Biofouling control by quorum sensing inhibition and its dependence on membrane surface

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ABSTRACT

Biofouling control by quorum sensing (QS) inhibition and the influence of membrane surface characteristics on biofilm formation and QS inhibition were investigated. *Pseudomonas putida* isolated from the bio-fouled reverse osmosis (RO) membranes in a real plant was used. Acylase was chosen as a model QS inhibitor. Bacteria on the membrane coupons were quantified with the heterotrophic plate count method. Cell distribution was imaged by a confocal laser scanning microscope. Results showed that biofilm formation on the membrane was reduced by acylase as it inhibits the activity of N-acylhomoserine lactone (AHL) which is a signal molecule of QS. It was also shown that membrane surface characteristics were influential factors affecting bacterial adhesion, biofilm formation, and QS inhibition.

Key words | acylase, biofouling, *Pseudomonas putida*, quorum sensing, reverse osmosis (RO) membrane

INTRODUCTION

The key steps of biofilm formation on the membrane surface are usually described as follows: (1) microorganisms are transported to the membrane and adhere to the membrane surface under the influence of physicochemical interactions with the membrane surface, (2) microbial cells aggregate into micro-colonies, (3) transcription of specific genes, which are required for the synthesis of the extracellular polysaccharides (EPSs), is activated, and (4) the sessile cells embedded in the EPSs matrix develop a biofilm (Costerton et al. 1999; Chen et al. 2004; Kim, T. H. et al. 2009). Various physicochemical methods have been reported to reduce membrane biofouling (Glater et al. 1994; Lee, W. N. et al. 2007). However, studies on the biofilm formation as well as biofouling control by biochemical methods are rather scarce. Recently, it has been known that quorum sensing (QS) plays an important role in biofilm development from the microcolonies. QS is a cell-to-cell communication system to express certain phenotypes through a cell density dependent manner (Costerton et al. 1999; Arevalo-Ferro et al. 2005; Gonzalez & Keshavan 2006). Bacteria use QS to regulate a variety of phenotypes, such as biofilm formation, toxin production, exopolysaccharide production, virulence factor production, and motility (Gonzalez & Keshavan 2006). In the case of biofilm formation, specific genes regulated by an auto-inducer (AI) express certain phenotypes such as changing the surface properties of the cell or fine-tuning the formation of cell aggregate in the biofilm development stage (Steidle et al. 2002; Arevalo-Ferro et al. 2005). Therefore, biofilm formation on the membrane surface can be controlled by inhibiting QS. Recently, it was presented that QS inhibition was successfully applied to control biofilm formation in a membrane bioreactor (MBR) system (Yeon et al. 2009).

Biofouling in a membrane system is influenced by membrane surface characteristics as the initial attachment of bacteria on the membrane surface is controlled by an interfacial interaction between bacteria and the membrane surface. For instance, biofilm formation occurs rapidly on the hydrophobic and less negatively charged surface due to the strong hydrophobic attraction and weak electrostatic repulsion (Kang et al. 2004; Pang et al. 2005). Therefore, for better application of biochemical methods to biofouling control, information on the surface characteristics of membranes is essential.

In this study, biofouling control by QS inhibition and the effect of membrane surface characteristics on bacterial
attachment, biofilm formation, and QS inhibition are discussed. *Pseudomonas putida* was chosen as an experimental strain, which was isolated from the real biofilm formed on the membrane used in a full-scale reverse osmosis (RO) plant. *P. putida* has been known to form biofilms by QS using N-acylhomoserine lactone (AHL) (Marques & Ramos 1993; Steidle et al. 2002; Arevalo-Ferro et al. 2005). The relationship between the membrane surface characteristics and bacterial adhesion was also investigated as the amount of adsorbed cells is one of the important factors affecting the rate of biofilm formation. For the first time, a direct application of QS sensing inhibition using a cross-flow RO test unit has been carried out and the influence of RO membrane surface characteristics on QS inhibition has been investigated.

**MATERIALS AND METHODS**

**RO membranes**

Commercial thin-film composite (TFC) RO membranes used were Dow-Filmtec SW30HRLE-400 (SW) and Toray TM820 (TM). Membranes were cut and stored in deionized (DI) water at 4°C with several changes of water prior to using in all experiments. More information on the membranes can be found elsewhere (Lee, E. et al. 2010; Lee, S. et al. 2011).

**Characterization of the RO membranes**

The membranes were characterized for chemical properties such as zeta potential, static contact angle, and dynamic hysteresis. Membrane zeta potential was determined by a streaming current electrokinetic analyzer (SurPASS, Anton Paar GmbH, Austria). The contact angle was measured with a goniometer (DM 500, Kyowa interface Science, Japan) through the method for equilibrium contact angle. Dynamic hysteresis was determined based on the Wilhelmy plate method. Detailed descriptions of these characterization methods can be found in our previous studies (Lee, E. et al. 2010, Lee, S. et al. 2011).

**Strain**

*P. putida* isolated from the bio-fouled RO membranes operated in a full-scale plant (Veolia Water Solution & Technologies Korea in Daesan, Korea) was used (Kim, S. J. et al. 2009). The strain was cultivated in growth media.

A fresh single colony of *P. putida* pre-grown on a LB agar plate at 37°C was inoculated in 10 mL LB broth and incubated for 18 h under vigorous agitation (160 rpm) at 37°C. The overnight grown cultures were collected by centrifugation at 3,000 rpm for 10 min, then the supernatant was decanted and the pellet was resuspended with 20 mL of 1× PBS (phosphate buffered saline) solution (pH 7.1) to wash the cells. After repeating these processes three times, the bacterial suspension with 1× PBS solution was diluted to make the optical density at 600 nm (OD₆₀₀) to be 0.1. Optical density was measured using UV spectrum at 600 nm (FEEM, Safire2, KSV, Germany).

**Acylase**

A QS inhibitor used was acylase known to inactivate AHL. Acylase I from porcine kidney (Sigma Aldrich) was used, which had a specific activity of 4,300 U/mg (1 U = 17 nmol N-acetyl-L-methionine (Ac-Met) deacylated per min at 25°C and pH 7 (Jiang et al. 2007)).

**Quantification of the microorganisms**

**Heterotrophic plate count (HPC) method**

The membrane coupon was picked up from the borosilicate glass carefully with tweezers as attached bacteria on the membrane should not be touched on the glass wall. Washing was carried out gently with 1× PBS solution several times and water drops were carefully removed by touching the edge of membrane coupons. The membrane coupon was transferred into a 15 mL falcon tube containing 5 mL of 1× PBS solution. Bacterial cells on the membrane surface were resuspended by sonication for 2 min with 3 min vortexing. Analyzing the total number of cells attached on membrane coupons was adapted from *Standard Methods* (APHA 2005).

**Confocal laser scanning microscope (CLSM)**

Microorganisms adhering to the membrane coupons were imaged using CLSM (LSM 5 Exciter, Carl-Zeiss, Germany). Pre-treated membrane coupons were put on slide glasses and water was wiped around the membrane coupon. Syto 9 (Molecular Probes, Invitrogen detection technologies, USA) was used for staining nucleic acid of the strain (Peeters et al. 2008). Three microliters of Syto 9 was diluted with 1 mL of DI water. Next, 100 μL of diluted Syto 9 was dropped on the membrane coupon and covered with a cover glass. The samples were wrapped with aluminium.
foil and incubated in the dark at room temperature for 1 h and then imaged by CLSM (Herzberg & Elimelech 2008). Each specimen was scanned at randomly selected positions. The CLSM images were generated using the Zeiss LSM Image Browser software.

**Bench scale cross-flow RO test**

Biofouling experiments were performed using a laboratory-scale cross-flow RO test unit. The total filtration area of the membrane is about 139 cm² (14.6 cm × 9.6 cm). Prior to each experiment, the RO unit was cleaned to remove all impurities and disinfected with 95% alcohol. The membrane was stabilized with DI water at the pressure of 35–41 bar and crossflow velocity of 0.3 L/min for 10–14 h until a constant permeate flux was obtained (14.7 μm/sec). After the stabilization, 1/100 diluted LB broth and 10 times diluted bacterial suspension adjusted to an OD₆₀₀ of 0.1 were inoculated into the feed reservoir. After 30 min for allowing cell and organic matter deposition to occur on the RO membrane, acylase stock solution was added.

**RESULTS AND DISCUSSION**

**Influence of membrane surface characteristics on bacterial adhesion**

RO membranes were characterized in terms of zeta potential, static contact angle, and dynamic hysteresis. As shown in Figure 1(a), SW is more negatively charged than TM. Figure 1(b) shows that SW is more hydrophilic than TM. Both membranes become more hydrophilic with increasing pH due to the deprotonation of functional groups (i.e., mostly –COOH). Figure 1(c) shows that the DH of SW is lower than that of TM. This suggests that the surface of TM is more heterogeneous than that of SW due to the uneven distribution of chemical surface charge as well as a physical peak-and-valley structure (Lee, W. et al. 2010; Lee, S. et al. 2011). These differences in the membrane surface characteristics are expected to affect bacterial attachments and, hence, the degree of biofilm formation and QS inhibition.

Figure 2 shows the number of microorganisms on the membrane surface counted by the HPC method after 48 h incubation. More than 100 times the number of microorganisms were attached on TM compared with SW. It is quite acceptable as TM is shown to be favorable to bacterial adhesion as its surface is less negative and more hydrophobic as presented in Figure 1. The CLSM images of
Microorganisms on SW and TM after 47 and 60 h incubation are presented in Figures 3(a), 3(c) and Figures 3(e), 3(g), respectively. Interestingly, microorganisms grew in a dispersed pattern on SW but in an aggregated pattern on TM. This observation is attributed to the fact that TM has a much more heterogeneous surface as shown in Figure 1. The results shown here clearly demonstrate that the bacterial adhesion is significantly influenced by the membrane surface characteristics.

A lot of microorganisms including *P. putida* are negatively charged and have a hydrophobic nature (Costerton *et al*. 1999). In case of SW, initial bacterial deposition is somewhat inhibited due to electronic repulsion between the membrane surface and microorganisms. Moreover, SW is relatively hydrophilic so that it does not tend to exhibit a strong hydrophobic interaction. However, bacterial deposition on TM is enhanced due to a strong hydrophobic interaction and weak electrostatic repulsion. Therefore, the rate of bacterial growth on SW could be slower than TM because the number of microorganisms deposited on SW is much less than TM. In addition, it can be noticed that the deposition pattern (i.e., aggregated vs. dispersed) of microorganisms on the membrane surface is directly related to the dynamic hysteresis. In the case of SW, elements related to the hydrophilic nature and negative charge are distributed on the surface quite uniformly so bacteria exist in a dispersed pattern (see Figures 3(a) and 3(e)). On the other hand, favorable sites for bacterial adhesion exist on TM due to its higher surface heterogeneity and, thus, there is a site-specific adhesion pattern on the TM surface (see Figures 3(c) and 3(g)).

**Biofouling control by quorum sensing inhibition**

In this study, to suppress the biofilm formation by *P. putida*, acylase was used as a QS inhibitor. Acylase hydrolyses the...
AHL amide bond that joins the lactone moiety and acyl side chain, releasing homoserine lactone and the corresponding fatty acid (Lin et al. 2003, Yeon et al. 2009). The number of bacteria on the membrane when acylase was added to the borosilicate glass containing the membrane coupon was quantified and compared with the control case (i.e., without acylase). Figure 4 shows the number of microorganisms on the membrane surface when 50 and 100 U/mL of acylase were added. The number of bacterial cells on membranes decreased in proportion to the concentration of acylase for both membranes. The quantity of microorganisms decreased from 57.1 to 42.9% for SW and 86.83 to 68.6% for TM when acylase concentration increased from 50 to 100 U/mL. It is clearly shown that acylase effectively inhibits biofilm formation in both membranes. However, there is a visible difference in the inhibition efficiency. The efficiency for SW was greater than TM. The explanation is that acylase can affect AI more effectively on SW because the number of bacteria attached on SW is much lower than that on TM as shown previously. The CLSM images taken at 47 h and 60 h (see Figure 3) also clearly show that acylase reduces the biofouling propensity for both membranes.

Biofouling and QS inhibition in RO experiments

Biofouling of inoculated P. putida on RO membranes

P. putida was inoculated after a stable initial permeate flux was acquired for 10–14 h stabilization. The permeate flux curve is commonly divided into three sections (Herzberg & Elimelech 2007, 2008). As shown in Figure 5, as soon as P. putida was injected, the permeate flux decreased drastically in less than 4 h (section A), the following slope was eased gradually for 4–15 h (section B). After 15 h, the flux decreased almost linearly without noticeable fluctuation (section C). During the first period (section A), it is likely that the radical flux decline resulted from the deposition of cells and organic matter and, thus, the formation of microcolonies. During the second period (section B), biofilm formation and development occurred where the permeate flux was slightly relieved. Here, the flux-decline behavior of the two membranes started to differentiate from each other due to the differences in the initial number of microorganisms attached. Membrane characteristics affected the bacterial attachment and, thus, TM showed more flux decline as a result of greater bacterial attachment followed by rapid biofilm formation and growth. In the last phase (section C), the flux seems to decrease linearly to about less than 20% of its initial value for both membranes.

Comparing the flux-decline behavior of SW and TM membranes (section A), the flux of TM decreased more rapidly after 3 h. It is likely that the microbial attachment in the cell of the cross-flow RO unit was induced by not only convection flow, but the interactions between the membrane surface and microorganisms (i.e., hydrophobic and electrostatic interactions). Therefore, more bacterial cells attached onto TM which has a stronger attractive force than SW due to its great hydrophobicity and lower zeta potential. In the following phase (section B), a

![Figure 4](image-url)  
Figure 4 | Cell concentration on SW30HRL-400 and TM820 membranes was counted by the HPC method. Bacteria were incubated for 48 h at 37 °C and 70 rpm with 50 and 100 U/mL of acylase (control is the absence of acylase).

![Figure 5](image-url)  
Figure 5 | Normalized flux-decline curves from bench-scale crossflow biofouling test using P. putida. Biofilm was grown on two RO membranes in 10-2 diluted LB broth. Experimental conditions were as follows: initial cell concentration of 10 times diluted bacterial suspension adjusted for OD600 of about 0.1, initial permeate flux of 14.7 μm/sec, cross flow velocity of 0.3 L/min, and temperature of 25 °C.
A distinguishable flux curve is observed between the two membranes, and the permeate flux of TM is less than that of SW. It is noticeable that a delicate difference in the number of microorganisms attached on the membrane in the previous phase leads to this great gap.

**Influence of acylase on bacterial biofouling of RO membrane**

Figure 6 shows the flux-decline behavior when 7 U/mL acylase (i.e., diluted about ten times) was added with the same experimental conditions shown in Figure 5. In the first period (section A), flux-decline behavior in the presence of acylase is similar to the control case (i.e., without acylase). This is because, in this initial period, flux decline is mainly due to bacterial and other foulants deposition whereas QS for biofilm formation has not yet been activated. In the second period (section B), it is observed that permeate flux decline with 7 U/mL acylase was reduced especially during 3–10 h compared with the control case. Results show that acylase acts as a QS inhibitor, so that the rate of biofilm formation is retarded. It may be noted that, in the dip-test, a high concentration of acylase is needed for effective contact with microbial cells on the membrane surface. In the later sections of B and C, the permeate flux decline was similar to the control case. This is attributed to the fact that acylase lost its enzymatic activation or could not handle the excess cells accumulated on the membrane due to the relatively long filtration time. In a recent study, it was shown that biofouling in MBR was successfully retarded by QS inhibition using 10 mg/L acylase (Yeon et al. 2009). In our study, it was shown that inhibiting QS by acylase with 7 U/mL (i.e., converted into about 21 mg/L) was effective for biofouling control in the bench-scale cross-flow RO test unit. These results show a good possibility of biochemical methods for more effective biofouling control when properly combined with physicochemical methods. In addition, more systematic studies are required to find out optimal conditions for effective QS inhibition including the amount and type of QS inhibitor with respect to application practices.

**CONCLUSIONS**

Biofouling propensity depending on membrane surface characteristics was investigated using *P. putida*. In the dip-test, where bacterial attachment was induced by contact through gentle shaking, biofouling propensity was higher for TM which has a more hydrophobic and less negatively charged surface. Then the retardation of biofilm formation by QS inhibition was investigated. It was demonstrated that biofilm formation was reduced by acylase through QS inhibition.

In the bench-scale RO experiments, similar to results from the dip-tests, biofouling propensity for SW was much less than TM. Moreover, biofouling could also be controlled by QS inhibition by acylase. The amount of acylase for QS inhibition in the RO test is much less than that in the dip-tests. The small amount of acylase used in the RO biofouling test was quite effective in inhibiting biofilm formation as acylase can be in close contact with the biofilm at the membrane surface in real filtration practices.

More applied research, for instance, a technique to enhance enzyme stability in the RO unit or a membrane
coating technique to hold the enzyme on the surface, are needed for further practical applications of QS inhibition to biofouling control.

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