Control of Membrane Biofouling in MBR for Wastewater Treatment by Quorum Quenching Bacteria Encapsulated in Microporous Membrane

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Supporting Information

ABSTRACT: Recently, enzymatic quorum quenching has proven its potential as an innovative approach for biofouling control in the membrane bioreactor (MBR) for advanced wastewater treatment. However, practical issues on the cost and stability of enzymes are yet to be solved, which requires more effective quorum quenching methods. In this study, a novel quorum quenching strategy, interspecies quorum quenching by bacterial cell, was elaborated and proved to be efficient and economically feasible biofouling control in MBR. A recombinant Escherichia coli which producing N-acyl homoserine lactonase or quorum quenching Rhodococcus sp. isolated from a real MBR plant was encapsulated inside the lumen of microporous hollow fiber membrane, respectively. The porous membrane containing these functional bacteria (i.e., "microbial-vessel") was put into the submerged MBR to alleviate biofouling on the surface of filtration membrane. The effect of biofouling inhibition by the microbial-vessel was evaluated over 80 days of MBR operation. Successful control of biofouling in a laboratory scale MBR suggests that the biofouling control through the interspecies quorum quenching could be expanded to the plant scale of MBR and various environmental engineering systems with economic feasibility.

INTRODUCTION

Over the past two decades, a membrane bioreactor (MBR) where a biological activated sludge process is combined with membrane filtration has emerged as an innovative technology for the effective wastewater treatment and reuse because it provides a treated effluent of high quality.1,2 However, the application of MBR has been hampered, particularly by membrane biofouling, which is closely associated with the naturally attached microbial growth on the membrane surface.3,4 Especially, biofouling control has been a critical research issue because around 60% of MBR operating cost is directly related to biofouling.2 Therefore, tremendous efforts to inhibit biofouling in membrane processes for water treatment have been made through engineering, material, and chemistry platforms.1,5 However, all these attempts are limited by the fact that they are essentially not able to uproot the natural biofilm formation on the membrane surface.

Since the regulation of microbial group behaviors by cell-to-cell communication (i.e., quorum sensing) was reported to be involved in the biofilm formation,6 the quorum quenching has been regarded as a fundamental approach of biofouling inhibition on diverse surfaces.7 Recently, the concept of bacterial quorum sensing was also introduced to an MBR as a new biofouling control paradigm. In detail, it was experimentally observed that quorum sensing is closely associated with the formation of a biofouling layer on the immersed membrane surface in a submerged MBR for wastewater treatment.8 Furthermore, AHL-acylase which degrades the N-acyl homoserine lactone (AHL) type quorum sensing signal molecules has proven its potential to inhibit biofouling when the AHL-acylase immobilized magnetic carriers were put into the submerged MBR9 or when the AHL-acylase was immobilized onto the nanofiltration membrane surface in the crossflow nanofiltration of microbial suspension.10

Practical issues of cost and stability of enzymes, however, are yet to be solved, which requires more effective quorum quenching methods. The purpose of this study was to devise and investigate the inhibition of quorum sensing in MBR by interspecies interference using quorum quenching bacteria. Application of bacterial quorum quenching can be more economic than enzymatic quorum quenching because the former has longer life span and does not need any enzyme
purification process. Two types of quorum quenching bacteria, a recombinant *Escherichia coli* which producing AHL-lactonase or quorum quenching *Rhodococcus* sp. isolated from a real MBR plant for wastewater treatment, were encapsulated into microporous membranes, respectively. The microporous membranes containing these functional bacteria were directly put into the MBR systems, respectively, to confirm the feasibility of interspecies quorum quenching which is definitely more economic than the enzymatic quorum quenching reported previously.

■ EXPERIMENTAL SECTION

Preparation of Microbial-Vessels. A microbial-vessel was designed to keep quorum quenching bacteria using polyethylene hollow fiber membrane (Econiity, Co. Ltd., Korea). The bottom side of the microbial-vessel was sealed with epoxy resin and the quorum quenching bacteria were packed into the microbial-vessel from the open top side using a peristaltic pump (Figure 1). The same type of vessel not containing bacteria was named as the vacant-vessel. Detailed specifications of the microbial-vessel are shown in Supporting Informaion (SI) Table S1.

**Quorum Quenching Bacteria.** Two types of quorum quenching bacteria were used for the quorum quenching experiment in MBR. One was AHL-lactonase-producing recombinant *E. coli*. The AHL-lactonase, AiiA, is known to hydrolyze the lactone ring of various AHL molecules. The AiiA gene from *Bacillus thuringiensis* serovar *kurstaki* HD263 was introduced into *E. coli* XL1-Blue using pMBP-His-Parallel1 vector. Before the recombinant *E. coli* was packed into the microbial-vessel, it was cultured on Luria–Bertani (LB) broth supplemented with ampicillin to maintain plasmids that provide AHL-lactonase producing system. The other one was obtained using an enrichment culture method. Activated sludge and biocake on the used membrane were taken respectively from the real MBR plant for wastewater treatment (Okcheon, Korea). The mixed bacteria from each sample (activated sludge or biocake) were seeded in a minimal medium containing AHL (2.5 mM N-butyryl-l-homoserine lactone or N-hexanoyl-l-homoserine lactone or N-(3-oxohexanoyl)-l-homoserine lactone) as a sole carbon source. After 3 days of incubation, 1% (V/V) transfer was made to a new minimal medium containing AHL. For each sample, this transfer procedure was done three times to confirm the isolation of bacteria which can live with only AHL as the carbon source. Then the final culture was spread on the LB agar to form single colonies. The single colony was incubated separately in the minimal medium containing AHL again, and then 16S rRNA gene sequences of cultured strains were analyzed.

**Strain Identification.** The 16S rRNA genes of the isolated strains were PCR amplified from the colonies using two universal primers H+ (5′-GAGTTTGATCCTGGCTCAG-3′) and E- (5′-AGAAAGGAGGTGATCCAGCC-3′). The PCR conditions were denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s. The sequencing of the PCR product was performed by an ABI3700 automatic sequencer (Applied Biosystems, U.S.). The sequence identification was performed using the EzTaxon server 2.1 (www.eztaxon.org). The 16S rRNA gene sequences of *Rhodococcus* sp. BH4, *Paenibacillus* sp. SYP2, *Enterobacter* sp. SHEB1, and *Micrococcus* sp. SHMC have been deposited in the GenBank database under accession numbers JN378528, JN378529, JN378530, and JN378531, respectively.

**Inoculums and MBR Operation.** Activated sludge from a wastewater treatment plant (Sihwa, Korea) was taken and acclimated to the synthetic wastewater before starting MBR. The composition of the synthetic wastewater in the batch experiment was as follows (mg/L): 8 glucose, 250; yeast extract, 12.5; bactopeptone, 12.5; (NH₄)₂SO₄ 125; K₂HPO₄ 300; 

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[Figure 1. Photograph and enlarged diagram of a microbial-vessel. Specifications of microbial-vessels are shown in SI Table S1.](#)

[Figure 2. Schematic diagrams of (a) the batch MBRs and (b) the continuous MBRs.](#)
KH₂PO₄, 300; MgSO₄, 2.25; FeCl₃, 0.2; NaCl, 1.75; CaCl₂, 0.2; CoCl₂, 0.6; and NaHCO₃, 37.5.

Two batch types of the MBR (Figure 2a), that is, the Control reactor with vacant-vessel and the microbial-vessel reactor with a microbial-vessel, were designed and operated under total recycle modes in which all permeates from the filtration membranes were recycled to the reactors. The working volume and the mixed liquor suspended solid (MLSS) concentration of each reactor were 800 mL and 200 mg/L respectively. Polyvinylidene fluoride (PVDF) hollow fiber modules (ZeeWeed 500, GE-Zenon, U.S.) with an effective area of 19 cm² were inserted in both reactors together with the vacant- or the microbial-vessel.

In addition, two continuous MBRs with 1.2 L working volume (Figure 2b) were operated in a way similar to that described by other MBR researchers.³,⁹ The composition of the synthetic wastewater in the continuous experiment was as follows (mg/L): glucose, 400; yeast extract, 14; bactopeptone, 115; (NH₄)₂SO₄, 104.8; KH₂PO₄, 21.75; MgSO₄, 15.63; FeCl₃, 0.075; CaCl₂, 2.45; MnSO₄, 1.8; and NaHCO₃, 255.5. The effective area of the hollow fiber membrane module (ZeeWeed 500) was 86 cm². Hydraulic retention time (HRT) and sludge retention time (SRT) were set to 12 h and 40 d, respectively. MLSS in both reactors were maintained within the range of 4500–5000 mg/L. MLSS and chemical oxygen demand (COD) were determined according to standard methods.¹⁵

The biocake (or biofilm) on a membrane specimen was stained with 100 μL SYTO 9 (Molecular Probes, U.S.; ex = 488 nm; em = 515/30 nm) for 20 min in the dark to visualize bacterial cells. After careful washing with distilled water, the stained biocake was observed using confocal scanning laser microscopy (CLSM, C1 plus, Nikon, Japan).

Measurement of Quorum Quenching Activity. The quorum quenching activity was measured by the degradation of AHLs in aqueous Tris-HCl buffer (pH 7, 50 mM). To determine the quorum quenching activity of the microbial-vessel, N-octanoyl-L-homoserine lactone (C₈-HSL) was added at a final concentration of 0.2 μM to the buffer. C₈-HSL was chosen because it was identified as one of the major signal molecules in our previous study.⁸ The microbial-vessel was then inserted into the buffer and the mixture was incubated at 30 °C with orbital shaking (200 rpm) for different lengths of time, as indicated. The remaining concentrations of C₈-HSL were measured using bioassay. In the activity test of the whole cell, C₈-HSL was added at a final concentration of 0.2 μM to the overnight bacterial culture, which was diluted to an optical density at 600 nm (OD₆₀₀) of 1.0.

Bioassay for Detecting AHL Molecules. All the AHLs were purchased from Sigma-Aldrich (U.S.). AHLs were detected using the indicating agar plate, which was made by mixing an overnight culture of Agrobacterium tumefaciens A136 (AHL biosensor),¹⁶ LB agar and X-gal. The samples were loaded into the well of the indicating agar plate and the concentrations of AHLs were calculated using relationship equations based on the color zone size and known amounts of AHLs.⁸,¹²,¹⁷

AHL Extraction and Detection. AHLs were extracted from the MBR broth as follows: 20 mL of sludge was centrifuged and the supernatant was mixed with an equal volume of acidified ethyl acetate (0.1% acetic acid). After shaking the mixture for 2 h, the upper layer was separated and dried in a rotary evaporator at 30 °C. The residues of each sample were suspended in 250 μL of methanol. Ten μL of each extracts was loaded on the indicating agar plate and they were incubated at 30 °C for 10 h. Samples were also analyzed by TLC-bioassay, as previously described.⁸

RESULTS AND DISCUSSION

Preparation of Microbial-Vessel with Recombinant E. coli. To decompose the AHL type quorum sensing signal molecules (i.e., quorum quenching) in the MBR, we applied recombinant E. coli expressing the heterologous AHL-lactonase gene (aiiA) for quorum quenching. Although the recombinant cell was constructed in such a way that isopropyl β-D-1-thiogalactopyranoside (IPTG) is needed for the overexpression of the AHL-lactonase, the bacterial cells showed quorum quenching activity which is needed to degrade AHLs even without IPTG induction (SI Figure S1). It is because pMal-His-Parallel1 vector has the tac promoter, a derivative of lac promoter, but the regulation of tac promoter is not tight enough. Hence the recombinant E. coli could produce AHL-lactonase without induction of IPTG. In this study, no IPTG was added to the recombinant E. coli for the application.

To continuously keep quorum quenching bacteria in the MBR, it was necessary to select an appropriate technique for the whole cell immobilization. Various polymer matrices such as alginate,¹⁹ agarose,¹⁰ and polyacrylamide¹¹ have been widely used for the entrapment of bacteria, but the unstability of...
polymer matrices and the leakage of cells have always been the limitations to the application. Therefore, we designed a microbial-vessel using hollow fiber (HF) membranes which can encapsulate quorum quenching bacteria inside the fibers (Figure 1). Because the nominal pore size of a microbial-vessel is 0.4 μm, smaller than the size of one bacterial cell, quorum quenching bacteria cannot escape from the microbial-vessel. On the contrary, the signal molecules can freely pass through the pores of microbial-vessel so as to be degraded by quorum quenching bacteria inside the microbial-vessel. On the contrary, the signal molecules can freely pass through the pores of microbial-vessel so as to be degraded by quorum quenching bacteria inside the microbial-vessel. On the contrary, the signal molecules can freely pass through the pores of microbial-vessel so as to be degraded by quorum quenching bacteria inside the microbial-vessel. Furthermore, the population of quorum quenching bacteria can be maintained in the reactor regardless of the periodic withdrawal of excess sludge from the MBR.

As shown in Figure 3, the degradation efficiency of C8-HSL with the microbial-vessel was measured to be 58% in the reaction time of 90 min when the microbial-vessel contains 360 mg of the recombinant E. coli. As the adsorption of C8-HSL on the vacant-vessel was negligible, the decrease in the concentration of C8-HSL was attributed mainly to its decomposition by the microbial-vessel.

Application of the Microbial-Vessel with Recombinant E. coli to the Batch MBR. The biofouling control in MBR by the microbial-vessel containing the recombinant E. coli was tested in the batch and the continuous modes, respectively (Figure 2). The extent of biofouling in each MBR was quantitatively evaluated by monitoring the increase of transmembrane pressure (TMP) under constant flux operation. In the batch MBR, it took about 28 h in the Control reactor with a vacant-vessel to reach the TMP of 25 kPa, whereas it took 39 h
in the microbial-vessel reactor to reach the same TMP (Figure 4). This result indicated the biofouling inhibition of the microbial-vessel encapsulating quorum quenching bacteria in the batch MBR.

To further confirm the relationship between quorum quenching and biofouling, AHL molecules were extracted by ethyl acetate from the broths of both reactors and their levels were compared with each other using A. tumefaciens A136. As shown in Figure 5a, the fade-out of blue color developments for the extract from the microbial-vessel reactor, unlike those from the Control reactor was observed, indicating that the AHL concentration was decreased in the microbial-vessel reactor. Then the extracts from both reactors were also analyzed by thin layer chromatography (TLC) and an AHL biosensor (A. tumefaciens A136) to identify the types of AHLs present in the reactors. Three different types of AHLs were detected in the extracts from the Control reactor, and following comparison with the Rf values of standard AHLs, they were confirmed to be C6-HSL, C8-HSL, and C10-HSL, respectively (Figure 5b). Yeon et al. also reported the presence of C6-HSL, and C8-HSL in the MBR. On the contrary, extracts from the microbial-vessel reactor did not show any AHLs in TLC analysis, which means that the concentrations of AHLs might be reduced to a level below the detection limit in the TLC-bioassay. From these results, we can conclude that the microbial-vessel encapsulating the quorum quenching bacteria degraded the AHL molecules, which resulted in the inhibition of biofouling, i.e., the delay of TMP rise-up by the quorum quenching mechanism in the batch type MBR (Figure 4).

**Table 1. List of Quorum Quenching Isolates from Biocake and Activated Sludge of the Real MBR Plant Operated at Okcheon in Korea**

<table>
<thead>
<tr>
<th>strain</th>
<th>colony morphology</th>
<th>identification (16S rRNA gene sequence homology)</th>
<th>carbon source</th>
<th>sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH4</td>
<td>ivory</td>
<td>Rhodococcus qinghensis djl-6 (100%)</td>
<td>C6-HSL</td>
<td>biocakea</td>
</tr>
<tr>
<td>SYP2</td>
<td>white</td>
<td>Paenibacillus turicensis MOL 722T (98.7%)</td>
<td>C6-HSL</td>
<td>biocake</td>
</tr>
<tr>
<td>SHEB1</td>
<td>white</td>
<td>Enterobacter ludwigii DSM 16688T (99.8%)</td>
<td>3-oxo-activated HSL</td>
<td>sludgeb</td>
</tr>
<tr>
<td>SHMC</td>
<td>yellow</td>
<td>Micrococcus lutus NCTC 2665T (99.7%)</td>
<td>C6-HSL</td>
<td>sludge</td>
</tr>
</tbody>
</table>

*aBiocake: a mixture of microorganisms on the submerged membrane surface. bActivated sludge: a mixture of microorganisms in the broth of a real MBR.*

**Figure 7.** Stability test for the quorum quenching activity of the microbial-vessel with recombinant E. coli. The microbial-vessel was taken out from the continuous MBR at the operating days of 0, 12, 25, and 80 to measure their quorum quenching activities. Error bar: standard deviation (n = 2).

**Figure 8.** Application of the indigenous quorum quenching bacteria isolated from a real MBR plant for wastewater treatment. (a) Comparison of the quorum quenching activity between quorum quenching bacteria; the recombinant E. coli (pMBP-His-aiiA), Paenibacillus sp. SYP2, and Rhodococcus sp. BH4. Control represents E. coli cells harboring the empty vector pMBP-His-parallel1. Error bar: standard deviation (n = 4). (b) TMP profile in the continuous MBRs. The vacant-vessels and the microbial-vessels with Rhodococcus sp. BH4 were inserted in the control and microbial-vessel reactors, respectively.
quenching, in the third cycle the vacant- and microbial-vessels were taken out of Reactor A and B, respectively and then they were interchanged. After two days of stabilization period, two reactors were run again using chemically cleaned membranes. Less biofouling, (i.e., slower TMP rise-up) was also observed in the reactor with the microbial-vessel (Reactor A) (Figure 6). There were variations in the rate of TMP rise-up in all cycles for both reactors. It was attributed to the continuous dynamic changes in the microbial community in the MBR.\(^\text{22}\) Despite those variations in the rate of TMP rise-up, the microbial-vessel always delayed the TMP rise-up substantially during all three cycles.

In a final step to ensure whether the recombinant \(E.\ coli\) inhibited biofouling by the quorum quenching mechanism or by other effects, we prepared a "control-vessel" containing \(E.\ coli\) harboring only an empty vector (pMBP-His-parallel1) without the \(aiiA\) gene which is unable to produce the AHL-lactonase. The TMP in the control-vessel reactor rose up much faster than that in the microbial-vessel reactor (SI Figure S2), indicating that the biofouling inhibition by quorum quenching occurred only in the MBR with the microbial-vessel containing the AHL-lactonase-producing \(E.\ coli\). In view of these results, it can be concluded that the microbial-vessel inhibited the biofouling on the membrane surface, and thus delayed the TMP rise-up through the quorum quenching effect.

**Stability of Quorum Quenching Activity in the Long Term Operation of MBR.** In the long term operation of MBRs (80 days), the same trend of TMP variations in each reactor were reproduced (data not shown). During that period, the microbial-vessel was intermittently taken out of the reactor to monitor its quorum quenching activity (Figure 7). There was a substantial variation in the quorum quenching activity of the microbial-vessel, which can be represented by the slope of each curve in a plot of the concentration of a signal molecule vs reaction time. It might be attributed to the continuous change in the population of live quorum quenching bacteria inside the microbial-vessel during the operation. Comparing the slope for the control (vacant-vessel) with those of the microbial-vessel, it has been concluded that quorum quenching activity of the microbial-vessel had been maintained for at least 80 days.

**Effect of Quorum Quenching on the Biodegradation of Organics in MBR.** Considering that quorum sensing regulates various microbial physiologies, possible side effects of the microbial-vessel should be checked. Consequently, the treatment efficiency of organics for both reactors which may be represented by the soluble CODs in the broth and permeate were monitored during the continuous reactor operations. As shown in SI Figure S3, the differences in COD profiles were negligible, suggesting that the insertion of the microbial-vessel did not affect the microbial activity for the biodegradation of the organics.

**Isolation of Indigenous Quorum Quenching Bacteria from a Real MBR Plant and Its Application to the Continuous MBR.** Even though the recombinant \(E.\ coli\) which producing AHL-lactonase was proved to be effective for the biofouling inhibition in MBR, two problems still remain which need to be overcome: one is that the recombinant \(E.\ coli\) can lose their activity without the antibiotics, while the other is that it may not survive well outside the laboratory, that is, in a real MBR. Therefore, we tried to find out indigenous quorum quenching bacteria which may inhabit a real MBR plant for wastewater treatment. In order to isolate indigenous quorum quenching bacteria, activated sludge and biocake were sampled from a real MBR plant for wastewater treatment being operated at Okcheon in Korea. AHL degrading bacteria were then isolated using the enrichment culture. 16S rRNA gene sequences of four quorum quenching isolates were analyzed and identified using the EzTaxon server (Table 1). Strain BH4 and SYP2 shared 100% and 98.7% sequence identity with the 16S rRNA gene sequences of \(Rhodococcus qingshengii\) djl-6\(^\text{7}\) and \(Paenibacillus turicensis\) 722\(^\text{7}\), respectively. As these two isolates degraded AHL molecules more efficiently than other strains, they were compared with the recombinant \(E.\ coli\) cell in terms of quorum quenching activity (Figure 8a). Because \(Rhodococcus\) sp. BH4 showed the highest degrading activity against C8-HSL, further study was done to test its quorum quenching ability in the continuous MBR. When the microbial-vessel containing \(Rhodococcus\) sp. BH4 inside was inserted in the MBR, TMP rise-up was substantially delayed as expected (Figure 8b) in two consecutive runs of the continuous MBR.

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**Figure 9.** The CLSM images of the biocake formed on the membrane surfaces in (a) the Control reactor and (b) the microbial-vessel reactor after 41 h of operation. The microbial cells were stained with SYTO9. Magnification 100x. Image size 1213 \(\mu m\) \(\times\) 1213 \(\mu m\).
Effect of Indigenous Quorum Quenching Bacteria on the Biocake Formation on the Membrane Surface in MBR. In order to confirm the quorum quenching effect on the inhibition of biofouling in more detail, the used membranes were taken out of both MBRs after 41 h of operation to measure total attached biomass (TAB) and to visualize biocake layers using CLSM, respectively (SI Figure S4). The amount of TAB in the control reactor was 17.7 mg, whereas that in the microbial-vessel reactor was only 9.1 mg. Moreover, from the CLSM image, it was clearly seen that the biocake formed on the used membrane in the microbial-vessel reactor (Figure 9b) was thinner and sparser than that in the Control reactor (Figure 9a). These data support the evidence of quorum quenching effect on the inhibition of biofouling.

In summary, we prepared the microbial-vessel containing recombinant quorum quenching bacteria (AHL-lactonase-producing recombinant E. coli) and used it successfully for the biofouling control by interspecies interference in MBR. In the continuous MBR operation, inserting the microbial-vessel into the MBR substantially delayed the TMP rise-up (i.e., membrane biofouling) without any deterioration of wastewater treatment performance. Furthermore, an indigenous quorum quenching bacterium, Rhodococcus sp. BH4, screened and isolated from a real MBR plant proved effective in biofouling inhibition by quorum quenching.

This new process for biofouling inhibition with the microbial-vessel is worth noticing because this technique could open new horizons, in that interspecies quorum quenching could be a novel technique for the control of biofouling in MBR for wastewater with mixed culture where a variety of microorganisms cohabit.

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