The Cultivation of Denitrifying Phosphorus Removal-aerobic Granular Sludge under Low Carbon Condition: Removal Performance and Microbial Characteristics

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Abstract. The sequencing batch reactor (SBR) was inoculated with fully granulated aerobic granular sludge (AGS). In this study, AGS was induced to enrich the denitrifying phosphorus-accumulating organisms (DPAOs) by gradually reducing the influent chemical oxygen demand (COD) concentration (550, 400, 160 mg/L). Here, the maximum denitrifying phosphorus removal efficiency of AGS and the microbial community were investigated. The removal efficiencies of TN and TP were greatly improved during the enrichment of DPAOs, increasing from 64.57% to 80.48% and 59.56% to 81.89%, respectively. Meanwhile, due to dense structure of AGS, aerobic and anoxic/anaerobic layers could be formed to promote the cultivation of DPAOs. The PPR and PUR of DPR-AGS were 6.02 mg TP/(g SS h) and 5.20 mg TP/(g SS h), respectively. Through high-throughput sequencing, predominant phosphorus accumulating organisms in AGS was the _Canadidatus Accumulibacter_, which had excellent denitrifying phosphorus removal capacity. The results indicated that DPAOs could be well enriched in AGS under low carbon condition.

Introduction

Aerobic granular sludge (AGS) is a microbial aggregate formed by spontaneous aggregation of microorganisms [1]. Compared with traditional active sludge (AS), AGS presents superior properties, such as regular shape, outstanding settling ability, high biomass accumulation and multiple biological functions [2, 3]. Moreover, due to the dense 3-D structure, AGS offers aerobic-anoxic-anaerobic microenvironments which further provide the coexistence of different bacterial groups for removing carbon, nitrogen, and phosphorus simultaneously [4]. Therefore, AGS has been considered as one of the less energy-consuming and most promising biological technologies in municipal wastewater treatment plants to solve the low available organic carbon contents in sewage [5].

Denitrifying phosphorus-accumulating organisms (DPAOs), which belong to phosphorus-accumulating organisms (PAOs), are capable of utilizing nitrate (NO$_3^-$) and/or nitrite (NO$_2^-$) as electron acceptors, instead of oxygen, to achieve satisfactory phosphorus uptake and nitrogen removal simultaneously [6]. AGS with the ability of denitrifying phosphorus removal is called denitrifying phosphorus removal-aerobic granular sludge (DPR-AGS) [7]. Compared with traditional biological nitrogen and phosphorus removal processes, denitrifying phosphorus removal requires less chemical oxygen demand (COD), demands less aeration, and minimizes sludge production [8, 9]. Due to the oxygen transfer limitation [11, 12], the structure of AGS is stratified into aerobic and anoxic/anaerobic layers. Under this condition, DPAOs can be cultivated and enriched in AGS when the operational condition is manipulated appropriately. Hence, by enriching DPAOs in AGS to strengthen the denitrifying phosphorus removal process, it could provide a simultaneous nitrogen and phosphorus removal system to treat low strength organic sewage, which is more economical and sustainable.
In this study, a process utilizing anaerobic/aerobic/anoxic SBR was investigated to enrich DPAOs in AGS under low carbon condition. The major purposes of this study were as follows: (i) to investigate the removal efficiencies of nitrogen and phosphorus during the enrichment of DPAOs; (ii) to explore the potential denitrifying phosphorus removal efficiency of DPR-AGS; (iii) to elucidate the variations in the richness and diversity of bacteria between DPR-AGS and traditional active sludge.

Materials and Methods

Reactor set-up and Operating Conditions

The inoculated AGS for experiments was obtained from a laboratory culture of two months, and it had an average particle diameter of 1.50 mm. Subsequently, cultured AGS was seeded into a SBR, and MLSS concentration, MLVSS/MLSS ratio and SVI were 5.0 g L\(^{-1}\), 0.76 and 30 mL g\(^{-1}\), respectively. The experiments were carried out in one SBR with inner diameter of 160 mm, height of 1000 mm and the volume exchange ratio of 60%, giving an effective volume of 16 L. The total cycle time was 8 h: 40 min for anaerobic feeding, operation time for anaerobic / aerobic / anoxic period in Table 1, 3 min for decanting and 23 min for idle. Each condition lasted 30 days. The temperature was controlled at 21 ± 3°C, and the pH was maintained between 6.5 and 8.5.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Anaerobic</th>
<th>Aerobic</th>
<th>Anoxic</th>
</tr>
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<tbody>
<tr>
<td>Condition I</td>
<td>90.00</td>
<td>230.00</td>
<td>90.00</td>
</tr>
<tr>
<td>Condition II</td>
<td>90.00</td>
<td>200.00</td>
<td>120.00</td>
</tr>
<tr>
<td>Condition III</td>
<td>120.00</td>
<td>170.00</td>
<td>120.00</td>
</tr>
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</table>

Table 1. The operational parameters for each phase during operation.

To begin with, more nutrients were added into reactor in order to promote the growth of microorganisms. Then nutrients were gradually decreased in synthetic wastewater to simulate the real municipal sewage. The sludge was fed with synthetic wastewater consisting of the following (mg L\(^{-1}\)): chemical oxygen demand (sodium acetate and glucose), 550 for 30 days, 400 for 30 days and 160 for the last 30 days; \(\text{NH}_4^+\text{N} (\text{NH}_4\text{Cl})\), 50 for 30 days and 40 for the last 60 days; \(\text{PO}_4^{3-}\text{P} (\text{KH}_2\text{PO}_4)\), 10 for 60 days and 5 for the last 30 days; \(\text{CaCl}_2\), 70; \(\text{MgSO}_4\), 28; \(\text{FeSO}_4\), 28; 5 mL of a concentrated solution of trace elements [10]. And the synthetic wastewater in the condition III was characterized as low carbon condition [11].

Experiments for Phosphorus Removal Efficiency

Phosphorus release rate (SPRR) and phosphorus uptake rate (PUR) were measured according to the method [12].

\[
\text{PPR} = \frac{\rho(TP)_t - \rho(TP)_0}{\text{MLSS}\cdot t} \quad \text{mg} / \text{g SS h}. \quad (1)
\]
\[
\text{PUR} = \frac{\rho(TP)_0 - \rho(TP)_t}{\text{MLSS}\cdot t} \quad \text{mg} / \text{g SS h}. \quad (2)
\]

Here \(\rho(TP)_0\) and \(\rho(TP)_t\) represent the concentration of TP at 0 and t h, respectively.

Evaluation of Microbial Community in AGS

High-throughput sequencing was used to determine the microbial diversity and composition of AGS and AS samples. On day 90, AGS samples were collected from the SBR and AS samples were collected from a secondary sedimentation tank in one municipal wastewater treatment plant (WWTP) for high-throughput sequencing, and immediately stored at -20 °C until their DNA was extracted. The genomic DNA of the mixed AGS sample was extracted with an E.Z.N.A.® Tissue DNA kit (Omega Biotek, Norcross, GA, USA), according to the manufacturer's instructions. The details of the procedures are available in the study [13].

Other Analytical Methods

The granule size was determined using a particle size analyzer (Mastersizer 2000). Evaluation of
COD, NH$_4^+$–N, NO$_3^-$–N, NO$_2^-$–N, TN, TP, and volatile suspended solid (VSS) contents was conducted in accordance with standard methods [14]. Some assays were performed in triplicate and the results were expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was used to test the significance of the results and $p < 0.05$ was considered to be statistically significant.

Results and Discussion

Reactor Performance during the Process of Enrichment

The removal efficiencies of COD, NH$_4^+$–N, TN and TP during the enrichment of DPAOs were shown in Figure 1. It was found that the removal efficiencies of COD and NH$_4^+$–N were almost stable and increased slightly throughout the whole operation period (Figure 1 a, b), maintaining above 95.08% and 83.41%, respectively. The results showed that the AGS still had good removal efficiencies of COD and NH$_4^+$–N on low strength sewage during the enrichment process.

The removal efficiencies of TN and TP were significantly increased from 64.57% to 80.48% (Figure 1 c), and 59.56% to 81.89% (Figure 1 d). It could be owing to the enrichment of DPAOs, which promoted the removal of nitrogen and phosphorus in the anoxic period [15]. On the condition III, the removal efficiency of TP was still raised and reached the peak (81.89%).

Figure 1. Reactor performance: the removal of COD (a) and NH$_4^+$–N (b) and TN (c) and TP (d) in the SBR during enrichment.

Performance of Denitrifying Phosphorus Removal

In order to explore the maximum denitrifying phosphorus removal efficiency of AGS after the enrichment of DPAOs under a low carbon condition, a static test was conducted to analyze the performance of anaerobic/anoxic phosphorus removal. As shown in Figure 2, due to the release of phosphorus in the anaerobic phase, TP concentration gradually increased, reached the peak (25.41 mg/L) at 50.00 min and maintained stable in the rest of anaerobic period. The average phosphorus release rate (PRR) of DPR-AGS was 6.02 mg TP / (g SS h) in the first 50 min of anaerobic phase. Nitrate (NO$_3^-$–N) were added into the reactor at the end of anaerobic phase (120 min), making the concentration of NO$_3^-$–N up to 36 mg/L. Nitrate was consumed in the first 50 min of anoxic phase and small amount of nitrite were accumulated. The average phosphorus uptake rate (PUR) in the first 50 min of anoxic phase was 5.20 mg TP/ (g SS h). Moreover, each 1 mg/L PO$_4^{3-}$–P was assimilated and 0.94 mg/L NO$_3^-$–N was removed as electron acceptors. At the end of the reaction, the removal efficiency of TP was 82.27%, indicating that the cultured DPR-AGS had outstanding denitrifying phosphorus removal efficiency.
Figure 2. Performance test of max denitrifying phosphorus removal efficiency

Error bars represent standard deviations (n=3).

Comparison between AGS and AS in Microbial Community

The microbial communities in AGS after the enrichment of DPAOs and traditional AS were analyzed through high-throughput sequencing. The effective sequences of the AGS and AS were 22770 and 24189 genomes. Meanwhile, the effective sequences were divided into 1463 and 2991 operational taxonomic units (OUT), respectively, which were based on the similarities of the domain values (0.96) (Table 2). The microbial phylotype levels characterized by the estimators Chao/Ace and Shannon/Simpson suggested that the AS showed the highest microbial diversity, due to more complex nutrients in municipal sewage than synthetic wastewater.

Table 2. Similarity-based OTUs and species diversity estimates for microbial communities in AGS and AS.

<table>
<thead>
<tr>
<th></th>
<th>Reads</th>
<th>OTUs</th>
<th>Coverage</th>
<th>Chao</th>
<th>Ace</th>
<th>Shannon</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>22770</td>
<td>1463</td>
<td>0.96</td>
<td>3242.02</td>
<td>4769.59</td>
<td>4.58</td>
<td>0.06</td>
</tr>
<tr>
<td>AS</td>
<td>24189</td>
<td>2991</td>
<td>0.96</td>
<td>6345.88</td>
<td>8485.62</td>
<td>6.31</td>
<td>0.04</td>
</tr>
</tbody>
</table>

a OTUs: operational taxonomic units. b Coverage: estimates the possibility that the next read will belong to a specific OTU. c Chao/Ace diversity estimator: total amount of OTUs estimated by infinite sampling. A higher number reflects more diversity. d Shannon/Simpson index: index to characterize species diversity.

As shown in Fig. 3, the proportion of phosphorus accumulating organisms to total bacteria was 20.68% in AGS, which was slightly more than it in AS (18.48%). The predominant phosphorus accumulating organisms in AGS were Canadidatus Accumulibacter, accounting for 9.95%, which had the ability of anaerobic/aerobic phosphorus removal and denitrifying phosphorus removal [16], indicating the excellent phosphorus removal by AGS under low carbon conditions. The predominant phosphorus accumulating organisms in AS was Acinetobacter, accounting for 15.88%, which only have the ability of anaerobic/aerobic phosphorus removal [17]. Although Canadidatus Accumulibacter in AS was 2.21%, AS was anaerobic/aerobic phosphorus removal on the whole.

As shown in Table 2, the diversity of microbial communities in AGS was higher than that in AS, indicating that AGS had a more diverse microbial community. The predominant phosphorus accumulating organisms in AGS were Canadidatus Accumulibacter, which had the ability of anaerobic/aerobic phosphorus removal and denitrifying phosphorus removal, while in AS, the predominant phosphorus accumulating organism was Acinetobacter, which only had the ability of anaerobic/aerobic phosphorus removal. Therefore, AGS had a more efficient phosphorus removal ability than AS.

Figure 3. Microbial community analysis in AGS (a) and AS (b). Percentage of representative abundance of genera (phosphorus accumulating organisms).
Conclusion

(1) With the decrease of influent COD concentration (550, 400, 160 mg/L), the removal efficiency of COD was stable, maintaining above 95.08%. The removal efficiency of NH$_4^+$-N was slightly increased by 6.80%. In addition, the removal efficiencies of TN and TP were greatly improved, increasing from 64.57% to 80.48 and 59.56% to 81.89%, respectively.

(2) Through experiments on the performance of anaerobic/anoxic phosphorus removal, DPR-AGS had higher PRR (6.02 mg TP / (g SS h)) in anaerobic period and PUR (5.20 mg TP/ (g SS h)) in anoxic period, indicating considerable capacity to remove phosphorus.

(3) *Canadidatus Accumulibacter* accounted for predominant phosphorus accumulating organisms in AGS, which had denitrifying phosphorus removal capacity. While traditional activated sludge remove phosphorus mainly by anaerobic phosphorus release and aerobic phosphorus uptake. The results indicated that DPAOs could be enriched in AGS under low carbon condition.

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