Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater

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ABSTRACT

Membrane biofouling was investigated in a laboratory-scale membrane bioreactor (MBR) to determine how microbial community structure influences the fouling process and the potential for quorum sensing. Differences in microbial communities of the mixed liquor and the membrane fouling layer were evaluated using pyrosequencing. During continuous MBR operation, macroscopic engineering parameters such as total cell concentration and organic removal rate were maintained at constant levels, however, microbial community structure was greatly changed showing a dynamic shift in the dominant bacterial species. The microbial composition in the membrane-biocake was very different from that in the mixed liquor and specific microbial groups such as the genera Enterobacter and Dyella were found to be closely associated with the initial and late biofouling stages, respectively. In parallel, in a culture-dependent study, a total of 61 isolates were obtained from the MBR. Both approaches indicate that Enterobacter cancerogenus is a dominant community member and that it is likely a contributor to membrane fouling in this particular MBR system. Furthermore, a bioassay with Agrobacterium tumefaciens A136 (Ti−) (pCF218) (pCF372) reporter strain demonstrated that this E. cancerogenus-like isolate can produce N-acyl homoserine lactone-type quorum sensing autoinducers, although an in-situ experiment would be needed to prove that QS mechanism is operative under actual operational conditions.

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1. Introduction

Drastic permeability loss caused by biofouling, e.g. formation of a biocake layer on the membrane surface, is the main obstacle restricting the development of membrane bioreactor (MBR) technology for advanced wastewater treatment. Biofouling is an intrinsically natural biological process of undefined mixed cultured communities on the surface of the membrane in the MBR. Therefore, it can be expected that information about microbial community structure is a prerequisite to a fundamental understanding of the biofouling phenomenon in the MBR. So far, the microbial community in the MBR has been investigated using various molecular techniques based on the 16S ribosomal RNA (rRNA) sequences such as fluorescence in situ hybridization (FISH) [1] terminal restriction fragment length polymorphism (T-RFLP) [2] and denaturing gradient gel electrophoresis (DGGE) [3]. Pyrosequencing technology can generate numerous DNA sequences in a single run, providing information on species richness and diversity in environments such as deep sea [4,5] and in biological wastewater treatment plant [6]. For membrane-based systems, such as a MBR, it can help to identify populations that are likely to play a role in biofouling.

In addition to the microbial community structure, the characteristics of individual microbial groups could play an important role in biofouling. So far, various microbial characteristics such as floc characteristics [7] and extracellular polymeric substances (EPS) [8] have been reported to be closely associated with fouling. However, all of those mentioned above are macroscopic parameters of total bacterial population and, therefore, not sufficient for describing the contribution of individual microbial groups to biofouling. Recently, Yeon and coworkers [9] have reported that quorum sensing (QS), regulation of bacterial group behaviors in a cell density dependent way, was closely associated with biofouling in the MBR. In detail, they found that at least three different N-acyl-homoserine lactone (AHL) signal molecules made up the mixed cultured biocake and successfully alleviated membrane biofouling in the MBR by disrupting the QS signal with quorum quenching acylase [10]. However, they couldn’t identify which microorganisms produced such signal molecules. In addition, Valle and coworkers [11] have isolated seven proteolytic bacterial strains producing compounds with AHL-like activity from the industrial wastewater treatment plant and also reported that external addition...
of AHLs at 2 μM to sludge samples generated changes in both community function (phenol degradation) and composition. Therefore, it can be said that information about the impact of intercellular signaling on microbial community parameters, such as species diversity and function would provide a clue to a better understanding of performance of biological wastewater treatment process.

In this study, we investigate the correlation between microbial population and membrane biofouling in a MBR from the view points of microbial community structure and QS activities. Community structures in both the mixed liquor and membrane-biocake at different biofouling phases were analyzed by pyrosequencing and subsequent automated bioinformatics pipelines. In parallel to pyrosequencing analysis, bacterial isolates were obtained from the MBR using a culture-dependent technique with various growth media and then each isolate was identified to compare its phylogeny with that of the pyrosequences. The QS activity of each isolate was also screened through a bioassay with genetically modified reporter strain and its proportion in the MBR community was evaluated.

2. Material and methods

2.1. Inocula and MBR operation

Seeding sludge was obtained from Si-Hwa wastewater treatment plant (Kyunggi Province, Korea) in January, 2009. It was acclimated in the laboratory scale MBR under continuous operation with synthetic wastewater for 3 months before community analysis. After acclimation, key operational parameters (mixed liquor suspended solids, COD removal efficiency, and profiles of transmembrane pressure, TMP) stabilized. Achievement of this operational "steady state" was a precondition for comparison of the microbial community composition of the mixed liquor to that of the foulant layer.

The laboratory scale MBR was constructed in a similar way to those described by other MBR researchers [9,12] and continuously operated at room temperature. Its schematic diagram is depicted graphically in Fig. 1. The composition of the synthetic wastewater was as follows (g/L): glucose, 1.0; yeast extract, 0.05; bactopeptone, 0.04; (NH₄)₂SO₄, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.3; MgSO₄, 0.009; FeCl₃, 0.0002; NaCl, 0.007; CaCl₂, 0.0002 and NaHCO₃, 0.15 (19). The membrane module was a hydrophilic polyvinylidene fluoride (PVDF) hollow fiber (ZeeWeed 500, GE-Zenon, US) with a nominal pore size of 0.04 μm. When the TMP reached 30 kPa, the membrane module was relieved with a new one. Other operation parameters were summarized in Table 1. 200 mL of sludge was withdrawn everyday from the MBR to adjust the solids retention time (SRT) to 30 days.

2.2. Pyrosequencing

DNA was extracted using a Mobio Soil kit according to the manufacturer’s instructions and 16S rRNA gene was amplified using barcode forward (5′-X-AC-GAGTTTGATCMTGGCTCAG-3′) and reverse primers (5′-X-AC-WTACCCCGCGCCGCGTCG-3) where X denotes an 8 nucleotide long barcode followed by a linker (AC) and unique barcode sequences uniquely designed for each sample [13]. PCR reactions were carried out in a thermocycler (MJ Research, Reno, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min and 20 s. The amplified 16S rRNA gene was purified using a resin spin column and 1 μg of each amplified product was mixed and subjected to pyrosequencing. Pyrosequencing was carried out at ChunLab, Inc. (Seoul, Korea) using a 454 Genome Sequencer FLX Titanium (Roche, Basel, Swiss).

2.3. Processing of raw nucleotide sequence reads

Sequence reads from samples were sorted by barcodes. Primer sequences were removed from raw sequence reads. The trimmed sequence reads were then processed to determine the microbial community structure and QS activities. Community structure and QS activities were characterized in Table 1. 200 mL of sludge was withdrawn everyday from the MBR and the total concentration was determined. The biomass concentration was maintained at 10000 mg/L.

2.4. Taxonomic assignment of sequence reads

Each sequence read was assigned to a taxonomic group using the EzTaxon-edatabase (http://www.eztaxon-e.org/) holding manually annotated 16S rRNA gene sequence information of both cultured type strains and uncultured representatives. In this database, representative phyotypes are designated as artificial species with artificially given specific epithets. For example, a specific epithet EU193120_s is given for the GenBank sequence entry EU193120 which plays a role as the type strain of an artificial species belonging to the genus Lysobacter. The suffix indicates the taxonomic level of the phyotype (s, species; g, genus; f, family; o, order; c, class). Individual sequence reads were taxonomically assigned according to the following criteria (x = similarity): species (>97%), genus (97–x>94%), family (94–x>90%), order (90–x>85%), class (85–x>80%) and phylum (80–x>75%). If the similarity was below the cutoff of ranks, the read was assigned to an unclassified group.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>MBR operating conditions.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working volume</td>
<td>6 L</td>
</tr>
<tr>
<td>Mixed liquor suspended solids</td>
<td>5200 (± 300) mg/L</td>
</tr>
<tr>
<td>Hydraulic retention time (HRT)</td>
<td>20 h</td>
</tr>
<tr>
<td>Solids retention time (SRT)</td>
<td>30 days</td>
</tr>
<tr>
<td>Membrane area</td>
<td>0.0008 m²</td>
</tr>
<tr>
<td>Membrane flux</td>
<td>15 L/m²/h</td>
</tr>
<tr>
<td>influent COD</td>
<td>1200 (± 180) mg/L</td>
</tr>
<tr>
<td>Organic loading rate</td>
<td>1.44 g COD/L/day</td>
</tr>
<tr>
<td>COD removal efficiency</td>
<td>98 (± 2) %</td>
</tr>
</tbody>
</table>
2.5. Estimation of species richness and diversity

Alignment was carried out using MOTHUR software package (http://schloss.micro.umass.edu/mothur/Main_Page). An aligned database of small subunit rRNA [14] was used as a template for alignment. The abundant-based estimator (ACE) [15] and Chao1 estimator [16] of each sample were calculated using the DOTUR (Defining Operational Taxonomic Units and Estimating Species Richness) function of MOTHUR [17].

2.6. Nucleotides sequence accession numbers

Sequences in this study were deposited in the NCBI short-read archive under accession numbers SRA009251. The accession numbers of isolates are GU272338–GU272398.

2.7. Isolation of single bacterial strain

Bacterial isolates were obtained using a culture-dependent technique with various growth media. Luria-Bertani (LB), Tryptic Soy Broth (TSB), Nutrient Broth (NB) and synthetic wastewater were used in this study. Samples such as mixed liquor and biomass detached from biocake were diluted in the ratio of 1:10^5 with sterilized water and spread on the agar plate of each growth medium. Especially, biocake was detached from the membrane module based on the procedure described by Lee and Park [18]. When colonies were grown on the agar, a single colony was picked up with a sterilized toothpick and spread again on the same agar to confirm the mono culture of the selected isolate. Then, colonies of the single isolate were taken with a sterilized loop, and suspended in 0.5 mL of sterilized glycerol (25%) to make frozen stock.

2.8. Sanger sequencing

16S rRNA gene sequences of each MBR isolate were determined by the Sanger method. In detail, DNA was extracted from the frozen stock of each MBR isolate using InstaGene Matrix (Bio-Rad, USA) and was used as the template in the PCR reaction with universal 27F/1492R primers for bacteria. The PCR amplification was comprised as follows: 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 60 s and elongation at 72 °C for 60 s. After PCR, unincorporated PCR primers and dNTPs were removed from PCR products using Montage PCR Clean up kit (Millipore, USA). Sanger sequencing was performed using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA). Sequencing products were resolved on Applied Biosystems 3730XL DNA analyzer (Applied BioSystems, USA).

2.9. Phylogenetic analysis of MBR isolates

The sequences of isolates were aligned manually against sequences obtained from pyrosequencing and EzTaxon database [19]. The retrieval of closely related sequences from pyrosequencing was done using megablast. The alignment and phylogenetic analyses were carried out using the JPHYDIT program [20] which is available at http://chunlab.snu.ac.kr/jphyddt and MEGA 4.0 [21]. Phylogenetic trees were inferred using the neighbor-joining method [22]. Evolutionary distance matrices were generated according to the maximum composite likelihood method [23].

2.10. Cross-feeding bioassay for the screening of QS active MBR isolate

MBR isolates producing QS autoinducers were screened using cross-feeding bioassay with Agrobacterium tumefaciens A136 (T1) (pCF218) (pCF372) reporter strain [24]. In detail, A. tumefaciens A136 and the MBR isolate to be tested were streaked in parallel with approximately 1 cm distance on LB agar covered with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). For each MBR isolate, the activation of tral-lacz fusion in the reporter strain was checked to confirm the production of N-acyl homoserine lactone (AHL) autoinducers.

2.11. Bioassay for the estimation of AHL level

The AHL level in a sample such as biofilm was estimated based on the procedure described by Yeon et al. [9]. Briefly, the indicating agar plate was prepared by mixing an overnight culture of A. tumefaciens A136 and LB agar in the ratio of 1:9. The indicating agar was also supplemented by antibiotics and covered with X-gal. Then, each sample to be tested was placed on the indicating agar. If the sample produces or contains AHLs, they diffuse vertically into the indicating agar, developing a blue color as a result. The AHL level was estimated quantitatively on the basis of the blue color length.

3. Results and discussion

3.1. MBR operation and sampling points for pyrosequencing

After 3 months of operation, the TMP profiles of the filtration runs showed good reproducibility. Pyrosequencing analyses were therefore conducted on biomass samples obtained in two consecutive filtrations. As shown in the 2nd operation in Fig. 2, the TMP showed a general tendency of a two stage increase in accordance with biofouling development phase on the membrane surface, an initial slow and gradual increase followed by an abrupt rise after a transition region [9]. The DNA of seeding sludge (S0) was extracted for pyrosequencing. After acclimation periods of 3 months, the DNA of suspended sludge from the mixed liquor (M0) was also extracted for pyrosequencing just before the 1st operation of the MBR. At the end of 30 h of the 1st operation, DNA samples were taken from both the mixed liquor (M30) and the biocake (B30) to analyze the microbial communities around the TMP transition point. DNA samples were also obtained from both the mixed liquor (M70) and the biocake (B70) at the end of 70 h of the 2nd operation for analysis of microbial communities at the highest TMP just before membrane cleaning.

3.2. Pyrosequencing data sets and microbial diversity analysis

A total of 88,128 sequence reads with valid bar codes were generated from the six samples. The number of sequence reads was reduced to 64,833 after processing. The average length of processed sequence reads was 432 bases. 90.16% of all processed reads were successfully assigned at genus level. The characteristics of data sets were summarized in Table S1.
Species richness indices in Table S2 clearly indicate that diversity and richness in the M70 community are much higher than those in other communities. In addition, rarefaction curves (Fig. S1) of all samples except M70 had reached the curvilinear phase, implying that the sampling in S0, M0, M30, B30 and B70 was sufficient to obtain total diversity and the additional sampling in M70 led to increased estimates of microbial diversity (Fig. S1-d).

During continuous MBR operation, macroscopic engineering parameters such as total cell concentration (MLSS) and COD removal rate were maintained at constant levels. However, microbial communities in both mixed liquor and biocake demonstrated high microbial diversities and dynamic changes of their compositions at each taxonomic level. Furthermore, specific phylotypes were dominant in each microbial community, which made us suspect a close association of microbial community structure with biofouling characteristics in the MBR.

3.3. Community structures in seeding sludge and mixed liquor in MBR after acclimation

Microbial composition at different taxonomic level and proportion of major phylotypes in each sample are summarized in Fig. 3 and Table S3 respectively. The major phylum groups in seeding sludge (S0) were Proteobacteria (60.78%) and Bacteroidetes (31.57%). Among Proteobacteria, the proportion of Alphaproteobacteria (20.71%) and Betaproteobacteria (28.62%) was much higher than that of Gammaproteobacteria (9.79%). Within Bacteroidetes, Flavobacteria (28.18%) was the major class group. After acclimation periods of 3 months, major phylum groups in mixed liquor (M0) were changed to Proteobacteria (72.52%) and Actinobacteria (16.65%). Alphaproteobacteria (31.78%) and Gammaproteobacteria (33.20%) constituted the major class groups among Proteobacteria. Most of the Alphaproteobacteria and Gammaproteobacteria were represented by Odysella (16.44%) / Rhizobiales (10.71%) and Enterobacteriales (8.95%) / Xanthomonadales (19.94%), respectively. The proportion of Betaproteobacteria dropped to 4.99%. Sinononas (8.82%) and Microbacterium (4.20%) represented most of the Actinobacteria in M0.

3.4. Community structures in mixed liquor and biocake in MBR at initial fouling stage

In the 1st MBR operation at 30 h, the TMP increased slightly from 6 to 13 kPa, which means that biocake started to form on the membrane surface. Therefore, both microbiomes of the mixed liquor and biocake at 30 h (M30, B30) were analyzed. Proteobacteria were the most dominant phylum in both M30 and B30 (72.79%, 73.05%), in a similar way to mixed liquor at the starting point (M0). Within Proteobacteria, Gammaproteobacteria was the most abundant class in both M30 (55.88%) and B30 (71.17%). The population of Gammaproteobacteria in M30 consisted mainly of two order groups of Enterobacteriales (43.65%) and Xanthomonadales (9.89%). On the other hand, Enterobacteriales (68.41%) was the only dominant Gammaproteobacteria phylotypes in B30. Alphaproteobacteria accounted for 11.71% and 1.45% of M30 and B30 respectively. Actinobacteria are the second dominant phylum in both M30 (22.36%) and B30 (12.72%). Actinobacteria in M30 were mainly composed of Sinononas (15.56%) and Microbacterium (3.55%). However, Sinononas was the only major Actinobacteria (11.22%) in B30. Bacteroidetes and Firmicutes showed higher proportions in B30 (7.35%, 6.11%) than in M30 (0.86% and 1.58%) and the main class was represented by Bacteroidales and Clostridiales, respectively. In order to identify a correlation between community structure and initial biofouling stage in MBR operation, the major genus groups in M30 were compared with those in B30. Fig. 5 shows clearly that community structure in the biocake (B30) was different from that in the

Fig. 3. Phylogenetic distribution of each microbial community in MBR at (a) phylum, (b) class, (c) order, and (d) family level, respectively.
mixed liquor (M30), although both originated from the same population of M. Enterobacter and Sinomonas are two major microbial groups in both M30 and B30. Enterobacter showed a much higher proportion in B30 (67.09%) than in M30 (40.17%). On the other hand, the proportion of Sinomonas in B30 (11.60%) was smaller than in M30 (16.89%). This suggests that a specific microbial group such as Enterobacter has a preference for attached growth on the membrane surface over the suspended growth in the mixed liquor in the MBR.

3.5. Community structures in mixed liquor and biocake at late fouling stage

In the 2nd MBR operation at 70 h, the TMP passed through the transition region and reached 30 kPa at which the extent of biofouling was so large that cleaning was required. Proteobacteria were the most dominant phylum in both mixed liquor (57.09%) and biocake (75.80%) at 70 h in a similar way to those at 30 h in the 1st MBR operation. Among Proteobacteria, Gammaproteobacteria was the most dominant in both M70 (38.53%) and B70 (68.46%). The population of Gammaproteobacteria in M70 and B70 consisted mainly of Xanthomonadales (27.96%) and Enterobacteriales (49.60%), respectively. The Alphaproteobacteria accounted for 13.57% and 4.87% of M70 and B70 respectively. The population of Betaproteobacteria represented 4.73% and 2.22% in M70 and B70 respectively.

Actinobacteria were the second dominant phylum and accounted for 30.94% and 16.68% of M70 and B70 respectively. Among Actinobacteria, Sinomonas was dominant in both M70 (19.46%) and B70 (13.25%). Microbacterium was the second most dominant Actinobacteria (8.39%) in M70.

In a similar way to M30 and B30, Firmicutes showed a much higher proportion in B70 (5.43%) than in M70 (0.51%). However, the major class in Firmicutes changed from Clostridia to Bacilli. Fig. 4 shows clearly that Enterobacter was still the most predominant genus in B70 (47.01%). Considering that the proportion of Enterobacter in the mixed liquor had dropped significantly from 40.17 (M30) to 8.24% (M70) during this period, it is probable that most of the Enterobacter in B70 was favored with the membrane surface, and that attachment was followed by growth within the biofilm. This coincides with previous results from M30 and B30. Sinomonas (14.09%) was the second most dominant group in B70. Unlike Enterobacter, the proportion of Sinomonas in mixed liquor increased from 16.89 to 22.19%. Furthermore, the distribution ratio between biofilm and mixed liquor at 30 and 70 h was calculated to be 0.68 (B70/M70 = 14.09/16.89) and 0.63 (B70/M70 = 22.19/27.96) respectively, which means that the preference of Sinomonas for biofilm was relatively constant. Therefore, it can be said that Sinomonas in B70 mainly came from convective deposition. Dyella was the third most dominant group in B70 and its proportion in mixed liquor increased from 6.14 to 22.94% in a similar way to Sinomonas. However, the ratio between biofilm and mixed liquor increased from 0.20 (B70/M70 = 1.20/6.14 at 30 h) to 0.50 (B70/M70 = 14.09/27.96 at 70 h), which implies an increased preference of Dyella for biofilm especially during the late fouling stage.

3.6. Phylogenetic analysis of MBR isolates

In addition to the analysis of community structure by culture-independent pyrosequencing, bacterial isolates were obtained through culture-dependent techniques and the phylogeny of these isolates was investigated. In detail, mixed liquor and biocake were taken from the MBR at the end of the 1st and 2nd MBR operations. A single bacterial strain was isolated from each mixed liquor and biocake sample using culture-dependent techniques with LB, TSB, NB, and synthetic wastewater medium, respectively. As a result, a total of 61 bacterial isolates were obtained and the closest known relatives are summarized in Table S4. The most dominant genus was Microbacterium (23 isolates), followed by Enterobacter (12 isolates). A phylogenetic tree based on evolutionary distance and neighbor joining was constructed with 16S rRNA gene sequences from MBR isolates, their closest relatives, and major pyrosequences (Fig. S2). Especially, isolate NP1MA, whose 16S rRNA gene sequences have 98.93% similarity with those of Enterobacter cancerogenus LMG 2693¹, was confirmed to be one of the dominant groups by a comparison with pyrosequencing results.

3.7. Identification of MBR isolate with QS activity

Cross-feeding bioassay results of MBR isolates are summarized in Table S4. Among a total of 61 MBR isolates, only E. cancerogenus-like isolate NP1MA (GU272338) showed a weak positive correlation (Fig. 5a). To see AHL production by E. cancerogenus-like isolate more clearly, the following experiment was conducted: a liquid culture of E. cancerogenus-like isolate (10 μL) was dropped on sterilized PVDF disc filters (25 mm diameter) placed on the NB agar and incubated at 30 °C for biofilm growth. At incubation times of 1, 12, 36, and 48 h, AHL levels at each incubation time were estimated by measuring the blue color length on the bioassay agar plate. As shown in Fig. 5b, biofilms at 36 and 48 h demonstrated much greater AHL levels than those at 12 and 24 h, which implies that E. cancerogenus-like isolate NP1MA (GU272338), one of the major microbial groups in the MBR, developed the high production rate of AHL autoinducers especially during biofilm maturation stage.

4. Conclusions

In this study, community structures in the lab scale MBR with synthetic wastewater were analyzed using pyrosequencing and a subsequent bioinformatics. At the same time, QS active microbes were isolated using culture dependent techniques and their phylogeny was compared with that of culture independent pyrosequences. Based on these results, the following conclusions about the close correlation between microbial community structure and biofouling in MBR were made:

1. Despite of the steady state operation in which biomass concentration and COD removal efficiency was maintained constantly, both mixed liquor and attached membrane-biocake showed a dynamic shift in the microbial population. In detail, the microbial composition in biocake was much different from that in mixed liquor even in the same time. Furthermore, specific microbial groups such as the genera Enterobacter and Dyella were found to be dominantly present in the biocake of initial and later fouling stage respectively, which implicate that only a few major players in whole microbial
community could be a main target of fouling control in MBR. Field-scale studies are needed to extrapolate these findings to field-scale MBRs to be able to generalize our findings and application to full scale system.

2. Total 61 bacterial isolates were obtained using culture dependent techniques. Among them, *E. cancerogenus* like isolate with QS activity was confirmed to be one of the dominant groups in MBR through the comparison of phylogeny of each MBR isolate with that of pyrosequences. *In-situ* experiments are needed to determine whether such a mechanism is in fact operative under actual operational conditions.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.desal.2011.09.030.

References


Fig. 5. Comparison of major genus groups in mixed liquor and biocake at each initial (M30, B30) and late biofouling stage (M70, B70). Percentage was calculated from the pyrosequencing data.