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Research

Effects of carbon to nitrogen ratio on the performance and stability of aerobic granular sludge

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ABSTRACT

The purpose of this study was to assess the nutrient salts removal efficiency and stability of the aerobic granular sludge (AGS) by change in C/N (carbon to nitrogen) ratio. The laboratory-scale experiments were performed to analyze the removal efficiencies for organic matter and contents of nitrogen, MLSS, sludge volume index, and extracellular polymeric substances (EPS) under C/N ratio conditions of 5.0, 10.0, 15.0, and 20.0. The microorganisms were observed using optical microscope and the microbial communities were analyzed using pyrosequencing. The increase in C/N ratio from 5.0 to 20.0 increased the organic matter and nitrogen removal efficiency to 95.9 and 79.1%, respectively. For the EPS contents, an influencing factor of granule stability, the polysaccharides to protein (PS/PN) ratio increased from 0.55 to 0.79. For the microbial community, the *Thauera* was the most common genus in ending phase occupying 63.7%. This microorganism is regarded as one contributing to organic matter degradation and improved production of EPS including AGS of microorganism, thus, may be an explanation of the results of this study such as increase in organic material in AGS and improvement of denitrification efficiency and contents of EPS with increase in C/N ratio.

Keywords: Aerobic granular sludge (AGS), Carbon to nitrogen ratio, Organic matter, Nitrogen, Microbial community

1. Introduction

The aerobic granulation is a self-immobilization process in which the loose biomass is converted into high-density compacted granule [1-4]. The aerobic granular sludge (AGS), compared to traditional activated sludge, has more excellent characteristics in terms of regular shape and compact structure, sedimentation, biomass accumulation, resistance to toxic compounds and high organic load, and removal efficiency of nutrient salts [5-8]. These made the AGS technique made to be widely used in treatments of high-load industrial wastewater as well as sewage [9-12]. The stability of AGS is the most important issue in applying AGS-based process in various fields and is related many factors including the composition of influent wastewater, carbon to nitrogen (C/N) ratio, food to microorganisms (F/M) ratio, composition of extracellular polymeric substances (EPS), and rate and temperature of aeration [4, 8, 13-17], among which, the C/N ratio of influent water is one of the most important factors for the growth of microorganism and biodegradation of nutrient salt [13, 18-20]. The application of AGS system, for example, into the treatment of wastewater treatment where the variance in wastewater is high was reported to be an important factor in decreased stability, degradation of granule and the growth of filamentous fungus [21-24]. Since the EPS degraded by microorganism and contributing to formation and stability of AGS are mainly composed of protein (PN) and polysaccharides (PS), the stability of granule is affected by change in EPS concentration and PS/PN ratio due to change in C/N ratio [25-28].

Mo et al. [29] analyzed the nutrient salts removal efficiency by operating the pilot plants of AGS-based sequencing batch process with capacity of 225 ton/day reported that the average C/N ratio was about 3.1 and the nitrogen removal efficiency decreased when the C/N ratio is under 2.0. Yae et al. [30] reported that, in the environment of average C/N ratio of 4.5, the stable operation of AGS-based process is possible by controlling variable operation conditions. Table S1 compares the AGS stability and pollutant removal efficiency by C/N ratio [23, 24, 29-31]. This test was, how-

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ever, performed in a restricted condition, and the further studies applying variant C/N ratio are necessary to achieve more accurate understandings of pollutant removal efficiency and stability mechanism of AGS by change in C/N ratio. The purpose of this study was, accordingly, to assess the effects of change in C/N ratio on the pollutant removal efficiency of AGS and stability of granule. As of 2017, the C/N ratio of domestic influent sewage is maintained in the range of 2.5-5.7 [32]. In addition, C/N ratio of domestic influent wastewater is maintained in the range of 6.1-20.9 [33]. Therefore, influent C/N ratio conditions of this study are to be subdivided from minimum 5.0 to maximum 20.0. For the purpose, the sequencing batch process was applied and the organic materials and nitrogen removal efficiency by change in C/N ratio were measured. This study intended to contribute to this field by providing detail information collected by monitoring physical characteristics and contents and composition of EPS for the stability test of AGS and analyzing the effect on the change in microorganism population.

2. Material and Methods

2.1. Synthetic Wastewater

The influent wastewater used in study was synthesized to satisfy the prescribed C/N ratios. The organic material, ammonias nitrogen, and alkaline were prepared using $CH_3COONa{\cdot}3H_2O$, NH_4Cl, and $NaHCO_3$ (SAMCHUN Chemical, Korea). The C/N ratio was set to increase gradually from 5.0, to 10.0, 15.0, and 20.0 by fixing the nitrogen concentration as 25 mg/L and changing the organic concentration. In various literatures, AGS has been reported as sludges larger than 0.2 mm [34-37]. The AGS used were those selected from ones cultured at laboratory-scale reactor to have size of over 0.2 mm (80 mesh/0.2 mm STS Sieve). The initial concentrations of mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and AGS were 2,430, 2,170, and 2,270 mg/L, respectively. The compositions during operation are shown in Table 1.

Table 1. Characteristics of the Influent

Parameters		Unit	Influent			
			Min.	Max.	Average	S.D.*
COD_{Cr}	(C/N: 5.0)	(mg/L)	122.3	127.7	125.3	1.7
	(C/N: 10.0)		247.3	254.3	250.6	1.9
	(C/N: 15.0)		372.3	377.3	375.6	1.4
	(C/N: 20.0)		498.3	506.3	501.4	2.0
Total Nitrogen		(mg/L)	23.6	25.6	25.1	0.3
Alkalinity		(mg/L)	186.0	224.0	202.0	5.4
pH		-	7.0	7.2	7.1	0.1

*S.D.: Standard deviation

2.2. Reactor and Experimental Set-up

The reactor used was the sequencing batch reactor (SBR) reported to be preferable in forming and maintaining AGS [38-40], which hash effective volume of 5 L (155 mm × 155 mm × 222 mm) and is made of acryl. The reactor was mounted with sampling port for collecting water and analysis and diffuser. The treated water was discharged through reactor middle port to apply volume exchange rate (VER) of 50% [41-43]. The operational condition of SBR was fixed as 6 cycles, thus the hydraulic retention time (HRT) was set as 8 h, to minimize the number of variables. The operation time of each phase were set as 10 min for filling and reaction, 210 min for aeration, 10 min for sedimentation, and 10 min for draw. The operation was performed by using programmable logic controller (PLC), a control program. The concentration of dissolved oxygen (DO) was maintained as 1.0-2.0 mg/L and the solid retention time (SRT) as 15-20 d. Table 2 shows the operation condition of the laboratory-scale SBR.

2.3. Analytical Methods

The chemical oxygen demand (COD) and total nitrogen (T-N) contents of the influent and treated water samples were analyzed using absorptiometry (DR-4000, Hach, USA) and the solids were analyzed using MLSS and MLVSS [44]. The sludge volume index (SVI_{30}) by change in C/N ratio was measured to assess the sedimentation characteristics of AGS [45]. Increasing sludge ratio of less than 0.2 mm reduces pollutant treatment efficiency. Therefore, various studies have been reported highlighting the importance of increasing AGS ratio [29, 46, 47]. The AGS/MLSS ratio was measuring for those of over and under 0.2 mm, separately, after collecting and selecting sludge (80 mesh/0.2 mm STS Sieve). The EPS contents of AGS were extracted using Formaldehyde and NaOH [48], and the extracted PN and PS were measured using Folin reagent [49] and Phenol-vitriol method [50]. The change in shape and surface characteristics of AGS were observed under the optical microscope (CX-31, Olympus, Japan) with magnitude of 40 after covering them with cover glass of 0.17 mm.

2.4. Microbial Community

The microbial communities were carried out a total of two analyzes by collecting the initial sludge at the start of the experiment (C/N ratio 5.0 conditions) and the sludge at the end of the experiment (C/N ratio 20.0). This experiment is composed of four phases. First, after completing sampling microorganism, DNA extraction (PowerMax® Soil DNA Isolation Kit) was performed and only DNA was measured using Fluorescence-based quantification technique of PicoGreen (Invitrogen, cat. #P7589), (Pass criteria: sample with concentration of over 1.0 ng/ul). Second, the library is fabricated by first PCR amplifying the target region and second one attaching the barcode (Nextra XT index kit). The main peak is checked again

Table 2. Experimental Conditions of Lab. Scale Reactor

Vol. (L)	Flow (L/day)	Cycles/day	pH	HRT(h)	SRT (d)	Temp. (℃)
5	15	6	7.0～7.2	8	15～20	23.8～24.2
Operation time for 1 cycle (min): Fill & React(10)→Oxic(210)→Settle(10)→Draw(10)						
Influent C/N ratio (mg COD_{Cr}/mg T-N): 5.0, 10.0, 15.0 and 20.0						

using chip photo and the Agilent Technologies 2100 Bioanalyzer to confirm the correct size of target. Third, the characterized samples were sequenced finally using sequencing by synthesis (SBS) technique of Illumina MiSeq® System. Fourth, the taxonomic assignment and statistical analysis were performed based on the sequencing date (Macrogen Inc., Seoul, Korea) [51-54].

3. Results and Discussion

3.1. Reactor Performance

The organic material removal efficiencies during whole operation period are shown in Fig. 1. The increase in C/N ratio tended to lead to increase in removal efficiency where the average organic material removal efficiencies under C/N ratios of 5.0, 10.0, 15.0, and 20.0 were 78.9, 86.7, 92.7, and 95.9%, respectively. It was intuitively confirmed, in particular, that the difference in removal efficiency between C/N ratios 5.0 and 10.0 was large, and this tended to be stabilized under C/N ratio of 10.0 and greater [55-57]. Kocaturk and Erguder [24] reported that the organic material removal efficiencies were kept over 90% under the conditions of higher C/N ratio (10, 20, and 30) and kept low under the lower C/N ratio, consistent with the results of this study. They explained that this is due to domination of heterotrophic bacteria, reporting that the wastewater with higher C/N ratio is more appropriate for organic matter removal. Considering the result of this study, therefore, the gradual increase in organic matter removal efficiency is deemed to be result of domination of heterotrophic bacteria [58-60]. Choi [61], contrary the results of this study, reported that the organic matter removal efficiencies were 89.4-95.6% under the C/N ratio of under 2.0 using AGS. These high efficiency under lower C/N ratio may be attributed to the high MLSS concentration (8,000 mg/L) compared to that of this study (3,720 mg/L).

The behavior of total nitrogen by change in C/N ratio showed that the increase in C/N ratio, as with organic matter removal efficiency, was related to increase in total nitrogen removal efficiency (Fig. 2). The total nitrogen removal efficiency of 57.5% under the lowest C/N ratio, 5.0, increased to 61.1, 69.6, and 79.1% for the C/N ratios of 10.0, 15.0, and 20.0, respectively. The three reasons

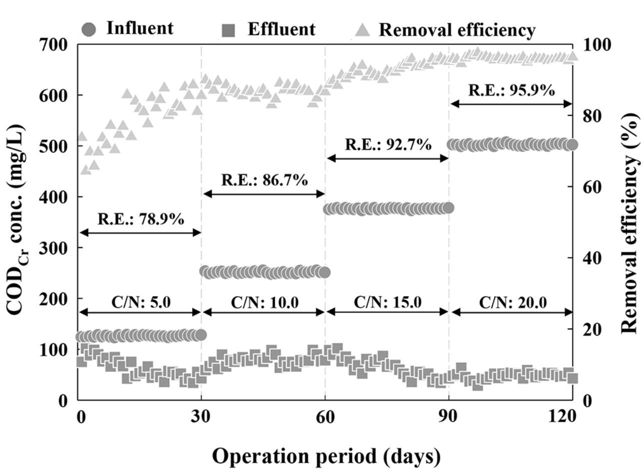

Fig. 1. Profile of COD_{Cr} concentration at different C/N ratio.

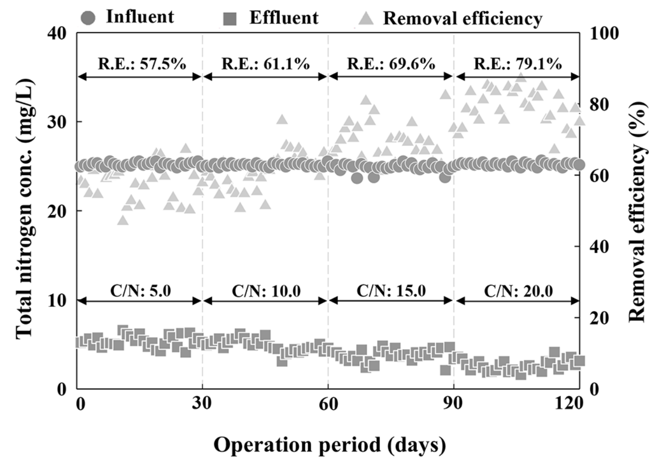

Fig. 2. Profile of total nitrogen concentration at different C/N ratio.

of these increase in removal efficiency by increase in C/N ratio despite the absence of anoxic reaction in whole processes are presented: denitrification reaction of nitrate nitrogen in the filling and reaction phase due to enough supply of organic matter needed for denitrification reaction; simultaneous nitrification and denitrification (SND) by AGS in the aeration reaction phase; the domination of aerobic or facultative anaerobes, which is described in the section 3.5 [62-64]. Mo et al. [29] emphasized the importance of controlling C/N ratio since the total nitrogen removal efficiency decreased under C/N of under 2.0. He et al. [65], to measure the nitrogen removal efficiency using AGS under the condition of low C/N ratio (4.0), induced simultaneous nitrification and denitrification reaction through controlling size of aeration and reported that the removal efficiency of 62.74% under aeration size of 1.5 L/min increased to 84.74% under that of 0.9 L/min. It means that the low removal efficiency observed under low C/N ratio in this study may be increased by controlling the size of aeration, suggesting that flexible operation and control strategies are needed to increase the nitrogen removal efficiency.

3.2. MLSS and SVI_{30}

Fig. 3 shows concentration of MLSS and AGS and behavior of AGS/MLSS ratio by condition. The initial concentration of MLSS and AGS and AGS/MLSS ratio were 2,430 mg/L, 2,270 mg/L and 93.4%, respectively. After the operation, the MLSS and AGS, under the condition of C/N ratio 5.0, tended to decrease down to 2,190 and 1,710 mg/L and accordingly the AGS/MLSS ratio to 78.1%. These patterns were reversed when the C/N ratio increased from 10.0 to 20.0, thus, the final values of them were 3,720 mg/L, 3,210 mg/L, and 86.3%. Kocaturk and Erguer [24] examined the effect of C/N ratio (7.5, 5.0, 3.5, 2.0, and 1.0) on the degradation of AGS and found that the decrease in C/N ratio increased the degradation of granule. In addition, it is also reported that when the C/N decreases from 7.5 to 5.0, the granule collapses and the mean diameter decreases by 0.7 mm. The decreased MLSS and AGS under relatively low C/N ratio (5.0) observed in this study may be attributed to the leakage of sludge during granule degradation due to unbalance of C/N ratio. Pijuan et al. [46] pointed out the importance of initial AGS/MLSS ratio for stable maintenance of AGS and Ni et al. [66]

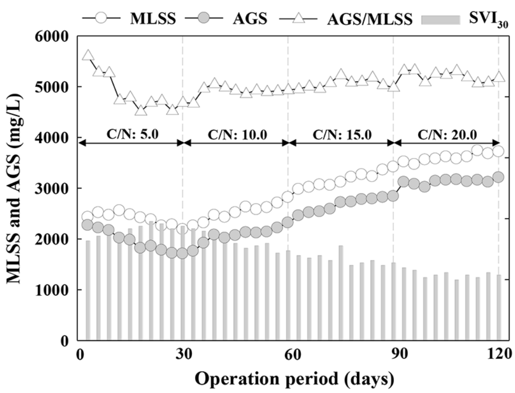

Fig. 3. Results of MLSS, AGS concentration, AGS/MLSS and SVI30 value at different C/N ratio.

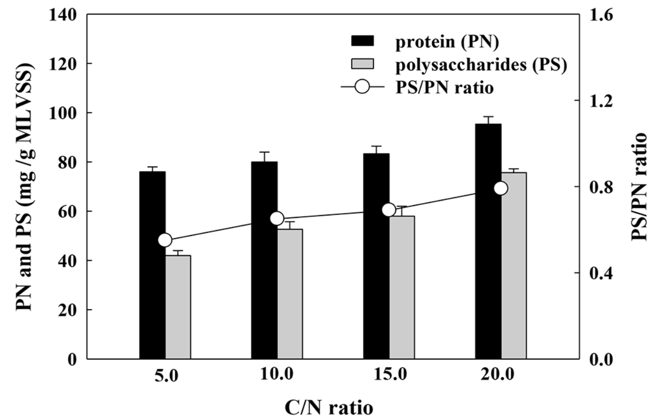

Fig. 4. Results of PN, PS concentration and PS/PN ratio value at different C/N ratio.

reported that the operation of over 300 days are needed to achieve AGS/MLSS ratio of 85%. The results of this study demonstrated that the C/N ratio, a factor described in Introduction to be influencing one of formation of AGS and it is suggested that the C/N ratio of over 5.0 is necessary for maintenance of granule.

The sedimentation characteristics (SVI_{30}) of sludge showed similar pattern with the concentration of MLSS and AGS. SVI_{30}, under C/N ratio of 5.0, increased from 82 mL/g of initial phase to 94 mL/g gradually due to formation of sludge with relatively poor sedimentation, however, decreased gradually with the change of C/N ratio from 10.0 to 20.0, reaching 54 mL/g at the ending of operation. These relatively poor sedimentation may be explained by degradation of granule described in above paragraph, and the increase in C/N ratio and the resultant increased portion of AGS is deemed to be reason of improved sedimentation. Wang et al. [67] performed low-strength wastewater treatment using AGS and found that at least 31 d are needed to achieve stable SVI_{30} after increase in initial phase. He et al. [68] reported the improved sludge concentration and sedimentation by decrease in aeration time. The maintenance of stable granule with excellent sedimentation by short sedimentation time (two minutes) was reported by He et al. [65].

3.3. Extracellular Polymeric Substances Analysis

The contents of PN, PS and PS/PN ratio showed different pattern with the increase in C/N ratio (Fig. 4). The contents of total EPS increased from 118 to 172 mg/g MLVSS with the increase of C/N from to 5.0 to 20.0, and the compose of PN and PS showed remarkable difference in each C/N ratio condition. The increase of PS from 42 to 76 mg/g MLVSS was stronger compared to those of PN from 76 to 96 mg/g MLVSS, and the resultant PS/PN ratio was shown to be 0.55 under condition of C/N ratio 5.0 and 0.79 under C/N ratio 20.0. The EPS was reported as importance factor in determining the formation and stability of AGS in many studies [69-71] and the contents of PS tended to be high in stable granule [72-74], consistent with this study showing increase in PS under high C/N ratio condition. The results of this study demonstrated that the gradual increase in C/N ratio has direct effects on the stability of AGS and also on the contents of PS, a constituent of EPS, making preferable condition for maintenance and stability of granule.

3.4. Microscopic Observation of AGS

Fig. 5 shows the observation, using optical microscope, of AGS under each condition. The hair-like objects of surface and filamentous fungus were more commonly observed in C/N 5.0 conditions than any other ones [75, 76]. The growth of the filamentous fungus has been reported to be observed in activated sludge and AGS and the sludge bulking has been pointed as a reason [77-79]. These results may be also explained by decrease in MLSS and the relatively small size of fungus in low C/N ratio condition is deemed to be due to decrease in AGS/MLSS ratio. Under the C/N ratio over 10.0, the size of fungus became bigger, reaching up to 1.0 mm and the shape was circular and dense. The degradation of granule in this study occurred more commonly in lower C/N

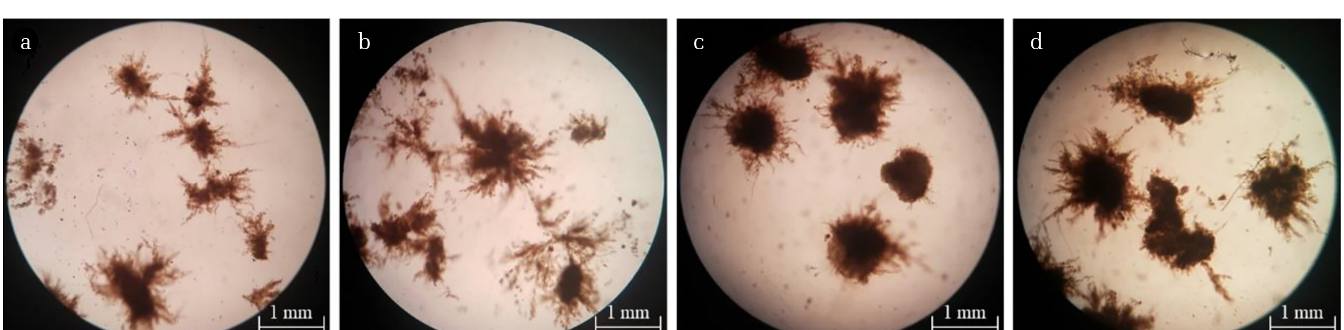

Fig. 5. Morphology observation of AGS at different C/N ratio, (a) 5.0; (b) 10.0; (c) 15.0; (d) 20.0 (40 magnification by optical microscope).

ratio, directly and indirectly confirming the importance of C/N ratio in stability of granule. It is recommended for future studies to investigate the C/N ratio controlling method (such as external carbon source) and operation method for stability of granule.

3.5. Microbial Communities of AGS

The operational taxonomic unit (OTU) of initial and end phases of experiment were 755 and 158, respectively, showing the decrease in diversity of microorganism. These may be explained by the gradual domination of heterotrophic bacteria that had adopted to microorganism degradation with the gradual increase in C/N ratio, decreasing the richness and diversity of microorganism [80-82]. The results of pyrosequencing analysis of microorganism

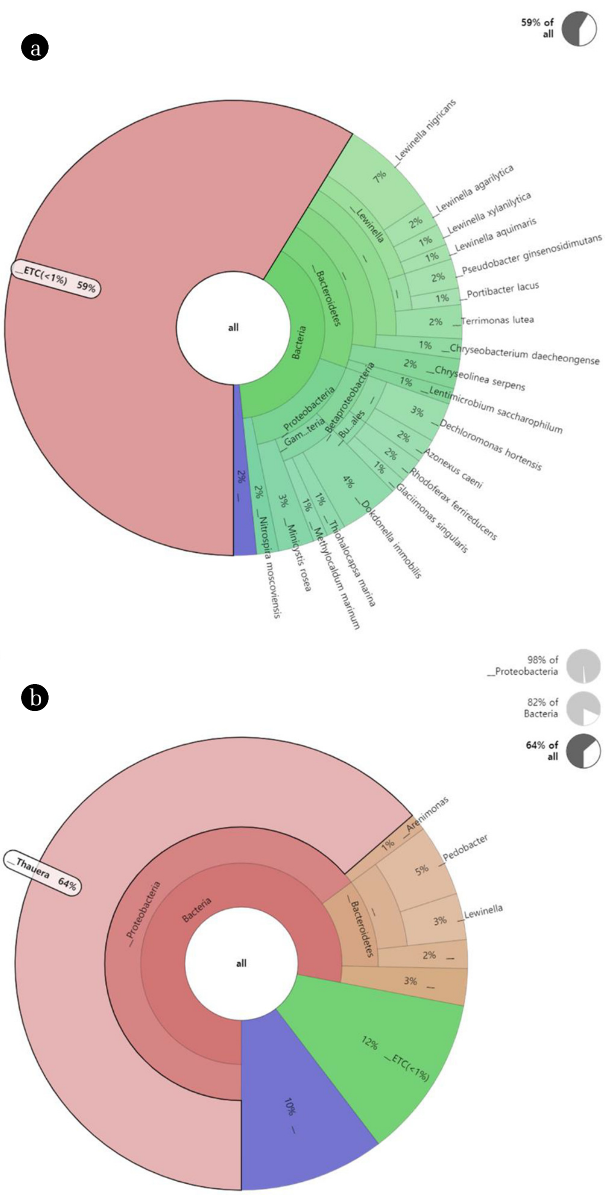

Fig. 6. Results of microbial communities using dynamic pie chart (Krona). (a) Initial phase AGS and (b) End phase AGS.

community of AGS, using the dynamic pie chart (krona), at initial and end phases showed difference between them (Fig. 6). The *Proteobacteria* reported to be most rich in the process of wastewater treatment under phylum condition were shown to be 34.8 and 70.2%, respectively, a highest increasing rate [83, 84]. These in *Bacteroidetes* were 37.4 and 10.9%, respectively, indicating decrease in the proportion. The *Bacteroidetes*, as with *Firmicutes*, was reported to show strong domination under condition of high salinity [85-88].

In terms of genus, *Thauera* occupied 0.01% in initial phase, however, increased to 63.7% in end phase, becoming most common one. *Thauera* is regarded as very important microorganism in the process of wastewater treatment [89], as aerobic denitrification one [90, 91], and as one contributing to improved production of EPS including AGS of microorganism [92-94]. The domination of *Thauera*, therefore, is suggested to be and explanation of the results of this study such as increase in organic material in AGS and improvement of denitrification efficiency and contents of EPS with increase in C/N ratio. The proportion of *Pedobacter*, knows as aerobic or micro-aerobic [95] and *Lewinella* known as organic chemical heterotrophic microorganism [96] were, at ending phase, 5.1 and 3.3%, respectively.

4. Conclusions

A study on nutrient salt removal efficiency and stability of AGS in various C/N ratio conditions was performed. The increase in C/N ratio from 5.0 to 20.0 increased the organic matter and nitrogen removal efficiency to 95.9 and 79.1%, respectively. MLSS and AGS tended to decrease under the C/N ratio of 5.0, however, increased under C/N ratio up to 20.0, reaching 3,720 and 3,210 mg/L in ending phase, resulting in AGS/MLSS ratio of 86.3%. The change in SVI_{30} showed similar pattern with MLSS where the value decreased from 82 to 54 mL/g under low C/N ratio. For the EPS contents, an influencing factor of granule stability, the PS/PN ratio increased from 0.55 to 0.79. The microscope observation showed more clear circular shape of sludge with the increase in C/N ratio. For the microbial community, the *Thauera* was the most common genus in ending phase occupying 63.7%. The gradual increase in C/N ratio, therefore, was confirmed to maintain the AGS stably in the operation of AGS-based biological treatment system by making heterotrophic bacteria (*Thauera*) as dominant. It is considered that the future studies are needed to examine the controlling of operation condition of AGS-based process and measurement of stability by using real wastewater with various C/N ratio.

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Author Contributions

H.G.K. (Ph. D.) conducted all the experiments. J.T.K. (Professor) and D.H.A (Professor) wrote the manuscript.

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Table S1. Comparison of AGS Stability and Pollutant Removal Efficiency by C/N Ratio

Substrate	Influent C/N ratio	AGS diameter (mm)	Organic matter removal efficiency (%)	Reference
Acetate, glucose	4.0	0.8	-	[23]
Acetate, glucose	1.0	0.2	-	[23]
Acetic acid	7.5	3.0	79.0	[24]
Acetic acid	5.0	2.3	68.0	[24]
Sewage	1.0-6.0	0.2	89.5	[29]
Sewage	4.5	1.0-1.5	92.6	[30]
Acetate	5.5	1.5	>90.0	[31]
Glucose	5.5	1.2	>90.0	[31]
Ethanol	5.5	0.7	>90.0	[31]