Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2013.780996

51 (2013) 6355–6361
September

Taylor & Francis
Taylor & Francis Group

Effects of enzymatic treatment on the reduction of extracellular polymeric substances (EPS) from biofouled membranes

Lan Hee Kim[a], Sung-Jo Kim[a], Chang-Min Kim[a], Myung Seop Shin[a], Seungho Kook[a], In S. Kim[a,b,*]

[a]*School of Environmental Science and Engineering, Gwangju Institute of Science and Technology (GIST), 261 Cheomdan-gwagiro (Oryong-dong), Buk-gu, Gwangju 500-712, South Korea*
Tel. +82 (62) 715-3381; Fax: +82 (62) 715-2434; email: iskim@gist.ac.kr
[b]*Center for Seawater Desalination Plant, Gwangju Institute of Science and Technology (GIST), 261 Cheomdan-gwagiro (Oryong-dong), Buk-gu, Gwangju 500-712, South Korea*

Received 8 November 2012; Accepted 8 January 2013

ABSTRACT

Biofouling is a major problem in a membrane-based water treatment system, because it causes a water flux decline and necessitates an increase in cleaning frequency. The objective of this study was to evaluate the effectiveness of an enzyme treatment method on the reduction of extracellular polymeric substances (EPS). For this enzyme treatment test, acylase I was used as it cleaves quorum sensing signal molecules and proteinase K degrading proteins. It was found that 5 μg/mL of acylase I, 100 μg/mL of proteinase K, and a combination of both enzymes (5 μg/mL of acylase I and 100 μg/mL of proteinase K) could remove 9.0, 56.6, and 33.7% of the bacteria on an reverse osmosis membrane, respectively. Proteinase K removed 33.6% of the EPS concentration per cell number. Although acylase I reduced bacteria numbers at high removal efficiency, it could not remove EPS—and, indeed, increased the number of cells (6.2×10^{-3} μg-EPS/cells) compared with the control sample (5.2×10^{-3} μg-EPS/cells). From the excitation emission matrix result, the peak intensities of UV, marine, and visible humic-like substances were decreased after enzyme treatment, but the amount of protein-like substances were not affected. For a more effective biofouling reduction, the enzyme treatment method should thus be accompanied by another EPS-degrading agent.

Keywords: Biofouling; *Pseudomonas aeruginosa*; Acylase I; Proteinase K; Extracellular polymeric substances

1. Introduction

It is recognized that membrane fouling causes the decrease in membrane process performance. Membrane fouling is caused by deposition or adsorption of solutes and growth of microorganisms (biofouling) [1]. In particular, biofouling have complex structure comprising attached bacteria and extracellular biofilm matrix containing polysaccharides, proteins, lipids, and DNA [2]. Because of the complex structure, it is difficult to

*Corresponding author.

Presented at The Fifth Desalination Workshop (IDW 2012), October 28–31, 2012, Jeju, Korea

remove even through application of physical and chemical control methods [3,4]. Recently, various biological control methods to prevent the attachment of bacteria have been developed [5].

Among biological control methods, methods to reduce extracellular polymeric substances (EPS) and quorum sensing (QS) signals have been studied because they are related to biofilm formation on the substratum (Table 1). QS is a process used to regulate gene expression when reaching the cell density threshold. Through the QS system, signal molecules including acyl homoserine lactones (AHLs) will be produced and contribute to the microbial attachment on the surface. EPS could reduce turbulent flow in close to the membrane surface and cause elevated concentration polarization and subsequent permeate flux decline [6]. Previously, QS systems were inhibited due to the interference of QS signals and signal-specific receptors [3,7–11]. AHLs bind to their specific receptor proteins and cause the expression of target genes. However, the AHL receptor could be interfered with by AHL analogs [8] and vanillin [3] due to fact that they can bind to the AHL receptor instead of AHL. In addition, the AHL concentration could be reduced by adding an AHL cognate antibody [7]. In addition, biofilm dispersal caused by the QS system was induced by nitric oxide (NO) [12].

Proteolytic enzymes, polysaccharase, and QS signal molecule hydrolases that degrade EPS and AHL have also been used for biofilm reduction [13–17]. The high removal efficiency and environmentally friendly properties of enzymes make them a promising agent for reducing biofouling on membranes. However, there have been few, if any, comprehensive studies about the effects of enzyme treatment on the EPS concentration. As such, this study will focus on the change of EPS concentration when acylase I and proteinase K enzymes were employed.

Table 1
Methods for biofilm reduction

Category	Kinds		Function	Substratum	Ref.
Quorum sensing (QS) regulation	Antibody		Inhibition of acyl homoserine lactones (AHL)-mediated QS	N.A.*	[7]
	Synthetic halogenated Furanone compound		Interfering with AHL-mediated QS	Cover glass	[8]
	Vanillin		Interference with AHL receptor	Polystyrene surface	[3]
	Polyphenols		Interference with bacterial QS	N.A.	[9]
	Sodium propionate		Inhibition of autoinducer-2 (AI-2) activity	N.A.	[10]
	D-amino acid		Induction of biofilm dispersal	Nylon membrane	[30]
	Garlic extract and *p*-coumaric acid		Antagonize the activity of QS receptors and p-coumaric acid fully inhibited QS	N.A.	[11]
	NO		Regulation of biofilm dispersal	96-well microtiter plate, slide glass	[12]
Enzyme treatment	Proteolytic enzymes	Proteinase K	Cleave the peptides bonds of aromatic, aliphatic, and hydrophobic compounds	96-well microtiter plate	[13]
		Trypsin	Hydrolyze lysine and arginine peptides	96-well microtiter plate	[14]
		Subtilisin	Inhibition of microbial adhesion and detachment of adhered bacteria	96-well microtiter plate	[15]
	Polysaccharase	Dispersin B	Hydrolysis of β-substituted *N*-acetylglucosamine which composed of slime. Detachment of biofilm	Polystyrene surface	[16]
	QS signal molecule hydrolase	AHL-lactonase	(1) Cleave the lactone ring of AHLs to produce acyl homoserines	N.A.	[17]
		AHL-acylase	(2) Hydrolyze the amide linkage of AHLs	RO membrane	[28]

*N.A.: Not Available.

2. Materials and methods

2.1. Preparation of artificial seawater and bacteria

Bacteria were isolated from raw seawater and fouled RO membrane samples. Raw seawater was sampled from Busan, Korea (35˚22´´N, 129˚22´´E). A laborarory-scale reverse osmosis (RO) membrane system was used to make the biofouled membrane; a commercial thin film composite RO membrane, RE8040-SHN (Woongjin Chemical Co. Ltd., Korea), was used in this process. To compare the potential for biofilm formation, the *Pseudomonas aeruginosa* PAO1 (*P. aeruginosa* PAO1) strain was provided by Seoul National University in Korea. This strain has been used in many studies as a model bacterium for biofouling tests [18,19]. In addition, *P. aeruginosa* P60 was used, which was isolated from seawater; P60 was previously used as a model bacterium [20]. Bacteria were incubated in Luria-Bertani (LB) broth (BD, USA), and artificial seawater was made using a sea salt reagent (Sigma-Aldrich, Inc., USA) and adjusted to a total dissolved solids (TDS) level of 35,000 mg/L (pH 8.0).

2.2. Selection of model bacteria

2.2.1. Biofilm assay on microtiter plate

A microtiter plate biofilm assay was used, following the method of Merritt et al. [21]. In brief, cultured bacteria strains were diluted to 1:100 in LB broth, and 100 μL of each diluted culture was then inoculated into each of three wells in a fresh microtiter plate and incubated for 3 days. Using a 0.1% crystal violet solution, the optical densities (ODs) were measured at 600 nm. The biofilm formation capabilities of each strain was subsequently classified into four categories: nonadherent, weakly, moderately, and strongly adherent [22].

2.2.2. Biofilm assay on RO membrane

To investigate the ability of a biofilm to form on an RO membrane, blank (with or without nutrients), PAO1, P60, F4, and F5 samples were prepared. In particular, F4 and F5 strains had been isolated from fouled membrane. RO membranes (3 cm × 3 cm) were attached to the cell culture bottle and 20 mL artificial seawater was added, including 200 μL of LB broth. Finally, 200 μL of cell culture (OD 1.0 at 600 nm) was inoculated and incubated for 4 days (37˚C, 150 rpm). After 4 days, membranes were taken from the cell culture bottle and washed with distilled water three times, and were then put in 6 mL of phosphate-buffered saline (PBS). After vortexing for 2 min, sonication was performed for 5 min to detach bacteria from the membrane. Total cell numbers were then obtained.

2.3. Enzyme treatment

To make a biofilm on an RO membrane, *P. aeruginosa* P60 was inoculated in a culture flask containing artificial seawater (TDS 35,000 mg/L, pH 8.0) and the RO membrane. After incubation for 4 days, RO membranes were taken out and washed with PBS three times. Acylase I (500–1,500 units/mg protein; Sigma-Aldrich, USA) and proteinase K (Sigma-Aldrich, USA) were selected as the AHL and EPS-degrading enzymes, at concentrations of 5 and 100 μg/mL, respectively. In another test, 5 μg/mL of acylase I and 100 μg/mL of proteinase K were mixed and applied. Enzymes were applied to microtubes containing the RO membrane and incubated at 37˚C for 2 h.

2.4. Total cell number analysis

To evaluate the biofouling reduction efficiency, total cell numbers were counted. Bacteria were stained with diamidino-2-phenylindole dye, and a fluorescent image was captured using an LSM5 and inverted confocal laser scanning microscopy (Zeiss, Germany).

2.5. Analysis of EPS concentration and composition

2.5.1. EPS extraction

EPS was extracted following the modified method established by Karunakaran and Biggs [22]. The culture supernatant after filter sterilization (0.2 μm filter) was used to extract EPS. The filter sterilized supernatant was added to a 3×volume of 100% ethanol and stored overnight at −20˚C. After storage, the mixture was centrifuged at 4,500 g for 20 min at 4˚C. The resulting pellet was then resuspended in 3 mL of sterile distilled water and left to dialyze against sterile distilled water overnight. The EPS was then dried using a freeze dryer; after drying, 2 mL of distilled water was added.

2.5.2. Measurement of EPS concentration

EPS in distilled water samples was filtered using a 0.45-μm filter (Advantec, USA) to analyze the total organic carbon (TOC) concentration; filtered EPS was diluted with distilled water and measured using a TOC analyzer (Shimadzu, Japan). Carbohydrate and protein which are main composition of EPS were also measured. Carbohydrate was assessed by following the method as established by Wu and Xi [23]. In brief, 80 μL of samples and standards were mixed with anthrone reagent (0.125% anthrone (wt./vol.) in 94.5% H_2SO_4 (v/v)). Samples were placed in a water bath at

100℃ for 14 min and cooled at 4℃ for 5 min. The absorbance was then measured using an ELISA reader (BioTek Instruments, USA) at 625 nm [24]. Protein was measured using a BCA assay kit (Thermo Scientific, USA).

2.6. Fluorescence analysis of dissolved organic matter

The fluorescence excitation emission matrix method [25] was employed to investigate the distribution of EPS composition. EPS samples were filtered using a 0.45-μm filter (Advantec, USA) and observed using a fluorescence spectrophotometer (Hitachi, Japan). Resulting excitation wavelengths were between 220 and 450 nm and emission wavelengths were 250–600 nm. Excitation emission matrix (EEM) peaks could be classified as B (EX_{max}/Em_{max} = 270–280/300–310; tyrosine-like, protein-like), T (EX_{max}/Em_{max} = 275/340; tryptophan-like, protein-like), A (EX_{max}/Em_{max} = 260/380–460; UV humic-like), M (EX_{max}/Em_{max} = 312/380–420; visible marine humic-like), and C (EX_{max}/Em_{max} = 350/420–480; visible humic-like).

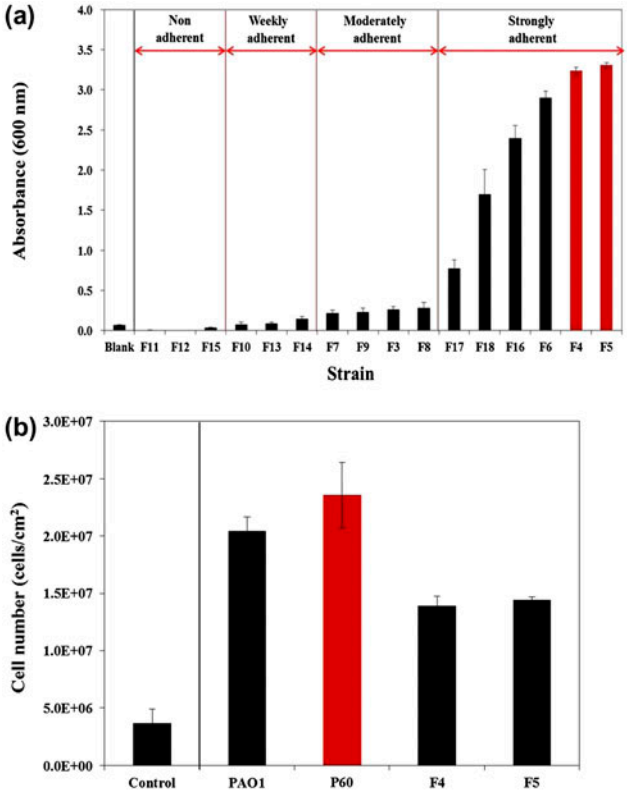

Fig. 1. Comparison of biofilm formation capabilities of isolated bacteria from (a) fouled membrane and (b) comparison of total cell numbers on biofilm formed membranes for different kinds of bacteria: PAO1, P60, F4, and F5.

3. Results and discussion

3.1. Selection of model bacteria

The biofilm formation potentials of various bacteria strains were investigated by analyzing the total number of cells. A total of 16 strains were isolated from the fouled membrane, with six strains showing four times the OD 600 nm values above the blank; these were defined as strongly adherent strains. Among them, F4 and F5 were selected as the most strongly attached bacteria (Fig. 1(a)). Compared with F4, F5, and *P. aeruginosa* PAO1, *P. aeruginosa* P60 formed more biofilms on membranes (Fig. 1(b)) and was therefore selected as the model bacterium in this study. Interestingly, *P. aeruginosa* PAO1 and P60 showed similar capabilities to attach onto membranes, with densities of 2.0×10^7 ($\pm 1.3 \times 10^6$) and 2.4×10^7 ($\pm 2.9 \times 10^6$) cells/cm^2, respectively. In addition, F4 and F5 also showed similar capabilities: 1.4×10^7 ($\pm 8.0 \times 10^5$) and 1.4×10^7 ($\pm 2.8 \times 10^5$) cells/cm^2, respectively. The F4 and F5 strains were identified as being from the same bacteria species, *Pseudomonas otitidis* (*P. otitidis*) strain MCC10330 (similarity 98%). Commonly identified bacteria on the fouled membranes were *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Flavobacterium*, and *Aeromonas* [26]. *P. otitidis* was also noted as being isolated from marine sediment [27].

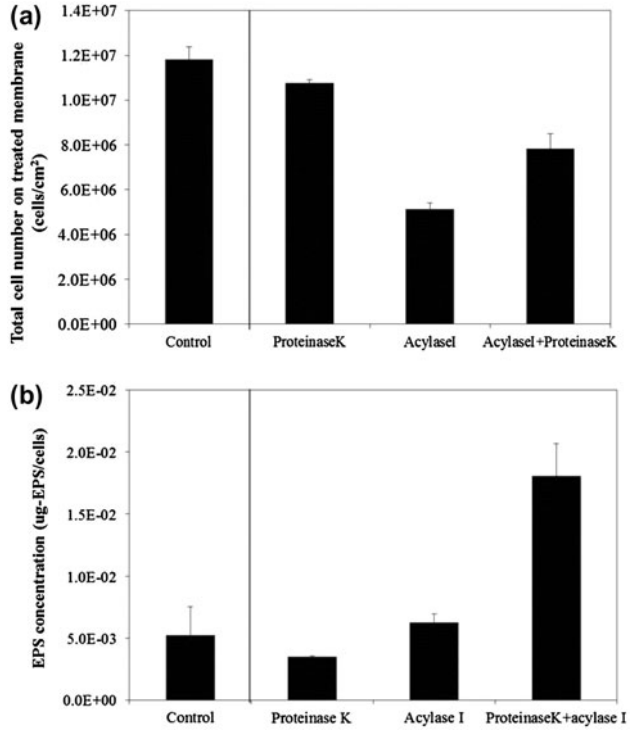

Fig. 2. Effects of enzyme treatment on (a) total cell number and (b) EPS concentration.

3.2. Effects of enzyme treatment on total cell number and EPS

In order to investigate the reduction of biofouling on RO membranes, acylase I (5 μg/mL), proteinase K (100 μg/mL), and their combination (5 μg/mL of acylase I and 100 μg/mL of proteinase K) were employed. The number of bacteria on the membranes decreased notably after enzyme treatment, with bacteria removal efficiencies of 9.0, 56.6, and 33.7% when treated with proteinase K, acylase I, and a combination of acylase I and proteinase K, respectively (Fig. 2(a)). In a recent study, 11.6 μg/mL of acylase I removed 57.1% of bacteria on an RO membrane [28], suggesting that acylase I is an efficient enzyme for biofouling reduction. However, the EPS concentrations could not be effectively removed here (Fig. 2(b)). Although acylase I showed a high bacteria removal efficiency, the EPS concentration (6.2×10^{-3} μg-EPS/cells) increased compared with the control sample (5.2×10^{-3} μg-EPS/cells), as the number of cells significantly decreased compared with the change of EPS concentration. The third sample was then treated with a combination of proteinase K and acylase I, which displayed both low bacteria and EPS removal efficiencies. Indeed, 3.00 mg/L of control EPS concentration was decreased to 1.89, 2.37, and 2.44 mg/L when treated with proteinase K, acylase I, and their combination, respectively. These results were comparable with the EPS concentration, in that acylase I and the combination of acylase I and proteinase K showed a low organic carbon removal efficiency. Previously, it was reported that released fatty acids and homoserine lactones produced by AHL hydrolysis could be utilized as an energy source for bacterial growth [29]; this phenomenon could exacerbate biofouling.

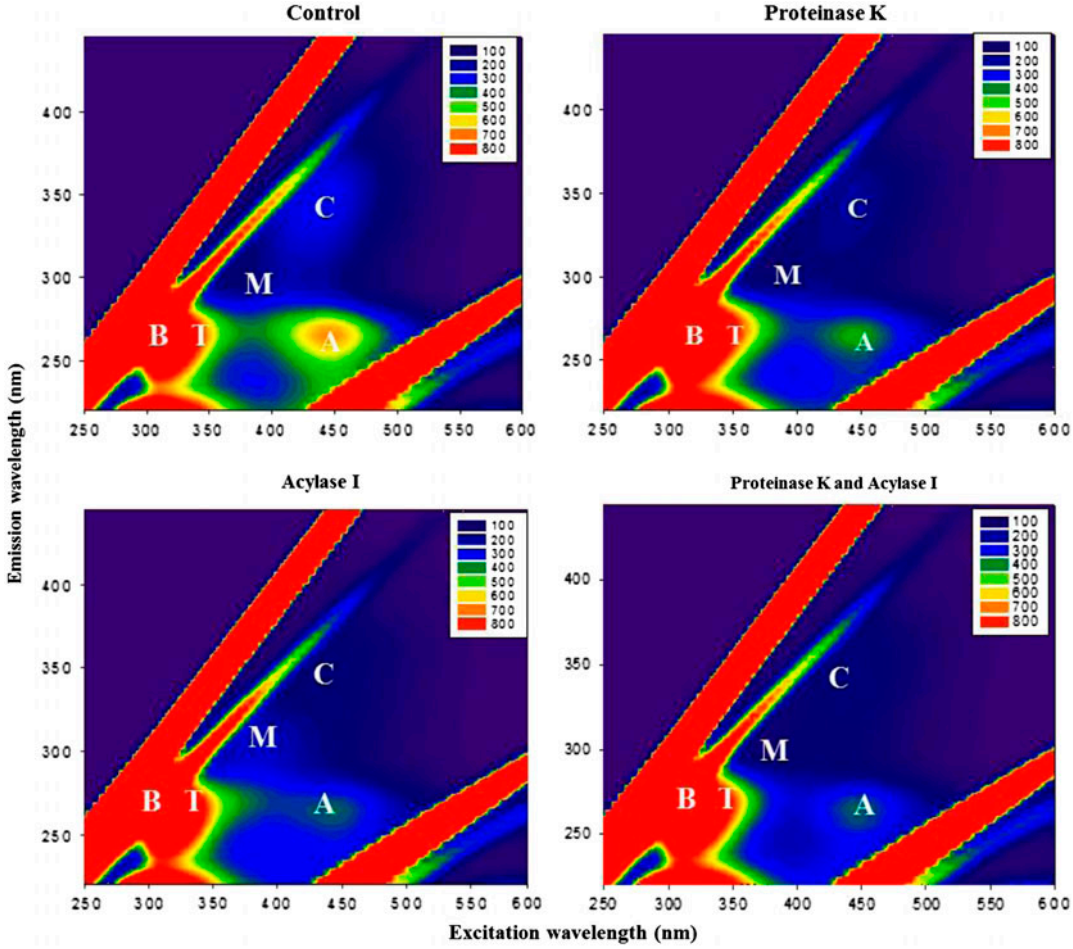

Fig. 3. EEM plots for extracted EPS after enzyme treatment with proteinase K, acylase I, and their combination. The x-axis represents excitation spectra and the y-axis represents emission spectra. (B: tyrosine-like and protein-like; T: tryptophan-like and protein-like; A: UV humic-like; M: visible marine humic-like; C: visible humic-like).

3.3. Fluorescence analysis of dissolved organic matter

EEMs provide information regarding the fluorescent dissolved organic matter composition of EPS in the wavelength-independent fluorescence maximum (Ex_{max}/Em_{max}) [26]. The main peaks of protein-like substances (peaks of B and T) and humic substances (peaks of A, M, and C) are shown in Fig. 3 [30]. Despite their complexity, however, the intensities of UV humic-like substances (A) and visible humic-like (C) peaks were decreased after enzyme treatment. For example, acylase I decreased the peak intensity of both A and C by 30.9 and 46.7%, respectively, compared with the control sample. In contrast, protein-like substances (B and T) and visible marine humic-like substances could not be removed by acylase I. Proteinase K removed UV humic-like, visible marine humic-like, and visible humic-like substances at 28.2, 26.5, and 32.0% efficiency. It was expected that proteinase K would also remove the protein-like substances (B and T); surprisingly, however, the intensities of B and T peaks actually increased by 16, and 51%, respectively. Humic substances were reported to decrease the proteolytic enzyme (pronase E) activity by interacting with the applied enzyme [30], possibly making it an effective agent for proteinase K activity. When both enzymes were applied, all humic substances decreased, with 39.3% (UV humic-like), 36.4% (visible marine humic-like), and 42.8% (visible humic-like) efficiency. These results confirm that proteinase K contributes to the reduction of visible marine humic-like substances and that acylase I contributes to the reduction of UV and visible humic-like substances.

4. Conclusions

In this study, acylase I and proteinase K were used to reduce biofouling. Biofouling on RO membranes was formed by *P. aeruginosa* P60, which was selected as a model bacterium. In a comparison of the bacteria removal efficiency, EPS could not be reduced by applying acylase I and a combination of acylase I and proteinase K enzymes. Subsequent EEM results showed that the intensities of humic substances were significantly decreased, though protein-like substances were not removed. We thus posited that the remaining humic substances could have an effect on enzyme activity; as such, an effective biofouling reduction agent should remove simultaneously bacteria and EPS. Nevertheless, we confirmed that the current form of enzyme treatment method is not an effective way to reduce biofouling. Therefore, more efficient enzymatic treatment method capable of increasing the EPS removal efficiency should be developed.

Acknowledgements

This research was supported by a grant (07SeaHero-A01-01) from the Plant Technology Advancement Program funded by the Ministry of Land, Transport and Maritime Affairs, Korea. The authors would also like to thank Prof. Cho's lab in the Gwangju Institute of Science and Technology for the use of their TOC analyzer.

References

[1] H.C. Flemming, G. Schaule, Biofouling on membranes-a microbiological approach, Desalination 70 (1988) 95–119.
[2] R.P. Schneider, L.M. Ferreira, P. Binder, E.M. Bejarano, K.P. Goes, E. Slongo, C.R. Machado, G.M.Z. Rosa, Dynamics of organic carbon and of bacterial populations in a conventional pretreatment train of a reverse osmosis unit experiencing severe biofouling, J. Membr. Sci. 266 (2005) 18–29.
[3] S. Kappachery, D. Paul, J. Yoon, J.H. Kweon, Vanillin, a potential agent to prevent biofouling of reverse osmosis membrane, Biofouling 26 (2010) 667–672.
[4] J.S. Vrouwenvelder, C. Picioreanu, J.C. Kruithof, M.C.M. van Loosdrecht, Biofouling in spiral wound membrane systems: Three-dimensional CFD model based evaluation of experimental data, J. Membr. Sci. 346 (2010) 71–85.
[5] Y. Xiong, Y. Liu, Biological control of microbial attachment: A promising alternative for mitigating membrane biofouling, Appl. Microbiol. Biotechnol. 86 (2010) 825–837.
[6] M. Herzberg, S. Kang, M. Elimelech, Role of extracellular polymeric substances (EPS) in biofouling of reverse osmosis membranes, Environ. Sci. Technol. 43 (2009) 4393–4398.
[7] G.F. Kaufmann, R. Sartorio, S.-H. Lee, J.M. Mee, L.J. Altobell, D.P. Kujawa, E. Jeffries, B. Clapham, M.M. Meijler, K.D. Janda, Antibody interference with *N*-acyl homoserine lactone-mediated bacterial quorum sensing, J. Am. Chem. Soc. 128 (2006) 2802–2803.
[8] M. Hentzer, K. Riedel, T.B. Rasmussen, A. Heydorn, J.B. Andersen, M.R. Parsek, S.A. Rice, L. Eberl, S. Molin, N. Høiby, S. Kjelleberg, M. Givskov, Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound, Microbiology 148 (2002) 87–102.
[9] B. Huber, L. Eberl, W. Feucht, J. Polster, Influence of polyphenols on bacterial biofilm formation and quorum-sensing. Zeitschrift für Naturforschung. C, J. Biosci. 58 (2003) 879–884.
[10] L. Lu, M.E. Hume, S.D. Pillai, Autoinducer-2-like activity associated with foods and its interaction with food additives, J. Food Prot. 67 (2004) 1457–1462.
[11] S.F. Bodini, S. Manfredini, M. Epp, S. Valentini, F. Santori, Quorum sensing inhibition activity of garlic extract and p-coumaric acid, Lett. Appl. Microbiol. 49 (2009) 551–555.
[12] N. Barraud, D.J. Hassett, S.-H. Hwang, S.A. Rice, S. Kjelleberg, J.S. Webb, Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*, J. Bacteriol. 188 (2006) 7344–7353.
[13] E.G.A. Fredheim, C. Klingenberg, H. Rohde, S. Frankenberger, P. Gaustad, T. Flaegstad, J.E. Sollid, Biofilm formation by *Staphylococcus haemolyticus*, J. Clin. Microbiol. 47 (2009) 1172–1180.
[14] P. Chaignon, I. Sadovskaya, C. Ragunah, N. Ramasubbu, J.B. Kaplan, S. Jabbouri, Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition, Appl. Microbiol. Biotechnol. 75 (2007) 125–132.
[15] C. Leroy, C. Delbarre, F. Ghillebaert, C. Compere, D. Combes, Influence of subtilisin on the adhesion of a marine bacterium which produces mainly proteins as extracellular polymers, J. Appl. Microbiol. 105 (2008) 791–799.
[16] J.B. Kaplan, C. Ragunath, K. Velliyagounder, D.H. Fine, N. Ramasubbu, Enzymatic detachment of Staphylococcus epidermidis biofilms, Antimicrob. Agents Chemother. 48 (2004) 2633–2636.

[17] F.M.I. Natrah, T. Defoirdt, P. Sorgeloos, P. Bossier, Disruption of bacterial cell-to-cell communication by marine organisms and its relevance to aquaculture, Mar. Biotechnol. 13 (2011) 109–126.
[18] J. Yu, Y. Baek, H. Yoon, J. Yoon, New disinfectant to control biofouling of polyamide reverse osmosis membrane, J. Membr. Sci. 427 (2013) 30–36.
[19] W. Lee, C.H. Ahn, S. Hong, S. Kim, S. Lee, Y. Baek, J. Yoon, Evaluation of surface properties of reverse osmosis membranes on the initial biofouling stages under no filtration condition, J. Membr. Sci. 351 (2010) 112–122.
[20] L.H. Kim, A. Jang, H.-W. Yu, S.-J. Kim, I.S. Kim, Effect of chemical cleaning on membrane biofouling in seawater reverse osmosis processes, Desalin. Water Treat. 33 (2011) 289–294.
[21] S. Stepanovic, D. Vukovic, I. Dakic, B. Savic, M. Svabic-Vlahovic, A modified microtiter-plate test for quantification of staphylococcal biofilm formation, J. Microbiol. Methods 40 (2000) 175–179.
[22] E. Karunakaran, C.A. Biggs, Mechanisms of *Bacillus cereus* biofilm formation: An investigation of the physicochemical characteristics of cell surfaces and extracellular proteins, Appl. Microbiol. Biotechnol. 89 (2011) 1161–1175.
[23] J. Wu, C. Xi, Evaluation of different methods for extracting extracellular DNA from the biofilm matrix, Appl. Environ. Microbiol. 75 (2009) 5390–5395.
[24] P.G. Coble, Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy, Mar. Chem. 51 (1996) 325–346.
[25] J.S. Baker, L.Y. Dudley, Biofouling in membrane systems—A review, Desalination 118 (1998) 81–90.
[26] K. Ramani, P. Saranya, S.C. Jain, G. Sekaran, Lipase from marine strain using cooked sunflower oil waste: Production optimization and application for hydrolysis and thermodynamic studies, Bioprocess Biosyst. Eng. 36 (2013) 301–315.
[27] M. Kim, S. Lee, H.-D. Park, S.-I. Choi, S. Hong, Biofouling control by quorum sensing inhibition and its dependence on membrane surface, Water Sci. Technol. 66 (2012) 1424–1430.
[28] Y.-J. Wang, J.R. Leadbetter, Rapid acyl-homoserine lactone quorum signal biodegradation in diverse soils, Appl. Environ. Microbiol. 71 (2005) 1291–1299.
[29] J.B. Jahnel, F.H. Frimmel, Comparison of the enzyme inhibition effect of different humic substances in aqueous solutions, Chem. Eng. Process 33 (1994) 325–330.
[30] H. Xu, Y. Liu, D-amino acid mitigated membrane biofouling and promoted biofilm detachment, J. Membr. Sci. 376 (2011) 266–274.