Microbial population dynamics and proteomics in membrane bioreactors with enzymatic quorum quenching

Hak-Woo Kim · Hyun-Suk Oh · Sang-Ryoung Kim · Ki-Baek Lee · Kyung-Min Yeon · Chung-Hak Lee · Seil Kim · Jung-Kee Lee

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Abstract Quorum sensing gives rise to biofilm formation on the membrane surface, which in turn causes a loss of water permeability in membrane bioreactors (MBRs) for wastewater treatment. Enzymatic quorum quenching was reported to successfully inhibit the formation of biofilm in MBRs through the decomposition of signal molecules, N-acyl homoserine lactones (AHLs). The aim of this study was to elucidate the mechanisms of quorum quenching in more detail in terms of microbial population dynamics and proteomics. Microbial communities in MBRs with and without a quorum quenching enzyme (acylase) were analyzed using pyrosequencing and compared with each other. In the quorum quenching MBR, the rate of transmembrane pressure (TMP) rise-up was delayed substantially, and the proportion of quorum sensing bacteria with AHL-like autoinducers (such as Enterobacter, Pseudomonas, and Acinetobacter) also decreased in the entire microbial community of mature biofilm in comparison to that in the control MBR. These factors were attributed to the lower production of extracellular polymeric substances (EPS), which are known to play a key role in the formation of biofilm. Proteomic analysis using the Enterobacter cancerogenus strain ATCC 35316 demonstrates the possible depression of protein expression related to microbial attachments to solid surfaces (outer membrane protein, flagellin) and the agglomeration of microorganisms (ATP synthase beta subunit) with the enzymatic quorum quenching. It has been argued that changes in the microbial population, EPS and proteins via enzymatic quorum quenching could inhibit the formation of biofilm, resulting in less biofouling in the quorum quenching MBR.

Keywords Membrane bioreactor (MBR) · Quorum sensing · Acylase · Pyrosequencing · Proteomics

Introduction

Membrane bioreactors (MBRs) have emerged as one of the innovative options for the treatment and reuse of wastewater. However, as biofilm formation (biofouling) on the membrane surface causes permeability loss, which is the most common and serious problem in MBRs, a number of studies have been performed to address this problem. However, many difficulties still remain in finding a solution to the biofouling problem as activated sludge in MBRs consists of undefined mixed culture communities. Furthermore, information on the microbial community in MBRs has been limited, even though it is critical for understanding membrane biofouling.

Recently, Yeon et al. (2009a) revealed that quorum sensing, communications between microorganisms using signal molecules like N-acyl homoserine lactones (AHLs; Fuqua et al. 1994; Fuqua and Greenberg 2002), is strongly related to biofilm formation in MBRs. Furthermore, they proved that the biofilm formation (biofouling) could be mitigated
substantially by the disruption of signal molecules (AHL) using a quorum quenching enzyme (acylase) even in MBRs with mixed cultures (Yeon et al. 2009b; Oh et al. 2012; Jahangir et al. 2012). However, the exact mechanisms by which the enzymatic quorum quenching can mitigate membrane biofouling are not yet fully determined. This is partly because changes in microbial communities before and after enzymatic quorum quenching in MBRs have not yet been revealed. In this context, pyrosequencing (Ahmadian et al. 2006) technology would be a golden opportunity to acquire the essential information necessary for identifying the mechanisms involved in enzymatic quorum quenching which results in the enhancement of MBR performance.

The aim of this study was to elucidate how and why quorum quenching can inhibit biofouling in MBRs. First, the pyrosequencing technique was used to analyze microbial communities in order to identify which species play key roles in the formation of biofilm on the membrane surface in MBRs. Second, as extracellular polymeric substances (EPS) are in close association with biofouling in MBRs, experiments on proteomics were conducted to monitor changes in protein composition before and after the enzymatic quorum quenching in MBRs.

**Materials and methods**

Preparation of magnetic enzyme carriers (MECs)

MIEX (ORICA, Australia) is a magnetic ion-exchange resin which was adopted as a magnetic core for enzyme immobilization. A magnetic enzyme carrier (MEC) was prepared in the same way as that described by Yeon et al. (2009b): First, the functionalization of MIEX was performed by layer-by-layer (LBL) deposition of an anionic polyelectrolyte (Polystyrene sulfonate, PSS) and a cationic polyelectrolyte (chitosan). And then the porcine kidney acylase I (EC-number 3.5.1.14, pI=5.8, Aldrich, USA) was covalently attached to the MIEX-PSS-chitosan using glutaraldehyde (GA) as a cross-linking agent.

The size distribution of MIEX and MEC ranged about 5 to 330 μm (Fig. S1) and the average sizes of MIEX and MEC were 188 (±1) and 184 (±3)μm, respectively. Three grams of MIEX and MEC was added to the control reactor with 7,000∼7,200 mg/L of MLSS and to the quorum quenching reactor with 6,600∼6,900 mg/L of MLSS, respectively. Thus the proportion of enzyme carriers (MEC) to microorganisms (MLSS) was 2.2∼2.3 in the quorum quenching reactor.

MBR operation

Activated sludge from a wastewater treatment plant (Sihwa, Korea) was used in MBRs after being acclimated to synthetic wastewater for more than 3 months. The composition of the synthetic wastewater 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.

Two lab scale MBRs, each with a 2.5-L working volume, the control MBR containing no enzyme but 3 g of MIEX, and the quorum quenching MBR containing 3 g of MEC, were run in parallel (Fig. 1). The hollow fiber membrane (GE-Zenon, USA) used for the filtration was a hydrophilic polyvinylidene fluoride (PVDF) with a pore size of 0.04 μm. Two membrane modules were immersed in each MBR. Synthetic wastewater was supplied continuously at a constant flow rate of 6.9 mL/min. The flux was maintained at 24 L/m2/h in each of the four membrane modules in two MBRs. The other operating parameters for two continuous MBRs are listed in Table 1.

![Fig. 1 Schematic diagram of MBR operation](image)
Sampling for microbial community analysis

Using pyrosequencing, analyses of microbial communities in both the broth (mixed liquor) and the biofilm on the membrane surface of each MBR was performed. The 16S rRNA genes of each sample were amplified as approximately 450 bp amplicon covering V1–V3 variable region of 16S rRNA gene. The differences in the microbial communities identified in the broth and biofilm from each MBR were analyzed, as were the differences between the control and quorum quenching MBRs. Samples of microorganisms were taken from the broths and from the biofilms on the used membranes at the early (numbered 1) and mature (numbered 2) stages of biofilm formation. As shown in Fig. 2, a total of ten samples were prepared for pyrosequencing. Five samples were taken from the control MBR (C start, C1 biofilm, C1 broth, C2 biofilm, and C2 broth) and another five samples were taken from the quorum quenching MBR (Q start, Q1 biofilm, Q1 broth, Q2 biofilm, and Q2 broth).

Pyrosequencing and microbial community analysis

DNA samples were extracted using a soil DNA extraction kit (MO Bio, USA) according to the manufacturer’s instructions. The amplification of the 16S rRNA genes and subsequent pyrosequencing was performed at Chunlab (Seoul, Korea). The 16S rRNA genes were amplified using bar-coded universal primers for each sample. The primer sequences are as follows: bacterial universal (27F: AGA GTT TGA TCM TGG CTC AG, 518R: WTT ACC GCG GCT GCT GG). Touch-down PCR was carried out using the conditions outlined in Table 2. Prepared samples were then analyzed by pyrosequencing, using the protocol outlined in a previous study (Lim et al. 2012). Total reads and OTUs data for ten samples are presented in Table S1. The contribution to resemblance among the samples was analyzed using SIMPER analysis. SIMPER analysis was carried out on microbial spatial location (broth vs. biofilm) and acylase

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**Table 1** Conditions of MBR operation

<table>
<thead>
<tr>
<th>Working volume</th>
<th>SRT</th>
<th>HRT</th>
<th>Feed flow rate</th>
<th>Membrane type</th>
<th>Membrane area</th>
<th>Flux</th>
<th>COD removal efficiency</th>
<th>MLSS</th>
<th>Feed COD</th>
<th>F/M ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 L</td>
<td>30 days</td>
<td>6 h</td>
<td>6.9 mL/min</td>
<td>PVDF, hollow fiber</td>
<td>232.8 cm² (116.4 cm² module × 2)</td>
<td>24 L/m²/h</td>
<td>Broth: Control MBR</td>
<td>Control MBR: 7,000–7,200 mg/L</td>
<td>Quorum quenching MBR: 6,600–6,900 mg/L</td>
<td>0.23–0.26</td>
</tr>
</tbody>
</table>

**Fig. 2** Transmembrane pressure (TMP) profiles and time of sample acquisition in a the control and b the quorum quenching MBR. (C start or Q start; start point in the control or quorum quenching MBR, Control 1 or Quorum quenching 1; early stage of biofilm development in the control or quorum quenching MBR, Control 2 or Quorum quenching 2; mature stage of biofilm development in the control or quorum quenching MBR)

**Table 2** Conditions used in touch-down PCR

<table>
<thead>
<tr>
<th>Step</th>
<th>°C</th>
<th>Min</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
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<td>Initial denaturation</td>
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<td>5:00</td>
<td>–</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>0:30</td>
<td>10</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>0:45</td>
<td>–0.5 °C/cycle</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1:30</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>0:30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>0:45</td>
<td>20</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1:30</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td>–</td>
</tr>
</tbody>
</table>
treatment (control vs. quorum quenching). The SIMPER was calculated using Primer 6 (Clarke and Warwick 2001).

Flow cell

A dual channel flow cell reactor (FC281: BioSurface Technologies, USA) was used for the comparison of proteins in biofilm with and without the quorum quenching (Fig. 3). The reactor consists of two channels, one for the control and one for quorum quenching. All of the flow cell devices were autoclaved before use at 121 °C for 30 min. Polysulfone membranes, each measuring 74×24 mm (Woongjin, Korea), were placed into two channels of the flow cell reactor after a 15-min ultraviolet treatment for disinfection. Each channel was then inoculated with 3 mL of the Enterobacter cancerogenus ATCC 35316 culture, which had been cultured in nutrient broth (BD, USA) for 1 day and diluted to an OD value of 1. The control channel was connected to an autoclaved nutrient broth containing one bag of raw ion exchange resin (3 g of Amberlite IRA-900 ion exchange resin, Aldrich, USA), while the quorum quenching channel was connected to a second autoclaved nutrient broth containing a bag of acylase-immobilized ion exchange resin. Each bag was made of polyamide with a pore size of 150 μm. For the preparation of the acylase-immobilized ion exchange resin, the resin was rinsed with a phosphate buffer (pH 7.0) and gently mixed at 40 rpm with an acylase solution (1,000 ppm) in phosphate buffer (pH 7.0).

Nutrient broth was circulated through each channel via peristaltic pump at 0.7 mL/min. After 7 days of operation, the flow cell unit was disassembled and a 0.5×0.5-cm fragment was cut from each used membrane. The biofilm which had formed on the used membrane surface was examined by confocal laser scanning microscopy (CLSM, C1 plus: Nikon, Japan) after staining with SYTO 9 fluorescent dyes. Z-section image stacks for 3D imaging were constructed by IMARIS software (BitplaneAG, Zurich, Switzerland).

Protein purification

The biofilm formed on the membrane surface in each flow cell channel was detached from the membrane and placed into deionized water. The biofilm suspension was then filtered through a 0.45-μm membrane. Proteins were precipitated using 80 % acetone at −20 °C followed by centrifugation at 12,000×g for 15 min. The protein pellets were resuspended in a buffer solution [20 mM Dithiothreitol (DTT), 2 % w/v IPG buffer, and 1 % w/v Bromophenol blue (BPB)]. The extracted proteins were then quantified using Bradford method.

Two-dimensional gel electrophoresis and analysis

Each protein sample (250 μg; control biofilm sample and quorum quenching biofilm sample) was loaded onto a 13-cm IPG strip (pH 4–7, GE Biosciences, USA) with sample buffer containing 20 mM Dithiothreitol (DTT), 2 % w/v IPG buffer, and 1 % w/v bromophenol blue (BPB). Following overnight rehydration of IPG strips at 50 V, each IPG strip was loaded onto a 12 % polyacrylamide gel (14×16×0.15 cm) and proteins were visualized using the silver staining method. The spot detection was performed using Progenesis SameSpots analysis software (Nonlinear Dynamics, UK).

Protein identification

The images of 2D SDS-PAGE gels are shown in Fig. S2. After selecting 30 spots from polyacrylamide gel by in-gel digestion for mass analysis, each one was analyzed by Nano LC with nanoAcquity™UPLC™ column (Waters, USA) using a binary solvent (water–acetonitrile with 0.1 % w/v...
formic acid) as the eluent. Each sample (5 μL) was loaded onto the column and the binary mixture solvent was eluted using a gradient from 2 % to 40 % of acetonitrile at a flow rate of 0.3 L/min. Mass spectrometry analysis of tryptic peptides was performed by Synapt™HDMS (Waters, USA) according to the manufacturer’s instruction. Protein identification was performed using MASCOT search engine (Matrix Science Inc., Boston, USA) to compare against the SwissProt protein database including the proteins from the genome of E. cancerogenus ATCC 35316 (PRJNA55079) rather than specific reference protein. The errors in peptide masses were in the range of 30 ppm. One missed tryptic cleavage site per peptide was allowed during the search. Proteins matching more than two peptides and with a MASCOT score higher than 30 were considered significant (p<0.05). Carboamidomethylation of cysteine was selected as the static modification and oxidation of methionine as the differential modification.

Analytical methods

Mixed-liquor suspended solids (MLSS) and chemical oxygen demand (COD) were determined according to the Standards Methods (American Public Health Association 1998). Extracellular polymeric substances (EPS) in the biofilm were filtered through a 0.45-μm hydrophilic membrane to separate suspended microbial flocs. Quantitative analysis of proteins and polysaccharides was performed using the modified Lowry and Phenol-sulfuric methods, respectively. Signal molecules (AHLs) in the reactor were detected by reporter strain, Agrobacterium tumefaciens A136 (Ti)(pCF218)(pCF372), as described in a previous study (Fuqua and Winans 1996). The activity and stability of the MEC were measured using the methods described by Yeon et al. (2009b).

Results

MBR operation

The rate of TMP rise-up is an important factor for evaluating the system performance in submerged MBRs as it is directly related to the rate of membrane fouling. In the control MBR, it took around 5 days to reach the TMP of 50 kPa (Fig. 2a); whereas in the quorum quenching MBR, it took around 12 days to reach the same TMP (Fig. 2b). The TMPs were monitored for two cycles in the control MBR, but only one cycle in the quorum quenching MBR. The patterns of TMP rise-up were confirmed to be reproducible in both MBRs by comparing those of the two membrane modules (control 1 and 2) in each MBR. In other words, the rate of membrane biofouling in the control MBR is twice as fast as the rate seen in the quorum quenching MBR. The COD removal efficiencies based on the broth COD were about 93 %, while based on the permeate COD they were about 98 % for the control and quorum quenching MBRs (Table 1).

Microbial community

The SIMPER analysis of all ten samples showed that the taxon resulting in the main differences between the broth and the biofilm was Gammaproteobacteria. The average abundances of class Gammaproteobacteria in broth samples and biofilm samples were 6.4 % and 47.9 %, respectively (Table S2). Moreover, Gammaproteobacteria included the order Enterobacteriales and Pseudomonadales, and these biofilm-specific taxa contain several microorganisms which are reported to play a role in quorum sensing (Mohamed et al. 2008). All three taxa were abundant in the microbial communities found in the biofilm, but not in those of the broth. This finding suggests that these biofilm-specific taxa play a crucial role in the formation of biofilm in MBRs.

However, the enzymatic quorum quenching did not significantly interfere with the entire microbial community, except for the development of biofilm. SIMPER analysis between control MBR and quorum quenching MBR showed that the average abundances in both the broth and biofilm samples (i.e., five samples in Fig. 2a) in the control MBR were not significantly different from those (i.e., five samples in Fig. 2b) in the quorum quenching MBR (Table S3). For example, even when the taxon contributing the most to the differences was class Gammaproteobacteria, the average abundances of this taxon in the quorum quenching and control MBRs were 21 % and 25.1 %, respectively, showing an overall difference of less than 5 %.

The abundance of biofilm-specific taxa in the Q2 biofilm was significantly lower than the taxa in the communities of other biofilms (Table S4). As shown in Fig. 4, the abundance of Gammaproteobacteria in the Q2 biofilm was 38 %, whereas those in the C1, C2, and Q1 biofilms were 52 %.

Fig. 4 The distribution of Gammaproteobacteria at a class level in broth and biofilm samples of control and quorum quenching MBRs
50 %, and 51 %, respectively. Likewise, when three species belonging to the *Gammaproteobacteria* taxon were selected (*Enterobacter*, *Pseudomonas*, and *Acinetobacter*) due to their quorum sensing characteristics (Williams et al. 2007), their contribution to the entire microbial community of the Q2 biofilm was 18 %, whereas those of the C1, C2, and Q1 biofilms were 35 %, 34 %, and 30 %, respectively (Fig. 5).

**EPS composition**

Comparing the total EPS mass (mg) per unit mass of biofilm (g) between the control and quorum quenching MBRs, the total EPS in the biofilm of the control MBR (C1 biofilm) was greater than that in the quorum quenching MBR (Q1 biofilm) at the early stage (Fig. 6a). The same result (i.e., EPS in C2 biofilm> Q2 biofilm) was observed at the mature stage. In addition, the proportion of proteins in the total EPS was found to be more important than that of polysaccharides (Fig. 6b, c), which confirmed previous findings (Yeon et al. 2009a).

Conversely, the mature biofilms (C2 biofilm and Q2 biofilm) contained larger amounts of EPS (either as proteins or polysaccharides) per unit mass of biofilm than the initial biofilms (C1 and Q1) for both MBRs. This indicates that microorganisms produced more proteins and polysaccharides per unit mass of microorganism as the biofilm formation progressed, and this phenomenon was more pronounced in the control MBR.

**Proteomics analysis**

A dual channel flow cell reactor (Fig. 3) was run to compare the biofilms formed in the control channel and the quorum quenching channel with regards to the architecture and proteomics of the biofilm. First, the thickness of biofilm produced was found to vary according to the location in the channel (i.e., inlet, middle, and outlet points) as shown in Fig. 7. However, the thickness of the biofilm in the quorum quenching channel was smaller than that in the control channel regardless of the location on the membrane surface.

Secondly, proteomic analysis was performed using a pure culture of *E. cancerogenus* ATCC 35316 in order to monitor changes in the structure of proteins in the biofilm both before and after the enzymatic quorum quenching. This microorganism was chosen because it was shown to be the most abundant quorum sensing strain in the biofilm formed on the surface of the membrane in MBRs (Lim et al. 2012). Although the genome of *E. cancerogenus* was not completed, approximately 4.6 Mbp of the genome was publicly available (PRJNA55079). We also recognize that the proteomics study using *E. cancerogenus* has limitations in representing the whole proteomics in MBR with mixed culture. However, it could be a practical alternative study to elucidate a part of the biofilm formation mechanisms in MBR.

After extracting proteins from the biofilm of each channel, 2D SDS-PAGE was performed by loading 250 μg of protein from each precipitated sample. After comparison of the gel images for proteins from the control channel and the quorum quenching channel, 30 spots were selected based on folding values of more than 1.5 for subsequent mass analysis using an image analysis program (Fig. S2).

Among the 30 spots selected, 15 proteins were identified by the above criteria. The molecular functions for the downregulated proteins (10 spots) and upregulated proteins (5 spots) are listed in Table 3. Three notable changes in the downregulated proteins appear to be associated with biofilm: (a) proteins from spots numbered 5, 17 (Outer membrane protein TolC), and 26 (Outer membrane protein OprG) were found to be in the outer membrane; (b) the protein in spot numbers 14 and 27 was shown to be flagellin which has a ciliary or flagellar motility function and is related to the formation of bacterial-type flagellum filaments; and (c) the proteins from spot numbers 5 and 18 are the ATP synthase beta subunit fragment.

**Discussion**

Microbial community structures in membrane biofilms are very different from those in the broth, regardless of quorum quenching (Table S2). This suggests that the membrane

![Fig. 5 (%) Proportions of Enterobacter, Pseudomonas, and Acinetobacter at a genus level in biofilm samples of control and quorum quenching MBRs](image-url)
biofilm is not formed solely by the deposition of the microbial flocs from the broth on the membrane surface, but that certain microbial groups, such as Gammaproteobacteria, have a preference for growth on the membrane surface over the suspended growth in the broth of the MBR. This has also been reported in a previous study (Lim et al. 2012).

In contrast, microbial communities in the control and quorum quenching MBRs were not significantly different...
Table 3  Downregulated and upregulated proteins in the biofilm of the quorum quenching channel compared with those of the control channel and their molecular functions

<table>
<thead>
<tr>
<th>Downregulated proteins</th>
<th>Upregulated proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spot number</strong></td>
<td><strong>Protein name</strong></td>
</tr>
<tr>
<td>5</td>
<td>ATP synthase beta subunit (Fragment)</td>
</tr>
<tr>
<td></td>
<td>Outer membrane protein TolC</td>
</tr>
<tr>
<td></td>
<td>Acetolactate synthase</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde-3-phosphate dehydrogenase, type I</td>
</tr>
<tr>
<td>8</td>
<td>Elongation factor Tu (Fragment)</td>
</tr>
<tr>
<td>9</td>
<td>Putative uncharacterized protein</td>
</tr>
<tr>
<td>14</td>
<td>Flagellin</td>
</tr>
<tr>
<td></td>
<td>Acetolactate synthase</td>
</tr>
<tr>
<td>17</td>
<td>Outer membrane protein TolC</td>
</tr>
<tr>
<td>20</td>
<td>Acetolactate synthase</td>
</tr>
<tr>
<td>26</td>
<td>Outer membrane protein OprG</td>
</tr>
</tbody>
</table>
This implies that the enzymatic quorum quenching does not seriously affect the general performance of an MBR in the removal of organics (Yeon et al. 2009b). In fact, the differences in the COD removal efficiency for the permeate and broth were negligible (less than 1%) between the control and quorum quenching MBRs (Table 1).

It is worth noting that the microbial community in the biofilm changed throughout the biofilm development in the quorum quenching MBR. Particularly, Gammaproteobacteria, which was the most abundant class in the biofilm, represented a comparatively lower proportion in the Q2 biofilm than in the other biofilms (C1, C2, and Q1 biofilms; Fig. 4). Three genera were selected for comparison of their proportions in various biofilms. These bacteria all belong to the Gammaproteobacteria, and are well-known quorum sensing bacteria (Williams et al. 2007). The total of all three genera was also lower in the Q2 biofilm than in the other biofilms (Fig. 4). This fact suggests that quorum sensing bacteria could represent a good target for enzymatic quorum quenching and, therefore, this could be a suitable way to reduce biofouling without any significant deterioration of MBR performance.

Many studies have reported that microorganisms can attach to solid surfaces more efficiently by producing EPS because of their sticky characteristics (Okabe et al. 1994; Langille et al. 2000; Choi et al. 2001; Kreft and Wimpenny 2001). However, the enzymatic quorum quenching retarded the production of EPS in the biofilm by approximately 2.6 times (Fig. 6). A lower production of EPS could cause microorganisms to detach from the membrane surface with greater ease, thereby mitigating the formation of biofilm. The fact that the TMP rise was also retarded by the addition of the enzyme acylase, which is able to disrupt AHL-like signal molecules, coincides with this result (Fig. 2). The reduction of EPS production by acylase can be seen in quorum sensing bacteria that generate AHL-like autoinducers (e.g., Enterobacter, Pseudomonas, and Acinetobacter; Mohamed et al. 2008). This might explain why the proportion of these bacteria in the Q2 sample decreased to 18% (Fig. 5).

It is worth noting that, even in the quorum quenching MBR, the total production of EPS per unit mass of biofilm also increased by approximately 2.1 times as the biofilm formation progressed from Q1 to Q2 (Fig. 6b, c). In the microbial world, there are other types of signal molecules besides AHL-like molecules (Dobretsov et al. 2011). Furthermore, the acylase used in this study could not degrade all types of AHLs (Xu et al. 2003). Consequently, it would be impossible to destroy all signal molecules in an MBR with only one enzyme, which explains the increase in EPS production even in the quorum quenching MBR.

The enzymatic quorum quenching apparently reduced the thickness of the biofilm, which is usually representative of the extent of membrane biofouling (Fig. 7). This coincides well with the delay in the TMP rise seen in the quorum quenching MBR (Fig. 2). The proteomic analysis also demonstrated the possible depression of protein expression related to biofilm formation (Table 3). Three proteins were identified to be outer membrane proteins, and the role of outer membrane proteins in biofilm formation has been previously studied (Sauer and Camper 2001; Abbas et al. 2007; Labbate et al. 2007; Tashiro et al. 2008; Vanoyan et al. 2010; Clemmer et al. 2011). These studies have shown that glycoprotein, an outer membrane protein, helps microorganisms to attach on the surface by its sticky characteristics. Therefore less outer membrane proteins can result in a less rigid biofilm. Two proteins were identified as flagellin. According to previous studies (Wolfgang et al. 2004; Giraud et al. 2010; Sawasdidoln et al. 2010; Sharma et al. 2010; Atkinson et al. 2011), the bacterial filament is important for attachment to the surface and the formation of biofilm. As a result, the lack of flagellin is considered to be

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein name</th>
<th>Molecular function</th>
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<tbody>
<tr>
<td>27</td>
<td>Flagellin</td>
<td>Ciliary or flagellar motility, bacterial-type flagellum filament, structural molecule activity</td>
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<tr>
<td></td>
<td>Putative uncharacterized protein</td>
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</tr>
<tr>
<td></td>
<td>Sugar fermentation stimulation protein homolog</td>
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*Molecular function: molecular function of proteins were searched from Protein Knowledge base (UniProtKB), http://www.uniprot.org*

(Table S3). This implies that the enzymatic quorum quenching does not seriously affect the general performance of an MBR in the removal of organics (Yeon et al. 2009b). In fact, the differences in the COD removal efficiency for the permeate and broth were negligible (less than 1%) between the control and quorum quenching MBRs (Table 1).
the reason for lower biofilm formation in quorum quenching channel. The role of the ATP synthase beta subunit in biofilm formation or quorum sensing has not been clearly explained, but some studies support that this protein is related to quorum sensing (Hasona et al. 2007; Zhu et al. 2008; Ahmed et al. 2009; Xiong and Liu 2010; Wen et al. 2011). For example, Xiong et al. (2010) claimed the possible involvement of ATP in granule aggregation.

The current study demonstrated that microbial communities in the control and quorum quenching MBRs were not significantly different. However, enzymatic quorum quenching decreased the proportion of quorum sensing bacteria with AHL-like autoinducers in the mature biofilm, and also substantially delayed the rate of TMP rise-up in the MBR. This could also be attributed to the mitigation of the EPS production of quorum sensing bacteria, thereby causing them to detach more readily from the membrane surface. Proteomic analysis revealed the possible depression of protein expression related to the microbial attachments to solid surfaces (i.e., the outer membrane protein, flagellin), as well as to the agglomeration of microorganisms (i.e., the ATP synthase beta subunit) with enzymatic quorum quenching.

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