Study on Anaerobic Ammoniumoxidation (ANAMMOX) Sludge Immobilized in Different Gel Carriers and Its Nitrogen Removal Performance

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ABSTRACT: To maintain Anammox biomass, different materials were used as carriers to immobilize Anammox sludge. The adsorptive property, anaerobic ammonia oxidation activity, and stability of the particles were evaluated. The results indicated waterborne polyurethane (WPU) was the most suitable immobilizing material. In a continuous flow experiment, we further investigated the resistance to impact load and the anaerobic ammonia oxidation activity by increasing the influent load. It was found that for WPU-immobilized particles, no effluent SS or particles fracture was observed during the 66-day operation, and the WPU-immobilized particles showed a strong capability to retain sludge and good stability in the long-term.

INTRODUCTION

THE ANAMMOX process is an effective microbial pathway to treat wastewater. In this process, NO$_2^-$ acts as the electron acceptor, and NH$_4^+$ becomes oxidized to N$_2$ under anaerobic conditions [1]. Compared with the conventional nitrogen removal system (nitrification-denitrification [2]), the ANAMMOX process has clear advantages: it does not require organic carbon and oxygen and produces less surplus sludge; these advantages all reduce operational cost [3–5]. However, ANAMMOX bacteria are autotrophic bacteria—they grow slowly and have a low cell yield; in addition, ANAMMOX bacteria are also affected by environmental conditions [6–7]. It is difficult for ANAMMOX biomass to remain in a reactor. Thus, there is difficulty in starting an ANAMMOX reactor, which limits its development [8]. Researchers have conducted extensive studies on the ANAMMOX technique and proposed techniques on starting an ANAMMOX reactor, such as the biological fluid bed and fed-batch [9–10]. However, the results from using these techniques are not ideal [11]. Therefore, retaining microbes and ensuring the biomass of ANAMMOX bacteria have become a primary direction in developing the ANAMMOX technique [12].

The embedded immobilization technique may be able to solve this problem, which is a new microbe immobilization technique in the modern bioengineering field. The embedded immobilization technique immobilizes free cells or enzymes in a constrained area through immobilization material such that the activity of cells or enzymes can be maintained and reused; the embedded immobilization technique provides a good retaining effect on microbes [13–15]. Using the embedded immobilization technique to immobilize ANAMMOX sludge can effectively prevent the loss of ANAMMOX bacteria and maintain the biomass in the reactor, and thus, this technique appears promising [16–18]. However, there are disadvantages with using the existing immobilization materials, such as insufficient mechanical strength, low bioactivity, and lack of stability during long-term operation [19–21]. This study uses waterborne polyurethane (WPU) [22], polyethylene glycol (PEG), carboxymethyl cellulose (CMC), and polyvinyl alcohol (PVA) as materials to immobilize ANAMMOX sludge, and the long-term mechanical stability, ANAMMOX performance, and shock-loading resistance were evaluated to provide a
basis for further study on the rapid start-up and stable operation of the embedded immobilized technique-based ANAMMOX process.

MATERIALS AND METHODS

Immobilization Materials

Sludge concentrate: the ANAMMOX sludge was obtained from a sequential batch reactor (SBR) in a laboratory that had been in stable operation for one year. The sludge taken from the reactor was first washed three times in deionized (DI) water to remove the residues on the surface of the sludge. Afterwards, the sludge was centrifuged at 500 r·m$^{-1}$ for 20 min. Analytically pure immobilization agents (PEG, WPU, CMC, PVA) and crosslinking agents/initiators (CaCl$_2$, H$_3$BO$_3$, potassium persulfate (KPS), and tetramethylenediamine (TMEDA)) were used.

Water Quality

Synthetic wastewater was used. Table 1 lists the main components of the wastewater. Trace elements I and II followed reference [23]. The pH of the water ranged from 7.1 to 7.84.

Preparation of Immobilized Granules

The preparation methods for all the immobilized granules are as follows (mass percentage):

- **WPU-immobilized granules**: 10% WPU solution and ANAMMOX sludge concentrate of equivalent volume were mixed homogeneously. The initiators TMEDA and KPS were added, and the solution was rapidly stirred. Approximately 30 min later, the mixed solution became a gel.

- **PEG-immobilized granules**: 10% PEG solution and equivalent volume ANAMMOX sludge were mixed. The initiators TMEDA and KPS were added after the mixed solution became a gel. The solid gel was cut into $3 \times 3 \times 3$ mm cubes, and WPU-immobilized granules were obtained.

- **CMC-immobilized granules**: Equivalent volumes of 3% CMC solution and ANAMMOX sludge concentrate were mixed. The mixed solution was added to 4% CaCl$_2$ solution drop by drop using a pipette. The solution was then stored in a 4°C refrigerator for crosslinking for 12 h to obtain spherical CMC-immobilized granules.

- **PVA-immobilized granules**: Equivalent volume of 8% PVA solution and ANAMMOX sludge concentrate were mixed homogeneously. The mixed solution was added to saturated H$_3$BO$_3$ solution drop by drop using a pipette. The solution was then stored in a 4°C refrigerator for crosslinking for 12 h to obtain spherical PVA-immobilized granules.

The prepared immobilized granules were collected and used for batch culture to determine their activity [24]. After 1 week of activation, the immobilized granules were removed for later use.

Mechanical Stability

To determine the mechanical stability of the immobilized granules, the mechanical strength, expansion coefficient, and swelling properties were used as evaluation indices. Mechanical strength: 30 immobilized granules of similar size of each material type were selected and placed in a 500-mL conical flask. Then, 400 mL of DI water was added to the reactor. The mixture was magnetically stirred at 500 r/min for 24 h, after which the ratio of intact immobilized granules to the original number of immobilized granules was determined. Expansion coefficient: 20 immobilized granules from each of the four material types were added to a 500-mL conical flask (400 mL DI water). The flask was slowly shaken at 30°C for 72 h; then, the diameters of the immobilized granules before and after treatment were measured by a vernier caliper. The ratio of the mean diameter of the immobilized granules after 72 h of treatment to the mean diameter of the original immobilized granules was the expansion coefficient. Swelling properties: 20 immobilized granules from each of the four material types were added to a 500-mL conical flask (400 mL synthetic wastewater). The flask was slowly shaken at 32°C for 1 week. The changes in the immobilized granules were observed.

<table>
<thead>
<tr>
<th>Main Components</th>
<th>Mass Concentration (mg·L$^{-1}$)</th>
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<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>32</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>142</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>283</td>
</tr>
<tr>
<td>KHCO$_3$</td>
<td>802</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>372</td>
</tr>
<tr>
<td>NaNO$_2$</td>
<td>502</td>
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<td>Trace element I</td>
<td>1 ml·L$^{-1}$</td>
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<tr>
<td>Trace element II</td>
<td>1 ml·L$^{-1}$</td>
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</tbody>
</table>
ANAMMOX Performance

The ANAMMOX performance of the immobilized granules was measured in a serum bottle. A total of 40 mL of activated immobilized granules (including approximately 20 mL of concentrated sludge) of each material type was added to a 500-mL serum bottle, which was covered with black plastic bags. Then, 400 mL of synthetic wastewater was added to the serum bottle. The bottle was magnetically stirred at 100 r/min. The serum bottle was air-stripped with purity N2 for 20 min to ensure an anaerobic environment, and the temperature was controlled at 30°C. The pH was not controlled. Sampling was conducted every 8 h from the serum bottle. A total of 20 mL of un-immobilized ANAMMOX sludge concentrate was used as the control group for the same experiments. The experiment was repeated twice, and the results were averaged.

Continuous-flow Experiment

The continuous-flow experiment used an up-flow anaerobic sludge blanket (UASB) reactor (Figure 1), which used the material polymethyl methacrylate (PMMA). The reactor contains three phase separators in the upper section with an effective volume of 17 L. A mesh was placed at the water outlet to prevent the immobilized granules from flowing out with the water. There was a water bath layer on the external wall of the reactor. The temperature in the reactor was maintained at 30°C with a water bath outside. After activation, immobilized granules (the amount was based on 20% of the volume fill ratio) were added to the reactor. The wastewater used in experiment was synthetic. The concentration of NH4⁺-N was between 40 and 200 mg/L, and the concentration of NO2⁻-N was between 50 and 180 mg/L; the concentrations of the other components are listed in Table 1. During the experiment, the hydraulic retention time (HRT) was 8h; such indices as NH4⁺-N, NO2⁻-N, and NO3⁻-N in the effluent were determined every day.

Scanning Electron Microscopy (SEM)

SEM analysis of immobilized granules: immobilized granules were removed from the reactor and washed, and 25% pentanediol was used to immobilize the granules for 1.5 h. Afterwards, the granules were washed three times in PBS. Subsequently, the granules were dehydrated in an ascending series of ethanol (volume fraction: 50%, 70%, 80%, 90%, and 100%); the duration of dehydration was 10–15min. Lastly, isoamyl acetate was used for displacement. After the granules were freeze-dried for 24 h, a 1500-nm thick metallic membrane layer was electroplated on the surfaces of the samples. A Hitachi S-4300 SEM was used for observation.

Testing Methods

The standard method was used to determine NH4⁺-N, NO3⁻-N, NO2⁻-N, TN, MLSS, and MLVSS [25]. A WTW/Multi 3420 multiparameter was used to determine the pH and temperature.

RESULTS AND DISCUSSION

Adsorption Properties

Figure 2(a) shows the adsorptions of NH4⁺-N on the different granules. The WPU granules had the highest adsorption rate for NH4⁺-N; their adsorption rates were 7.1% at 32 h and 7.3%, at 40 h. The PVA granules had
the lowest adsorption rate for NH$_4^+$-N; their adsorption rate was only 2.7% at 40 h. Figure 2(b) shows that there were significant differences among the materials in terms of the adsorption of NO$_2^-$-N. The WPU granules’ adsorption rate reached 19.1% at 40 h; the PEG, PVA, and CMC granules all had extremely low adsorption rates for NO$_2^-$-N that did not surpass 5%, and the PVA granules had the lowest adsorption rate, which was only 1.1% at 40 h.

The experiments revealed that the four types of immobilization materials all had certain adsorption properties for NH$_4^+$-N and NO$_2^-$-N; however, the adsorption rates were not high and reached equilibrium rapidly. Therefore, after 1 week of activated culture, all types of immobilized granules reached their adsorption equilibrium for NH$_4^+$-N and NO$_2^-$-N. As a result, the adsorption properties had only a slight impact on the subsequent experimental results; therefore, the properties could be neglected.

Stability of the Immobilized Granules

Table 2 lists the mechanical stability indices of the four types of immobilized granules. After 1 week of stirring in synthetic wastewater, the CMC-immobilized granules and the PVA-immobilized granules became soft and fragile. The mechanical strength of the CMC-immobilized granules was the lowest; after 24 h of high-speed rotation, almost all the CMC-immobilized granules had broken. The WPU-immobilized and PEG-immobilized granules exhibited the best stability; after 1 week of stirring, these granules exhibited essentially no change. The expansion coefficient of the WPU-immobilized granules was 1.02; after 24 h of high-speed rotation, no granules had broken. The PEG-immobilized granules also exhibited relatively good stability, though the stability was less than that of the WPU-immobilized granules; after high-speed rotation, 14% of the granules had broken. The expansion coefficient of the PVA-immobilized granules was 1.31; the PVA-immobilized granules also exhibited good stability. However, a phenomenon was observed in the PVA-immobilized granules in which the granules stacked together. Thus, considering all indices, the order of the mechanical stability from strongest to weakest was WPU > PEG > PVA > CMC.

The differences in the mechanical stability were primarily caused by the properties of the different immobilization materials. PVA is a synthetic polymer and has good physical properties; as an immobilization material, the PVA-immobilized granules inherited the merits of PVA. However, these granules contain many hydrophilic hydroxyl groups in their chemical structure, which results in the PVA-immobilized granules exhibiting certain water swelling properties and auto-condensing tendencies [26]. Therefore, PVA-immobi-
lized granules tended to conglutinate, which decreased the specific surface area of the immobilized granules and thus affected their stability. CMC-immobilized granules have no clear spherical shell, and thus, these granules exhibit a good mass transfer performance but have a loose chemical structure; the CMC-immobilized granules had the lowest stability and are not well-suited as an immobilization material. PEG and WPU are synthetic polymeric material and thus exhibit good bio-compatibility and excellent mechanical strength [27]. When cross-linked, these materials form a stable gel. However, the materials have a different relative molecular mass and chemical structure; the space of the WPU three-dimensional network structure is relatively small, which not only ensures the mechanical strength of the WPU-immobilized granules but also absorbs less water when swelling. Therefore, the WPU-immobilized granules exhibited a better mechanical stability than the PEG-immobilized granules and are considered the best material among the four types.

ANAMMOX Performance of the Immobilized Granules

Figure 3(a) shows the variation curve of the removal rate of NH$_4^+$-N with respect to time for the different immobilized granules. The removal rate of NH$_4^+$-N increased first rapidly and then slowly in both the control group and the immobilization group, which was because the initial concentration of NH$_4^+$-N was high and the ANAMMOX rate was fast; later, the substrate concentration decreased, which decreased the reaction...
rate. Between 0 and 8 h, the removal rate of NH$_4^+$-N in the control group and CMC-immobilized granules increased the fastest. After 16 h, the removal rate for the WPU- and PEG-immobilized granules surpassed the removal rate of the control group and CMC-immobilized granules. After 40 h, the removal efficiency decreased in both the control group and the immobilization group. Eventually, at 50 h, the NH$_4^+$-N in all the reactors was completely removed. The variation pattern of the concentration of NO$_2^-$-N was essentially the same as the variation pattern of NH$_4^+$-N. Between 0 and 8 h, the removal rates for NH$_4^+$-N and NO$_2^-$-N in the control group (and CMC group) were greater than in the immobilization group, which was primarily because the immobilization material prevented mass transfer between the materials in the water and the ANAMMOX bacteria in the immobilized granules. After 16 h, the removal rates for NH$_4^+$-N and NO$_2^-$-N in the WPU- and PEG-immobilized granules were greater than in the control group, which benefited the more concentrated biomass in the immobilized granules and resulted in a higher activity of the ANAMMOX bacteria.

Between 8 and 32 h, the substrate completely dispersed into the immobilized granules. An analysis was performed for this period, and the average removal rates for NH$_4^+$-N and NO$_2^-$-N were as follows (from fastest to slowest): WPU > PEG > CMC > PVA. By comparison with the variation curve of the yield of NO$_3^-$-N with respect to time in Figure 3(c), the ratios of the removed amount of NO$_3^-$-N to the removed amount of NH$_4^+$-N of the WPU-, PEG-, PVA-, and CMC-immobilized granules were 1.204, 1.292, 1.328, and 1.537, respectively. The ratios of the yield of NO$_3^-$-N to the removed amount of NH$_4^+$-N were 0.337, 0.325, 0.185, and 0.229, respectively, which were both extremely similar to the ratios of the control group. Thus, these immobilized granules exhibited good ANAMMOX performance.

The ANAMMOX properties of the different immobilized granules are related to the different chemical structures and the form of the cross-linked immobilized granules. For each PVA-immobilized granule, there was a homogenous spherical shell; the exterior was relatively dense, and thus, the mass transfer resistance was large. In addition, the conglutination of granules resulted in a decrease in specific area, which resulted in a poor mass transfer property; therefore, the ANAMMOX performance of the PVA-immobilized granules was the poorest. The spherical shell formed on the surface of each CMC-immobilized granule was relatively thin, and the mass transfer resistance was relatively small. After cross-linking, the size of the network structure formed inside was relatively large. However, because CMC-immobilized granules have no stable structure, they easily shatter; shattered granules lose the immobilized advantages, and thus the performance of CMC-immobilized granules is similar to that of un-immobilized ANAMMOX sludge. The WPU- and PEG-immobilized granules contained no clear shell on the surface of each immobilized granule and therefore had good mass transfer performance and contained a looser three-dimensional network structure. The WPU-immobilized granules exhibited a better ANAMMOX performance compared with the PEG-immobilized granules. Figures 4(a) and 4(b) show × 4.0 k and × 8.0 k SEM images of the surfaces of the WPU-immobilized granules. Figure 4(a) shows that there are many channels on the surface of each WPU-immobilized granule; bacteria were divided into different areas by the channels. Figure 4(b) shows that these bacteria had a spherical shape, and there were volcanic crater-like concaves on the two sides of each bacterium, which indicates that these were typical ANAMMOX bacteria. Figure 4(c) shows the SEM image of the inside of a WPU-immobilized granule after being cut open; it can be seen that a large amount of ANAMMOX bacteria grew along the channels inside of the granule. There-

![Figure 4. SEM images of the WPU-immobilized granules: (a) surface × 4.0 k; (b) surface × 8.0 k; and (c) inside × 2.5 k.](image-url)
fore, the WPU-immobilized granules had the best mass transfer property and ANAMMOX activity.

Continuous-flow Experiment

To study the nitrogen removal performance and stability of WPU-immobilized granules during long-term operation, a continuous-flow experiment was developed. The HRT was 8h, the influent \( \text{NH}_4^+ \)-N was varied between 40 and 200 mg/L, and \( \text{NO}_2^- \)-N was varied between 50 and 180 mg/L. Using \( \text{NH}_4^+ \)-N as a metric, Fig. 5 shows that between 0 and 20 days, the removal load of \( \text{NH}_4^+ \)-N was less than 0.5 kg·m\(^{-3}\)/L because the immobilized granules were under a domestication period. Then, the activity of the immobilized granules increased. The influent concentration increased the removal load of \( \text{NH}_4^+ \)-N, which had also been increasing. On the 66th day, the removal load of \( \text{NH}_4^+ \)-N was 0.455 kg·m\(^{-3}\)/L, and the removal rate was up to 77%. In addition, the effluent of the reactor was always clear during the experimentation period, and no suspended solids (SS) were detected. None of the immobilized granules broke. The WPU-immobilized granules exhibited a good sludge retaining ability and mechanical stability.

CONCLUSIONS

By comparing WPU-, PEG-, PVA-, and CMC-immobilized granules, the WPU-immobilized granules exhibited an extremely strong shock-loading resistance and mechanical stability during long-term operation, in which none of the WPU-immobilized granules broke. The WPU-immobilized granules exhibited good sludge retaining ability and mechanical stability. WPU has significant advantages compared with the other materials and is suitable for use as an immobilization material for ANAMMOX sludge.

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