Proteins causing membrane fouling in membrane bioreactors

Taro Miyoshi, Yuhei Nagai, Tomoyasu Aizawa, Katsuki Kimura and Yoshimasa Watanabe

ABSTRACT

In this study, the details of proteins causing membrane fouling in membrane bioreactors (MBRs) treating real municipal wastewater were investigated. Two separate pilot-scale MBRs were continuously operated under significantly different operating conditions; one MBR was a submerged type whereas the other was a side-stream type. The submerged and side-stream MBRs were operated for 20 and 10 days, respectively. At the end of continuous operation, the foulants were extracted from the fouled membranes. The proteins contained in the extracted foulants were enriched by using the combination of crude concentration with an ultrafiltration membrane and trichloroacetic acid precipitation, and then separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The N-terminal amino acid sequencing analysis of the proteins which formed intensive spots on the 2D-PAGE gels allowed us to partially identify one protein (OmpA family protein originated from genus *Brevundimonas* or *Riemerella anatipestifer*) from the foulant obtained from the submerged MBR, and two proteins (OprD and OprF originated from genus *Pseudomonas*) from that obtained from the side-stream MBR. Despite the significant difference in operating conditions of the two MBRs, all proteins identified in this study belong to β-barrel protein. These findings strongly suggest the importance of β-barrel proteins in developing membrane fouling in MBRs.

Key words | characterization of foulants, membrane bioreactor, membrane fouling

Taro Miyoshi (corresponding author)
Yoshimasa Watanabe
Research Center for Environmental Nano and Bio Engineering in Hokkaido University,
N13W8, Kita-ku, Sapporo 060-8628,
Japan
E-mail: *t-miyoshi@pegasus.kobe-u.ac.jp*

Taro Miyoshi
Department of Chemical Science and Engineering,
Center for Membrane and Film Technology,
Kobe University,
1-1 Rokkodai, Nada-ku, Kobe 657-8501,
Japan

Yuhei Nagai
Katsuki Kimura
Division of Built Environment,
Hokkaido University,
N13W8, Kita-ku, Sapporo 060-8628,
Japan

Tomoyasu Aizawa
Division of Biological Sciences,
Hokkaido University,
N10W8, Kita-ku, Sapporo 060-0810,
Japan

INTRODUCTION

Membrane bioreactors (MBRs) have various advantages over conventional wastewater treatment technologies, such as complete removal of suspended solids and reduced footprint; therefore, they are increasingly popular for wastewater treatment (Kraume & Drews 2010). At present, however, wider application of this technology is hampered by its high operation and maintenance costs, which are mainly attributed to the development of membrane fouling. To establish a strategy for controlling membrane fouling, the mechanisms by which membrane fouling develops need to be elucidated.

Membrane fouling develops as a result of interaction between membrane and constituents causing membrane fouling (foulants). Therefore, the characteristics of both membrane and foulants are obviously important for better understanding of fouling mechanisms. Nonetheless, information on the characteristics of foulants is particularly limited at present. In a number of previous studies dealing with membrane fouling in MBRs, together with polysaccharides, proteins were also identified as one of the important foulants (Metzger *et al.* 2007; Kimura *et al.* 2008a; Miyoshi *et al.* 2009; Tang *et al.* 2010; Tian *et al.* 2011). At present, detailed information on the characteristics of proteins involved in membrane fouling in MBRs is quite limited. We previously identified two proteins involved in membrane fouling developed in pilot-scale MBRs treating real municipal wastewater as OprD and OprF, originated from genus *Pseudomonas* (Miyoshi *et al.* 2012). However, the number of proteins identified is still insufficient, and therefore, more proteins that cause membrane fouling in MBRs operated under various operating conditions should be identified to obtained comprehensive knowledge about the characteristics of proteins which are detrimental to the operation of MBRs.

In this study, we attempted to identify the proteins causing membrane fouling in two pilot-scale MBRs treating real municipal wastewater. These two MBRs were operated under significantly different operating conditions; one MBR

doi: 10.2166/wst.2015.282

was equipped with a submerged membrane module whereas the other was operated with a side-stream membrane module. At the end of operation, proteins that caused membrane fouling were extracted from the fouled membranes and subjected to the identification of proteins through determining the N-terminal amino acid sequences. On the basis of the results obtained in this study, the proteins responsible for developing membrane fouling are discussed.

MATERIALS AND METHODS

Continuous operation of pilot-scale MBRs

We operated two pilot-scale MBRs with different operating conditions. The first MBR (MBR-1) was a submerged MBR; MBR-1 was operated as a baffled MBR (BMBR), in which both nitrification and denitrification proceed in a single bioreactor (Kimura *et al.* 2008b). The other MBR (MBR-2) was a side-stream MBR equipped with a tubular membrane module. The inside of the bioreactor of MBR-2 was also separated by baffles and was operated in a manner similar to a BMBR (Hoque *et al.* 2012). As a result, excellent removal of total nitrogen was also achieved in MBR-2 (Hoque *et al.* 2012). In both MBRs, membranes fabricated by polyvinylidene fluoride (PVDF) were used. The operating conditions of each MBR are summarized in Table 1; MBR-1 and MBR-2 were continuously operated for 20 and 10 days, respectively. Both MBRs were installed at an existing municipal wastewater treatment facility (Soseigawa Wastewater Treatment Center, Sapporo, Japan) and operated continuously for several weeks using the raw wastewater delivered from the inlet of the primary sedimentation basin of the facility. The characteristics of raw wastewater can be found elsewhere (Kimura *et al.* 2008b).

Identification of proteins causing membrane fouling

At the end of the continuous operation, the membrane modules were disassembled and the foulants that caused membrane fouling in each membrane module were extracted by soaking the fouled membranes in NaOH solution with a pH of 12 overnight. In a preliminary test, the extraction of foulants by NaOH solution reduced membrane filtration resistance by approximately 50% (data not shown). Proteins remaining after extraction by NaOH solution can be further extracted using a solution comprised of sodium dodecyl sulfate (SDS). However, such secondary extraction using strong protein solubilizer did not result in apparent restoration in membrane permeability, partially due to the small quantity of proteins remaining after the extraction with NaOH solution (data not shown). These results obtained in our preliminary test suggest that the major proteins contributing to the development of membrane filtration resistance could be extracted using NaOH solution.

After the extraction was completed, the NaOH solution containing the extracted foulants was collected. The proteins contained in the extracted foulants were then enriched using protocols reported previously (Miyoshi *et al.* 2012). Briefly, at first, the proteins contained in the extraction solution were crudely concentrated using an ultrafiltration (UF) membrane made of low-protein binding regenerated cellulose with a nominal molecular weight cutoff of 10 kDa (YM-10; Millipore, Bedford, MA, USA). Then, the proteins contained in the concentrated solution were precipitated in 10% (w/v) trichloroacetic acid (TCA), incubated on ice for 1 h, and recovered by centrifugation (15,000 rpm; 5 min). The protein pellets were washed twice in 80% (v/v) ice-cold acetone and then air-dried. The dried protein pellets were subjected to separation using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The pellets were resuspended in 50 μL of sample solubilization buffer comprising 5 M urea, 1 M thiourea, 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS; amphoteric surfactant), 1% (w/v) Triton X-100 (nonionic surfactant), and 1% (w/v) dithiothreitol. After ultrasonication, the solubilization buffer containing resuspended pellets was placed on a vortex shaker. The solubilization buffer was then centrifuged at 15,000 rpm for 30 min at 4 °C to remove suspended matter, and then, 40 μL of the supernatant was applied to the 2D-PAGE separation. For first-dimension separation of the 2D-PAGE, 75-mm immobilized pH gradient strips (AgarGEL

Table 1 | Operating conditions of MBRs

	Membrane type	Membrane material	Pore size (μm)	Bioreactor configuration	HRT (h)	SRT (days)	Membrane flux ($m^3/m^2/day$)
MBR-1	Flat sheet	PVDF	0.1	Baffled reactor	3	20	0.8
MBR-2	Tubular	PVDF	0.01	Baffled reactor	4	40	2.0

with a pH range of 3e10; Atto Corporation, Tokyo, Japan) were placed in an isoelectric focusing (IEF) unit (discRun, Atto Corporation, Tokyo, Japan) and focused for 210 min with a constant voltage of 350 V. Following IEF, proteins were fixed by soaking the strips in 2.5% TCA solution for 1 min. TCA was then eliminated by soaking the strips in milli-Q water. After that, the strips were equilibrated with a solution comprising 50-mM Tris–HCl, pH 6.8, 2% SDS, and 0.001% bromophenol blue for 10 min. Strips were then loaded onto cast 12.5% polyacrylamide gels (E-D12.5L, Atto Corporation, Tokyo, Japan), and electrophoresis was performed at 20 mA for 90 min. The gels were stained with Coomassie Brilliant Blue (EzStain AQua; Atto, Tokyo, Japan).

After 2D-PAGE, the separated proteins were electroblotted onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). The spots of the selected proteins were cut out and then subjected to N-terminal amino acid sequencing analysis. The N-terminal amino acid sequences were determined by automated Edman degradation using a pulsed-liquid sequence analyzer (Procise 492, Perkin Elmer, Foster City, CA, USA). Amino acid sequences were searched using protein blast at http://blast.ncbi.nlm.nih.gov/ using the default settings of the 'Search for short, nearly exact matches' function in September 2014.

RESULTS AND DISCUSSION

Identification of proteins involved in membrane fouling in pilot-scale MBRs

At the end of the continuous operation, the foulants that caused membrane fouling in each MBR were extracted from the fouled membranes. Then, proteins contained in each foulant were enriched by the combination of a crude concentration using a UF membrane and TCA precipitation method. The enriched proteins were subjected to separation using 2D-PAGE. Figure 1 shows the 2D-PAGE gel images of the proteins extracted from the fouled membranes used in the two pilot-scale MBRs. In both 2D-PAGE gels, the proteins were well separated and formed intensive spots, indicating that proteins contained in the extracted foulants were successfully enriched. After the separation using 2D-PAGE, several proteins which formed intensive spots were recovered from the gels and the N-terminal amino acid sequences of recovered proteins were analyzed using the Edman degradation reaction.

In total, 15 and 16 proteins were successfully recovered from the 2D-PAGE gels of the foulants obtained from MBRs-1 and -2, respectively. The results of the N-terminal amino acid sequencing analysis are summarized in Table 2. Unfortunately, none of the amino acid sequences determined in this study completely matched any protein sequences in a blast search. However, the amino acid sequence of protein-12 obtained from MBR-1 was highly matched to seven proteins with a query coverage of 93%. The corresponding *E*-values (description of the random background noise) were sufficiently low: 3e-05 for two proteins and 2e-04 for the others. All were OmpA family proteins originated from genus *Brevundimonas* or *Riemerella anatipestifer*. For protein-4 obtained from MBR-2, nine proteins recorded the highest match with a query coverage of 86%. Although *E*-values for these proteins were relatively high (0.015 for three proteins and 0.076 for the other six proteins), all proteins with the highest match were completely occupied by membrane protein originated from genus *Pseudomonas*. Two of the nine proteins were assigned as OprD. On this basis, it can be said that protein-4 obtained from MBR-2 is partially identified as OprD originated from genus *Pseudomonas*. The amino acid sequence of protein-10

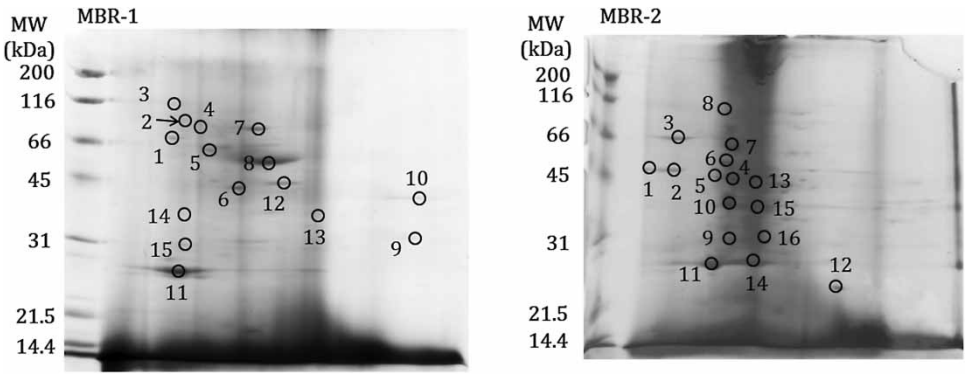

Figure 1 | 2D-PAGE gel images of the proteins extracted from the fouled membranes.

Table 2 | N-terminal amino acid sequence of proteins causing membrane fouling

	Protein	Amino acid sequence
MBR-1	5	NXLLFPYFTTXXGXX
	6	EPNGXXGAIDAXXXX
	7	XGXTPATDNLXFPYI
	8	NSLLFPYFTTATGAQ
	11	ATVILDGVTKQQGVV
	12	EPNGWYGAVDAGYHX
	14	ATVTINGSTTPFALL
	15	ANVTINGTXXXXXXX
MBR-2	1	AFVVGGVDFGAPGSH
	2	XXKAGTPFTLAGXLA
	3	XIVTXHVAAVQQMYV
	4	XXQDDAKGFVEDSXL
	10	QGQGAVEXELNYGKX
	12	SXSVKXMPEHXEXXX
	14	AFVVGGVDFGAPGSH

obtained from MBR-2 matched with a query coverage of 93% to eight proteins (the corresponding *E*-value was 2e-03). As with the case of protein-4 obtained from MBR-2, all proteins with the highest match belong to membrane protein originated from genus *Pseudomonas*. Two of the eight proteins were assigned to OprF, and therefore, it is very likely that protein-10 obtained from MBR-2 was closely related to OprF.

Characteristics of identified proteins

For the source organisms of protein-12 obtained from MBR-1 (OmpA family protein), six of the seven highly matched proteins were assigned as bacteria belonging to genus *Brevundimonas*. This genus was formerly categorized as one of the subgroup of genus *Pseudomonas* (rRNA group IV; Kersters *et al.* 1996). At present, however, as tools for genetic analysis have improved, the classification has been revised. Currently, the genus *Pseudomonas* species is restricted to the former rRNA group I, which belongs to γ-proteobacteria, and the former rRNA group IV, which belongs to α-proteobacteria, was reclassified in a new genus *Brevundimonas* (Kersters *et al.* 1996). *Brevundimonas* is only very distantly related to *Pseudomonas*. The bacteria belonging to *Brevundimonas* were often detected in activated sludge or other related wastewater treatment processes (Li & Li 2009; Srinandan *et al.* 2011; Amorim *et al.* 2014; Janeczko *et al.* 2014), and therefore, the presence of *Brevundimonas* in our MBR is not surprising. The source organism of the other identified protein from the amino acid sequence of protein-12 of MBR-1 was *Riemerella anatipestifer*. This organism is a well-known pathogen for ducks and geese, but related species were also detected in wastewater treatment processes (Li & Li 2009). Therefore, as with the case of genus *Brevundimonas*, the presence of bacteria related to *Riemerella anatipestifer* is also highly possible. The source organisms of the two proteins identified in the foulant obtained from MBR-2 were bacteria belonging to genus *Pseudomonas*. Among the plausible organisms, aquatic organisms which had been detected in activated sludge (e.g., *Pseudomonas fluorescens*; Drysdale *et al.* 1999) were included. The two proteins detected in the foulant obtained from MBR-2 (i.e., OprD and OprF originated from genus *Pseudomonas*) had also been detected in our previous study (Miyoshi *et al.* 2012). They have β-barrel structures and are embedded in the outer cell membrane comprised of lipopolysaccharides (LPSs). OprF is widely considered as an ortholog of OmpA with significant amino acid similarity in C-terminal domains (Brinkman *et al.* 2000). On this basis, it can be said that similar proteins were consistently detected in the foulants obtained from pilot-scale MBRs treating real municipal wastewater.

The operating conditions of the pilot-scale MBRs operated in this study and our previous study (Miyoshi *et al.* 2012) differed significantly. The MBRs operated in our previous study had a single aerobic bioreactor (i.e., denitrification were not promoted). In contrast, the two pilot-scale MBRs operated in this study had a baffled bioreactor in which both nitrification and denitrification were promoted in a single chamber (Kimura *et al.* 2008b; Hoque *et al.* 2012). Therefore, it can be expected that the structures of microbial the community involved in the biological treatment differed significantly between the MBRs operated in this study and those operated in our previous study. In addition, the configurations of the membrane module were also significantly different. In our previous study, submerged MBRs equipped with hollow-fiber membrane were used. The MBR-1 operated in this study was also a submerged MBR, but this MBR was equipped with a flat-sheet membrane. Conversely, the MBR-2 operated in this study was a side-stream MBR, in which the flow regime around the membrane is entirely different from that of the submerged MBR. The nominal pore sizes of the membrane used in each MBR were also different: 0.1 μm for MBR-1 (this study), 0.01 μm for MBR-2 (this study), and 0.4 μm for the submerged MBRs operated in our previous study. Despite the significant differences in operating conditions mentioned above, similar proteins were repeatedly detected in the foulants that caused membrane fouling in pilot-scale MBRs treating real municipal wastewater. The findings obtained in this study suggest that the β-barrel proteins are very widely involved in the membrane fouling in MBRs, irrespective of operating conditions. As discussed in our previous paper (Miyoshi *et al.* 2012), it is highly possible that the β-barrel proteins surviving in

mixed liquor suspension are contained in the small debris of the outer membrane of the source bacteria, which is comprised of an LPS bilayer, since such proteins become more resistant to proteolysis by protease if they associate with LPSs (Freulet-Marriere *et al.* 2000). The information on the existence form (including surrounding circumstances) of the β-barrel proteins involved in membrane fouling in MBR would be of critical importance for elucidating the fouling mechanisms in MBRs. Further study on this issue is therefore needed.

CONCLUSIONS

In this study, proteins causing membrane fouling in MBRs operated under different operating conditions were investigated in detail. The proteins contained in the foulants extracted from the fouled membranes used in the two different pilot-scale MBRs treating real municipal wastewater were separated by 2D-PAGE. The N-terminal amino acid sequencing analysis of the proteins that formed intensive spots on the 2D-PAGE gels allowed us to partially identify one protein from MBR-1 (submerged MBR) and two proteins from MBR-2 (side-stream MBR). Although operating conditions of the two pilot-scale MBRs differed significantly, the identified proteins obtained from both MBRs belong to β-barrel proteins. The results obtained in this study strongly suggest that β-barrel proteins are widely involved in membrane fouling in MBRs.

ACKNOWLEDGEMENTS

We thank Mr Tomohiro Hirose from the Equipment Management Center in Hokkaido University for his assistance in N-terminal amino acid sequencing analysis. This research was financially supported by a Grant-in-Aid for the 'Megaton Water System' Project from the New Energy and Industrial Technology Development Organization (NEDO), Japan and by a Grant-in-Aid for Research Activity start-up from the Ministry of Education.

REFERENCES

Amorim, C. L., Maia, A. S., Mesquita, R. B., Rangel, A. O., van Loosdrecht, M. C., Tiritan, M. E. & Castro, P. M. 2014 Performance of aerobic granular sludge in a sequencing batch bioreactor exposed to ofloxacin, norfloxacin and ciprofloxacin. *Water Res.* **50**, 101–113.

Brinkman, F. S. L., Bains, M. & Hancock, R. E. W. 2000 The amino terminus of *Pseudomonas aeruginosa* outer membrane protein OprF forms channels in lipid bilayer membranes: correlation with a three-dimensional model. *J. Bacteriol.* **182** (18), 5251–5255.

Drysdale, G. D., Kasan, H. C. & Bux, F. 1999 Denitrification by heterotrophic bacteria during activated sludge treatment. *Water SA* **25** (3), 357–362.

Freulet-Marriere, M. A., El Hamel, C., Chevalier, S., De, E., Molle, G. & Orange, N. 2000 Evidence for association of lipopolysaccharide with *Pseudomonas fluorescens* strain MF0 porin OprF. *Res. Microbiol.* **151** (10), 873–876.

Hoque, A., Kimura, K., Miyoshi, T., Yamato, N. & Watanabe, Y. 2012 Characteristics of foulants in air-sparged side-stream tubular. Membranes used in a municipal wastewater membrane bioreactor. *Sep. Purif. Technol.* **93**, 83–91.

Janeczko, A. K., Walters, E. B., Schuldt, S. J., Magnuson, M. L., Willison, S. A., Brown, L. M., Ruiz, O. N., Felker, D. L. & Racz, L. 2014 Fate of malathion and a phosphonic acid in activated sludge with varying solids retention times. *Water Res.* **57**, 127–139.

Kersters, K., Ludwig, W., Vancanneyt, M., De Vos, P., Gillis, M. & Schleifer, K. -H. 1996 Recent changes in the classification of the pseudomonads: an overview. *Syst. Appl. Microbiol.* **19** (4), 465–477.

Kimura, K., Miyoshi, T., Naruse, T., Yamato, N., Ogyu, R. & Watanabe, Y. 2008a The difference in characteristics of foulants in submerged MBRs caused by the difference in the membrane flux. *Desalination* **231** (1–3), 268–275.

Kimura, K., Nishisako, R., Miyoshi, T., Shimada, R. & Watanabe, Y. 2008b Baffled membrane bioreactor (BMBR) for efficient nutrient removal from municipal wastewater. *Water Res* **42** (3), 625–632.

Kraume, M. & Drews, A. 2010 Membrane bioreactors in waste water treatment – status and trends. *Chem. Eng. Technol.* **33** (8), 1251–1259.

Li, A. J. & Li, X. Y. 2009 Selective sludge discharge as the determining factor in SBR aerobic granulation: numerical modelling and experimental verification. *Water Res.* **43** (14), 3387–3396.

Metzger, U., Le-Clech, P., Stuetz, R. M., Frimmel, F. H. & Chen, V. 2007 Characterisation of polymeric fouling in membrane bioreactors and the effect of different filtration modes. *J. Membr. Sci.* **301** (1–2), 180–189.

Miyoshi, T., Aizawa, T., Kimura, K. & Watanabe, Y. 2012 Identification of proteins involved in membrane fouling in membrane bioreactors (MBRs) treating municipal wastewater. *Int. Biodeter. Biodegr.* **75**, 15–22.

Miyoshi, T., Tsuyuhara, T., Ogyu, R., Kimura, K. & Watanabe, Y. 2009 Seasonal variation in membrane fouling in membrane bioreactors (MBRs) treating municipal wastewater. *Water Res.* **43** (20), 5109–5118.

Srinandan, C. S., Shah, M., Patel, B. & Nerurkar, A. S. 2011 Assessment of denitrifying bacterial composition in activated sludge. *Bioresour. Technol.* **102** (20), 9481–9489.

Tang, S., Wang, Z., Wu, Z. & Zhou, Q. 2010 Role of dissolved organic matters (DOM) in membrane fouling of membrane bioreactors for municipal wastewater treatment. *J. Hazard. Mater.* **178** (1–3), 377–384.

Tian, Y., Chen, L., Zhang, S. & Zhang, S. 2011 A systematic study of soluble microbial products and their fouling impacts in membrane bioreactors. *Chem. Eng. J.* **168** (3), 1093–1102.

First received 15 February 2015; accepted in revised form 19 May 2015. Available online 2 June 2015