Analytical Technologies for Water Treatment Biofilms

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Abstract

When treating wastewater by using microbe ability to purify water, it is important to maximize their biological functions. In the field of advanced waste water treatment, experiments for the artificial control of biofilms formed with microbes have recently received high attention because these biofilms are the source of major purification functions for wastewater treatment. There are currently new research programs in progress for wastewater treatment technologies by combining the different fields of studies like genetic engineering and biochemical engineering.

In order to attain long-term stability of waste water treatment systems and achieve further improvement of water processing efficiencies, we are now working on basic technology development of the analysis of biofilms and research of biofilms application to waste water treatment systems. As one of our R&D results, we will acquire information on the ecological structure of anaerobic ammonium oxidizing bacteria granule and on its forming mechanisms.

1 Preface

In order to maintain long-term stability in biochemical wastewater treatment systems and improve water processing efficiencies, it is necessary to control the parameters of environmental factors to control the wastewater treatment tank as well as examine the ecological structure of biofilms and its functions[(1)]. Through a joint research program with Nagoya University, we established a biofilm analysis technology as a basic technology for biological wastewater treatment and are working on its application to our wastewater treatment systems.

As an example of an application of biofilm analysis technology, this paper introduces an analytical result of anaerobic ammonium oxidizing bacteria biofilms cultured in our wastewater treatment systems.

2 Biological Wastewater Treatment

2.1 Water Purification by Microbes

In wastewater treatment, the most common treatment method is biological. It artificially and efficiently promotes a part of natural water purifications. Under a biological treatment method, a complicated microbial ecosystem is formed by various kinds of microbes such as bacteria, fungi, protozoa, micrometazoa, etc., and removes organic substances.

We will explain the organic substances intake mechanism and the relationship among microbes in wastewater. Bacteria and fungi function to remove organic substances from wastewater, but protozoa do not take up any organic substances. The program, however, takes up floating bacteria and fungi. Just like protozoa, micrometazoa do not remove organic substances, but take up dead bodies of the protozoa and bacteria[(2)]. In a natural environment, bacteria plays a major role in removing organic substances from wastewater, produces biofilms, and helps various kinds of bacteria live together. Fungi, protozoa, and micrometazoa cling to these biofilms and proliferate there so that they finally establish microbial biofilms that effectively work its advanced water purification function[(3)].

2.2 Structure of Biofilms

In general, biofilms denote microbial structures and have a cubic construction consisting of extracellular secretion that covers microbes, their clearances, and surface layers. Extracellular secretion contains various kinds of cell-derived molecules. Major substances are polysaccharides, polypeptide,

and extracellular Deoxyribonucleic Acid (DNA)[4]. Microbial coagulants (floc) generated in activated sludge systems and also self-granulation products (granule) produced by anaerobic ammonium oxidizing bacteria are a type of biofilm[5]. Even in the inside of a biofilm, combinations of species and functions of living microbes can differ from each other according to the spatial difference, lapse of time, and environmental factors. Each biofilm can be considered as a multicellular organism with an abundant flexibility[6].

2.3 Analysis of Biofilms by Using Molecular Biological Approach

Biofilms are mostly composed of bacteria groups for which an isolation culture is difficult to achieve. For group analysis of such microbial bacteria, phylogenetic sorting analysis (bacteria flora analysis) based on nucleotide sequencing of bacteria DNA is widely used. Based on the result of this analysis, we carried out a systematic identification and diversity evaluation for bacteria existing in biofilms.

In this case, gene arrangement common to the target bacteria is identified, a gene probe marked with pigment is produced, and fluorescence in situ hybridization "Fluorescence In Situ Hybridization (FISH) analysis" is performed in order to detect the target microbes and analyze the spatial distribution.

In regard to the extracellular DNA that is a key element of biofilms, it is possible to analyze a polysaccharide component by using binding specificity belonging to lectin.

By using analysis technologies for biofilms as shown in the **Table 1**, we analyzed and evaluated the biofilms that support waste water treatment. In order to maintain good conditions of biofilms for wastewater treatment, we aim to realize the advanced treatment of a wastewater treatment system and long-term stable operation of the system. This will be made real by controlling the environment of the biofilms.

Table 1 List of Analysis Technologies for Biofilms

Typical biofilm analysis technologies are shown. To fit with the analysis of object conditions, properties of a biofilm and the type of bacteria, it is necessary to change pretreatment method and analytical approach.

Items of biofilm analysis	Technological details
Grasp the bacterial flora analysis	By gene analysis, species of biofilm composing bacteria.
Quantitative PCR	Quantity of objective microbes existing in biofilms is measured.
FISH analysis	Grasp the state of spatial distribution of objective microbes existing in biofilms.
Analysis of extracellular polymeric substances	Grasp of the type of polysaccharides secreted by bacteria and polysaccharide distribution in biofilms.

3 Example of the Results of Biofilms Analysis

3.1 Anaerobic Ammonium Oxidizing Bacteria Granule

As an example of biofilm analysis, we will introduce here a result of analysis of a biofilm (granule) that is produced by anaerobic ammonium oxidizing bacteria. Nitrogen wastewater treatment using anaerobic ammonium oxidizing bacteria (autotrophic bacteria) has been paid much attention as an excellent alternative technology that can replace a conventional denitrification treatment method using heterotrophic bacteria. Advantages of nitrogen treatment processes using anaerobic ammonium oxidizing bacteria are: (1) the running cost can be lowered than that of processes using heterotrophic bacteria and (2) overall size of a plant can be made more compact.

Fig. 1 shows an outline diagram of the system for this experiment. Inorganic synthetic waste water containing NH_{4+} and NH_{2-} was continuously supplied from a lower part of the reactor. Under the condition that the inner temperature was 35℃, the reactor was controlled so that the nitrogen loading rate was maintained within the range of 4.0 to 6.0kg-N/m³/day. **Fig. 2** shows anaerobic ammonium oxidizing bacteria granule cultured in this reactor. Anaerobic ammo-

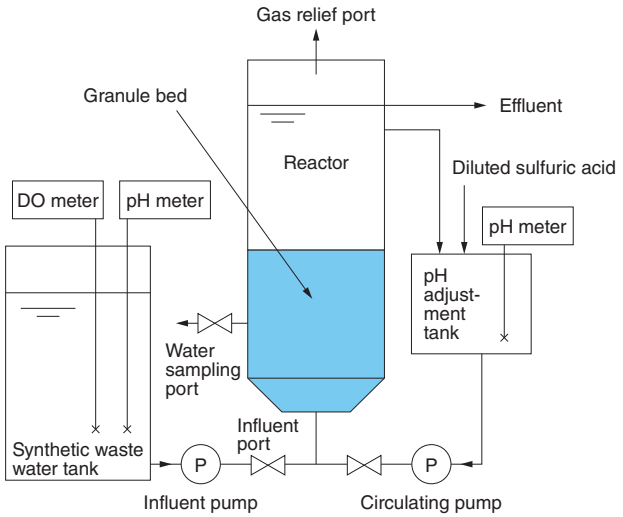

Fig. 1 Outline Diagram of Wastewater Treatment System Using Anaerobic Ammonium Oxidizing Bacteria Granule

Synthetic wastewater is supplied from reactor bottom and nitrification is performed in the granule bed filled with anaerobic ammonium oxidizing bacteria granules. Granules are always fluidized by the effect of upward streams caused by synthetic wastewater influent.

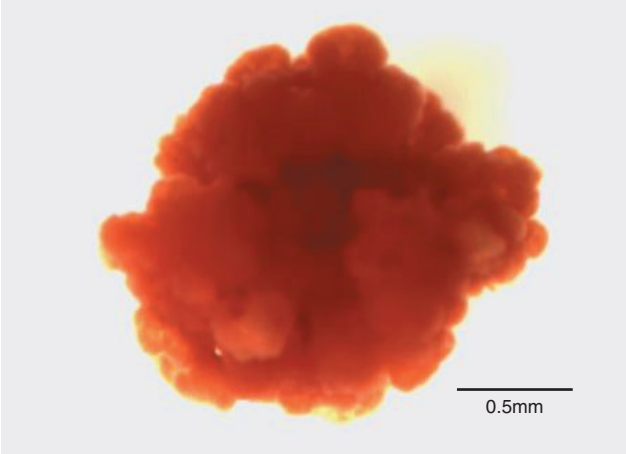

Fig. 2 Anaerobic Ammonium Oxidizing Bacteria Granule

Anaerobic ammonium oxidizing bacteria have a nature to produce red spherical granules. Granules have a strong nature of solid-liquid separation and microbes living there are solidified at a high density. Granules offer various advantages such as a high settling velocity in waste water treatment.

nium oxidizing bacteria perform self-granulation streaming with the effect of a self-generated denitrification gas. The obtained granule had a size of 0.2 to 5mm in diameter. Using image analysis software, grain diameters of 500 granules were measured and classified into a grain size distribution scale of classes at the intervals of 0.5mm. As a result, it was clear that the class of 1.5 to 2.0mm in diameter is most common. Granules of this class were therefore adopted for analysis.

3.2 Bacteria Organizing Granule

In order to grasp the bacteria type of shape granules, bacterial genetics were extracted from granules to decipher the domain where the inherent gene arrangement is reserved for respective bacteria. **Table 2** shows the result of bacterial flora analysis based on bacterial gene information. It is known that granules are composed of at least nine types of bacteria other than Candidatus Kuenenia stuttgartiensis that belongs to anaerobic ammonium oxidizing bacteria. Using the quantitative Polymerase Chain Reaction (PCR) method, the rate of existence of anaerobic ammonium oxidizing bacteria was calculated based on the gene quantity of all bacteria composing granules and that of anaerobic ammonium oxidizing bacteria. As a result, it was clear that about 60% belongs to anaerobic ammonium oxidizing bacteria. Regarding the granules cultured in our reactor shown in **Fig. 1**, anaerobic ammonium oxidizing bacteria are the dominant species. Given the above, it is suggested that our reactor is suitable

Table 2 Result of Bacterial Flora Analysis for Anaerobic Ammonium Oxidizing Bacteria Granules

The results of investigation into bacteria with high homology show that ten types of bacteria were detected from six divisions that compose granules.

Division	Species	Homology (%)
Planctomycetes	Candidatus Kuenenia stuttgartiensis	99～100
	Plancomyces brasiliensis	99～100
	Phycisphaera mikurensis	99～100
Proteobacteria	Zoogloea sp. UNPF89	99～100
	Zoogloea sp. UNPF36	99～100
Chloroflexi	Thermanaerothrix daxensis	93～99
	Bellilinea caldifistulae	95～99
Ignavibacteria	Ignavibacterium album	88～95
Bacteroidetes	Rhodothermus marinus	94～99
Armatimonadetes	Fimbriimonas ginsengisoli	95～99

for the enrichment culture of anaerobic ammonium oxidizing bacteria and it is capable of high-speed nitrification treatment.

The gene extraction yield from a biofilm can greatly affect the analytical result of bacterial flora analysis and a quantitative polymerase chain reaction.

We adopted an approach of gene extraction from granules where both treatments, an ultrasonic homogenizer and freeze-fracture treatment, are used. Microbes are then dispersed inside biofilms by this ultrasonic homogenizer treatment and extracellular secretion covering the respective microbes can be separated by freeze-fracture treatment. As a result, the gene extraction yield is improved by approximately 15% and we could further reduce the effect of extracellular secretions with analysis blocking matter. In so doing, we improved analytical accuracy.

3.3 Distribution of Anaerobic Ammonium Oxidizing Bacteria

Fig. 3 shows observation and analytical images of granules using a microscope. In order to grasp the distribution of anaerobic ammonium oxidizing bacteria existing in granules, a section of granule with a thickness of about 20μm was sliced by using a frozen section producing machine. The obtained section was then put into FISH analysis with the use of a gene probe for anaerobic ammonium oxidizing bacteria and a gene stain solution for bacterial detection. The result of FISH analysis is shown in (a) and (b). We came to learn that anaerobic ammonium oxidizing bacteria produce a great number of

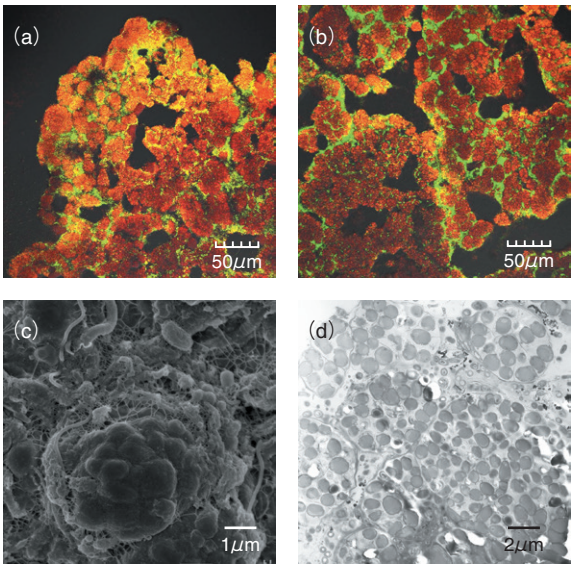

Fig. 3 Observation and Analytical Images of Anaerobic Ammonium Oxidizing Bacteria Granules

(a) Granule peripheral area (b) Inner image of FISH analysis without any remarkable difference between both. Anaerobic ammonium oxidizing bacteria are shown in yellow-red and symbiotic bacteria are shown in green. (c) Image of granule observed through a Scanning Electron Microscope (SEM). Coagulant of micrococci in the center was presumed as a colony of anaerobic ammonium oxidizing bacteria. (d) Image of granule observed through a Transmission Electron Microscope (TEM). Symbiotic bacteria are living in the periphery of a coagulant body of micrococci that were assumed as anaerobic ammonium oxidizing bacteria.

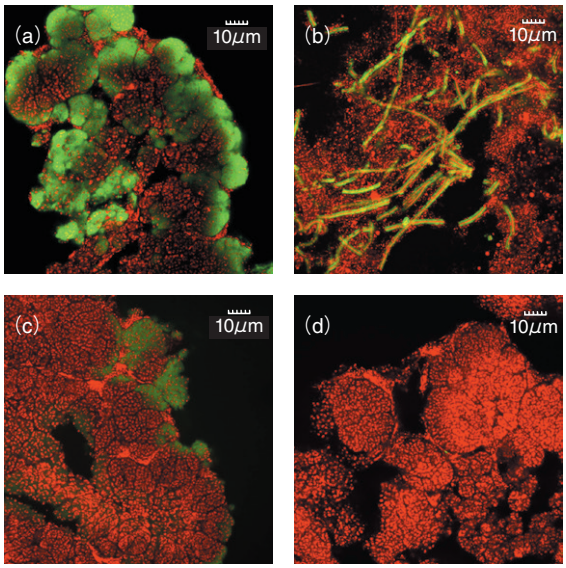

Fig. 4 Result of Extracellular Polymeric Substance Analysis Using the Bonding Singularity of Lectin

Sugar chains are shown in green and bacteria in red. Photos (a) to (d) show mutually different lectins from which sugar chains were detected.

coagulant bodies within granules in sizes ranging from several to tens of μm. Further, there are bacteria living together with anaerobic ammonium oxidizing bacteria around the coagulant bodies. Similar distribution can be observed around the periphery and inside of granules. The result of observation on the granule surface by using a scanning electron microscope is shown in (c). Micrococci predicted to be anaerobic ammonium oxidizing bacteria were observed to create a coagulant body with a diameter of 10μm. Around the periphery of this body, symbiotic bacteria like filamentous bacteria, cocci, and bacilli appeared to be attached. The result of observation inside the granule using a transmission electron microscope is shown in (d). We observed the presence of symbiotic bacteria formed around a coagulant body by anaerobic ammonium oxidizing bacteria. According to the result of our observation and analysis using electron microscopes we observed that there is a possibility that anaerobic ammonium oxidizing bacteria plays a role in producing aggregate and connecting aggregate by some symbiotic bacteria. We confirmed the presence of symbiotic bacteria that are key to granule formation.

Through continuous grasping of the distribution of anaerobic ammonium oxidizing bacteria and symbiotic bacteria in granules, we aim to establish operation and management technologies such as (1) finding the preliminary phenomena before the deterioration of system performance of wastewater treatment systems or (2) recovering operation from the failure.

3.4 Analysis of Extracellular Secretion Using Lectin

Lectin is mainly extracted from plants. Since it has a property of bonding only with specific sugar chains, it is utilized for sugar chain structure analysis. We adopted lectin to analyze polysaccharide that is a main component of extracellular secretion. Fig. 4 shows a result of extracellular polymetic substance analysis using the bonding singularity of lectin. In the same manner as for the FISH analysis approach shown in Fig. 3, a thin section was sliced from granule, extracellular polymeric substances were stained with lectin, and bacteria were stained with a gene bonding reagent. According to the type of lectin, the position of the stained part was different in extracellular secretion. This suggests that major components of extracellular secretion for composing granules are a certain type of substance containing acidic amino sugar. There is a very high possibility that this substance is essential for the anaerobic ammonium oxidizing bacteria to create coagulants. We aim to realize high-speed wastewater

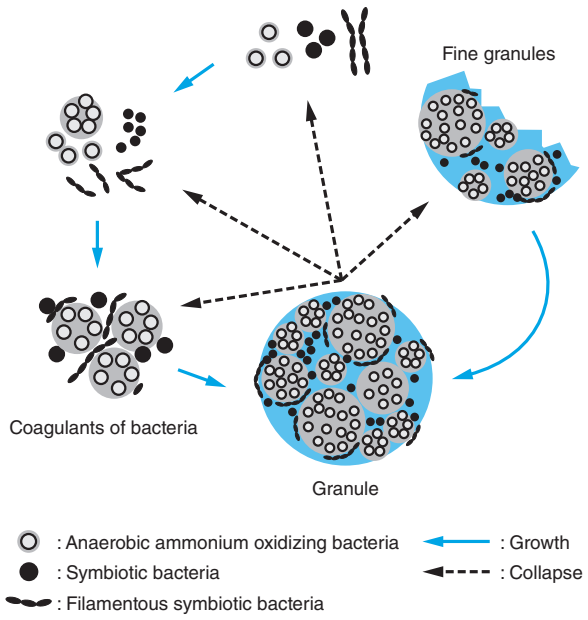

Fig. 5 **Anaerobic Ammonium Oxidizing Bacteria Granule Forming Mechanism**

Granules are mainly composed of anaerobic ammonium oxidizing bacteria, symbiotic bacteria, and filamentous symbiotic bacteria. These bacteria are considered to gather together and grow into 3D granules.

treatment by (1) activating metabolic pathways where acidic amino sugar contained substances of anaerobic ammonium oxidizing bacteria are synthesized and (2) activating the cell division of anaerobic ammonium oxidizing bacteria.

3.5 Mechanism of Granule Formation

Through biofilm analysis and microscope observation, we worked on analyzing the mechanism of granule formation of anaerobic ammonium oxidizing bacteria. Fig. 5 shows a mechanism of granule formation in a reactor which we derived from the various analysis results. Granules form and collapse repeatedly. When a granule collapses, anaerobic ammonium oxidizing bacteria, symbiotic bacteria, and filamentous bacteria are discharged. These bacteria are then coagulated and grown to form a granule again. When granules grow and get larger in size, the supply of substrate into their inside becomes harder, thus lowering the activation of bacteria existing on the inside. We predict, from this result, that it becomes impossible to secrete protein and polysaccharides to maintain biofilms which leads to the collapse. We confirmed that there was a large volume of fine granules and bacterial coagulants which were generated as a result of granule collapse in the reactor. We suspect that these substances grow with time into larger granules. Fine granules showed a condition where the overall body was covered with extracellular secretion and bacterial coagulants showed a state in which bacteria themselves stuck to each other. At start-up period of wastewater treatment facilities where anaerobic ammonium oxidizing bacteria are employed, it is essential to make granules in the tank become high density as soon as possible. This time study suggests a possibility that start-up time can be shortened than that of conventional practice if we take measures to let bacterial coagulants and fine granules remain inside the tank.

4 Postscript

This paper introduced a case study example of our research program which uses our biofilm analysis technologies. According to the result of our biofilm analysis, we obtained information about the ecological structure of biofilms produced by anaerobic ammonium oxidizing bacteria and on the biofilm forming mechanisms.

Our next step is to try to apply our developed technologies to water treatment biofilms cultured in a pilot plant using actual wastewater. By establishing biofilm control and a management method, our wastewater treatment system can attain long-term stable operation and improve the efficiencies of processing.

Lastly, we would like to express our deepest thanks to Professor Katsutoshi Hori, of Nagoya University for his kind guidance and suggestions during the development of our biofilm analysis technologies.

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