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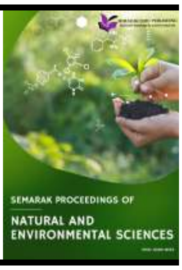

Revealing the Bacterial Community Structure of Aerobic Granular Sludge for Agro-based Wastewater Treatment

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ABSTRACT

This study examined the development and composition of bacterial communities in an aerobic granular sludge bioreactor using Illumina high-throughput sequencing. Mature granules with a compact structure were obtained after 90 days of bioreactor operation, with the organic loading rate varying between 2.5 and 3.3 kg COD m^{-3} d^{-1}. Cocci-shaped microbes were observed to cover the outer surface of granules. Reliable COD and simultaneous nitrogen and phosphorus removal efficiencies were achieved during the steady-state period. Metagenomic sequencing analysis revealed that *Pseudomonas* (34.05%) was the most dominant genus, followed by *Bacteroides* (10.27%) and *Clostridium* (2.68%). Several microorganisms were identified as functional bacterial groups responsible for pollutant removal, contributing to organic and nutrients biodegradation. Exopolysaccharide-producing bacteria, accounting for 70.71% of the total abundance, appeared to be responsible for the formation of granules, and the stability of the microbial granulation reactor.

Keywords: Aerobic granular sludge; bacterial community; next generation sequencing

1. Introduction

Aerobic granular sludge (AGS) is a favorable biological wastewater treatment technology that has shown excellent performance in the treatment of high strength wastewater due to its advantages of fast settling velocity, dense microbial quantity, compact and strong structure. Simultaneous carbon, nitrogen and phosphorus removal can be achieved by granular sludge through degradation, nitrification, denitrification, and phosphorus uptake. Aerobic granules are aided by bacterial consortia consisting of both heterotrophs and autotrophs. These bacteria often reside within different layers of aerobic granules, which are capable of oxic, anoxic and anaerobic metabolisms.

Detailed investigations have been conducted by several researchers using microscopic analysis and biomolecular techniques to gain better understanding of the mechanisms underlying AGS formation [1]. The bacterial community structure has a significant influence on sludge performance and granulation. Notably, the morphology of AGS is highly influenced by reactor operation, feeding composition and association of microbial community. It was reported that the phosphorus accumulating organisms *Accumulibacter*, which contributes to the formation and stabilization of AGS, increased in abundance from 0.4% in the initial sludge to 10.2% after 60 days of cultivation [2].

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Although numerous studies have explored bacterial diversity, limited research has been performed on revealing the bacterial community during aerobic granulation process conducting the agro-based industrial wastewater treatment, nor on identifying the functional exopolysaccharide bacterial groups within the biological granular reactor. Therefore, the objective of this study was to investigate granular sludge formation using real agro-based industrial wastewater for simultaneous organic and nutrients removal. In addition, the bacterial population structure and characteristics during the aerobic granulation were investigated using high-throughput next generation sequencing.

2. Methodology

2.1 Experimental set-up

The experimental set-up and bioreactor operation were conducted following the procedures described by [3]. At the end of the experiment, a complete genomic analysis consists of DNA extraction and high-throughput sequencing was carried out to characterize the microbial composition of the biomass. Finally, the granule layered structure and the exopolysaccharides (EPS) producing bacteria in the bioreactor were evaluated.

2.2 DNA extraction

The granules samples were collected from the bioreactor on day 90, when the sequencing batch bioreactor operation system achieved steady-state condition. The microbial genomic deoxyribonucleic acid (DNA) of the aerobic granular sludge sample was extracted by alkaline lysis method using GF-1 Soil Sample DNA Extraction Kit (Vivantis, Malaysia) according to the manufacturer’s protocol. The bacterial diversity was determined by next generation sequencing procedures (Illumina, USA). The extracted DNA was first purified by electrophoresis where its solution was processed into a 1% (w/v) agarose gel, and the DNA was visualized under ultraviolet (UV) light. The isolated DNA was evaluated via spectrophotometry to measure the concentration and for further purification using Nanodrop 1000 V3.7 Spectrophotometer (Thermo Scientific, USA).

2.3 High-throughput sequencing

The prepared DNA of aerobic granular sludge was sequenced using Illumina high-throughput sequencing on the HiSeq 2000 platform through paired-end sequencing on one lane to generate 100 bp paired-end DNA fragment sequences in fastq format. The raw sequences contaminated by adapter or containing three or more unknown nucleotides (‘N’) were removed via the quality control pipeline. In the quality control pipeline options, the artificial replicate sequences produced by sequencing artifacts were also removed following [4]. The data were filtered based on sequence ambiguity base (non-ACGT) count when no quality score information is available. In addition, removal of low-quality sequences was done using a modified dynamic trim with the lowest phred score = 15 was counted as a high-quality base and sequences were trimmed when 5 bases were below the above-specified quality. This was to ensure that all the data sets generated during the next generation sequencing using the Illumina HiSeq 2000 were of high quality.

The quality-filtered Illumina reads of the aerobic granular sludge samples were submitted to the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST), v3.3.7 server at http://metagenomics.anl.gov/. The functional annotation is under accession name of UTM wastewater treatment plant project:5465. MG-RAST uses similarity-based binning algorithms which refer to the process of sorting DNA sequences into groups that might represent an individual genome or genomes from closely related organisms. For functional annotation, the reads were searched against GenBank database with the default settings on MG-RAST of a maximum e-value of 10^{-5}, a minimum identity of 60% and a minimum alignment length of 15 measured in amino acid for protein and base pair for RNA databases. Finally, the diversity of the consortium in the SBR was assessed.

3. Results & discussion

3.1 Granule layered structure

The structure of aerobic granules was suggested build with different layers of biomass regions as illustrated in Figure 1. The bacterial distribution in each biomass layers, namely aerobic, anaerobic, and facultative anaerobic, was quantified and expressed as a percentage. The structured layers of aerobic granules, consisting of aerobic, anaerobic, and facultative microbial, observed in this study were consistent with the findings of [5]. The typical depth of aerobic zone ranged between 50 to 200 µm, while anaerobic zone was detected between 800 to 900 µm from the granule surface. However, the depth of each zone varies depending on oxygen penetration into the granule and the microbial metabolism activity. The microorganism's association and bacterial community assembly in every layer is essential for the formation of granules to obtain compact, dense, and stable granular sludge. The bacterial community structure is also responsible for the degradation of organic matter and nutrients removal during wastewater treatment.

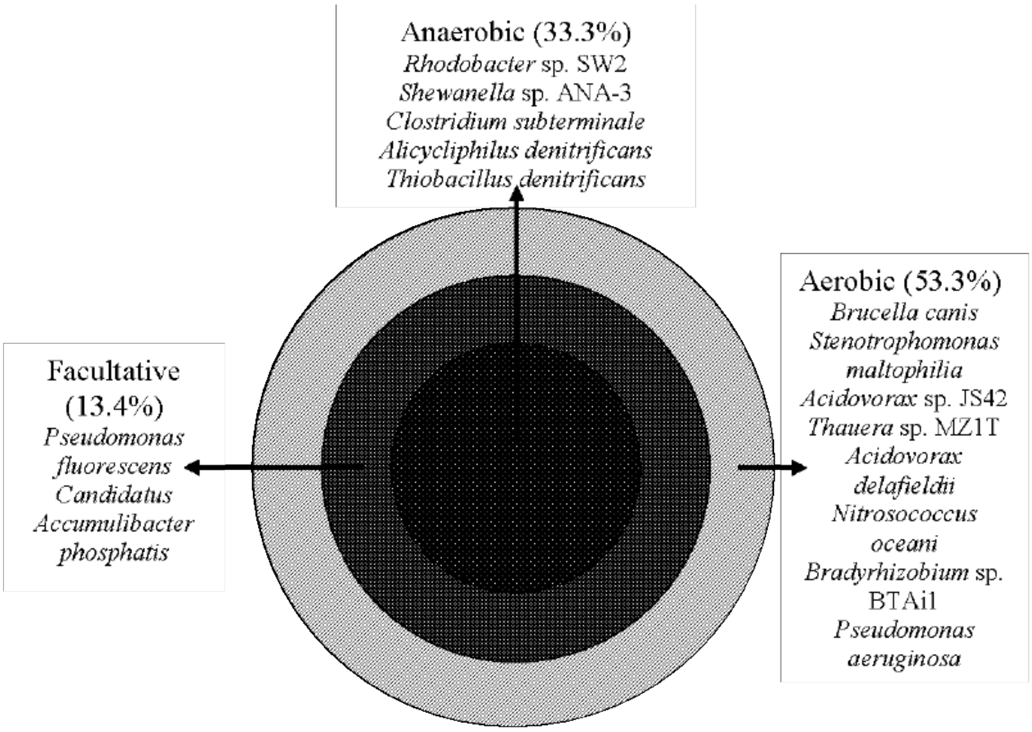

Fig. 1. Distribution of identified bacteria in aerobic granular sludge

From the illustration of layered structure of the granules, 53.3% of the identified bacteria were aerobic bacteria that dominated the outer layer of the aerobic granules. These bacteria are fast-growing heterotrophs, where both oxygen and ammonia concentrations and detachment rates are high. These bacteria dominated the outer layer of the aerobic granules, which were in close proximity to a high oxygen supply. Meanwhile, 13.4% of the identified bacteria were facultative bacteria and those facultative and anaerobic bacteria grown in the core of the granules. The anaerobic conditions are beneficial for the survival of facultative bacteria and strengthens the removal capacity of nitrogen and phosphorus in a single SBR cycle. Most denitrifying bacteria are facultative, and these bacteria use organic carbon as the electron donor for energy generation and growth. It was also found that 33.3% of the bacteria identified were anaerobic bacteria. Significantly, anaerobic bacteria have been found in various aerobic granulation systems and based on bacteria growth preferences, it is suggested that the inner part of the granules is favourable for the growth of these types of bacteria.

3.2 Exopolysaccharide-producing bacteria

Exopolysaccharide (EPS) is one of the important factors that enhance the aggregation of bacterial cells during sludge granulation process, and it is secreted by microbes as the general attributes of the bacteria’s exposed environment. The higher number of microorganisms in an AGS leads to a higher amount of EPS secretion, which offers an advantage by improving granule density, structural properties and settling velocities. EPS, a sticky material that is secreted by microorganisms as metabolic products that facilitate the cell-to-cell adhesion to aggregate the microbial biomass, which is crucial for start-up of granulation process.

Table 1 lists the EPS producing bacteria identified in the aerobic granular sludge bioreactor. The total percentage abundance of EPS producing bacteria in aerobic granular sludge was 70.71%. The high percentage of EPS bacterial population in aerobic granules lightened the assumption of high EPS bacterial existence in SBR, which supported the granulation of aerobic granular sludge. This indicates that the EPS bacteria population is related to the physiological state of bacteria which is affected by the operating conditions to develop aerobic granules. As the EPS producing microbial, *Pseudomonas* (34.05%), *Bacteroides* (10.27%), *Clostridium* (2.68%), *Citrobacter* (2.19%), *Flavobacterium* (1.92%), *Agrobacterium* (1.70%), *Escherichia* (1.52%), *Rhizobium* (1.33%), and *Thauera* (1.28%) were the most abundant bacteria identified in aerobic granular sludge.

Table 1
Phylogenetic classification of the bacteria involved in aerobic granulation at genus level

EPS microbial	Abundances (%)
Pseudomonas	34.05
Bacteroides	10.27
Clostridium	2.68
Citrobacter	2.19
Flavobacterium	1.92
Agrobacterium	1.70
Escherichia	1.52
Rhizobium	1.33
Thauera	1.28
Azotobacter	1.08
Vibrio	1.27

Sphingomonas	1.11
Serratia	1.01
Yersinia	1.08
Rhodococcus	0.96
Enterobacter	0.85
Proteus	0.32
Hyphomonas	0.88
Klebsiella	0.87
Acinetobacter	0.83
Aeromonas	0.62
Bacillus	0.60
Chryseobacterium	0.59
Arthrobacter	0.42
Pantoea	0.39
Corynebacterium	0.37
Bifidobacterium	0.16
Legionella	0.15
Staphylococcus	0.11
Lactobacillus	0.10

Thauera organisms possess numerous physiological properties in biological nutrient removal systems of wastewater treatment plants. These bacteria produce EPS and store polyhydroxyalkanoates (PHA) when exposed to high organic loads under aerobic conditions, forming flocs or biofilms. Feast-famine regimes and high shear stress applied on the reactor system would also trigger and stimulate EPS production during granulation process. *Thauera* sp. have been described as a floc-forming bacterium and could produce copious quantities of EPS from relatively simple short chain fatty acids. [6] reported that a high abundance of Betaproteobacteria such as *Thauera, Zoogloea* and other unidentified *Rhodocyclaceae* are present in hydrophobic population of aerobic granular sludge.

Phylum Firmicutes identified in aerobic granular sludge included *Bacillus, Clostridium* and *Staphylococcus*, which produced EPS for microbial aggregation. These genera accounted for 0.60%, 2.68% and 0.11% of the abundance in aerobic granules, respectively. Hence, the existence of EPS producing bacteria from the phylum Firmicutes in aerobic granular sludge is beneficial for granulation. Meanwhile, prokaryotes with gram-positive rods bacteria such as *Bacillus* and Clostridium with rigid cell walls could secrete EPS that are widespread among the microorganisms. Mixed culture consortium of *Staphylococcus* and *Pseudomonas* were able to produce high amounts of exopolysaccharides. A significant number of cells can produce exopolysaccharides associated with biofilm and granular formation. The Pseudomonas genus also contains versatile bacteria capable of performing both nitrification and denitrification.

4. Conclusions

A laboratory SBR was fed with high-strength wastewater (OLR of 2.5-3.3 kg COD m^{-3} d^{-1}) and operated for 90 days to identify the community assembly during aerobic granulation. In-depth analysis of the bacterial communities, using high-throughput sequencing, was performed to investigate the interplay between the microbial community structure, reactor performance and the stability of granular sludge. The diversity of bacterial communities was obtained in the bioreactor when it reached steady state conditions, presumably due to the complexity of substrates in the influent and as the granules formed. The genus that mostly dominated the bacterial communities is *Pseudomonas* by the end of the experiment. These genera have similar roles, namely EPS production, nitrification, and denitrification. EPS plays an important role in granulation process and formation, the aerobic condition selects for heterotrophic bacteria, while the anaerobic feeding favours bacteria with storage capability. In every functional group, numerous persistent and common species were also found (aerobic, anaerobic, facultative, nitrifying bacteria group). This implies that, regardless of fluctuated OLRs, functionally redundant microbiomes accumulated during aerobic granulation.

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