Submission for *International Biodeterioration & Biodegradation*

Identification of proteins involved in membrane fouling in membrane bioreactors (MBRs) treating municipal wastewater

Taro Miyoshi[1,*], Tomoyasu Aizawa[2], Katsuki Kimura[3], Yoshimasa Watanabe[1]

1: Research Center for Environmental Nano and Bio Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo 060-8628, Japan
2: Division of Biological Sciences, Hokkaido University, N10W8, Kita-ku, Sapporo 060-0810, Japan
3: Division of Built Environment, Hokkaido University, N13W8, Kita-ku, Sapporo 060-8628, Japan

**Corresponding author*
Tel & Fax: +81-11-706-6267
E-mail: jms-mbr@eng.hokudai.ac.jp

Abstract

The proteins that caused membrane fouling in a continuous operation of MBRs treating real municipal wastewater were investigated in detail. We continuously operated two identical pilot-scale MBRs under different solid retention times (SRTs) and extracted the foulants at the end of the operation. Regardless of the operating conditions, proteins were dominant components in the foulants extracted from the fouled membranes. The extracted proteins were subjected to the separation with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the identification through the N-terminal amino acid sequencing analysis. The proteins concentrated by the combination of the crude concentration using an ultra-filtration (UF) membrane and trichloroacetic acid (TCA) precipitation were separated and visualized well on 2D-PAGE gels. The results of 2D-PAGE analysis indicated that the compositions of proteins that caused membrane fouling significantly differed depending on the SRT, although such differences cannot be seen in the amino acid composition analysis. Analyzing selected 2D-PAGE spots by N-terminal amino acid sequencing analysis led to the identification of two well-characterized outer membrane proteins originating from *Pseudomonas* genus, namely OprF and OprD. To our knowledge, this is the first successful identification of proteins that have caused membrane fouling in continuous operations of MBRs treating real wastewater.
Key words: Membrane bioreactor, Membrane fouling, Foulant characterization, Proteins

1. Introduction

Membrane bioreactors (MBRs) have many advantages over the conventional wastewater treatment technologies (Yamamoto et al. 1989; Stephenson et al. 2000) and are therefore becoming increasingly popular for wastewater treatment (Kraume and Drews 2010). To use MBRs more efficiently, however, problems associated with membrane fouling should be addressed. An understanding of fouling mechanisms is indispensable for controlling membrane fouling in MBRs. There have been many studies on membrane fouling in MBRs, and a considerable number of papers have been published (Meng et al. 2009; Drews 2010). Despite those intensive efforts, fundamental knowledge on fouling mechanisms in MBRs has been insufficiently accumulated (Drews 2010).

The insufficient understanding of fouling mechanisms in MBRs is mainly attributed to the lack of information on the constituents that cause membrane fouling (foulants). It is widely accepted that organic macro-molecules such as soluble microbial products (SMPs) and extracellular polymeric substances (EPSs), which are released during cell lysis, diffuse through the cell membrane, or are excreted for some purposes, are the compounds primarily responsible for developing membrane fouling in MBRs (Judd 2007; Wang et al. 2008). In the early studies on membrane fouling in MBRs, the relationships between concentrations of such microbial products evaluated with conventional colorimetric methods (Lowry et al. 1951; Dubois et al. 1956) and fouling rate were intensively investigated. Although reasonable relationships between microbial products concentrations and fouling rates were seen in some studies (Ng et al. 2006; Rosenberger et al. 2006; Trussel et al. 2006), other researchers reported that development of membrane fouling cannot be explained by concentrations of microbial products determined by the conventional analytical methods (Drews et al. 2008; Kimura et al. 2009; Miyoshi et al. 2009). These discrepancies suggested that some fractions of organic matter contained in mixed liquor suspension contribute more to membrane fouling than the others (Drews 2010; Miyoshi et al. 2010).

Recently, many researchers have investigated contributions of specific fractions of microbial products on membrane fouling. Attention has been given to various fractions such as utilization-associated products (UAP) (Jiang et al. 2010; Tian et al. 2011), biopolymer cluster (BPC) (Wang and Li 2008; Sun et al. 2011), and transparent exopolymer particles (TEP) (de la Torre et al. 2008). At present, however, actual contributions of these fractions to the development of membrane fouling are still unclear, and therefore, no universal fouling

indicator has been proposed (Drews 2010). This is probably because, in many studies focused on membrane fouling in MBRs, constituents responsible for membrane fouling were characterized by comprehensive analyses (e.g., concentrations of carbohydrates and proteins, excitation-emission matrices (EEM) analysis, Fourier transform infrared spectra analysis, building block analyses such as monosaccharide, amino acid composition analyses, etc.). Information on detailed characteristics of individual constituents causing membrane fouling is particularly useful for investigating the interactions between membrane and organics or among organics, giving us deeper insight into fouling mechanisms. However, such information cannot be obtained by the comprehensive analyses mentioned above.

In a number of previous studies, together with polysaccharides, proteins were also identified as one of the major foulants in MBRs (Metzger et al. 2007; Kimura et al. 2008a; Miyoshi et al. 2009; Tang et al. 2010; Tian et al. 2011). Identifying the major proteins causing membrane fouling will give us detailed information on the structures, the properties, and even the functions of the proteins that play important roles in developing membrane fouling. Recently, many key proteins in activated sludge systems have been identified through metaproteomic analyses, in which proteins are separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or two dimensional-PAGE (2D-PAGE) and selected proteins are identified by mass spectrometric (MS) analysis or N-terminal amino acid sequencing analysis (Wilmes and Bond 2004; Park and Helm 2008; Park et al. 2008; Silva et al. 2012). Applying techniques used in such analyses to foulants extracted from membranes fouled in MBRs would enable us to identify proteins that cause membrane fouling in MBRs. Very recently, Huang et al. (2012) identified several proteins contained in a gel layer accumulated on membrane surfaces during a batch filtration of anaerobic activated sludge. Although their work clearly demonstrated the applicability of such analyses for investigating proteins involved in membrane fouling, it is thought that a gel layer formed during the batch filtration of anaerobic activated sludge, which contains a microorganism whose genome sequences are fully determined (i.e., *Ralstonia eutropha*), is irrelevant to membrane fouling in real MBRs: most MBRs are aerobic, and genetic information on microorganisms contained in the mixed liquor suspension is generally unavailable.

Membrane fouling can be divided into two categories: physically reversible and irreversible fouling (Kimura et al. 2004). The former can be controlled as long as efficient physical membrane cleaning is applied. In contrast, the latter develops even when efficient physical cleaning is carried out. Control of physically irreversible fouling is particularly important. To establish a strategy for controlling physically irreversible fouling, proteins contained in

foulants responsible for physically irreversible fouling should be investigated. The foulants involved in the development of physically irreversible fouling are totally different from the gel layer formed on the membrane surface that was investigated by Huang et al. (2012), and at present, proteins contained in the foulants causing physically irreversible fouling in a continuous operation of an MBR have not been identified yet.

In this study, we identified selected proteins causing physically irreversible fouling in a continuous operation of MBRs. Pilot-scale MBRs treating a real municipal wastewater were operated continuously. At the end of the continuous operation, the foulants that caused physically irreversible fouling were extracted from the fouled membranes, and proteins contained in the extracted foulants were subjected to the separation with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the identification through the N-terminal amino acid sequencing analysis. To our knowledge, this is the first successful identification of proteins causing membrane fouling in a continuous operation of real MBRs. The results obtained in this study would be a break-through for advanced investigations of membrane fouling in MBRs.

2. Materials and methods

2.1 Continuous operation of pilot-scale MBRs

Three pilot-scale MBRs operated continuously at an existing municipal wastewater treatment facility (Soseigawa Wastewater Treatment Center, Sapporo, Japan). All three MBRs were fed with the same raw wastewater delivered from the inlet of the primary sedimentation basin of the facility. The characteristics of the raw wastewater of this plant can be found elsewhere (Miyoshi et al. 2009). Two of the three MBRs (MBRs 1 and 2) were identical and operated in parallel (Miyoshi et al. 2011). MBRs 1 and 2 were equipped with a hollow-fiber micro-filtration (MF) membrane module made of polyvinylidene fluoride (PVDF) with a total surface area of 1.3 m^2 and nominal pore size of 0.4 μm (Mitsubishi Rayon Engineering, Tokyo, Japan). These MBRs were operated with different solid retention times (SRTs) (MBR1: 12 days, MBR2: 49 days). Other than SRT, the two MBRs had exactly the same operating conditions. Therefore, any differences between these two MBRs can be attributed solely to the difference in SRT. The membrane flux was fixed at 0.8 m^3/m^2/day (33.3 L/m^2/h), and intermittent filtration (12 min filtration and 3 min pause) was carried out. The hydraulic retention time (HRT) was 5 h for both reactors. As a result, the mixed liquor suspended solid (MLSS) concentrations of MBR 1 and MBR 2 were 1.3 g/L and 9.8 g/L, respectively. These two MBRs continuously operated for 15 days.

The third MBR (MBR 3) was operated with a baffled MBR configuration (Kimura et al. 2008b). MBR 3 was equipped with 6.8 m^2 flat-sheet micro-filtration (MF) membranes (Toray, Tokyo, Japan). The membrane was made of PVDF and had a nominal pore size of 0.1 μm. Ten flat-sheet membrane elements were inserted in the reactor. Five of the ten membrane elements were operated with a membrane flux of 0.4 m^3/m^2/day (16.7 L/m^2/h), whereas the other elements were operated with a membrane flux of 0.8 m^3/m^2/day. Intermittent filtration (15 min filtration and 1 min pause) was also carried out. HRT and SRT were set around 2.9 h and 35 days, respectively. As a result, MLSS concentration in the reactor was 16.4 ± 1.4 g/L. The foulant obtained from the membrane element operated with a membrane flux of 0.8 m^3/m^2/day was used for investigating the appropriate pretreatment protocols for concentrating and purifying the proteins contained in the extracted foulant.

In all three pilot-scale MBRs, the biomass was allowed to acclimatize to the operating conditions for more than three months. After acclimatization of biomass had been confirmed, continuous operation of MBRs was initiated with new membranes. The degree of membrane fouling was evaluated from the membrane filtration resistance calculated by the following equation:

$$J = \frac{\Delta P}{\mu R_t} = \frac{\Delta P}{\mu (R_m + R_f)}$$

where J is the membrane permeate flux ($m^3/m^2/s$), ΔP is the transmembrane pressure (Pa), μ is the viscosity of permeate (Pa s), R_t is the total filtration resistance (m^{-1}), R_m is the intrinsic membrane resistance (m^{-1}), and R_f is the fouling resistance (m^{-1}). When membrane fouling became significant, the membrane modules were taken out from the reactor and were physically cleaned by spraying pressurized water on the membrane and surface wiping with a sponge. The physical membrane cleaning conducted in this study was efficient and almost all physically reversible fouling can be eliminated (Miyoshi et al. 2009).

2.2 Extraction of foulants from fouled membranes

At the end of the continuous operation of the pilot-scale MBRs, foulants that caused physically irreversible fouling were extracted from the fouled membranes. To ensure that a cake layer that had accumulated on the membrane was removed, the surface of the fouled membrane was gently wiped with a sponge prior to the extraction of the foulant. The wiped membrane fibers were then cut into small pieces (1~2 cm). The foulant was extracted from the fouled membrane by soaking the membranes (effective membrane surface area of 0.74 m^2) in a sodium hydroxide solution (2 L) at 30°C for 24 h. During the extraction, the solution

containing the pieces of fouled membrane was gently mixed by a shaker. The pH of the extraction solution was set at 12.

After extraction, the concentrations of total organic carbon (TOC), total protein, and total carbohydrate of the solutions containing the extracted foulants were measured. TOC concentration was determined using a TOC analyzer (TOC-V, Shimadzu, Kyoto, Japan). The Lowry method (Lowry et al. 1951) and the phenol-sulfuric acid method (Dubois et al. 1956) were used to determine the concentrations of total proteins and total carbohydrates, respectively. Bovine serum albumin (BSA) and glucose were used as standards for the measurements of total proteins and total carbohydrates concentrations, respectively. Amino acid composition of extracted foulants was determined using L-8900 (Hitachi, Tokyo, Japan) equipped with ninhydrin-detection system. Before amino acid contents were measured, proteins were subjected to acid hydrolysis (6 N HCl, 24 h, 110°C).

2.3 Pre-concentration of proteins contained in extracted foulants

Crude concentration of proteins extracted from the fouled membrane was performed in a manner similar to that proposed by Tanoue (1995). SDS was added to solutions containing the extracted foulants so that the final concentration was 0.01%. The solution was then concentrated in a continuously stirred dead-end filtration cell (UHP-62K; Advantec Toyo, Tokyo, Japan) equipped with an ultra-filtration (UF) membrane made of low-protein binding regenerated cellulose with a nominal molecular weight cutoff of 10 kDa (YM-10; Millipore, Bedford, MA). 1000 mL of the solution containing the extracted foulant was concentrated to 100 mL. After the concentration with a UF membrane, 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 (15000 rpm; 5 min). The protein pellets were washed twice in 80% (v/v) ice-cold acetone and then air-dried.

2.4 Separation of proteins using polyacrylamide gel electrophoresis

The dried pellets were subjected to the separation using SDS-PAGE or 2D-PAGE. SDS-PAGE with a 12% polyacrylamide separation gel and 5% condensation gel was performed by the method of Laemmli (1970). The pellets were resuspended in 40 µL of SDS-PAGE sample buffer containing 50 mM Tris-HCl pH 6.8, 2% SDS, 6% 2-mercaptoethanol, and 10% glycerol and heated at 95°C for 5 minutes. After centrifugation (15000 rpm; 5 min) at 4°C, 20 µL of the supernatant was applied to SDS-PAGE. For 2D-PAGE separation, 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 (DTT). After an ultrasonication, the solubilization buffer containing resuspended pellet was placed on a vortex shaker. The solubilization buffer was then centrifuged at 15000 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 (IPG) strips (AgarGEL with a pH range of 3-10; 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 MQ 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 (BPB) 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).

2.5 Protein identification using N-terminal amino acid sequencing analysis

After 2D-PAGE, 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 the 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 on December 2011.

3. Results and discussion

3.1 Development of membrane fouling in pilot-scale MBRs

Changes in total filtration resistance determined for MBRs 1 and 2 can be found elsewhere (Miyoshi et al. 2011). In MBR 1 (short SRT), the development of both physically reversible and irreversible fouling were significant. In contrast, the development of both types of membrane fouling was insignificant in MBR 2 (long SRT). Many researches investigating membrane fouling in MBRs treating real wastewater also reported that membrane fouling became significant as SRT decreased (Kimura et al. 2005; Ng et al. 2006; Rosenberger et al. 2006; Miyoshi et al. 2009). Detailed information on characteristics of foulants that caused membrane fouling in these two MBRs is important for elucidating how the difference in SRT affected the development of membrane fouling. Therefore, the foulants that caused physically

irreversible fouling were extracted from the fouled membranes at the end of the continuous operation, and then their characteristics were investigated.

3.2 Characteristics of extracted foulants

Table 1 lists the amount of organic matter extracted from the fouled membranes at the end of the continuous operation of MBRs 1 and 2. For both MBRs, more proteins than carbohydrates were extracted from the fouled membranes. This indicates that, regardless of the operating conditions, proteins were the dominant components in the foulants extracted in this study. Previous studies reported that carbohydrates in SMP are the major components responsible for membrane fouling (Kimura et al. 2005; Rosenberger et al. 2006; Trussel et al. 2006). Recent reports, however, pointed out that proteins are also important foulants in MBRs (Metzger et al. 2007; Kimura et al. 2008a; Miyoshi et al. 2009; Tang et al. 2010; Tian et al. 2011). The results obtained in this study agreed with the recent findings indicating that proteins should also be considered in the development of membrane fouling in MBRs.

To investigate the characteristics of proteins that caused physically irreversible fouling in each MBR, amino acid compositions of the extracted foulants were analyzed. The results are shown in Fig. 1. The amino acid composition analysis gave us more detailed insights into the characteristics of proteins causing physically irreversible fouling in the MBRs than the conventional colorimetric analyses (e.g., Lowry method). The foulants obtained from the two MBRs had similar amino acid compositions. This suggests that the proteins causing the physically irreversible fouling in these two MBRs were similar, which turned out to be false as discussed below.

3.3 Concentration and separation of proteins contained in extracted foulants

The proteins that caused physically irreversible fouling in each MBR were separated using polyacrylamide gel electrophoresis to investigate their characteristics in further detail. Generally, pre-concentration is indispensable for applying the proteins collected from membranes. Huang et al. (2012) concentrated the proteins contained in a gel layer formed on the membrane surface by the precipitation with ammonium sulfate. In their study, this pretreatment worked very well, and consequently, clear and distinct bands were seen in an SDS-PAGE gel. This might be attributed to high protein content in their sample suspension. The gel layer formed on membrane surface during batch filtration of anaerobic activated sludge was likely to have been dominated by proteins (relative dominances of other organic compounds such as polysaccharide or humic substance were likely to have been low), and these proteins can easily be collected from a membrane surface with high protein

concentration. In the case of foulants causing physically irreversible fouling, however, the concentrations of organic matter in the solutions containing the extracted foulants are generally insufficient for the concentration by ammonium sulfate precipitation. In addition, the extracted foulants generally contain impurities (e.g., polysaccharides or humic substances) with higher relative dominances. This would be an additional hindrance in concentrating the proteins contained in the extracted foulants. Obviously, an alternative method is required for successfully concentrating proteins that caused physically irreversible fouling in a continuous operation of MBRs.

Silva et al. (2012) investigated strategies to concentrate and enrich the proteins contained in bound and soluble EPS, including lyophilization, crude concentration with UF membrane or dialysis membrane coated by a highly absorbent powder of polyacrylate-polyalcohol, and precipitation methods. They mentioned that the combination of crude concentration with UF or dialysis membrane and precipitation method was a promising strategy for concentrating proteins contained in both bound and soluble EPS, whereas lyophilization did not work well mainly due to the formation of an insoluble powder. In our experiment, no obvious protein band was seen in the SDS-PAGE gel to which the lyophilized foulant was applied as well (Fig. 2 (a)). In the next step, we applied the combination of crude concentration with UF membrane and precipitation method. This protocol has also been applied successfully to the concentration of proteins contained in seawater (Tanoue et al. 1995; Miyoshi and Suzuki 2004). For selecting appropriate precipitation method, we examined various protein precipitation methods including ammonium sulfate precipitation (saturation level at 80%), acetone precipitation (80% (v/v)), and TCA precipitation (10% (w/v)) in preliminary test, and TCA precipitation was found to be the most effective for precipitation the proteins concentrated by a UF membrane (data not shown).

Fig. 2 (b) shows the SDS-PAGE gel image of the extracted foulant concentrated the combination of the crude concentration with UF membrane and TCA precipitation. Although the banding pattern in Fig. 2 (b) was slightly smeared, many protein bands were clearly detected in this SDS-PAGE gel. As reported by Silva et al. (2012), the smeared banding pattern is probably explained by the existence of organic constituents that can interfere in obtaining well-contrasted bands. Another possible explanation for the smeared banding pattern is that the proteins extracted from the fouled membrane have been degraded. However, the latter explanation is less likely because, as discussed below, clear and distinct spots were detected in the gel used for the separation by 2D-PAGE. In the SDS-PAGE gel presented on Fig. 2 (b), there were too many intensive protein bands to be identified individually. As a

consequence, it was very difficult to subject them to subsequent N-terminal amino acid sequencing analysis. Obviously, unlike the case of proteins contained in a gel layer formed on a membrane surface (Huang et al. 2012) or in bound/soluble EPS (Silva et al. 2012), further separation is required to identify proteins contained in the foulant that caused physically irreversible fouling in a continuous operation of pilot-scale MBR treating real municipal wastewater. In this study, first, we applied a sequential precipitation with ethanol and TCA to the foulant obtained from MBR 3. Although this purification reduced the complexity of the sample to some extent and enabled us to analyze the N-terminal amino acid sequences of two proteins, no protein was successfully identified through the database search (see Fig. S1 and Table S1). Further separation was required to identify the proteins causing physically irreversible fouling in MBRs. Therefore, we applied the 2D-PAGE separation as an alternative to SDS-PAGE in the following investigations.

Fig. 3 shows 2D-PAGE gel images of the foulants obtained from MBRs 1 and 2. In contrast to the separation using SDS-PAGE (Fig. 2 (b)), many clear and distinct spots were seen in the 2D-PAGE gels. This indicates that degradation of proteins was not significant at least for the major proteins that caused physically irreversible fouling in the pilot-scale MBRs. The foulant obtained from MBR 1 had a considerably different spotting pattern from the foulant obtained from MBR 2, indicating that composition of the proteins that caused membrane fouling differed depending on the operating conditions. This suggests that the dominant fouling mechanism in MBR 1 was different from that in MBR 2 (Miyoshi et al. 2011). This difference cannot be discovered by amino acid composition analysis.

To identify the proteins separated by the 2D-PAGE, the proteins present in intensive spots were subjected the N-terminal amino acid sequencing analysis. Totals of 17 and 15 proteins were recovered from the 2D-PAGE gels of the foulants obtained from MBRs 1 and 2, respectively. Many proteins which have similar molecular weights were successfully separated based on their isoelectric points in 2D-PAGE. The application of 2D-PAGE instead of SDS-PAGE is particularly advantageous for the N-terminal amino acid sequencing analysis of the proteins extracted from fouled membranes.

3.4 Identification of proteins with N-terminal amino acid sequencing analysis

As reported by Wilmes and Bond (2004), database matches of results of MS analysis generated from environmental samples for which complete metagenome sequences are not available do not produce useful information. The information on amino acid sequences is particularly useful for reliably identifying proteins collected from environmental samples by

using a homology search, especially when genetic information of source organisms is unavailable. In this study, we applied the N-terminal amino acid sequencing analysis by using Edman degradation reaction, since this method is one of the most reliable methods for determining amino acid sequences, and has been successfully applied for characterizing novel proteins even in recent years (Fodil et al. 2011; 2012). Table 2 summarizes the results of N-terminal amino acid sequencing analysis. The amino acid sequences were determined properly for eight of the 17 proteins for MBR 1 and three of the 15 proteins for MBR 2. The amino acid sequence of protein-6 detected in the foulant obtained from MBR 1 completely matched 45 protein sequences in a blastp search. Most proteins that have the same sequences as protein-6 obtained from MBR 1 were OprF originated from *Pseudomonas* genus. The source organisms registered in the database include aquatic organisms such as *Pseudomonas flurescens*, *Pseudomonas migulae*, and *Pseudomonas mandelii*. The amino acid sequence of protein-10 obtained from MBR 1 completely matched 32 proteins. All were OprD originated from *Pseudomonas* genus, including *Pseudomonas flurescens*, which is an aquatic organism as mentioned above. For protein-2 obtained from MBR 2, no protein sequence with a complete match was found in the database. However, the proteins with the highest match (93%, corresponding E-value (description of the random background noise) = 2e-04) were completely occupied by OprF from *Pseudomonas* genus. As with the case of protein-6 of the foulant extracted from MBR 1, aquatic organisms such as *Pseudomonas flurescens*, *Pseudomonas migulae*, and *Pseudomonas mandelii* were included in the source organisms in the database. The other proteins listed in Table 1 did not return any positive hits in the database analysis, indicating that these proteins originated from unsequenced organisms.

Both OprF and OprD have β-barrel structures and are embedded in the outer cell membrane, which is comprised of lipopolysaccharide (LPS) (Biswas et al. 2007; Staatsuma and Soares 2009). These proteins form channels for transporting substrates through the outer membrane and have considerably similar structures, properties, and functions. Although the structure of the outer membrane proteins is usually very stable (Kleinschmidt 2006), the formation of native structure requires a hydrophobic environment, which is usually provided by bilayers of LPS in activated sludge.

To be embedded in the LPS bilayers, these proteins contain hydrophobic residues. However, most such residues are associated with an LPS layer and do not contact with the water phase. The hydropathy index proposed by Kyte and Doolittle (1982) is widely used for describing hydrophobicity of amino acid. Hydrophilic amino acids have low values (e.g., -4.5 for arginine or -3.9 for lysine), and high values are assigned for hydrophobic amino acids (e.g.,

4.5 for isoleucine or 4.2 for valine). A gravy score can be calculated by taking the average hydropathy values of the amino acids comprising a protein. Therefore, higher gravy values indicate that the proteins have a more hydrophobic nature. The gravy values of the proteins identified in this study (OprF: -0.56, OprD: -0.45) were relatively low, especially compared with α-helix proteins (e.g., bacteriorhodopsin originating from *Halobacterium salinarum*; gravy value = 0.72), indicating that these proteins have a relatively hydrophilic nature. Therefore, it is hard to expect that hydrophobic interaction was the dominant mechanism for the attachment of these proteins onto the membrane.

Based on the molecular weights evaluated by the locations of spots in the 2D-PAGE gel, both OprF and OprD detected in this study were likely to have been intact, suggesting that they have high resistance to microbial degradation in an activated sludge system. This is in accordance with the discussion on the basis of the 2D-PAGE gel images suggesting that degradation of proteins was not significant for the major proteins that caused physically irreversible fouling in the pilot-scale MBRs. Generally, proteins with their native structure have higher resistances to proteolysis by proteases. As mentioned above, the structures of outer membrane proteins are usually very stable when embedded in an LPS bilayer (Biswas et al. 2007). In addition, it was reported that outer membrane proteins become more resistant to proteolysis by protease if they associate with LPS (Freulet-Marrière et al. 2000). Based on the features of these proteins mentioned above, the outer membrane proteins surviving in mixed liquor suspension were most likely contained in small debris of outer membranes comprised of an LPS bilayer rather than dissolved individually. To investigate the fouling mechanisms in which the proteins identified in this study were involved, information is needed on the interactions between these proteins and other organic (and/or inorganic) compounds in mixed liquor suspension.

In this study, we successfully identified the proteins that caused physically irreversible fouling in a continuous operation of MBRs treating real municipal wastewater. On the basis of the results obtained in this study, however, it was difficult to discuss the difference in membrane fouling developed in the two pilot-scale MBRs operated with different SRTs, since similar proteins were detected in the foulants obtained from the two MBRs. To investigate the difference in membrane fouling in the MBRs operated with different operating conditions, the number of identifiable proteins should be increased. For many proteins recovered from the 2D-PAGE gels (nine from MBR 1 and 12 from MBR 2), unfortunately, N-terminal amino acid sequences could not be determined. One reason might be the N-terminals of these proteins were blocked by some functional groups. If an N-terminal of a protein is modified by some

functional groups, Edman degradation reaction does not occur. In this case, de-blocking of N-terminal would be an effective measure for determining the N-terminal amino acid sequences. The difficulties in determining N-terminal amino acid sequences of the proteins contained in the extracted foulant could also be attributed to the sensitivity of the analysis. Generally, analyzing peptide fragments with mass spectrometry (MS) is more sensitive than the N-terminal amino acid sequencing analysis with Edman degradation reaction. Recent development of MS analysis coupled with peptide *de novo* sequencing enables us to predict amino acid sequences without reference database (Schneider and Riedel 2010). Many researchers identified proteins by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Wilmes and Bond 2004) or matrix-assisted laser desorption/ ionization-tandem time-of-flight mass spectrometry (MALDI-TOF/TOF/MS) analyses (Kang et al. 2009; Wang et al. 2010; Silva et al. 2012). The application of these techniques would enable us to increase the number of identifiable proteins, and therefore will be an important topic in the future study.

Many researchers reported that the characteristics of foulant differed depending on the types of membrane (Choi and Ng 2008; Miyoshi et al. 2010; Hoque et al. 2012; Huang et al. 2012). This suggests that proteins causing membrane fouling would also be different depending on the membrane characteristics. In this study, unfortunately, we did not succeed to identify the proteins that caused membrane fouling in MBR 3 which was equipped with flat-sheet membranes. It would be interesting to identify proteins causing membrane fouling in membranes with different membrane characteristics. Such information is very useful to develop an anti-fouling membrane.

Another issue that should be considered is how the identified proteins contribute to the development of filtration resistance. Detection of some specific compounds in extracted foulants does not necessarily indicate that these compounds are detrimental foulants that cause severe membrane fouling. We previously demonstrated that affinity chromatography is a powerful tool for evaluating contributions of specific compounds for developing filtration resistances (Miyoshi et al. 2010). Identification of proteins contained in extracted foulants allows us to produce antibodies that are specific for the amino acid sequences of the identified proteins. Such antibodies can be applied to affinity chromatography. By conducting affinity chromatography with antibodies that are specific for the amino acid sequences of the identified proteins, their contributions to development of filtration resistance can be evaluated specifically. Identifying proteins contributing greatly to the development of filtration resistance will lead us to improve our understanding on fouling mechanisms through the

investigations on possible interactions between these proteins and membrane, or these proteins and other organic/inorganic compounds contained in mixed liquor suspension.

Identifying proteins with high fouling propensity will also be practically useful. Understandably, constituents that actually cause membrane fouling will be more accurate fouling indicators than surrogate indexes such as SMP, BPC, and TEP concentrations. Since variation in concentration of proteins for which information on amino acid sequence is available can be monitored specifically, proteins identified to have higher fouling propensity could be used as fouling indicators that will represent fouling propensities of mixed liquor suspension more precisely than the other fouling indicators proposed in previous studies. It would also be interesting to use proteins with high fouling propensities as model compounds for investigating membrane fouling. In many previous studies, commercially available model proteins such as bovine serum albumin (BSA) or lysozyme were used as model proteins responsible for membrane fouling (Hughes et al. 2007; Susanto et al. 2008; Wang et al. 2009). In actual membrane filtration processes, however, these commercially available model compounds are less likely to be involved in the development of membrane fouling. The investigation with proteins detected in foulants obtained from actual MBRs (actual foulants) should be realistic, and they will provide insight into membrane fouling that is more precise. The results obtained in this study should be a breakthrough for enabling the advanced investigations mentioned above.

4. Conclusions

We investigated the details of proteins that caused physically irreversible fouling in a continuous operation of MBRs treating real wastewater. The proteins contained in the foulants extracted at the end of a continuous operation of pilot-scale MBRs were separated and visualized well on the 2D-PAGE gel after the concentration by the combination of the crude concentration with a UF membrane and TCA precipitation. The results of 2D-PAGE analysis indicated that the compositions of proteins that caused physically irreversible fouling significantly differed depending on the SRT. This enabled us to subject them to N-terminal amino acid sequencing analysis separately. Analyzing selected 2D-PAGE spots by N-terminal amino acid sequencing analysis led to the identification of two well-characterized outer membrane proteins originating from *Pseudomonas* genus, namely OprF and OprD. The identified proteins may be used as fouling indicators in MBRs or model compounds for investigating membrane fouling. To our knowledge, this is the first successful identification of proteins that have caused membrane fouling in continuous operations of MBRs treating real

wastewater.

Acknowledgements

We thank Mr. Tomohiro Hirose of 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 “Mega-ton 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.

Reference

Biswas, S., Mohammad, M.M., Patel, D.R., Movileanu, L., van den Berg, B., 2007. Structural insight into OprD substrate specificity. Nature Structural and Molecular Biology 14, 1108-1109.

Choi, J.-H., Ng, H.Y., 2008. Effect of membrane type and material on performance of a submerged membrane bioreactor. Chemosphere 71, 853-859.

De la Torre, T., Lesjean, B., Drews, A., Kraume, M., 2008. Monitoring of transparent exopolymer particles (TEP) in a membrane bioreactor (MBR) and correlation with other fouling indicators. Water Science and Technology 58, 193-1909.

Drews, A., Vocks, M., Bracklow, U., Iversen, V., Kraume, M., 2008. Does fouling in MBRs depend on SMP? Desalination 231, 141-149.

Drews, A., 2010. Membrane fouling in membrane bioreactors – Characterisation, contradictions, cause and cures. Journal of Membrane Science 363, 1-28.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., 1956. Colorimetric determination of sugars and related substances. Analytical Chemistry 28, 350-356.

Fodil, D., Badis, A., Jaouadi, B., Zaraî, N., Ferradji, F.Z., Boutoumi, H., 2011. Purification and characterization of two extracellular peroxidasesfrom *Streptomyces* sp. strain AM2, a decolorizing actinomycetes responsible for the biodegradation of natural humic acids. International Biodeterioration and Biodegradation 65, 470-478.

Fodil, D., Jaouadi, B., Badis, A., Nadia, Z.J., Ferradji, F.Z., Bejar, S., Boutoumi, H., 2012. A thermostable humic acid peroxidase from *Streptomyces* sp. strain AH4: Purification and biochemical characterization. Bioresource Technology 111, 383-390.

Freulet-Marrière, M.A., El Hamel, C., Chevalier, S., Dé, E., Gérard, M., Orange, N., 2000. Evidence for association of lipopolysaccharide with *Pseudomonas fluorescens* strain MF0 porin OprF. Research in Microbiology 151, 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. Separation and Purification Technology 93, 83-91.

Huang, Y.-T., Huang, T.-H., Yang, J.-H., Demodar, R.A., 2012. Identification and characterizations of proteins from fouled membrane surfaces of different materials. International Biodeterioration and Biodegradation 66, 47-52.

Hughes, D.J., Cui, Z., Field, R.W., Tirlapur, U.K., 2007. Membrane fouling by cell-protein mixtures: In situ characterisation using multi-photon microscopy. Biotechnology and Bioengineering 96, 1083-1091.

Jiang, T., Kennedy, M.D., Schepper, V.D., Nam, S.-N., Nopens, I., Vanrolleghem, P.A., Amy, G., 2010. Characterization of soluble microbial products and their fouling impacts in membrane bioreactors. Environmental Science and Technology 44, 6642-6648.

Judd, S., 2007. The status of membrane bioreactor technology. Trends in Biotechnology 26, 109-116.

Kang, Y.-M., Prewitt, M.L., Diehl, S.V., 2009. Proteomics for biodeterioration of wood (*Pinus taeda* L.): Challenging analysis by 2-D PAGE and MALDI/TOF/TOF/MS. International Biodeterioration and Biodegradation 63, 1036-1044.

Kimura, K., Hane, Y., Watanabe, Y., Amy, G., Ohkuma, N., 2004. Irreversible membrane fouling during ultrafiltration of surface water. Water Research 38, 3431-3441.

Kimura, K., Yamato, N., Yamamura, H., Watanabe, Y., 2005. Membrane fouling in pilot-scale mambrane bioreactors (MBRs) treating municipal wastewater. Environmental Science and

Technology 39, 6293-6299.

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, 268-275.

Kimura, K., Nishisako, R., Miyoshi, T., Watanabe, Y., 2008b. Baffled membrane bioreactor (BMBR) for efficient nutrient removal from municipal wastewater. Water Research 42, 625-632.

Kimura, K., Naruse, T., Watanabe, Y., 2009. Changes in characteristics of soluble microbial products in membrane bioreactors associated with different solid retention times: Relation to membrane fouling. Water Research 43, 1033-1039.

Kleinschmidt, J.H., 2006. Folding kinetics of the outer membrane proteins OmpA and FomA into phospholipid bilayers. Chemistry and Physics of Lipids 141, 30-47.

Kraume, M., Drews, A., 2010. Membrane bioreactors in waste water treatment - Status and trends. Chemical Engineering and Technology 33, 1251-1259.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology 157, 105-132.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265-275.

Meng, F.G., Chae, S.-R., Drews, A., Kraume, M., Shin, H.-K., Yang, F., 2009. Recent advance in membrane bioreactors (MBRs): Membrane fouling and membrane material. Water Research 43, 1489-1512.

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. Journal of Membrane Science 301, 180-189.

Miyoshi, T., Suzuki, S., 2004. Degradation of outer membrane proteins of *Synechococcus* sp. *in vitro* and *in situ*. Journal of Oceanography 60, 825-833.

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

Miyoshi, T., Tanaka, I., Tsuyuhara T., Watanabe, E., Aizawa, T., Kimura, K., Watanabe, Y., 2010. Fouling potentials of polysaccharides in MBRs assessed by lectin affinity chromatography. Water Science and Technology 61, 1787-1792.

Miyoshi, T., Aizawa, T., Kimura, K., Watanabe, Y., 2011. Characteristics of proteins involved in membrane fouling in membrane bioreactors (MBRs) treating municipal wastewater: the application of metaproteomic analyses. Desalination and Water Treatment 34, 150-155.

Ng, H.Y., Tan, T.W., Ong, S.L., 2006. Membrane fouling of submerged membrane bioreactors: impact of mean cell residence time and the contributing factors. Environmental Science and Technology 40, 2706-2713.

Park, C., Helm, R.F., 2008. Application of metaproteomic analysis for studying extracellular polymeric substances (EPS) in activated sludge flocs and their fate in sludge digestion. Water Science and Technology 57, 2009-2015.

Park, C., Novak, J.T., Helm, R.F., Ahn, Y.–O., Esen, A., 2008. Evaluation of the extracellular proteins in full-scale activated sludge. Water Research 42, 3879-3889.

Rosenberger, S., Laabs, C., Lesjean, B., Gnirss, R., Amy, G., Jekel, M., Schrotter, J. -C., 2006. Impact of colloidal and soluble organic material on membrane performance in membrane bioreactors for municipal wastewater treatment. Water Research 40, 710-720.

Schneider, T., Riedel, K., 2010. Environmental proteomics: Analysis of structure and function of microbial communities. Proteomics 10, 785-798.

Silva, A.F., Carvalho, G., Soares, R., Coelho, A.V., Barreto Crespo, M.T., 2012. Step-by-step strategy for protein enrichment and proteome characterisation of extracellular polymeric

substances in wastewater treatment systems. Applied Microbiology and Biotechnology 95, 767-776.

Staatsuma, T.P., Soares, T.A., 2009. Characterization of the outer membrane protein OprF of Pseudomonas aeruginosa in a computer simulation. Proteins 74, 475-488.

Stephenson, T., Judd, S., Jefferson, B., Brindle, K., 2000. Membrane bioreactors for wastewater treatment. IWA publishing, London.

Sun, F.-Y., Wang, X.-M., Li, X.-M., 2011. Change in fouling propensity of sludge in membrane bioreactors (MBRs) in relation to the accumulation of biopolymer clusters. Bioresource Technology 102, 4718-4725.

Susanto, H., Arafat, H., Janssen, E.M.L., Ulbricht, M., 2008. Ultrafiltration of polysaccharide-protein mixtures: Elucidation of fouling mechanisms and fouling control by membrane surface modification. Separation and Purification Technology 63, 558-565.

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. Journal of Hazardous Materials 178, 377-384.

Tanoue, E., 1995. Detection of dissolved protein molecules in oceanic waters. Marine Chemistry 51, 239-252.

Tanoue, E., Nishiyama, S., Kamo, M., Tsugita, A., 1995. Bacterial membranes: Possible source of a major dissolved protein in seawater. Geochimica et Cosmochimica Acta 59, 2643-2648.

Tian, Y., Chen, L., Zhang, S., Zhang, S., 2011. A systematic study of soluble microbial products and their fouling impacts in membrane bioreactors. Chemical Engineering Science 168, 1093-1102.

Trussel, R.S., Merlo, R.P., Hermanowicz, S.W., Jenkins, D., 2006. The effect of organic loading on process performance and membrane fouling in a submerged membrane bioreactor treating municipal wastewater. Water Research 40, 2675-2683.

Wang, B., Xu, M.-Y., Sun, G.-P., 2010. Extracellular respiration of different amounts azo dye by *Shewanella decolorationis* S12 and comparative analysis of the membrane proteome. International Biodeterioration and Biodegradation 64, 274-280.

Wang, X.-M., Li, X.-Y., 2008. Accumulation of biopolymer clusters in a submerged membrane bioreactor and its effect on membrane fouling. Water Research 42, 855-862.

Wang, Z., Wu, Z., Yin, X., Tian, L., 2008. Membrane fouling in a submerged membrane bioreactor (MBR) under sub-critical flux operation: Membrane foulant and gel layer characterization. Journal of Membrane Science 325, 238-244.

Wang, Z., Song, Y., Liu, M., Yao, J., Wang, Y., Hu, Z., Li, Z., 2009. Experimental study of filterability behavior of model extracellular polymeric substance solutions in dead-end membrane filtration. Desalination 249, 1380-1384.

Wilmes, P., Bond, P. L., 2004. The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. Environmental Microbiology 6, 911-920.

Yamamoto, K., Hiasa, M., Mahood, T., Matsuo, T., 1989. Direct solid-liquid separation using hollow fiber membrane in an activated sludge aeration tank. Water Science and Technology 21, 43-54.

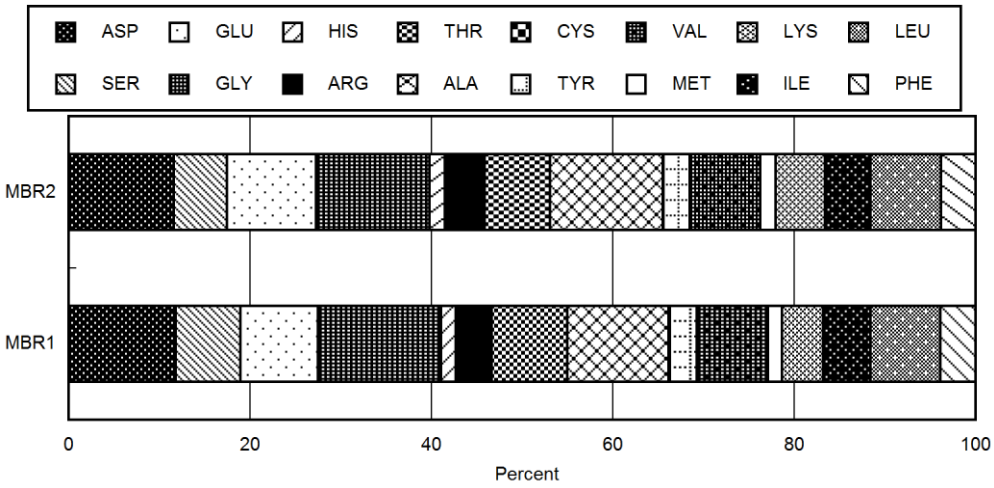

Fig. 1. Amino acid composition of extracted foulants.

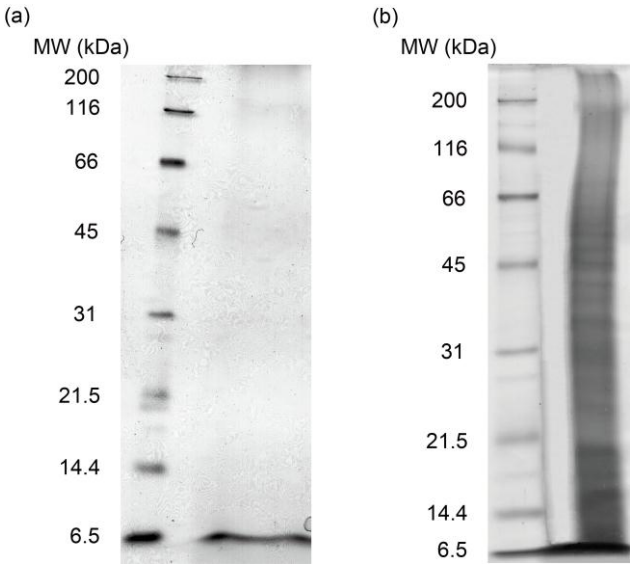

Fig. 2. SDS-PAGE gel images of extracted foulant concentrated with (a) lyophilization and (b) combination of crude concentration with UF membrane and TCA precipitation.

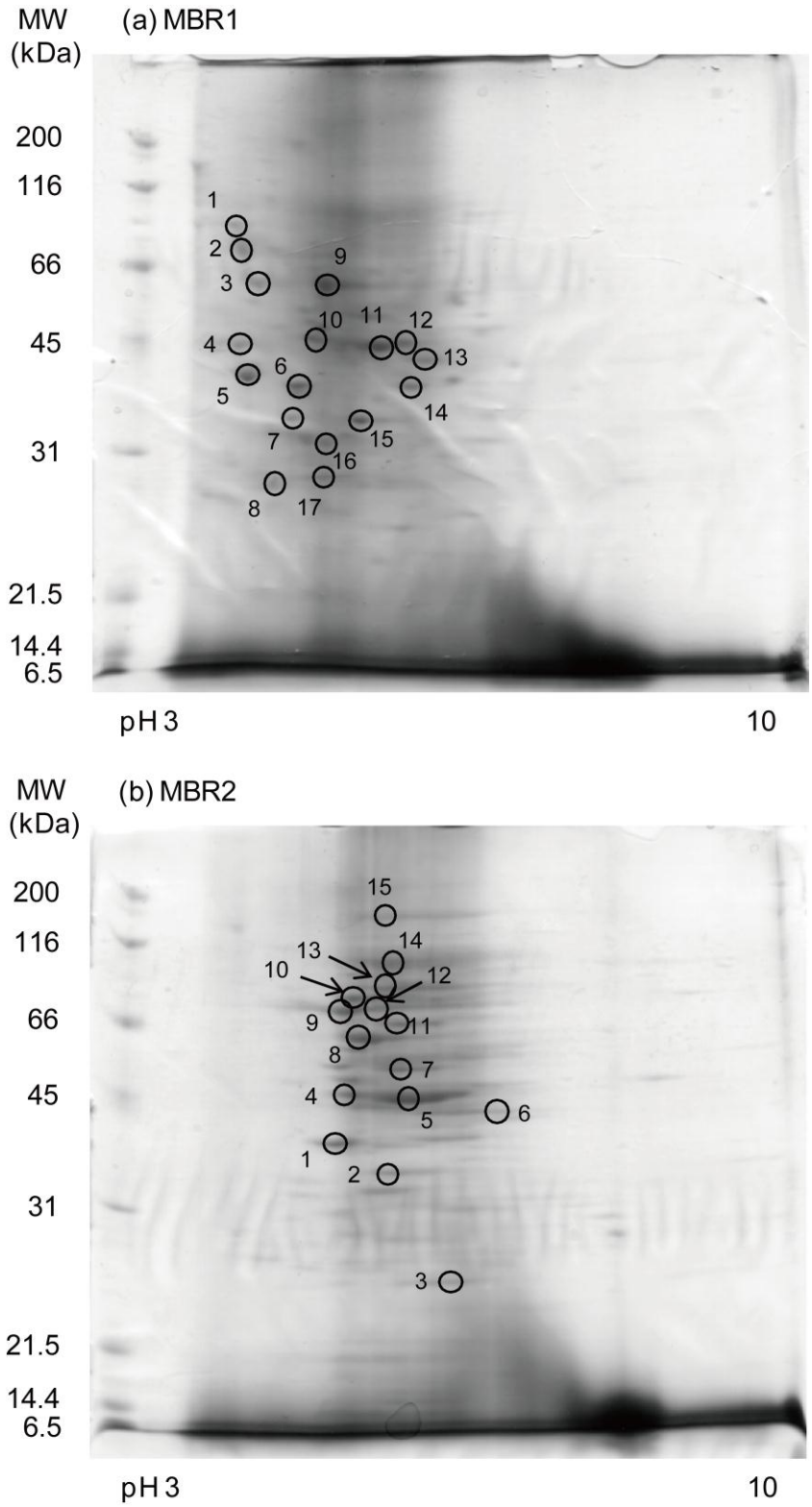

Fig. 3. 2D-PAGE gel images of foulants extracted from fouled membranes, used in (a) MBR 1 and (b) MBR 2.

Table 1 Amounts of organic matter extracted from fouled membranes.

	TOC (mg/m^2)	Carbohydrate (mg/m^2)	Protein (mg/m^2)
MBR1	74.8	40.2	190.8
MBR2	48.3	22.9	89.0

Table 2 Results of N-terminal amino acid sequencing analysis.

	Protein	Amino acid sequence													
MBR1	1	Ser	Asn	Val	Asn	Pro	His	Ser	Ser	Cys	Ser	Ile	Trp	Asp	Thr	Gln
	4	Gly	Ile	Thr	Ile	Asn	Val	Pro	Asp							
	5	Asp	Gly	Ile	Thr	Ile	Asn	Ile	Val	Pro	Asp	Ala	Ile	Ser	Arg	Thr
	6	Gln	Gly	Gln	Gly	Ala	Val	Glu	Ile	Glu	Gly	Phe	Ala			
	7	Ala	x	Leu	Thr	Gln	Val	x	Val	Arg	Phe	Asp	Arg	Met		
	10	Ala	Pro	Phe	Val	Ser	Asp	Gln								
	14	Val	Phe	x	Asp	x	Asp	x	Asp	Leu						
	15	Gln	Gly	Ala	Val	Glu	x	Glu	Leu	Phe						
MBR2	2	Gln	Gly	Gln	Gly	Ala	Val	Glu	Ile	Glu	Leu	Phe	Ala	Lys	Lys	Glu
	3	Val	Gln	Gly	x	Val	Glu	Gly	x	Leu	Phe	x	Lys	x	Gln	
	9	Glu	x	Phe	Ala	Glu	Leu	x	Glu	x	x	Leu	x	Arg		