, each holding 50 mL. We divided the tubes into two groups. One group was designated as the control and was kept at normal room temperature for a period of 12 h. Meanwhile, the experimental group was frozen at −20 °C for an equivalent duration, followed by thawing at room temperature. The aforementioned method was used for measuring the sedimentation performance, and the supernatant obtained was examined to determine the levels of protein and polysaccharide present within it. The specific anammox activity (SAA) of samples before and after freezing and thawing was determined according to the previous method [27]. Sludge samples before and after freeze–thaw were added into bottles with 100 mL of liquid volume. To eliminate O_2, both the gas and liquid phases were purged with argon, and the bottles were firmly sealed with butyl rubber caps. The initial substrate concentrations were 10 mg/L of NH_4^+–N and 10 mg/L of NO_2^-–N, with the NH_4^+–N, NO_2^-–N, and NO_3^-–N concentrations being periodically monitored throughout the experimental period. The SAA is determined by calculating the maximum slope of the substrate concentration decrease over time, which is then correlated with the corresponding biomass concentration per vial.

2.6. Measurement of Other Indicators

The influent and effluent samples were collected and filtered through 0.45 μm filters for nitrogen detection. The concentrations of ammonium, nitrate, and nitrite were measured using a Discrete Chemistry Analyzer Clever Chem Anna (DeChem-Tech, Hamburg, Germany). Oxygen and pH sensors WTW Multi 3420 (WTW Company, Munich, Germany), were used to detect the DO and pH. The concentrations of MLSS were measured using the standard methods [28]. The particle size was measured using a Beckman Coulter Particle

Counting and Particle Size Analyzers Multisizer 4 e (Beckman Coulter, CA, USA), and measurements were conducted for each sample in triplicate.

3. Results and Discussions

3.1. Form Aggregates of Free Anammox Bacteria through Cross-Liking

A calcium treatment experiment was conducted to verify whether free anammox bacteria could aggregate together, and it was found that free anammox bacteria can aggregate to enlarge their size through cross-linking.

We considered that if the ALE on the surface of anammox bacteria can cross-link with Ca^{2+} in water, the higher concentration of Ca^{2+} will provide more cross-linking sites to strengthen the cross-linking and form a larger entity. Therefore, a comparison of the particle size and sedimentation performance of four types of sludge (control, sludge treated with additional calcium for 0.5 h ($Ca_{0.5\,h}$), sludge with room temperature treatment for 12 h ($RT_{12\,h}$), and sludge treated with additional calcium for 12 h ($Ca_{12\,h}$)) was conducted. Our findings show that $Ca_{0.5\,h}$ displayed no difference in particle size compared to the control (Figure 1a), as the flocs formed were easily dispersed by the test fluid. According to the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, the intercellular interaction forces are the sum of repulsive electrostatic forces, attractive van der Waals forces, and hydration interaction [29]. Van der Waals interaction plays a significant role when the distance between two microbial cells is short enough (less than 0.1 μm), and hydrophobic interactions assume a predominant role with increasing distance [30]. Calcium addition, however, introduced negligible differences in the hydrophobicity of EPS [31]. Functional groups in EPS, such as -COOH, -OH, and $-NH_2$, are electron donors whereas Ca^{2+} is an electron acceptor. The electrostatic repulsion reduced by electron-donor interactions between Ca^{2+} and functional groups is an important factor influencing intercellular interaction forces. In this study, the reduction of electrostatic repulsive force caused by increased Ca^{2+} concentration dominated intercellular interaction among microcolonies, while its small absolute value limited the influence [20,32]. The weak energy between the microcolonies caused the entirety of vulnerability. Consequently, the decrease in electrostatic repulsion caused by the calcium addition is not a crucial factor in the formation of a bigger entirety. Our results indicate that the particle size increased significantly when the reaction time was extended to 12 h. Specifically, the particle size is 60.47 μm, increased by 411.45% compared with the control group, 298.27% compared with $Ca_{0.5\,h}$, and 370.34% compared with $RT_{12\,h}$. In past research, the successful cultivation of free anammox bacteria occurred when 95% of the bacteria had a particle size below 50 μm [33]. However, single anammox bacteria have a particle size of only 0.8–1.1 μm [5]. These findings suggest that free anammox bacteria do not exist independently but as microcolonies, enveloping the microcolonies with EPS containing ALE. A higher calcium concentration can increase cross-linking sites between ALE and calcium, forming a more stable entity within sufficient reaction time. The sedimentation performance also showed similar trends. The supernatant transmittance for the control, $Ca_{0.5\,h}$, $RT_{12\,h}$, and $Ca_{12\,h}$ are 15.91%, 30.70%, 28.42%, and 47%, respectively (Figure 1b).

In addition, previous studies have demonstrated that the increased Ca^{2+} levels stimulated cyclic dimeric guanosine monophosphate (c-di-GMP) synthesis by promoting the abundance of diguanylate cyclase (DGC), resulting in a higher content of EPS [34]. Elevated EPS concentrations catalyze the development of microbial aggregates [35]. To eliminate the influence of calcium on EPS concentration shifts, we compared the EPS concentration of untreated and calcium-treated specimens. The result showed no difference in EPS content after calcium treatment for 12 h (Figure 2). The effect of changes in EPS concentration caused by calcium on aggregation can be ignored in this study. In summary, as the concentration of Ca^{2+} increases, the electrostatic repulsion between microcolonies decreases, leading to interaction and aggregation between them in a relatively short time. This interaction force is weak, and the structure formed is easily destroyed. However, since elevated Ca^{2+} concentrations provide more cross-linking sites, larger and more stable aggregates of free anammox bacteria can be formed by providing sufficient reaction time without external

hydraulic interference. The above experiments also hint that reaction time and the number of cross-linking sites are both important factors affecting cross-linking. The thought was employed to enhance cross-linking, which will be discussed subsequently.

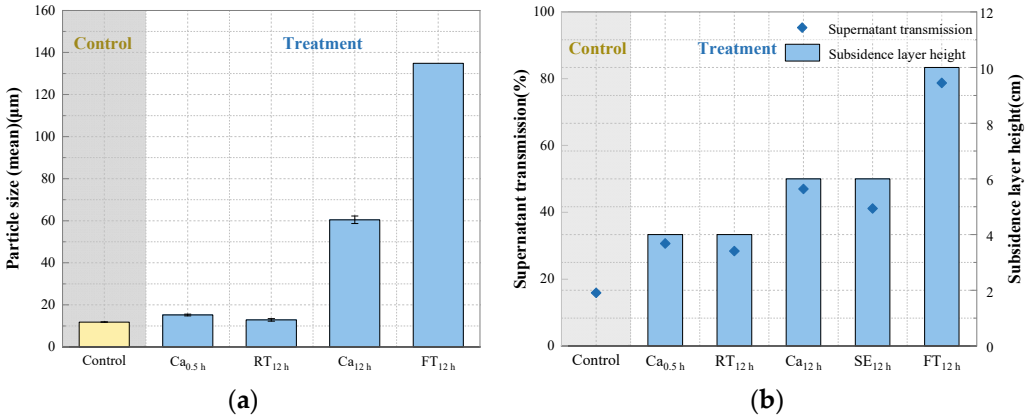

Figure 1. The particle size and the settling performance of free anammox bacteria with different operational parameters: control, calcium treatment for 0.5 h ($Ca_{0.5\,h}$); room temperature treatment for 12 h ($RT_{12\,h}$); calcium treatment for 12 h ($Ca_{12\,h}$); S-EPS extraction and static for 12 h ($SE_{12\,h}$); freeze–thaw treatment for 12 h ($FT_{12\,h}$). (**a**) The particle size of free anammox bacteria with different operational parameters. (**b**) The sedimentation layer height and supernatant transmission with different operational parameters.

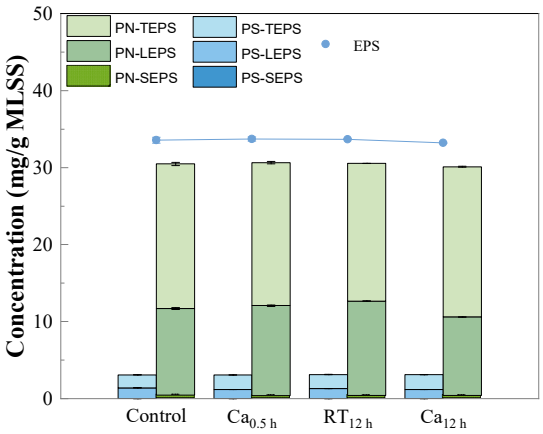

Figure 2. Extracellular protein and polysaccharide contents of anammox sludge with different operational parameters: control; calcium treatment for 0.5 h ($Ca_{0.5\,h}$); room temperature treatment for 12 h ($RT_{12\,h}$); calcium treatment for 12 h ($Ca_{12\,h}$).

3.2. More Cross-Linking Sites to Strengthen the Cross-Linking

S-EPS extraction and freeze–thaw experiments were conducted to verify if more cross-linking sites could strengthen ALE cross-linking with Ca^{2+}, and the sedimentation performance of free anammox bacteria was significantly improved after S-EPS extraction and freeze–thaw.

The S-EPS extraction experiment was designed to expose more ALE to provide more cross-linking sites between anammox microcolonies. In this study, the LB-EPS content was significantly higher than the S-EPS content, with LB-EPS accounting for 37.64% and S-EPS accounting for 1.38% of the total EPS (Figure 2). Since ALE is a typical component of EPS, ALE content would be more abundant in LB-EPS than S-EPS. After S-EPS extraction, the trend in the sample-fitting curve was similar to that of the control, and the degree of increase gradually decreased over time (Figure 3). However, S-EPS extraction positively impacted both the aggregation rate and percentage of anammox bacteria, as demonstrated by an increase in the aggregation percentage from 56.86 ± 1.39% to 67.92 ± 1.57%, and an increase

in the aggregation rate from 0.26 ± 0.01 $h^{(-1)}$ to 0.27 ± 0.01 $h^{(-1)}$ (Table 1). This indicated that S-EPS extraction stimulated the aggregation of anammox microcolonies, which was also verified using the sedimentation performance. The supernatant transmittance increased from 15.91% to 41.15%, and the settling layer height increased from 0 cm to 4 cm after S-EPS extraction. S-EPS extraction exposes LB-EPS containing more ALE, providing more sites for Ca^{2+} cross-linking in water. More cross-linking sites promote microcolonies binding and forming more stable aggregates of free anammox bacteria with better sedimentation performance. Although the content of LB-EPS was much higher than S-EPS, no additional Ca^{2+} was added in the S-EPS extraction test, resulting in insufficient Ca^{2+} cross-linked with ALE, which limits the improvement of sedimentation performance. If the concentration of Ca^{2+} improves after removing S-EPS, aggregates of free anammox bacteria with better sedimentation performance may be reaped. Compared with LB-EPS, TB-EPS accounts for a higher proportion of EPS and seems to contain more ALE, which can better promote cross-linking and aggregation. Nonetheless the S-EPS layer exhibits a loose structure, which facilitates its removal solely through centrifugation, presenting a user-friendly and eco-friendly approach without the need for chemical agents. On the other hand, the LB-EPS layer delivers a strong adhesion to cell surfaces, making its extraction more complex and expensive, involving methods such as the formaldehyde-NaOH or heating techniques [36]. This extraction process may also adversely trigger environmental contamination and impact the activity of the cells. Notably, S-EPS extraction does not significantly affect the living cell ratio compared to LB-EPS extraction [21]. In summary, S-EPS extraction through centrifugation would be a practical and effective strategy that would enhance aggregation and form aggregates of free anammox bacteria, providing an optimal balance of economic and performance benefits.

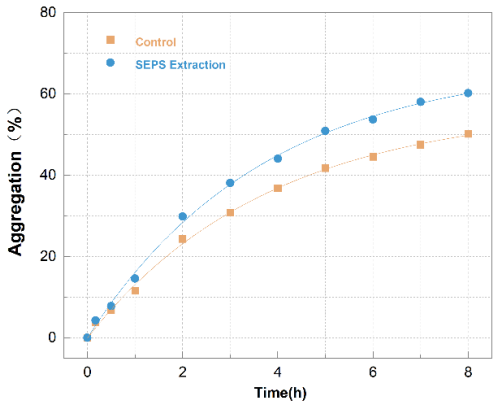

Figure 3. Aggregation ability of anammox bacteria before and after extraction of S-EPS.

Table 1. Kinetic parameters of microbial cell aggregation before and after extraction of S-EPS (a, before S-EPS extraction; b, after S-EPS extraction).

Samples	K_1 (h^{-1})	A_e (%)	R^2
a	0.26	56.86	0.998
b	0.27	67.92	0.998

The freeze–thaw experiment was designed to provide more contact points between anammox microcolonies through concentration while ALE and Ca^{2+} contents remained constant. Following the freeze–thaw treatment, the sludge displayed an irregular hollow network structure, and the particle size increased to 138.8 μm. After applying mechanical force for a minute, the particle size measured 134.9 μm (Figure 1a). This result suggests that the structure developed by the freeze–thaw treatment presents superior physical durability. During freezing, local ALE and Ca^{2+} concentrations increase due to the reduction of free water content caused by the formation of ice crystals. Under low-temperature

concentrations, ALEs can be close to each other and form regular and ordered binding regions through more network cross-linking sites. The freezing process formed stable structures without reducing their physical strength upon thawing. Previous research has shown that the freeze–thaw treatment of sodium alginate solution led to a frozen gel with an storage modulus more than 100 times higher than before freezing [37]. The sedimentation performance after freezing is also improved. Specifically, the supernatant transmittance increased from 15.91% to 78.74%. The sedimentation layer height increased from 0 cm to 10 cm. Upon freeze–thawing, the supernatant of sludge presents a soft yellow appearance, with a thick consistency and small foam, accompanied by increased protein and polysaccharide content. The results suggest that freezing can cause the rupture of anammox bacteria cells, allowing large quantities of intracellular protein and polysaccharides to be released (Figure 4a). Furthermore, a comparison was conducted on the specific anammox activity before and after this process, revealing the disappearance of activity after freeze–thawing (Figure 4b). These results could be attributed to the relatively slow process of freezing that permits the continuous growth of intracellular ice crystals, disrupting the cell structure and causing irreversible damage. Even though active anammox bacteria have been discovered in ocean ice studies, sustaining their viability in frozen laboratory conditions is challenging. Therefore, it is crucial to enhance the method used to maintain the activity of anammox bacteria, given that while freeze–thawing can increase the particle size, it results in the inactivation of bacteria.

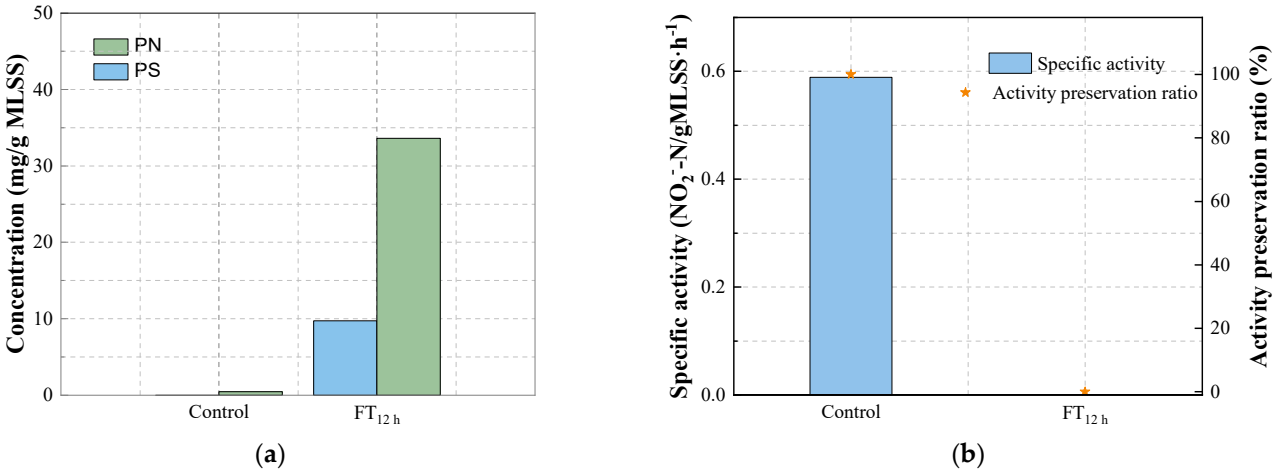

Figure 4. (**a**) Protein and polysaccharide contents in sludge supernatant before (control) and after freeze−thaw for 12 h ($FT_{12\,h}$). (**b**) Anammox activity before and after freeze−thaw for 12 h ($FT_{12\,h}$).

3.3. Longer Cross-Linking Time to Strengthen the Cross-Linking

Static experiments were conducted to verify the effect of time on the Ca^{2+} cross-linking with ALE, and the results showed that the sedimentation performance improved with the extension of time under static conditions.

The sedimentation performance was continuously improved with the extension of static time (Figure 5a). Specifically, supernatant transmittance was 13.28% at T = 0 h, 39.65% at 24 h, and 56.38% at 84 h, respectively. The sedimentation layer height was 0 cm at T = 0 h, 8 cm at 24 h, and 10 cm at 84 h, respectively (Figure 5b). As previously stated, the utilization of commercial alginate as a cross-linking agent to immobilize microorganisms demonstrated that extending the cross-linking time could greatly enhance the mechanical stability of the structure. For example, the gel beads prepared are usually left overnight in immobilized anammox tests [8]. ALE has similarities with commercial alginate and can cross-link with Ca^{2+} in the water. When not subjected to external hydraulic forces, extended periods of standing time result in longer cross-linking times. Thus, cross-linking reactions could continuously occur at these unchanged cross-linking sites between anammox microcolonies. The mechanical strength of the resultant structure was increased and the sedimentation

performance was improved. It should be noted that in the actual anammox reactor, the size of microcolonies cannot increase indefinitely due to disturbances, such as hydraulic shear force in the reactor. The static experiments showed that achieving the aggregation of anammox microcolonies requires sufficient reaction time, which explains why the free anammox bacteria mainly exist in the MBR. In addition to achieving complete biomass retention, the MBR is a continuous flow reactor with no settling time, resulting in insufficient contact between anammox microcolonies and insufficient cross-linking of ALE within EPS on the surface of microcolonies.

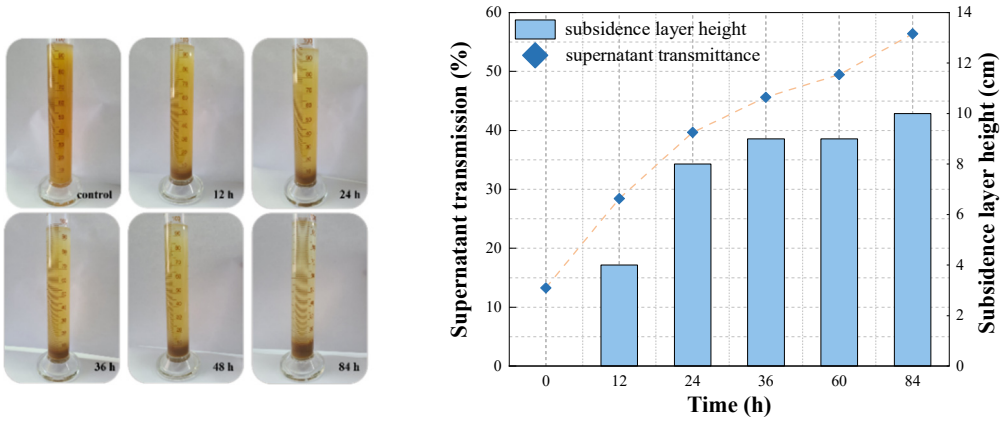

Figure 5. (**a**) Images of the free anammox bacteria with different static times. (**b**) The sedimentation performance of anammox bacteria with different static times.

3.4. Implications of This Work

The anammox bacteria primarily depend on forming dense aggregates (biofilms or granules) to boost the retention and deposition rate of available biomass. Unfortunately, the low growth rate of anammox bacteria in such aggregation morphology causes a long start-up time of engineering. While free anammox bacteria exhibit a faster growth rate, poor sedimentation limits their application. Our study revealed that free anammox bacteria demonstrate the capability to aggregate, which was confirmed by a significant increase in the size of anammox bacteria after increasing Ca^{2+} concentration. Furthermore, enhancing the cross-linking sites through S-EPS extraction and freeze–thaw treatment while facilitating adequate cross-linking times through static experiments promotes cross-linking and ultimately leads to improved sedimentation performance. It should be noted, however, that freeze–thaw treatment can render the bacteria inactive. Based on the experimental results, a practical method was proposed to improve sedimentation performance through forming aggregates of free anammox bacteria. The method includes a tertiary organization regime, consisting of S-EPS extraction to expose more ALE as the primary base, followed by Ca^{2+} addition for cross-linking with ALE as the second level, and ultimately minimizing the perturbation of external forces on the bacteria. This method has several advantages, such as a simple process, minimal use of chemical reagents, cost-effectiveness, environmental friendliness, and practical applicability in engineering. Consequently, this work provided feasible training for improving the working efficiency of the anammox system.

4. Conclusions

This study confirmed the possibility of forming aggregates of free anammox bacteria to improve sedimentation performance. Calcium addition demonstrated that Ca^{2+} cross-linking with ALE is the main contributor to the formation of aggregates of free anammox bacteria. S-EPS extraction and freeze–thaw increased ALE and Ca^{2+} concentration to strengthen cross-linking and sedimentation. However, freezing caused bacteria breakdown and activity disappearance. The static experiment proved that the reaction time also

affected the cross-linking effect, and the sedimentation performance increased over time. A practical method was proposed to improve sedimentation performance through forming aggregates of free anammox bacteria.

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