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GRANULAR ACTIVATED CARBON AS NUCLEUS FOR FORMATION OF ANAMMOX GRANULES IN AN EXPANDED GRANULAR-SLUDGE-BED REACTOR

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ABSTRACT

Granular activated carbon (GAC) was used in an Anammox process treating synthetic wastewater with the aim of evaluating its use as a growth nucleus to enhance granule formation. In an expanded granular-sludge-bed reactor, granules were formed demonstrating excellent retention characteristics with an average settling velocity of 200 m h^{-1} (5 cm s^{-1}) over a startup period of only 38 days. The time required for formation of Anammox granules under the experimental conditions in this study, was thus demonstrated to be much shorter than that of others. A genetic analysis of the organisms in the granular bed revealed an abundance of Paenisporosarcina uncultured bacterium similar to other known Anammox bacteria.

Keywords: Anaerobic ammonium oxidation (Anammox); GAC; Granulation;

1. Introduction

Since the original report of anaerobic ammonium oxidation (Anammox) in 1995 (Mulder *et al.*, 1995), this innovative technology has undergone rapid development for use in wastewater treatment (Kuenen, 2008; Susanne, 2014). Anammox biomass granules were first observed in a full-scale rector (van der Star *et al.*, 2007) around six years ago; subsequently, very high nitrogen loading rates (NLRs) up to 27 g l^{-1} d^{-1} have been successfully treated by an expanded granular-sludge-bed (EGSB) reactor using Anammox granules (Chen *et al.*, 2014). Those results are some 50 times higher than that of conventional denitrification processes, which are typically below 0.5 g l^{-1} d^{-1}. Though the use of Anammox granules for wastewater treatment has been well documented (Alvarino *et al.*, 2015), the initial formation of Anammox granules, which can take several months to years, is a subject that is often overlooked. In addition, even apart from the granulation question, Anammox bacteria have a doubling time of approximately 11 days (Kuenen, 2008), which is even longer than that of methane producing bacteria (MPB). From an engineering standpoint, enhancing Anammox granule formation is one of the most important and challenging factors in this field.

The granulation process can be divided into two steps, aggregation of inert particles and biofilm formation on the inert aggregates. To accelerate the granulation process, the addition of inert particles has been considered for the formation of methane-producing granules. Synthetic and natural polymers such as synthetic Percol 763, chitosan, and a cationic polymer have been reported to enhance granulation (Tiwari

et al., 2005; Ni *et al.*, 2015), but the results have not been consistent and the granules were smaller than those of a typical granular sludge (Xing *et al.*, 2015). Thus, an appropriate material to use as an inert seed for granulation of Anammox biomass is urgently needed. The preferred characteristics of such a material would include having a high specific surface area, relatively high specific gravity, near spherical shape and low production cost.

In this study, granular activated carbon (GAC) was used for evaluation of its effectiveness as a seed for granule formation. GAC has a porous microstructure suitable for retention of bacteria and a specific gravity slightly heavier than that of water, and was thus thought to be a suitable candidate for use in an Anammox reactor treating high-strength wastewater. Genetic analysis using the 16S rRNA gene was employed to characterize the microbial population of the GAC-based Anammox granules.

2. Materials and methods

2.1. Anammox reactor

The reactor had an inner diameter of 14 cm with a total liquid volume of 10 l including a reaction zone of 8 L and a settling zone of 2 l (Fig. 1). The reactor was made of acrylic resin and had a water jacket for temperature control. Sampling ports were located at heights of 3, 17, 20 and 25 cm above the reactor bottom. Part of the effluent was collected in a 5-L container (with mixer and heater) for use as recycle. The pH was adjusted by an online pH controller (TPH/T-10, Tengine, China) using 0.5 mol l^{-1} H_2SO_4. The reactor was enclosed in a black-vinyl sheet to inhibit growth of photosynthetic bacteria and algae.

The reactor was operated in up-flow mode, with influent introduced at the bottom using a peristaltic pump (BT100-2J, LongerPump, China). A recirculation pump (BT600-2J, LongerPump, China) was used to dilute the influent with the treated wastewater in the 5-l recycle container.

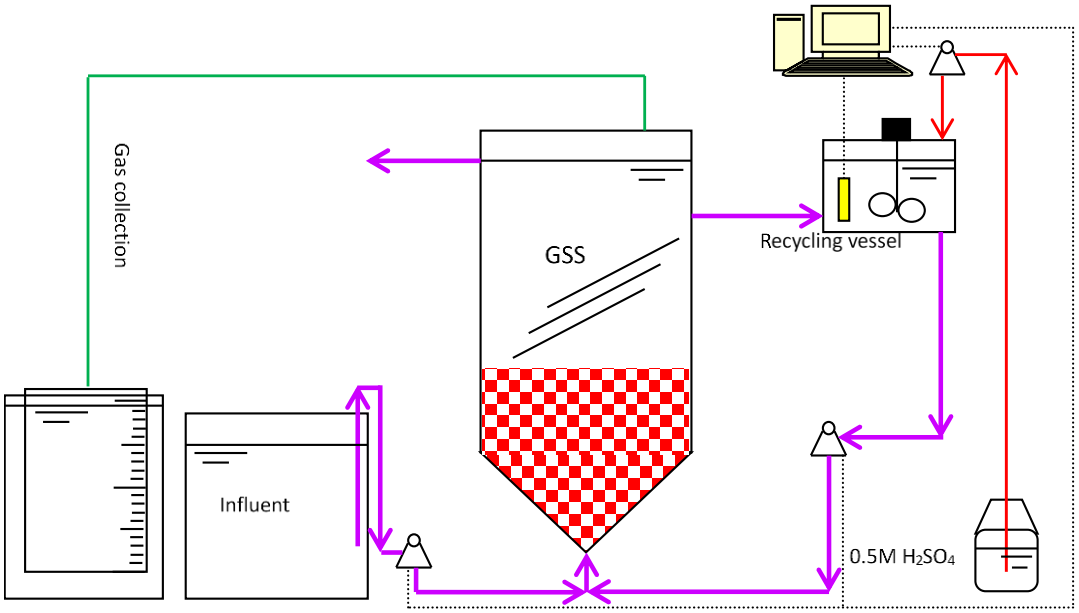


Figure 1. Schematic view of the Anammox reactor system

Symbols: GSS, Gas Solid Separator.

2.2. Seed sludge

The Anammox seed sludge used in the reactor was generously provided by Kumamoto University (Zhang *et al.*, 2014). The initial seeding concentration (mass of mixed liquor suspended solids (MLSS) per liter) was set at 2 g MLSS l^{-1}.

2.3. GAC

The original GAC (Ruicheng, China) had an average diameter of 2-3 mm, apparent specific gravity of 0.45, and a porous microstructure suitable for retention of bacteria. The GAC bed was easily expanded, being only slightly heavier than water. Prior to use, the GAC was black in color and had an average settling velocity of 150 m h^{-1}. In total, 2 L of GAC were added to the reactor in this study.

2.4. Feed media

The reactor was fed with synthetic wastewater with a nitrite to ammonium molar ratio of 1.0-1.2. Anammox nutrient medium consisted of 200-1000 mg-N l^{-1} $(NH_4)2SO_4$, 200-1000 mg-N l^{-1} $NaNO_2$, 1500 mg l^{-1} $KHCO^3$, 50 mg l^{-1} KH_2PO_4, 200 mg l^{-1} $MgSO_4{\cdot}2H_2O$, 226 mg l^{-1} $CaCl_2{\cdot}2H_2O$, and 1 ml of trace element solution I and II (Zhang *et al.*, 2014). The influent storage tank was flushed with nitrogen gas to maintain DO under 0.5 mg l^{-1}, and Na_2SO_3 was added to a concentration of 40 mg l^{-1} (shown to be harmless to Anammox bacteria, data not shown) to keep the DO level close to zero.

2.5. Analytical method

NO_2-N was measured by the colorimetric method according to Standard Methods (4500- NO_2-, B; APHA, 1995). NH_4-N was measured by the phenate method according to Kanda (1995). Total nitrogen (TN) was determined by the persulfate method (4500-N, C; APHA, 1995) using the UV spectrophotometric screening method (4500-NO_3-, B; APHA, 1995) for quantification of TN as NO_3-N (the oxidization product of the persulfate digestion). NO_3-N (of the original sample) was determined by calculation of the difference of TN and the sum of NO_2-N and NH_4-N. The pH was measured by using a pH meter (9010, Jenco, USA), and dissolved oxygen (DO) was measured by using a DO meter (6010, Jenco, USA).

The suspended solids (SS) content was determined according to Standard Methods (2540-Solids, D; APHA, 1995). The total sludge content was estimated as mixed-liquor suspended solids (MLSS). For determination of MLSS, a sludge sample of known volume was washed twice by centrifuging at 1000 X g for 15 min with decanting and re-suspending in deionized water and then dried to a constant weight at 105 °C, with cooling to room temperature under desiccation prior to weighing.

2.6. Scanning electron microscopy (SEM)

Samples were first washed in a 0.1 M phosphate buffer solution (pH 7.4) for 5 min. Then samples were hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the buffer solution. Next, samples were washed in the buffer solution three times for 10 min each and then fixed for 90 min in a 1.0% OsO_4 solution prepared with the buffer solution. After washing samples three times for 10 min each in the buffer solution, they were dewatered for 10 min each in serially graded solutions of ethanol at concentrations of 10%, 30%, 50%, 70%, 90%, and 95%. SEM observations were conducted by using a scanning electron microscope (JSM-6380LV, JEOL, Tokyo).

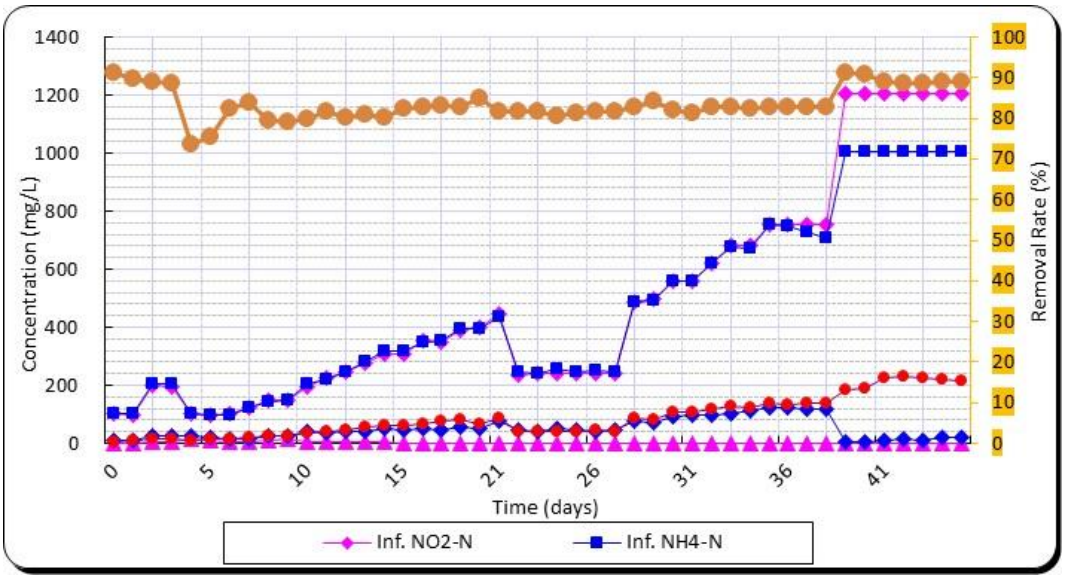
2.7. DNA extraction and high-throughput 16s rRNA gene pyrosequencing

After 139 days of operation, the particle based granules were taken out from the Anammox reactor. A granular sludge sample was first ground with a pestle under liquid nitrogen. Meta-genomic DNA was extracted using the E.Z.N.A. Soil DNA Kit (OMEGA Biotec. D5625-01, USA) according to the manufacturer's instructions. Amplification of the 16S rRNA gene was performed using primers 27F (forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (reverse primer: 5'-TTACCGCGGCTGCTGGCAC-3'). PCR was carried out according to the following thermocycling parameters: 120 s initial denaturation at 95°C, 25 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, 5 min final elongation at 72°C, then held at 10°C until halted by user. Duplicate PCR products were pooled and purified using the AXYGEN gel extraction kit (Axygen, USA) (Isanta *et al.*, 2015).

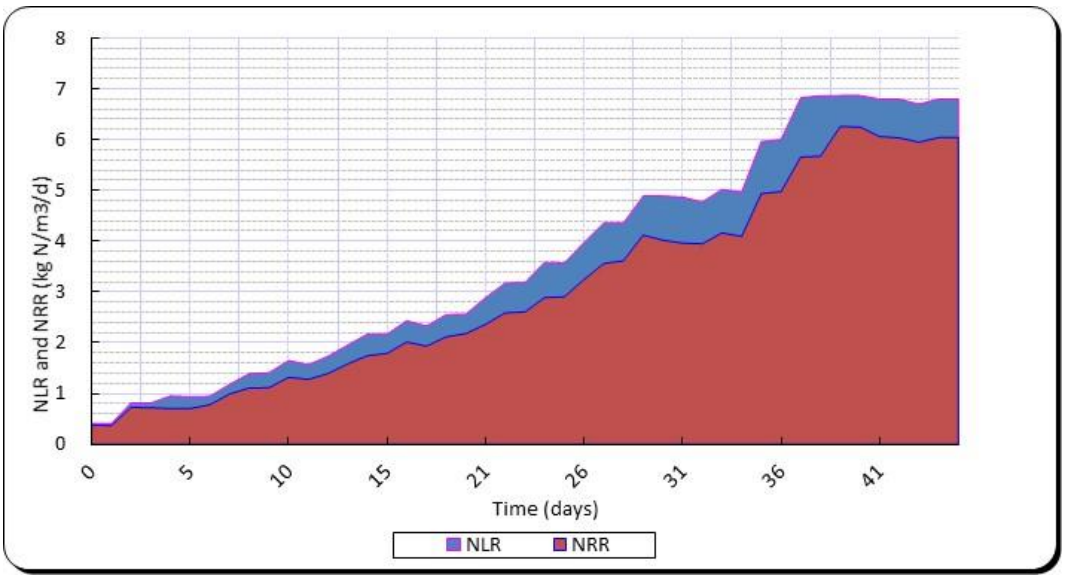
Pyrosequencing was carried out by a 454 Life Sciences Genome Sequencer FLX Titanium instrument (Roche). Sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 distance limit (equivalent to 97% similarity) using the MOTHUR program.

3. Results

3.1. Reactor performance



(a)



(b)

Figure 2. Reactor performance during the study

The start-up period for the Anammox reactor was considered to be from day 0 to day 35 (Fig. 2), during which time the NLR was increased from 0.5 to 6 kg-N/m^3/day by shortening the HRT from 12 to 1.6 h and increasing the influent TN from 174 to 490 mg l^{-1}. The corresponding NRR achieved on day 35 was 4.8 kg

N/m^3/day corresponding to a TN removal efficiency of 84%, which is corroborative of the stoichiometric expectation for a complete Anammox reaction (Kuenen, 2008).

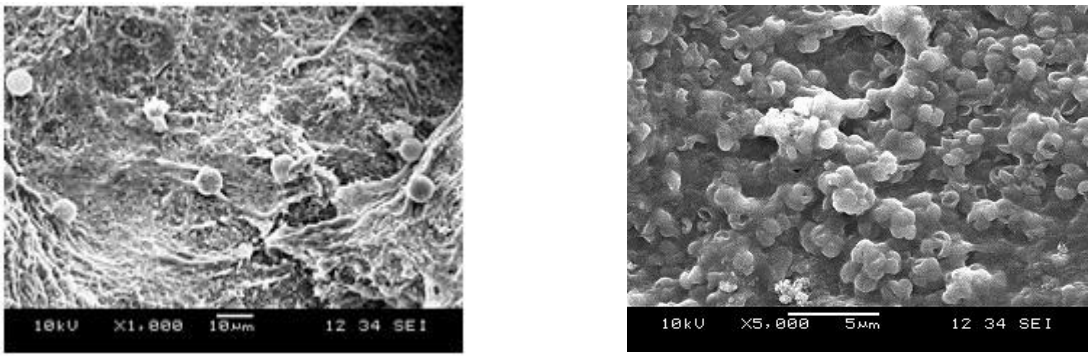
Subsequently, from day 35 to day 46 (Fig. 2), influent TN concentrations were increased stepwise to 1000 mg l^{-1}, 1500 mg l^{-1}, and 2200 mg l^{-1} to probe the treatment potential of the EGSB reactor under a constant NLR of 6 kg-N/m^3/day (maintained by decreasing the influent flow rate).

Throughout the study, the temperature in the reactor was maintained at 33±1°C, and the DO concentration was held below 0.5 mg l^{-1}. Furthermore, the effluent pH (7.6-8.2) was notably higher than that in the influent (6.9-7.2), which is due to consumption of acidity in the Anammox reaction (Kuenen, 2008).

3.2. GAC granules

Figure 3. Appearance of GAC on days 1, 15, 35

At first the GAC was black in color and the Anammox sludge was red. The variations in appearance were recorded using a digital camera at 15 to 20-day intervals throughout the study (Fig. 3). Within one month, the GAC had become red due to the growth of Anammox bacteria in the biofilm attached to the GAC. The matured, red GAC-seeded granules (Fig. 3) had an average settling velocity 200 m h^{-1} (5 cm s^{-1}), compared to typical values for biomass granules of 73 to 88 m h^{-1} (Tang *et al.*, 2011). Some fragments from crushed granules washed-out of the reactor, which suggests that the increased density of the mature GAC-based granules is instrumental in enhancing sedimentation and retention of the essential biomass, which could also reduce the occurrence of floating loss due to gas generation activity.



day 1 day 38

Figure 4. SEM microphotograph of GAC

The Anammox biomass granules seeded with GAC were similar in appearance to granules reported by others (Abma *et al.*, 2007). SEM micrographs (Fig. 4) indicate that a relative large numbers of bacteria were attached to the GAC nuclei. The spaghetti theory suggests that filamentous microorganisms form a frame or mesh by which other bacteria such as cocci are trapped and retained, which leads to granule growth, as appears to be the case here.

4. Discussion

Seed particles have often been employed to enhance the granulation process in traditional UASB reactors. Imajo *et al.*, (2004) studied the feasibility of granulation in an Anammox process using granules from a UASB reactor as seed material, by which an effective granule bed of the extremely slow-growing Anammox microorganisms was achieved in approximately 25 weeks. Compared to the original full-scale UASB reactor (Abma *et al.*, 2007), the start-up period was greatly reduced, though nonetheless still considered prohibitively long. Comparatively, the formation of an Anammox granular bed in this study using a GAC seed was accomplished within 5 weeks while gradually increasing the NLR to 6 kg-N/m^3/d. In addition, the temporary flux of high effluent ammonia concentrations that would be expected due to the degradation of the seed granules from a UASB reactor was avoided during this study due to seeding only with GAC.

Although the functions of co-existing bacteria in Anammox consortia are largely unknown, the Uncultured bacterium clone KIST-JJY024 and Uncultured bacterium cloneFN-11 are considered to be key organisms in the granulation process. In this study, though, the GAC is thought to have provided the necessary environmental characteristics for growth of Anammox bacteria, such as the protective niches with growth-supportive surfaces. Nonetheless, the co-existing organisms soon adapted to the conditions employed here and obtained prevalence in the consortium. Over most of the study, only 25% of the total clones were identified as Anammox bacteria (maintained until the final period of the study when the influent TN was greater than 2 g l^{-1}). Very high TN removal rates, though, were obtained indicating that the Anammox bacteria in the granular medium were uninhibited and highly active, obtaining a specific removal rate of 1.44 g TN/L GAC/day.

As shown in Fig. 3(b), the influent TN concentration was increased to 2200 mg l^{-1} with the nitrogen removal efficiency above 89% and the NRR at 6 kg N/m3/d (process hydraulic retention time of 8.8 h) near the end of the study. High NO2-N concentrations are inhibitory to Anammox bacteria (Carvajal-Arroyo *et al.*, 2014); thus, highly concentrated nitrogen wastewater is usually diluted prior to treatment in Anammox reactors (Yamamoto *et al.*, 2011). However, this practice is forbidden by law for wastewater treatment in China. Accordingly, the GAC-seeded Anammox reactor as demonstrated here could have a very effective application for high-strength wastewaters.

The predominant bacteria in this study are Uncultured bacterium clone KIST-JJY024, Uncultured bacterium cloneFN-11, Uncultured bacterium clone KIST-JJY012, and Uncultured bacterium clone: AnSal-09, which is a population balance clearly different from others cases, which shows that the GAC seed acts well not only as a biofilm carrier by replacing the structural function of filamentous growth, but also by evidently stimulating the growth of Uncultured bacterium clone KIST-JJY024 and Uncultured bacterium clone FN-11, which are well known as key components of Anammox granulation and may have been involved in extending the granulation process external to the GAC. Thus, it is considered that the added GAC seed used in this study can function effectively as nucleus particles by which the formation of granules can be readily enhanced.

5. Conclusions

Using GAC as seed for granulation of Anammox biomass, startup of an EGSB reactor was achieved in over a five-week period. The red, matured granules demonstrated excellent retention characteristics with an average settling velocity of 200 m h^{-1} (5 cm s^{-1}). The potential roll of GAC in providing a medium well suited for growth of co-existing bacteria and thus enhancing granule formation was discussed.

Acknowledgements

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