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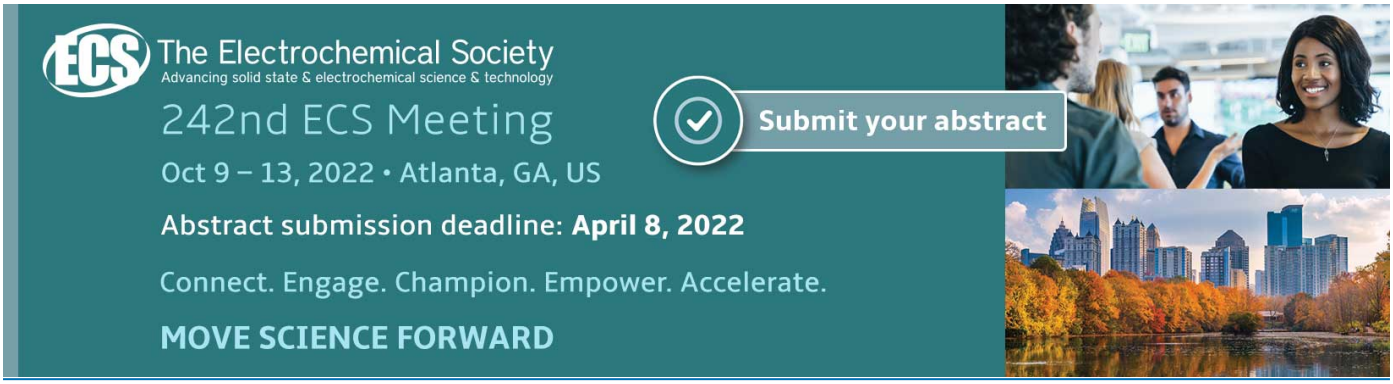

Identification and role of microbial species developed in aerobic granular sludge bioreactor for livestock wastewater treatment

I Othman[1], M H Ab Halim[1,2*], A N Anuar[2], N H Rosman[1], H Harun[3], S M Noor[2], Z Ujang[2] and M van Loosdrecht[4]

[1] School of Civil Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, Johor, Malaysia
[2] Malaysia-Japan International Institute of Technology, Universiti Teknologi Malaysia, Kuala Lumpur, Malaysia
[3] Faculty of Engineering Technology, Universiti Tun Hussein Onn, Johor, Malaysia
[4] Faculty of Applied Sciences, Delft University of Technology, Delft, Netherland

*Corresponding author e-mail: hakim_90@rocketmail.com; mohdhakim@utm.my

Abstract. The purpose of the microbial diversity studies conducted is to discover species composition, structure, bacterial distribution, spatial activity as well as the function and role of the microbial. A laboratory-scale sequencing batch reactor with a working volume of 2 L was used to develop aerobic granular sludge to treat livestock wastewater. The seed sludge was taken from municipal wastewater treatment plant, while the wastewater was collected from cattle farm at Primaternak Enterprise, Johor, Malaysia. The composition and diversity of microbial community in the seed sludge and aerobic granules were explored using next generation sequencing analysis. Based on the findings, the details of phylogenetic bacterial which consists of phylum, class, order and family were identified and compared between the seed sludge and aerobic granules. The metagenome DNA sequencing analysis has revealed an abundance of microbial diversity in the seed sludge and 8 hours aerobic granular sludge samples. The metagenome analysis discovered wide variety of microorganism including archaea, bacteria, eukaryote, and virus. Bacteria has been evaluated as the most dominant microbial in both seed sludge and aerobic granules. Acidovorax sp JS42 was found to be the most abundance bacteria species in seed sludge while Thauera MZIT was the most abundance bacteria species in aerobic granules. Whereas, Bacteroides, Flavobacterium, Comamonas, Pseudomonas and Acinetobacter were the most abundance bacteria that responsible in developing aerobic granules were observed to be higher in aerobic granules compared to the seed sludge. The results from this study indicated that distinct differences of microbial community from the seed sludge and aerobic granular sludge were observed clearly, which provided some evidence of the granulation process.

1. Introduction

Aerobic granular sludge (AGS) has recently appeared as a promising technology in biological wastewater treatment to be correlated to microbial diversity. Each species of microorganism found in the system executes different functioning roles in the development of AGS structure and the efficiencies of the treatment. This has been revealed by researchers in the previous studies [1-7]. It was shown that the presence of specific microorganism in the system led to stable granular sludge and capable in utilized polluting matter in wastewater. The interaction between microorganisms with the

environment at the initial stage of the granulation process play a significant role in the assurance of successful cultivation of AGS. Furthermore, the microorganisms that grow within AGS prevent to be washed out due to excellent in settling properties. However, the microbial diversity are sensitive to operational conditions of SBR such as settling time, shear force, cycle time, organic loading rate and etc. Therefore, thorough study of microbial diversity is needed to discover the species composition within the system during granulation.

Different types of microbes residing in different types of wastewater and sludge. Livestock wastewater contains high microbial diversity including bacteria, archaea, virus, pathogenic microorganisms and parasite eggs which represents a serious environmental threat that might affect water body [8]. Nevertheless, livestock wastewater contains abundant supply of the nutrients required by microorganism. As the environmental conditions change, the dominant microbial also change. The changes of microbes' physiology transform the dispersed sludge to a dense AGS. Therefore, different operational strategies have led to different AGS characteristics and removal performances. Moreover, the efficiencies of the treatment depend on the conditions benefit the proliferation of microorganism. As been reported by Liu et al. [9], the high abundance of microorganism, the higher pollutant removal rate.

The correlation between the abundance of microorganism shifted from the seed sludge and AGS during granule formation were investigated in this study. Investigation on factors that may affect the mechanisms during the initial stage of the granulation process should be considered as a crucial aspect to be explored. The purpose of the microbial diversity studies is to discover species composition, structure, bacterial distribution, spatial activity as well as the function and role of the microbial.

2. Materials and Methods

2.1. Wastewater Collection and Sludge Sampling

The sampling location of the wastewater was at a cattle farm at Primaternak Enterprise, Pekan Nenas, Johor, Malaysia. The selected location was the closest to the laboratory which is less than 15 km to preserve the microbial activity during sampling. Raw livestock wastewater was fed directly into the reactor after screening and sieving process. A fresh activated sludge (seed sludge) was taken from an aeration tank in Indah Water Konsortium (IWK) municipal wastewater treatment plant at Taman Perindustrian Lima Kedai, Johor. The location was close to the laboratory for easy handling of sludge samples and maintaining microbial activity.

2.2. Laboratory-scale Reactor Design

A laboratory-scale SBR used to develop AGS was based on the reactor configuration proposed by Nor Anuar [10] and Muda [11]. The cylindrical column type SBR with a total volume of 5 L and a working volume of 2 L was used in the experimental study. The column reactor was designed with an internal diameter of 8 cm and a total height of 100 cm. A set of two peristaltic pumps was used to feed and to discharge the wastewater within the reactor system. The influent was introduced into the reactor through a port located at the bottom of the column. The reactor was continuously aerated via porous air stones located at the bottom of the reactor. The wastewater effluent was collected at an outlet port located at the middle of the reactor height with 60% volumetric exchange ratio. The reactor system was programmed using timer for continuous operation.

2.3. Operational Conditions of the System

The routine cycle in the SBR consisted of five phases, namely filling, reacting, settling, decanting and idling periods. The livestock wastewater was filled from the bottom of the reactor with flow rate of 0.2 L min^{-1} and the filling mode was kept non-aerated. There were aerobic and anaerobic conditions during the reacting phase. At this phase, the livestock wastewater flow into the tank was cut off (flow rate is zero), meanwhile aeration and mixing continued. Air was introduced through a fine air bubble diffuser at the bottom of the reactor during aerobic condition. The airflow rate was set to 1 L min^{-1} in all experiments. During anaerobic condition, airflow pump was turned off. The settling phase was operated with a minimum time (5 minutes). After that, the clarified supernatant was decanted from the reactor as effluent.

2.4. Microbial Identification

Several steps were involved in microbial identification in the seed sludge and AGS sample which included DNA extraction, DNA sequencing and stastical analysis. Abundance microorganisms present in the samples from the reactor were investigated by sequencing the extracted DNA. Briefly, sequencing analysis for the extracted DNA samples were generated during the next-generations sequencing (NGS) using Illumina HiSeq2000 paired end sequencing on one lane. This approach captured the whole and complete genomes of the entire microorganism in the population. Metagenomic analysis is a molecular technique to identify the genes present in a bacterial population without assembling individual bacterial genomes. A small fraction (average 2%) of the data uploaded in the Metagenomics Rapid Annotation Subsystem Technology (MG-RAST) server failed the QC pipeline, while the rest (average 98%) were successfully uploaded and annotated. The mean sequence length best paired (bp) for all samples were 101 ± 0 bp. The quality control (QC) of the sequences was 97.3% and used by the MG-RAST server for further analysis.

Metagenomics Rapid Annotation Subsystem Technology (MG-RAST) is a data repository server, an analysis pipeline and a comparative genomics environment. Its fully automated pipeline provides quality control, feature prediction and functional annotation. MG-RAST server is an automated analysis platform for metagenomes providing quantitative insights into microbial populations based on sequence data. All data generated by MG-RAST can also downloaded, shared and published within the portal. A gene bank was used as the source of annotation for further analysis due to its extensive database. Hence, sequences were compared to Genebank databases using the BLAST server at the National Centre of Biotechnology (NCBI).

3. Results

3.1. Classification of Microbial Community during Granulation

Microbial community in the seed sludge and AGS sample were explored and analyzed with the output of phylogenetic structures and microbial community diversity in the system. Figure 1 depicts the taxonomic result of seed sludge and AGS in term of the relative abundance percentage of microbial domain distribution existed in the samples. These results indicated that the major populations in the seed sludge were noticeably different from those in the AGS. From the analysis, microbial distribution in both samples were includes archaea, bacteria, eukaryote and virus. Significantly, AGS sample was more diverse than seed sludge sample. In this study, about 97.8 % of bacteria in seed sludge shifted to 96.8% of bacteria in AGS. Lesser amount of bacteria in AGS shows that only bacteria involved in the development and removal performance present in AGS. Meanwhile, increased of archaea in AGS was revealed from 1.0 % to 1.5%, eukaryote from 0.6% in the seed sludge to 0.8% in AGS and viruses from 0.6% to 0.9%.

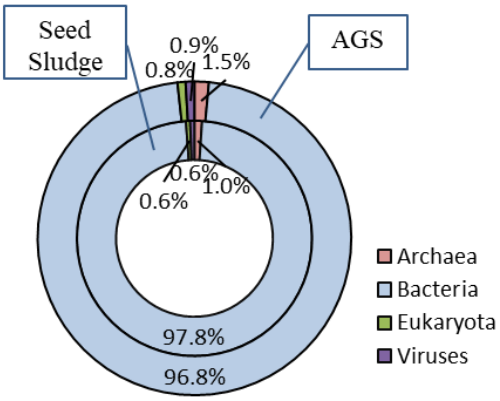

Figure 1. Microbial domain distribution percentage in seed sludge and AGS.

3.2. Bacteria Community during Granulation

The composition and diversity of microbial community in the seed sludge and AGS were explored using next generation sequencing (NGS) analysis. Figure 2 depicts the phylogenetic profiling of bacteria community in the seed sludge and AGS including phylum, class, order and family of bacteria. The figure illustrated the top 30 classification and abundance of bacterial diversity from phylum to family level of bacteria during aerobic granulation. Bacterial community were diverse in both seed sludge and AGS.

The microbial community composition in seed sludge and AGS demonstrated dominancy of Proteobacteria followed by Bacteroideted and Actinobacteria as shown in Figure 2a. Meanwhile, four major subclasses of Proteobacteria are AlphaProteobacteria (α-Proteobacteria), BetaProteobacteria (β-Proteobacteria), GammaProteobacteria (γ-Proteobacteria) and DeltaProteobacteria (*δ*-Proteobacteria) were dominant in both samples. Burkholderiales, Rhizobiales and Rhodobacterales order shows higher percentage in AGS compared to seed sludge. Comamonadaceae, Burkholderiaceae, and Pseudomonadaceae family were dominant in both samples.

At phylum level, Proteobacteria has found to be dominant taxonomy discovered in seed sludge and AGS with no significant different which were 67.6 % and 66.6% respectively. It shows the highest abundance of Proteobacteria phylum compared to other phylum. Meanwhile, the retrieval of Bacteroides (12.3% in seed sludge and 6.7% in AGS) phylum was grown in the seed sludge and AGS at second highest abundance after Proteobacteria. Actinobacteria phylum were abundance as well with percentage of 7.4% in AGS and 4.8% in seed sludge samples. Figure 3 represents the tree profile of overall assigned bacteria phylum including Actinobacteria, Bacteroidetes, Chloroflexi, Deferribacteres, Firmicutes and Verrucomicrobia. The major populations of bacteria in the seed sludge were slightly different from those in AGS. The seed sludge and AGS consisted of 28 phylum of bacteria. The bacteria found in the seed sludge were mostly preserved throughout the granulation. However, the total abundance of the seed sludge phylum greater than in AGS with 2 433 361 and 1 666 287 respectively.

At class level, four major subclasses of Proteobacteria such as α–Proteobacteria, β–Proteobacteria, γ–Proteobacteria and δ–Proteobacteria. It was shown that β–Proteobacteria and δ–Proteobacteria constitute a large fraction of Proteobacteria in AGS with percentage 24.5% and 8.0 % respectively.

Burkholderiales is suborder of β–Proteobacteria were observed to be the dominant population in AGS with percentage of 34.3% and 30.4% in seed sludge. The second highest order of Rhizobiales represent high percentage of 14.9% in AGS than 11.8% in seed sludge. Rhodobacterales order was found highest in AGS compared to seed sludge with small difference percentage of 8.1% and 7.9% respectively.

In phylogenetic analysis, Comamonadaceae, Burkholderiaceae and Pseudomonadaceae were dominant in family level. The most abundance family member in AGS was Comamonadaceae with percentage of 20.1% followed by Burkholderiaceae and Pseudomonadaceae with 9.4% and 4.4% respectively.

Further investigation of the abundance microbial species in seed sludge and AGS were summarized in Table 1 and Table 2. The bacteria species were dominated in the seed sludge rather than archaea, eukaryote, and virus in both samples. Therefore, this study have been focusing on the properties and role of bacteria. The top 15 most abundance microbial species were evaluated in term of properties of each species. Wastewater treatment system provided a suitable operation condition which favour the growth of certain bacteria species and maybe detrimental to the growth of other bacteria. The typical properties of top 15 bacterial species in seed sludge listed in Table 1.

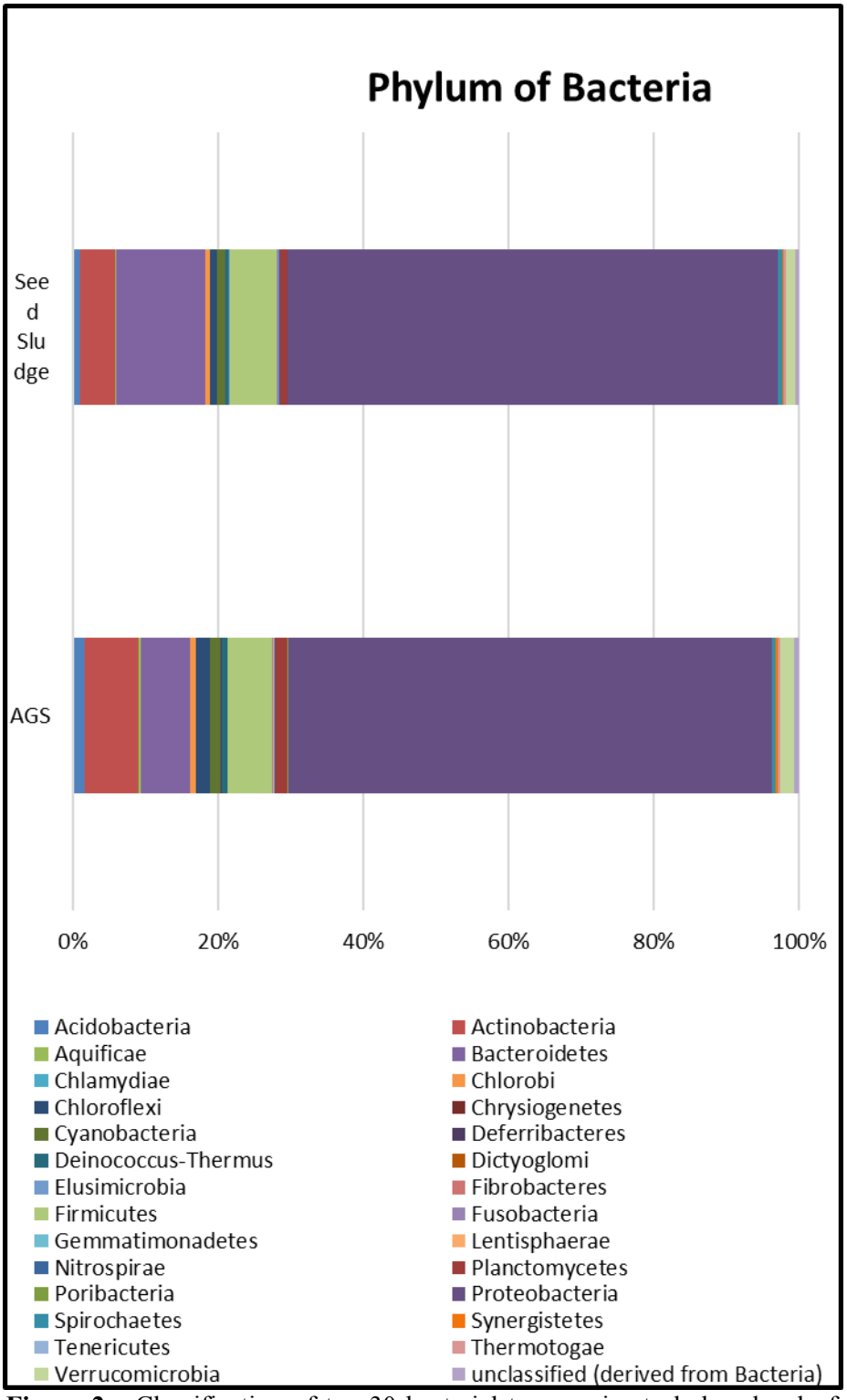

Figure 2a. Classification of top 30 bacterial taxonomic at phylum level of seed sludge and AGS.

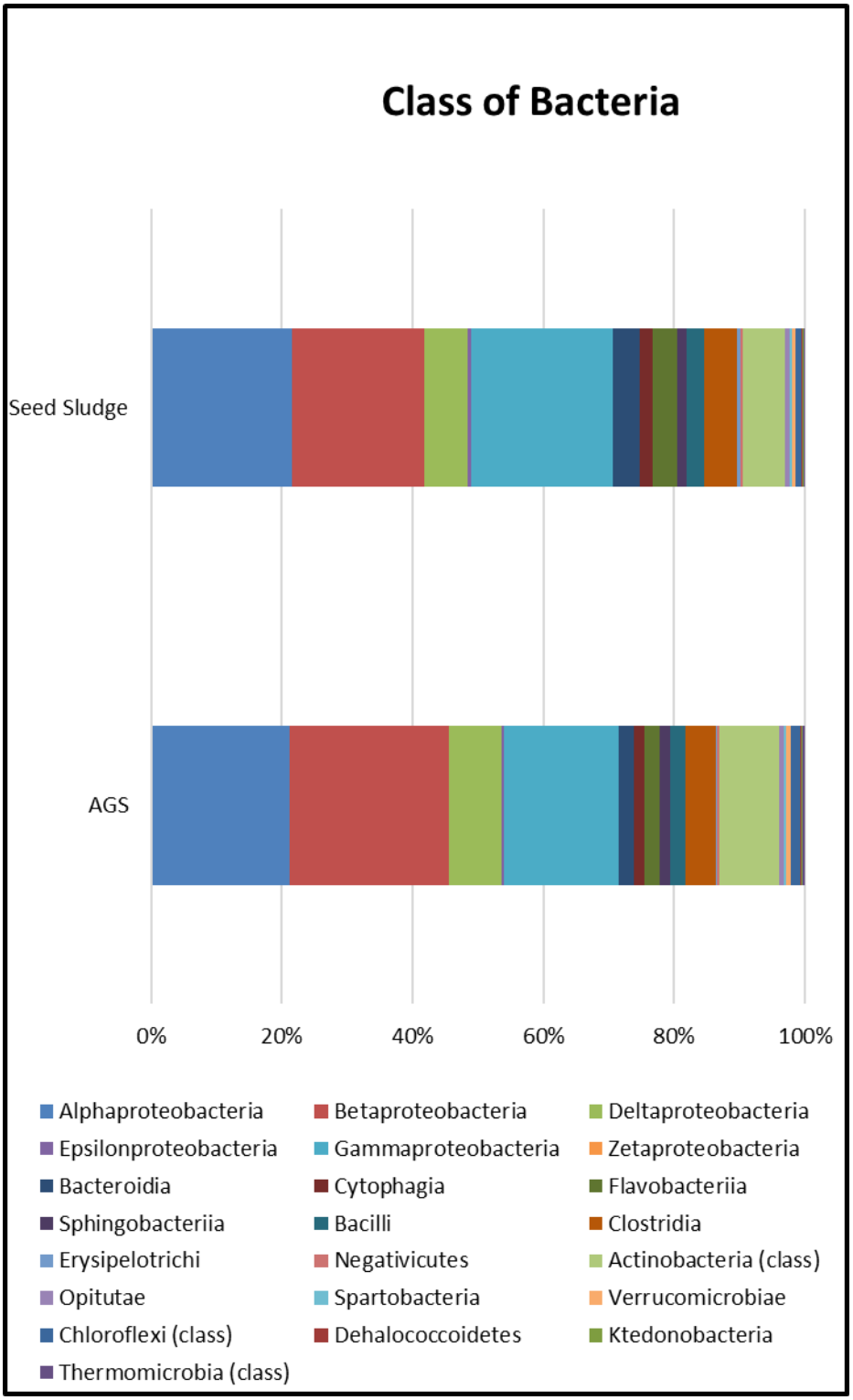

Figure 2b. Classification of top 30 bacterial taxonomic at class level of seed sludge and AGS.

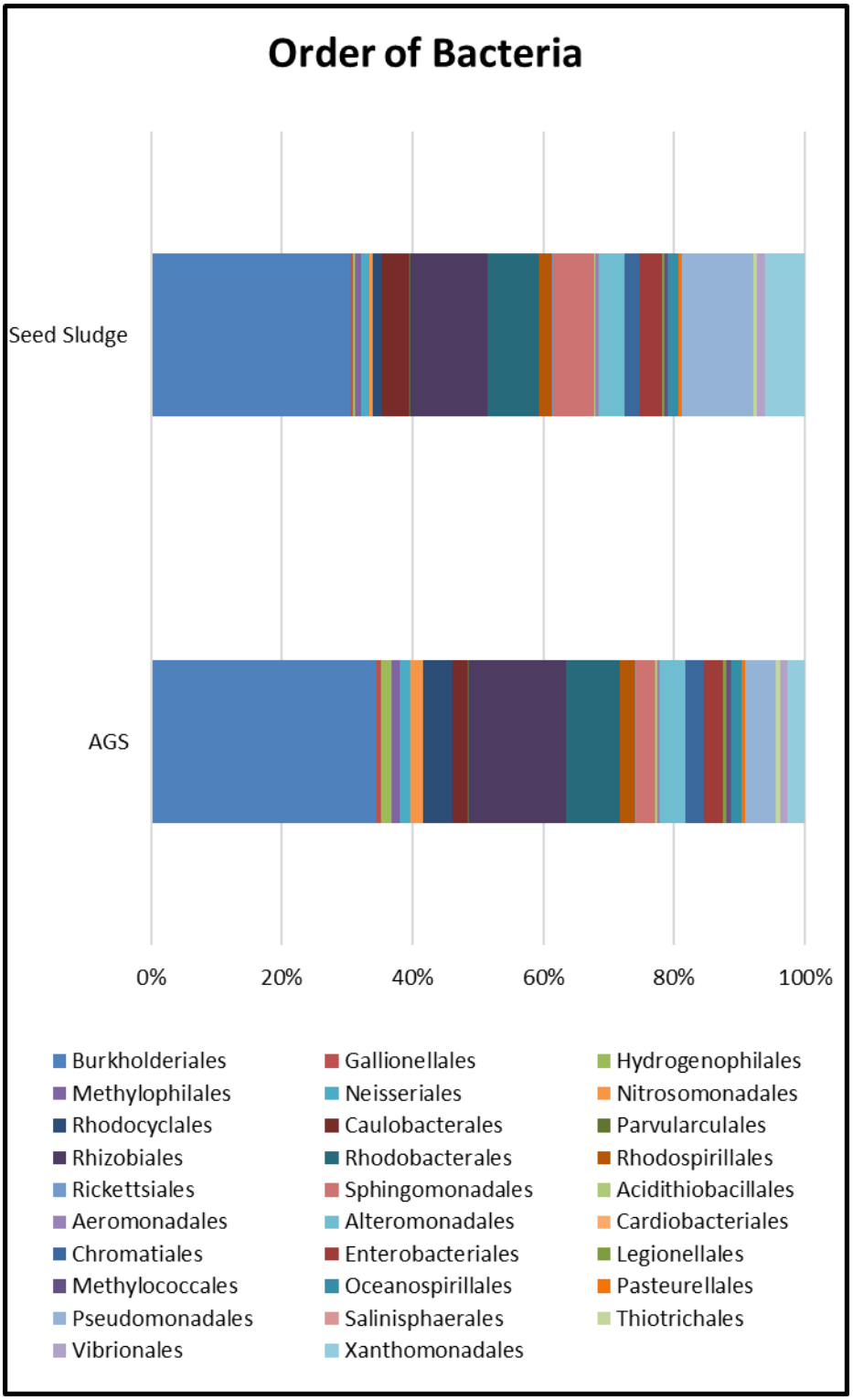

Figure 2c. Classification of top 30 bacterial taxonomic at order level of seed sludge and AGS.

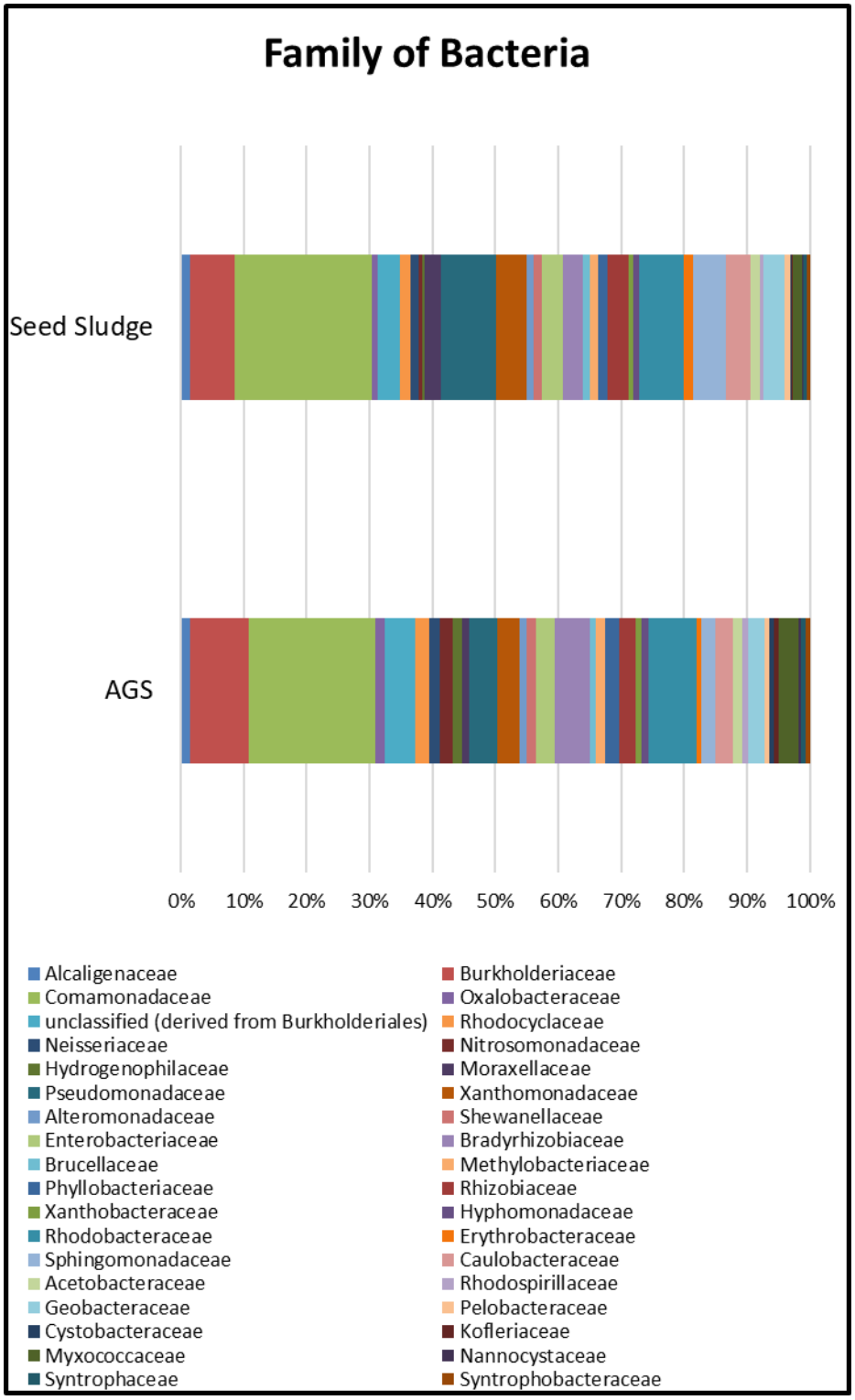

Figure 2d. Classification of top 30 bacterial taxonomic at family level of seed sludge and AGS.

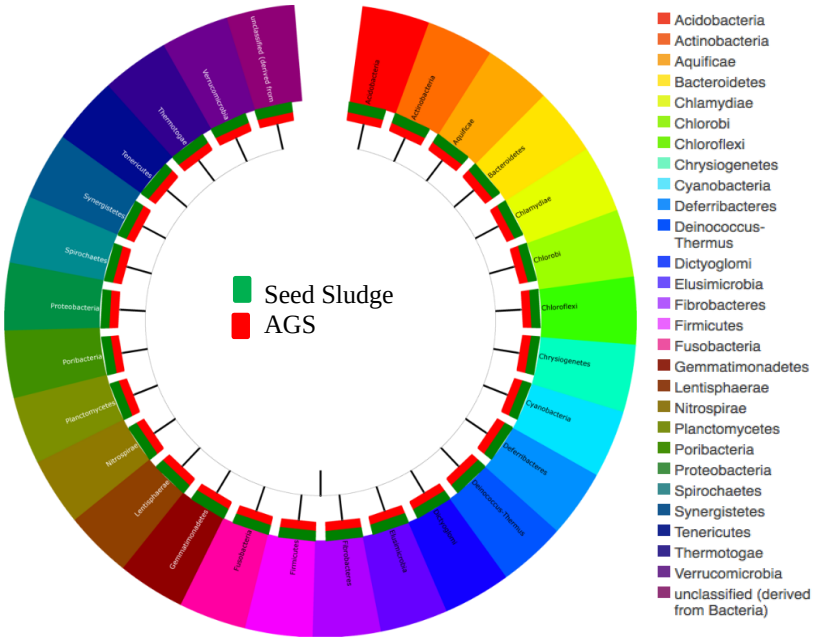

Figure 3. The tree profile of overall bacteria phylum in the seed sludge (green) and AGS (red).

Table 1 Top 15 the most abundant bacteria species from the seed sludge in the SBR.

No	Species	Properties	References
1	***Acidovorax sp. JS42*** Abundance: 504708	Gram negative bacterium Rod-shaped Class of β–*Proteobacteria* Nitroaromatic compound-degrader Most abundance in activated sludge	[12]
2	***Acidovorax citrulli*** Abundance: 399769	Gram negative bacterium Straight to slightly curved rods Aerobic metabolism Utilized carbon source Bacterial fruit blotch	[13]
3	***Polaromonas sp. JS666*** Abundance: 353393	Aerobic metabolism Dechlorinating microorganisms Motile Utilized carbon source Able to degrade xenobiotic compounds	[14]
4	***Flavobacterium johnsoniae*** Abundance: 340326	Gram negative bacterium Rapid gliding motility Aerobic metabolism Utilized polysaccharides	[15]
5	***Albidiferax ferrireducens*** Abundance: 313444	Gram negative bacterium Short rod-shaped Motile Facultatively anaerobic metabolism Able to degrade organic substrate	[16]

No	Species	Properties	References
6	***Comamonas testosterone*** Abundance: 265290	Gram negative bacterium Bacillus-shaped Motile, flagellated, non-spore forming Aerobic metabolism The strongest chromate-reducing bacteria Able to degrade polycyclic aromatic hydrocarbons such as phenanthrene, naphthalene and anthracene Enrich from municipal wastewater	[17]
7	***Methylibium petroleiphilum*** Abundance: 262289	Gram-negative Rod shaped with size from 0.5-2.0 μm Motile and is non-pigmented, aerobic bacteria Found in sites contaminated with aromatic hydrocarbon Able to degrade toxic organic compounds	[18]
8	***Novosphingobium aromaticivorans*** Abundance: 248561	Gram negative bacterium Rod-shaped bacterium Non spore forming rods with single polar flagellum Motile Able to degrade aromatic compounds such as phenol, aniline, nitrobenzene and phenanthrene	[19]
9	***Variovorax paradoxus*** Abundance: 237675	Gram negative bacterium Straight to slightly curved rods Motile, flagellated Aerobic metabolism Able to degrade toxic or complex chemical compounds	[20]
10	***Pseudomonas aeruginosa*** Abundance: 221367	Gram negative Rod-shaped bacterium Facultative bacterium Found in environments such as soil, water, humans, animals, plants, sewage and hospitals Catabolize a wide range of organic molecules, including organic compounds such as benzoate Able to degrade polycyclic aromatic hydrocarbons	[21]
11	***Verminephrobacter eiseniae*** Abundance: 221107	Gram-negative Rod-shaped bacterium Motile with one or more flagella Heterotrophic and aerobic Inhabits the earthworm nephridia (excretory organs present in every segment of the worm)	[12]

No	Species	Properties	References
12	***Leptothrix cholodnii*** Abundance: 213811	Gram-negative Straight rods Motile by one polar flagellum Aerobic bacterium Grow to form filamentous cells Able to oxidize manganese and iron	[22]
13	***Polaromonas naphthalenivorans*** Abundance: 210750	Gram-negative Non-spore forming Non-Motile coccus (without flagella) Aerobic heterotroph Able to remove naphthalene (polycyclic aromatic hydrocarbon) Able to utilize glucose	[20]
14	***Pseudomonas stutzeri*** Abundance: 121123	Gram-negative Rod-shaped bacterium Diverse metabolism Motile Plays role in nitrogen cycle for denitrification process	[23]
15	***Pseudomonas fluorescens*** Abundance: 112822	Gram-negative Anaerobic condition Metabolic capabilities Contribute to plant growth Able to degrade various pollutants and toxins including styrene and polycyclic aromatic hydrocarbons	[7, 24]

Table 2. Top 15 the most abundant bacteria species from the AGS in the SBR.

No	Species	Properties	References
1	***Thauera sp.MZ1T*** Abundance: 208859	Gram-negative bacterium Rods shape Motile Strictly aerobic Chemoorganotrophic using various organic acids, amino acids, and aromatic and aliphatic compounds as sole substrates Enriched groundwater aquifers, rivers, lakes, and pond sediments that are contaminated with aromatic or aliphatic organic compounds or toxic inorganic compounds naturally Able to produce abundant exopolysaccharide and degrade various aromatic compounds with nitrate as electron acceptor	[25,26]

No	Species	Properties	References
2	***Acidovorax sp. JS42*** Abundance: 185041	Gram negative bacterium Rod-shaped Class of β–*Proteobacteria* Nitroaromatic compound-degrader Most abundance in activated sludge	[12]
3	***Methylibium petroleiphilum*** Abundance: 144267	Gram negative bacterium Rod shaped Motile and non-pigmented aerobic bacteria Found in sites contaminated with aromatic hydrocarbon Able to degrade toxic organic compounds	[18]
4	***Alicycliphilus denitrificans*** Abundance: 113317	Gram negative bacterium Rod shaped Motile and non-spore-forming Facultative anaerobe Capable of degrading hydrocarbons such as nitrate or chlorate in environments under both oxic and anoxic conditions	[27]
5	***Leptothrix cholodnii*** Abundance: 106766	Gram-negative bacterium Straight rods Motile by one polar flagellum Aerobic bacterium Grow to form filamentous cells Able to oxidize manganese and iron	[22]
6	***Dechloromonas aromatic*** Abundance: 80140	Gram-negative bacterium Rod shaped cells Motile, forming flagella Found in aquatic and aquatic sediment habitats Oxidizes aromatic compounds such as toluene, benzene and chlorobenzene	[28]
7	***Delftia acidovorans*** Abundance: 69012	Gram-negative Straight to slightly curved rods Motile by means of polar or bipolar flagella Does not produce endospores Aerobic with a strict respiratory metabolism with oxygen as the terminal electron acceptor Found in soil, sediment, water, crude oil, oil brine, various clinical samples and activated sludge Reduces nitrate to nitrite and does not denitrify	[29]

No	Species	Properties	References
8	***Nitrosomonas eutropha*** Abundance: 65463	Gram-negative bacterium pleomorphic shaped cells Anaerobic bacterium Motile, forming flagella Ammonia-oxidizing bacteria Utilize nitrite as an electron acceptor and H2 as reductant	[30,31]
9	***Comamonas testosterone*** Abundance: 56279	Gram negative bacterium Bacillus-shaped Motile, flagellated, non-spore forming Aerobic metabolism The strongest chromate-reducing bacteria Able to degrade polycyclic aromatic hydrocarbons such as phenanthrene, naphthalene and anthracene Enrich from municipal wastewater	[17]
10	***Cupriavidus metallidurans*** Abundance: 47325	Gram-negative Rod shaped Motile and non-spore forming bacteria Facultative anaerobic Found in industrial sediments or wastes, which contain high heavy metal concentration Able to resist toxic heavy metals	[32]
11	***Chitinophaga pinensis*** Abundance: 44475	Gram negative bacterium Bacillus-shaped Filamentous Aerobic bacterium Biomass degrader and chitin degradation Efficiently degrade plant-derived carbohydrates	[33]
12	***Myxococcus xanthus*** Abundance: 42284	Gram negative bacterium Rod-shaped Motile and ubiquitous soil bacterium Abundant in the soil reaching high densities per gram of soil Secretes many compounds that have antibiotic properties, such as myxalamid during predation	[34]
13	***Pirellula staleyi*** Abundance: 36578	Gram negative bacterium Bacillus-shaped Motile cells Aerobic heterotrophic bacterium High inorganic sulphate concentration	[35]

No	Species	Properties	References
14	***Methylococcus capsulatus*** Abundance: 35592	Gram negative coccus-shaped bacterium Aerobic bacterium Oxidize greenhouse gas Capable of nitrogen fixation Able to perform methane oxidation	[30]
15	***Pseudomonas aeruginosa*** Abundance: 33769	Gram negative Rod-shaped bacterium Facultative bacterium Found in environments such as soil, water, humans, animals, plants, sewage and hospitals Catabolize a wide range of organic molecules, including organic compounds such as benzoate Able to degrade polycyclic aromatic hydrocarbons	[21]

4. Discussions

4.1. Microbial Community in Seed Sludge and AGS

The shifted and changes in microbial communities throughout the granulation process was due to the operational parameter in the treatment system such as settling, flocculation and floc formation characteristics. This is in agreement with More et al. [36], the changes of the operational parameter in the treatment system control the microbial diversity.

Archaea consists of greater domain distribution in AGS compared to seed sludge in the system. These results are consistent with Dang et al. [37] whereby the diversity of archaea in AGS was higher compared to the seed sludge. Nevertheless, as previously revealed by Winkler et al. [38], archaea were not found in the flocculent sludge but were present in small amount in the granular sludge. This is presumably due to AGS offer better growing conditions for archaea. Moreover, archaea were found to be favourable in maintaining pH conditions for the formation of inorganic precipitates by converting volatile fatty acids to methane [39].

In the present study, eukaryota found as the minority population in the seed sludge and AGS samples. In general, eukaryota organisms fulfill a wide variety role in biomass conversion, water clarification process and involved in the formation and structure of AGS (Weber et al., 2007). Additionally, eukaryote involved in synthesize proteins and nuclei acids. Meanwhile, eukaryota organisms increased in AGS probably due to the role of eukaryota organisms in developing the AGS. Stalked ciliates of the eukaryota organisms were involved in the granulation process. As been reported by Li et al. [40], low organic loading rate (OLR) favourable to eukaryote species to the formation of large filamentous granules.

The detection of pathogenic viruses in the wastewater is a fundamental component of health monitoring. It is vital to significantly reduce and remove the pollutants including viruses and pathogenic organisms in wastewater treatment. However, increasing in virus organism in AGS have been observed and may result in waterborne diseases caused by pathogens and viruses [41]. The small amount of virus increase in AGS can be neglected. Furthermore, biological treatment processes are not always successful in removing pathogens and viruses, hence chemical disinfectants is required to be added to the treatment process [42].

The most dominant microbial in the seed sludge and AGS sample were bacterial community. The evolution of bacteria in the seed sludge during formation of AGS were identified in this study. The diverse bacterial community were nominated in the seed sludge while the percentage of bacterial community decreased in AGS. Basically, bacteria are the most versatile of all organisms in terms of

their nutrient requirement, mechanism, cell lysis and metabolic activity [38]. Moreover, bacterial community that resided in the seed sludge and AGS were not equally distributed. Some of the bacteria were sharing the same functionality and interacting to each other. Therefore, the ideal condition is required for microbial communities to grow excellent aggregation bacteria. Furthermore, ecological niches are important in order to maintain the bacterial diversity in the system [43].

4.2. Bacterial Diversity in Seed Sludge and AGS

Bacteria diversity in seed sludge and AGS were significantly different at all taxonomy levels. This showed there were immense diversity of bacteria in both samples. The diverse bacterial community present in the system signified in the formation of AGS [44]. Typically, the major populations of bacteria in seed sludge appeared differently from those in AGS. The bacteria that found in the seed sludge were mostly preserved throughout the granulation. Therefore, high selection pressure facilitating the growth of certain bacteria which survive in nature by cellular motility. This is consistent with the finding obtained by Hu et al. [45] which reported the essential of cellular motility with the ability to seek out favourable environment and avoid hazardous situation.

In Phylum level, Proteobacteria was the dominant bacteria in both seed sludge and AGS. Similar observation was reported by Jiang et al. [24] that the dominancy of Proteobacteria implicated in phenol degradation in activated sludge. Under a strong selective pressure of short settling time at 8 hours cycle time, the microorganism was washed out together with light biomass and the dense biomass retained in the reactor together forming microbial colony to develop the AGS [46]. This further indicated that the high abundance of Proteobacteria in the sludge could be regarded as a functionally dominant and might have contributed significantly to development of granules by secreting EPS. Nevertheless, dominancy of Proteobacteria in the system were crucial for biodegradation of organic pollutant such as dyes and aromatic compounds [47].

Bacteroides can produce acetic acid, succinic acid, propionic acid and a mixture of gasses [48]. Concurrently, excessive Bacteroides phylum can destroyed the structure and stability of the aerobic granules [49]. Moreover, high abundance of Bacteroides play an important role in nitrogen and phosphorus removal. Furthermore, Bacteroides has been detected at the core of the granule where oxygen is limited [4]. Additionally, Guo et al. [50] confirmed that a number of bacteria classified under the phylum of Bacteroides were the main EPS inducer to increase cell hydrophobicity during the flocculation and granulation process of aerobic granules.

The abundance of Actinobacteria in AGS contribute to the producing intracellular storage compound and able to remove sulphate in wastewater [51]. The present of Actinobacteria were beneficial for the development of AGS. Dahalan [4] found that Actinobacteria signified in phototrophic condition used to develop photosynthetic AGS. Furthermore, Song et al. [52] reported that Actinobacteria have important roles in the formation of aerobic granular sludge when all the bacteria extracted from the aerobic granular sludge belonged to the class of Actinobacteria.

4.3. Role of Abundance Bacteria in Aerobic Granulation

Generally, the mechanism of AGS formation is influenced by various factors including the selection pressures in the SBR which promote immobilization of microbial in seed sludge to finally form AGS. The accumulation and aggregation of microbial during granulation process enhanced the formation of granules. The presence of EPS has a significant influence on the microbial aggregates such as surface charge, flocculation, settling properties and adsorption ability [53]. Nevertheless, EPS accelerate the formation of microbial aggregates such as AGS through binding cell closely [54]. EPS is produced and secreted by microbial and can affect the morphology of AGS and also improving the efficiency of wastewater treatment. Therefore, high microbial diversity gives high amount of EPS secretion.

Table 3 listed the most abundance of AGS developing bacteria present in seed sludge and AGS. High total percentage abundance of the AGS developing bacteria was determined in developed AGS with 68.95% compared to the percentage of 25.10% in the seed sludge. These results proved that the dominancy of AGS developing bacteria are highly related to the formation of AGS in livestock wastewater. As determined, most of the AGS producing bacteria responsible in secreted EPS consists of protein, polysaccharides, humic acid, nuclei acid and carbohydrates. However, environmental niche

was also major consideration for the growth of AGS developing bacteria. Further, the AGS developing bacteria Bacteroides, Flavobacterium, Comamonas, Pseudomonas and Acinetobacter were the most abundance bacteria observed in the AGS compared to the seed sludge.

Bacteroides were the most abundance of AGS developing bacteria in AGS with greater differences in percentage of 17.26%. It is appealing to correlate microbial evolution to the formation of Bacteroides. High diversity of Bacteroides in AGS presumably due to gradual development of Bacteroides due to ecological niche in the system. More precisely, the growth of microorganisms was promoted by the nutrient of livestock wastewater. In addition, Bacteroides were found at a depth of 800 μm in the granules has been discovered by [48] by using fluorescence in situ hybridization (FISH). The bacteria grown in the core layer of granules was beneficial for the strengthened of AGS structure. As described by Gao et al. [55], facultative and anaerobic bacteria resided in the core of granules.

Flavobacterium, Comamonas and Pseudomonas dominated the reactor after granulation but less diverse in the seed sludge. This is consistent with the finding by Li et al. [17] that discovered the existence of those bacteria in the granules. Flavobacterium and Pseudomonas are significant in utilizing polysaccharides and known for their production of glue-like extracellular polymers and ability to bind cells together [17]. Significantly, distribution of EPS over the entire AGS is very important to structuring the granules. The evolution in microbial community during granulation towards the end of the study could be due to the microbial attachment and detachment processes allowing in the formation of AGS. On the contrary, Flavobacterium and Pseudomonas were previously reported to have no contribution towards AGS formation [56]. Furthermore, Comamonas bacterium was revealed in contributing to the granule stability [57]. Although Hyphomicrobiaceae were not dominant in AGS, it also appeared to be responsible for forming and maintaining the granule structure Zhang et al. [58].

4.4. Role of Bacteria in Removal Performance

The majority of bacteria were either dominant species in the seed sludge or gradually migrated as dominant culture in AGS. More than 90% of bacteria were found to be dominant in the seed sludge and AGS hence bacteria play the most important role in degrading organic and nutrient removal in the wastewater. Significantly, most of the bacteria potential in the degradation of COD, TN and TP whereas some of bacteria have not discovered yet. Table 4 summarize the potential degrader bacteria or organic and nutrient removal in the seed sludge and AGS. The total amount of performance degrader bacteria in AGS were 30.4% higher than in the seed sludge.

Table 3. AGS developing bacteria in the SBR system of seed sludge and AGS.

No	AGS developing bacteria	Properties	% abundance		References
			SS	AGS	
1	*Bacteroides*	able to produce acetic acid, succinic acid, propionic acid and gasses	4.55	21.81	[48,59]
2	*Flavobacterium*	able to produce EPS utilize polysaccharides export protein across cytoplasmic membrane	2.48	14.38	[15]
3	*Comamonas*	high extrapolysaccharide content structuring and stabilize AGS	3.97	11.21	[24]
4	*Pseudomonas*	produce glue-like extracellular polymers	2.38	9.35	[60]

No	AGS developing bacteria	Properties	% abundance		References
			SS	AGS	
5	*Acinetobacter*	exhibited high auto-aggregation potential with interconnecting fibrils rapidly form AGS	1.02	6.87	[61]
6	*Thiobacillus*	oxidize sulphur compounds, allowing it to grow on a much larger variety of nutrient denitrifying bacteria able to transforming sulphide into elemental sulfur	7.58	2.16	[62]
7	*Escherichia*	able to transforming sulphide into elemental sulphur	0.71	1.28	[52]
8	*Hyphomicrobiaceae*	glued together by EPS to form granules excrete EPS, allowing attachment to surface-associated nutrients	0.87	0.79	[63]
9	*Rhodococcus*	produce EPS that lower the cell surface hydrophobicity and function as a hydrophilin	1.02	0.48	[64]
10	*Lactobacillus*	assimilation of carbohydrate producing EPS	0.35	0.37	[59]
11	*Citrobacter*	opportunistic pathogens capable degrading color of wastewater	0.18	0.25	[11]
Total % abundance of AGS developing bacteria			**25.10**	**68.95**	

Table 4. Performance degrader bacteria in the SBR system of seed sludge and AGS.

No	AGS developing bacteria	Properties	% abundance		References
			SS	AGS	
1	*Thauera*	Biological organic oxidation Nitryfying/denitrifying process	2.90	14.75	[65]
2	*Comamonas*	Denitrifying bacteria Aromatic compound degrader	3.97	11.21	[66]
3	*Pseudomonas*	Heterotrophic ammonium oxidation COD and phosphorus degrading bacteria Denitrifying bacteria Heterotrophic nitrifier and aerobic denitrifier	2.38	9.35	[60]
4	*RhodoPseudomonas*	Photosynthetic bacteria Able to utilize organic compounds	3.97	6.14	[4,67]

No	AGS developing bacteria	Properties	% abundance		References
			SS	AGS	
5	*Dechloromonas*	Anaerobically synthesize PHA Aerobically accumulate polyphosphate Enhanced biological phosphate removal	8.06	5.66	[58]
6	*Candidatus Solibacter*	GAO bacteria Breaking down organic carbon Remove nitrate and nitrite	3.81	5.15	[32]
7	*Candidatus Accumulibacter*	PAO bacteria Remove phosphorus Take up phosphorus	2.60	3.38	[68]
8	*Nitrobacter*	Capable of utilizing organic compounds Oxidation of nitrite to nitrate	1.61	2.80	[38]
9	*Desulfovibrio*	Sulfate-reducing bacteria Nitrogen degrading bacteria	2.26	2.26	[69]
10	*Nitrospira*	Remove nutrients Ammonia and nitrite oxidising bacteria	0.02	1.28	[70]
Total % abundance of degrader bacteria			**31.58**	**61.98**	

In the present study, performance degrader bacteria including Thauera, Comamonas, Pseudomonas, RhodoPseudomonas, Candidatus Solibacter, Candidatus Accumulibacter, Nitrobacter and Nitrospira revealed a greater diversity in the AGS than in seed sludge. Biological denitrification processes convert nitrates into nitrogen gas and nitrous oxide [71]. Denitrification is important to reduce concentration of TN in the effluent discharge of wastewater treatment system. The denitrifying bacteria proliferate and develop into mature AGS in this study were Thauera, Comamonas, Pseudomonas, Candidatus Solibacter, Nitrobacter, Desulfovibrio and Nitrospira. Majority of the performance degrader bacteria were belong to the nitrifying and denitrifying bacteria. According to the Zhang et al. [58], Thauera and Nitrospira were mainly responsible for nutrient removal. Meanwhile, Liu et al. [72] examine that the present of Thauera essential in the COD removal. Furthermore, Thauera have been discovered secreted EPS including galacturonic acid and amino sugar [26]. Thauera was also observed capable of gelatinously agglomerating in the liquid medium [73]. Satisfyingly, Thauera was observed with high percentage of 14.75% in AGS sample which promising the efficiencies of nutrient removal and also development of AGS.

Candidatus Accumulibacter is important in accumulating phosphate in wastewater and known as PAO. In the study PAO bacteria shows greater percentage in AGS as compared to in the seed sludge. This revealed that the development of AGS led to the good nutrient removal performance in the system. PAO used both nitrite and nitrate for phosphorus removal in AGS which is crucial for biological nutrient removal (BNR) process. For instance, Candidatus Accumulibacter proliferated as soon as AGS formed under anaerobic conditions [74]. Candidatus Accumulibacter mainly located at the outer layer of granules with the ability to use oxygen and nitrate for phosphate uptake [75]. In contrast, Accumulibacter community only able to reduce nitrite but not nitrate [76].

During aerobic conditions, nitrification is the dominant process while anaerobic conditions involved denitrification process using autotrophic nitrifying and heterotrophic denitrifying bacteria. Furthermore, Nitrosomonas, Nitrobacter and Nitrospira were reported as the primarily bacteria that involved in the TN removal of high ammonia concentration [77]. However, Nitrobacter and Nitrospira

present the less percentage amount of bacteria in AGS as well as in the seed sludge. In this study, Nitrosomonas also consists of limited bacterial amount in the AGS hence, the amount of Nitrosomonas have been neglected. Although greater amount of other nitrifying and denitrifying bacterial in the AGS, unsatisfactory of TN removal performance have been observed. This proved that Nitrosomonas, Nitrobacter and Nitrospira play important role in TN removal performance than other nutrient degrader bacteria present in this study.

5. Conclusions

The metagenome DNA sequencing analysis has revealed an abundance of microbial diversity in the seed sludge and 8 hours AGS samples. The metagenome analysis discovered wide variety of microorganism including archaea, bacteria, eukaryote, and virus. Bacteria has been evaluated as the most dominant microbial in both seed sludge and AGS. The results from this study also indicated that distinct differences of microbial community from the seed sludge and AGS was obviously observed which provided some evidence of the granulation process. The details of phylogenetic bacterial consists of phylum, class, order and family were identified and compared between the seed sludge and in AGS. This shows the evolution of bacterial population was change towards the formation of AGS. Acidovorax sp JS42 was found to be the most abundance bacteria species in seed sludge while Thauera MZIT was the most abundance bacteria species in AGS. Whereas, Bacteroides, Flavobacterium, Comamonas, Pseudomonas and Acinetobacter were the most abundance bacteria that responsible in developing AGS were observed to be higher in AGS compared to the seed sludge. Meanwhile, the performance degrader bacteria including Thauera, Comamonas, Pseudomonas, RhodoPseudomonas, Candidatus Solibacter, Candidatus Accumulibacter, Nitrobacter and Nitrospira revealed a greater diversity in the AGS than in seed sludge. This has been confirmed that the formation of AGS was important for the removal performances since the greater diversity of bacteria population residing in the granules.

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