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Metagenomic Analysis of Bacteria Community in Activated Sludge of Domestic Waste Water Treatment Plant

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Abstract. Biological treatment of the domestic waste water relies on the functioning of microbial community in its conventional activated sludge. The pyrosequencing of 16S rRNA approach is used to monitor the bacterial population pattern. The viable bacteria community directly related to performance of activated sludge is also evaluated by using Ethidium monoazide (EMA) treatment comparison with non-treatment sample prior to sequence 16S rRNA gene. The fecal bacteria group *Spirochetes* and unassigned "other" bacteria are totally disappear in the viable community. Half of the relative abundance of *Actinobacteria* and *Chloroflexi* are also overestimated in non-EMA treated sample. In conclusion, the bacterial community in activated sluge share similar viable pattern at phylum level. Moreover, qPCR is applied to quantify the 16S rRNA gene copies of population.

Keywords: Bacterial community, 454pyrosequencing, EMA, qPCR

1. Introduction

The extremely high microbial diversity, high cell density and diverse functions of activated sludge are the interesting point for the microbiological studies [1]. Conventional activated sludge is the main performance of biological treatment system that is widely used for the domestic and industrial waste water for the removal of organic matter [2], [3]. Activated sludge contains many microorganisms that can transform and metabolize organic and inorganic substances into environmentally acceptable compounds. The bacterial population studies in activated sludge are revealed by many molecular methods such as DGGE (denaturant gradient gel electrophoresis) together with cloning and T-RFLP (terminal-restriction fragment length polymorphisms). [4], [5] However, these methods have limitations that could not cover the whole microbial populations especially for samples containing high bacterial diversity. The detected number of clones form libraries was in average of 20-100, and the number of operational taxonomic units obtained range from 25-65 from wastewater samples [6], [7]. Pyrosequencing is the current efficient approach to overcome all these limitations [8] These methods are DNA-based methods and DNA from the dead cells community can also be extracted, as DNA still remain for years after cell dead, and contribute in the defining community. It is important to know the viable community out of the whole community of activated sludge as they are essential and directly related to the performance and make the waste water treatment more efficient.

In this study, we used Ethidium monoazide (EMA) treatment prior to DNA extraction from the activated sludge samples in combination with pyrosequencing. EMA has the ability to penetrate dead cells with compromised membrane whereas intact membranes of live cells exclude its ability [9]. Once inside the cells, it has explained theoretically that upon exposure of light, the azide group of EMA has covalently cross linking to the nucleic acids once inside the cells. One researcher also has reported that dead bacteria DNA

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was directly cleaved into fragments after EMA treatment followed by visible light irradiation [10]. Quantitative PCR is also applied for the quantitative measurement of 16S rRNA gene copies to enhance the applicability of EMA treatment more reliable. Based on this study, almost all of bacterial community in the activated sludge share similar viable pattern and contribute to biological treatment and depend on each other.

2. Material and Methods

2.1. Wastewater treatment plant description

The wastewater treatment plant covered 251,000 residents living in an area of 2653.56 hector. It has total capacity of 11290 m^3 domestic wastewater per day. The raw sewage had a biological oxygen demand (BOD) of 220mg/L and chemical oxygen demand (COD) of 130 mg/L and total suspended solid of 200 mg/L. Chlorination was used for disinfection purpose and the concentration was ca 1mg/L of 12% NaClO solution and residence time is about 20 mins.

2.2. Activated sludge sample preparation

The sludge samples were taken from the aeration tank of the domestic waste water treatment plant in Tokyo, Japan. The samples were brought to the laboratory for immediate processing on the same day of collection. Homogenization was applied to the samples for 10 mins at room temperature for the ease extraction of DNA from the environmental samples like wastewater sludge. The samples were centrifuged at 8000 rpm for 4˚C and the pellet was suspended with 10 ml of bacterial free water. The samples were diluted with bacterial free water to OD_{600nm} measurement of 0.5 to 1.

2.3. EMA treatment

Ethidium monoazide (EMA), DNA intercalating dye was applied to the sample as they have behaviour to bind covalently to the DNA of dead cells which has compromised cell wall. Ethidium monoazide (Sigma Aldrich) was dissolved in Milli-Q unltrapure water to get a stock solution of 1 mg/ml concentration and stored at -20˚C.The EMA stock solution was added to the activated sludge sample to reach final concentration of 100 µg/ml. Once the EMA has been added to the samples, the samples were exposed to the LED light for 30 mins at a distance about 2cm and the samples were kept on ice according to the manufacturer protocol. After light exposure, the samples were centrifuged at 10,000xg for 5 mins at 4˚C and then the pellet was washed with bacterial free water and centrifuged again at the same condition to wash remains of EMA. The final pellet is subjected to DNA extraction.

2.4. DNA extraction

Both activated sludge samples with EMA stain and without EMA stain are treated with enzymatic treatment such as 10 µl of Lyzozyme (1mg/ml), 40 µl of Achromopeptidase (0.2ng/ml) and 50 µl of Protease K (1mg/ml) at 55˚C for 0.5 hr, 37˚C for 1 hr and 55˚C for 0.5 hr respectively. For all samples in this study, DNA was extracted by Phenol/Chloroform beads-beating method.

2.5. qPCR

The total 16S rRNA gene were quantified by quantitative PCR (qPCR) using the universal primers 341F (CCTACGGGAGGCAGCAG) and 534R (ATTACCGCGGCTGCTGG). To generate standard curve, the 16S rRNA gene of *Desulfovibrio desulfuricans* ATCC 13699 was amplified with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3') and cloned into Escherichia coli XL 1-Blue using with pGEM-T vector system (Promega, WI) according to manufacturer's instruction. Clones were selected and confirmed for the presence of inserts by PCR using the pGEM-T primer set [11]. Transformants containing the recombinant plasmid were selected and subjected to plasmid extraction with Pure Yield Plasmid Miniprep System (Promega, WT). The purified recombinant plasmid DNA was measured for concentration with NanoDrops 2000 spectrophotometer (Thermo Scientific) and used as standard for the total 16S rRNA gene. Quantitative PCR was performed on a StepOne real-time PCR system (Applied Biosystems). Each 20-µl real-time PCR mixture consisted of 10 µl of SYBR qPCR mix (Thunderbird, Toyobo co, Japan), 200 mM of each primer 341F and 534R, and 2 µl of template DNA The

thermal cycling conditions included a holding stage for 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C.

2.6. 454 pyrosequencing and data analysis

The 16S rRNA gene universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') were used to amplify approximately 500 bp of the variable regions V1 to V3. Different tags were attached to the forward primer in order to distinguish different samples. Components for reaction mixture are 27F and 519R, dNTP, 10x Ex Taq buffer, Ex Taq and sample DNA. The conditions for PCR were as follows: an initial denaturation step at 95 ℃ for 5 min, followed by 30 cycles of amplification (denaturation at 95 ℃ for 30 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 60 s), and a final extension step for 5 min at 72 ℃. PCR products were purified with NucleoSpin Gel and PCR clean up kit (Clontec lab, Inc. Takara, Japan), and all samples were multiplexed by combining 10 ng of purified DNA from each. Roche 454 GS-FLX Titanium pyrosequencing was performed by Hokkaido System Science Co., Ltd. All 16S rRNA gene pyrosequencing reads were analyzed using QIIME, version 1.9.0.

3. Results

3.1. Description of domestics wastewater treatment plant

In this Fig. 1 showed the sampling site of activated sludge sample used in this research.

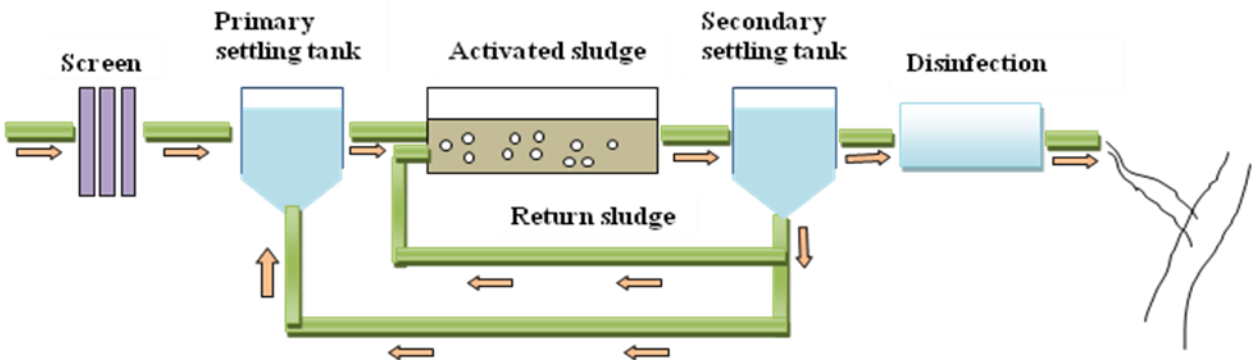

Fig. 1: General description of domestic wastewater treatment plant

3.2. Quantification of 16S rRNA gene

In this study, the applicability of EMA effect on the pure laboratory strains of dead cells of *E.coli* K12 and dead and live cells of and Staphylococcus aureus 003 was conducted with different concentration of EMA 0 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml in concentration. There was no obvious decrease of 16S rRNA gene copy number in the bacteria samples treated with low concentration of EMA (0, 1 and 10 µg/ml). Based on the experiment, the EMA concentration 100 µg/ml is selected to use for the actual environmental samples. The qPCR result showed that there were 10^8 copies of 16S rRNA gene in activated sludge and 10^7 copies from out of total are from the viable bacterial community. The results are the mean of three independent reactions.

3.3. Pyrosequencing analysis

After filtering the low quality reads, there are 2095 pyrosequencing reads for AS and 5502 reads for the AS (EMA) sample. The average read lengths of AS is 463 bp and AS (EMA) is 459 bp. Rarefaction curves were obtained and showed that the observed species richness in the sludge samples were high at this sequencing depth although the curves were not totally saturated for all samples. The result showed that the bacterial community in the activated sludge are depend on each other and performed the treatment process. The classification of sequencing reads at phylum level with and without EMA treatment is shown in Fig. 2 (a) and (b). Phylum constituting less than 1% of reads in the two samples is combined and annotated as "other". The most dominant phylum is *Proterobacteria* at an average percentage of 70%. The second most dominant bacteria group was *Bacerioidetes* followed by *Actinobacteria* and *Chloroflexi*. "Unassigned" bacteria and *Spirochaetes* groups are almost the least representing group in the activated sludge.

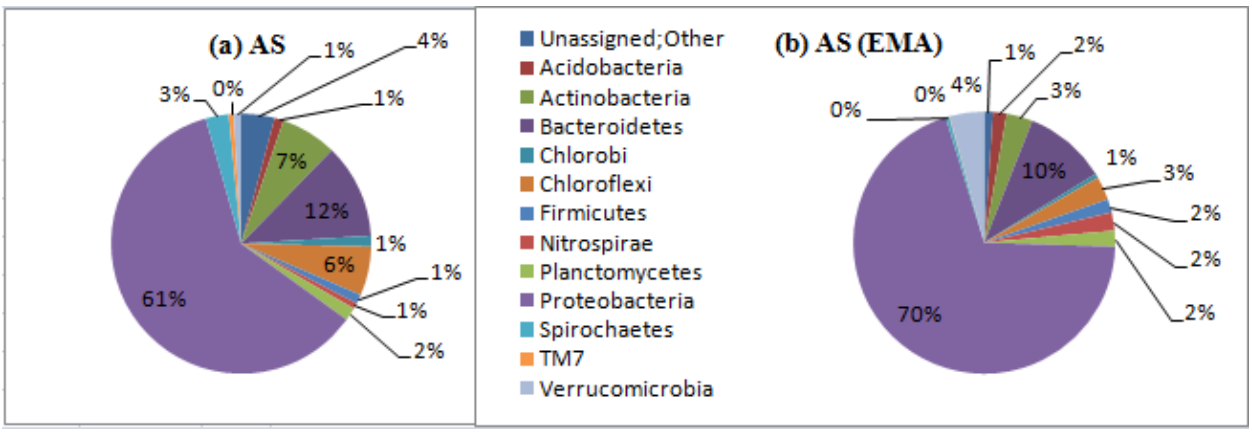

Fig. 2: Abundance at a phylum level (a) activated sludge without EMA treatment (b) activated sludge with EMA treatment

4. Discussion

The dye EMA is selectively amplified DNA from living cells and make loss of dead cells' DNA by intercalating to the double stranded DNA and this principle is reported in many studies [12], [13] The applicability of EMA is also reported in other study such as for the bacterial diagnosis. [14] However, there is only one study using Propidium monoazide (PMA) to know the viability community and heat-tolerant bacteria in activated sludge [13]. In this study, *Firmicutes* was found to be only 2%. Obligate anaerobic bacteria could not survive in aerobic tank. Moreover, the fecal anaerobic bacteria group *TM7* and *Spirochaetes* are totally disappeared in activated sludge sample, suggesting that the dominant bacteria population in raw sewage might not play an important during wastewater treatment. Interestingly, unassigned "other" bacteria are contribute to dead community in AS and also agreement with the previous study [15].

As shown in Fig. 1 the phylum *Proteobacteria* is the most abundant in the activated sludge, accounting for 61% in without EMA treated samples and it is increased to 70% in EMA treated samples as few percentage of other less dominant bacteria groups like *Actinobacteria*, *Bacteroides* and *Chloroflexi* are alive. Moreover, it is undoubtedly that the viable percentage of *Proteobacteria* is high as the sludge dominant bacteria classes are β, α and δ *Proteobacteria* [16]. For instance, phosphate accumulating bacteria *Rhodocyclales*, ammonia-oxidizing *betaproteobacteria* were belonged to *Proteobacteria* Phylum. In this study, *Actinobacteria* were representing the least viability group. Some researchers reported that *Actinobacteria* were efficient for removal of BOD and some heavy metals and most of their potential isolates were from raw wastewater samples but not from the activated sludge [17]. It is likely that the BOD and some heavy metals removal efficiencies were not carried out by Actinobacteria group in this study. In contrast, other researchers also reported that *Actinobacteria* were played an important role in sludge bulking and foaming and became the focus of interest [18], indicating that there was no sludge bulking problem in the treatment plant of this study. As the regular monitoring results of the discharge effluent of the WWTP also reached the required standard. In overall, β, α and δ *Proteobacteria* as well as the Bacteroidetes and the Actinobacteria were most frequently observed groups in activated sludge although they can be vary in representing percentage according to spatial and strength of raw wastes in individual treatment plant.

However, in this study, it's difficult to discuss the relationship between each bacterial group population and the performance of activated sludge accurately, our results suggesting the pyrosequencing approach in combination with the use of EMA is an alternative technology to address in monitoring the performance of activated sludge by reducing DNA from dead community in near future.

5. Acknowledgements

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