**Application of glyco-blotting for identification of structures of
polysaccharides causing membrane fouling in a pilot-scale membrane
bioreactor treating municipal wastewater**

Katsuki Kimura^{*1}, Shin-Ichiro Nishimura², Risho Miyoshi³,
Asiful Hoque¹, Taro Miyoshi⁴ and Yoshimasa Watanabe⁴

¹*Division of Environmental Engineering, Graduate School of Engineering, Hokkaido
University, Sapporo 060-8628, Japan*

²*Graduate School of Life Science, Hokkaido University, Sapporo 001-0012, Japan*

³*Medicinal Chemistry Pharmaceuticals, LLC, 1-715, N7W4, Kita-ku, Sapporo 060-0807,
Japan*

⁴*Center for Environmental Nano and Bio Engineering, Hokkaido University, Sapporo
060-8628, Japan*

^{*}Corresponding author.

Tel & Fax: +81-11-706-6271

Email: kkatsu@eng.hokudai.ac.jp

Abstracts

A new approach for the analysis of polysaccharides in membrane bioreactor (MBR) is proposed in this study. Enrichment of polysaccharides by glyco-blotting, in which polysaccharides are specifically collected via interactions between the aldehydes in the polysaccharides and aminoxy groups on glycoblotting beads, enabled MALDI-TOF/MS analysis at a high resolution. Structures of polysaccharides extracted from fouled membranes used in a pilot-scale MBR treating municipal wastewater and those in the supernatant of the mixed liquor suspension in the MBR were investigated. It was found that the overlap between polysaccharides found in the supernatants and those extracted from the fouled membrane was rather limited, suggesting that polysaccharides that dominate in supernatants may not be important in membrane fouling in MBRs. Analysis using a bacterial carbohydrate database suggested that capsular polysaccharides (CPS) and/or lipo-polysaccharides (LPS) produced by gram-negative bacteria are key players in the evolution of membrane fouling in MBRs.

Keywords

Wastewater treatment; capsular polysaccharides; lipo-polysaccharides; glyco-blotting; MALDI-TOF/MS

1. Introduction

Membrane bioreactors (MBRs) are promising technologies for future municipal wastewater treatment due to their high performance and their suitability for satellite/decentralized systems (Asano et al., 2006). However, membrane fouling, which results in the decline of membrane permeability in long-term operations, remains a major concern. Many attempts have been made to control membrane fouling in MBRs, none of which have turned out to be very effective (Malaeb et al., 2013). Because of this, MBRs are still not a mainstream technology in municipal wastewater treatment.

Detailed information on foulants is indispensable for the effective control of membrane fouling in MBRs. The consensus is that the soluble and colloidal fractions of the mixed liquor suspension in MBRs play an important role in the development of membrane fouling (Defrance et al., 2000; Bouhabila et al., 2001; Yamato et al., 2006; Fan et al., 2006; Teychene et al., 2008). In particular, the involvement of polysaccharides in membrane fouling in MBRs has been reported (Kimura et al., 2005; Rosenberger et al., 2006). In previous studies where ^{13}C NMR analysis was carried out for foulants extracted from MBRs, it was shown that polysaccharides were quantitatively dominant in the foulants (Kimura et al., 2005; Kimura et al., 2008). In the majority of the relevant studies, polysaccharides were measured by colorimetric methods such as the phenol sulfuric acid method (Dubois et al., 1956) as a sum parameter. However, such methods might not be informative enough to predict the operational performance of MBRs (Al-Halbouni et al.,

2009; Kimura et al., 2009). Rather, it should be considered strange if a sum parameter of polysaccharides can describe membrane fouling in MBRs, considering the fact that polysaccharides in the mixed liquor suspension of an MBR are not at all uniform: a tremendous diversity of polysaccharides in the suspension is expected, although such diversity has not been yet revealed. The authors have shown that characteristics of foulants in MBRs vary depending on operational parameters such as F/M ratio, membrane flux, membrane materials, cross-flow conditions and temperature (Kimura et al., 2005; Yamato et al., 2006; Kimura et al., 2008; Miyoshi et al., 2009; Hoque et al., 2012). To a certain extent, this reflects the diversity of polysaccharides in MBRs. It is therefore interesting to determine the structures of the polysaccharides that are important in the development of membrane fouling in MBRs.

A previous study partially revealed the diversity of polysaccharides in MBRs by using lectin-affinity chromatography (Kimura et al., 2012), demonstrating the presence of polysaccharides with high fouling potentials. In the same study, structures and origins of polysaccharides with high fouling potentials were suggested on the basis of MALDI-TOF/MS analysis. In the trial MS analysis, however, the quality of the MS spectra was not high, likely due to insufficient purification of the samples. As a result, the information on the structures and origins of polysaccharides obtained in that study was limited and biased by many factors.

Knowledge of the diversity of the polysaccharides in MBRs is still quite limited, and it is

therefore difficult to establish effective methods for controlling membrane fouling in MBRs. Increasing the amount of basic information available is crucial. Recently, the “glycoblotting method”, a practical and highly sensitive method for oligosaccharides enrichment, was developed and can be used for the analysis of biological samples such as sera, cells, and egg whites (Kita et al., 2007; Miura et al., 2008; Furukawa et al., 2008; Amano et al., 2010; Miura et al., 2010). In this method, oligosaccharides are selectively captured by selective chemical ligation between the aldehyde group at the reducing terminus of the oligosaccharide and an aminooxy group displayed on glycoblotting beads, followed by labeling the molecules to increase sensitivities in the subsequent MALDI-TOF/MS analysis.

The present study applies the glyco-blotting method to the oligosaccharides obtained by the partial hydrolysis of polysaccharides that cause membrane fouling in a pilot-scale MBR. Further examination of the structures and origins of polysaccharides that cause membrane fouling in the MBR were carried out. This study describes the first trial that applies the enrichment method to wastewater polysaccharides that display extreme heterogeneity. Due to the effectiveness and high efficiency of the method, as demonstrated, structures of polysaccharides that caused membrane fouling in the MBR could be suggested with a high degree of accuracy.

2. Materials and Methods

2.1 Sample collection

112

113 All samples were obtained from the pilot-scale MBR installed at the Soseigawa
114 Wastewater Treatment Plant (Sapporo, Japan), which is described in detail elsewhere
115 (Kimura et al., 2005; Kimura et al., 2008; Hoque et al., 2012). Wastewater examined in
116 this study was classified as “weak”: average total organic carbon (TOC) concentration
117 and total nitrogen (T-N) concentration during the experiments were 40.6 mg/L and 26.7
118 mg/L, respectively. During the sampling periods, the sludge retention time was set at 30
119 days. The mixed liquor suspended solids (MLSS) concentration in the biological tank
120 was 12 g/L on average. The pilot-scale MBR used in this study was a side-stream type
121 equipped with tubular membranes made from PVDF polymer. The nominal pore size of
122 the membrane was 0.05 μm .

123

124 Extraction of foulants from the fouled membranes was performed three times in
125 May-August in 2011. The three samples are designated Foulant 1, 2 and 3 hereafter.
126 Before the sampling campaign, the MBR had been operated for >3 months under the
127 same conditions as those used in this study. Therefore, sufficient acclimatization of
128 biomass in the reactor could be assumed. After operating for 10-20 days with a membrane
129 flux of 42 LMH, the membrane module was disconnected from the system and was
130 subject to foulant extraction. New membranes were used in each experiment.

131

132 Supernatants of the mixed liquor suspension of the MBR (Supernatants 1-3) were also
133 collected a few days before the membranes were disassembled for foulant extraction (i.e.,

Foulants 1-3).

2.2 Extraction of foulants

Prior to foulant extraction, membrane modules were disassembled. Each membrane tube was opened by using a stainless cutter knife, and the surface of active layer was gently wiped with a sponge. This was carried out to eliminate bias caused by the cake on the membrane surface and to focus on irreversible fouling, which is important to be controlled (Kimura et al., 2004, 2005). Macroscopic deposition of particles on the membrane surface (i.e., formation of cake layer) can be avoided by implementing efficient physical cleaning such as intensive backwashing or the introduction of granular materials (Siembida et al., 2010). As for the present operations, it was confirmed that the contribution of the cake to the total filtration resistance was minor in the MBR, thanks to the effective physical cleaning of the membrane provided by two-phase flow (Hoque et al., 2012). However, sponge cleaning was carried out in this study to assure that foulants causing irreversible fouling were investigated. Wiped membrane specimens were then immersed in an alkaline solution (NaOH, pH 12) for 24 hours at 25 °C to obtain Foulants 1-3. Concentrations of polysaccharides in Foulants 1-3 were assessed by the phenol sulfuric acid method. The concentrations of polysaccharides in Foulant 1, 2 and 3 were 23.6, 6.1 and 23.6 mg/L, respectively. Extracted foulants were then partially hydrolyzed by hydrochloric acid (Kimura et al., 2012) to obtain oligosaccharides for subsequent MS analysis. Hydrolyzed samples were kept in a freezer until the next step.

2.3 Enrichment of oligosaccharides by glyco-blotting

Enrichment of oligosaccharides by glyco-blotting was carried out by following the established method (Hirose et al., 2011). Aliquots of BlotGlycoH beads (500 μ L, Sumitomo Bakelite, Tokyo, Japan) in a 10 mg/mL suspension with water were placed into a well of a MultiScreen Solvinert filter plate (Millpore, Billerica, MA), and the water was removed by vacuum. The sample mixtures (20 μ L) containing hydrolyzed oligosaccharides were applied to the well, followed by the addition of 180 μ L of 2% acetic acid in acetonitrile. The plate was incubated to dryness at 80 °C for 45 min in a thermostat to capture oligosaccharides in samples onto beads via stable hydrazone bonds. The plate was washed with 200 μ L of 2 M guanidine-HCl in ammonium bicarbonate, followed by washing with the same volume of water and 1% triethylamine in methanol. Each washing step was performed twice. Unreacted hydrazine functional groups on beads were capped by incubation with 10% acetic anhydride in methanol at 25 °C for 30 min. The solution was removed by vacuum, and then the beads were washed twice with 200 μ L of 10 mM HCl, methanol and dioxane, successively. On-bead methyl esterification of carboxyl groups in sialic acids was conducted via incubation with 100 mM 3-methyl-1-p-tolyltriazene in dioxane at 60 °C for 90 min to dryness. Then, the bead was serially washed with 200 μ L of dioxane, water, methanol, and water. The oligosaccharides blotted on beads were subjected to the trans-iminization reaction with 20 μ L of 50 mM O-benzyloxyamine hydrochloride (BOA), followed by treatment with

178 180 μ L of 2% acetic acid in acetonitrile for 45 min at 80 °C. The BOA-labeled

179 oligosaccharides were eluted via treatment with 100 μ L of water.

180

181 2.4 MALDI-TOF/MS analysis

182

183 The recovered BOA-labeled oligosaccharides were mixed with an equivalent volume of

184 the liquid matrix solution (2,5-dihydroxybenzoic acid (DHB): DHB (Na) = 9:1, 10

185 mg/mL in 30% acetonitrile), followed by the deposition of 2.5 μ L of the mixture on an

186 MTP 384 target plate (polished steel TF, Bruker Daltonics) and dried under vacuum to

187 create crystals of the analytes. Then, the analytes were subjected to MALDI-TOF/MS

188 analysis using an Ultraflex III (Bruker Daltonics) in reflector, positive ion mode,

189 typically totaling 200 x 5 shots. The software provided with the analytical system (Flex

190 Analysis version 3, Bruker Daltonics, Bremen, Germany) was used to pick up peaks from

191 the MS spectra.

192

193 2.5 Identification of structures of oligosaccharides based on peaks in MS spectra

194

195 Plausible compositions of oligosaccharide structures were determined by calculations

196 with mass values obtained from the mass spectra. The GlycoMod tool (GlycoMod

197 Website) was used for this calculation. GlycoMod is a program designed to find all

198 possible compositions of a glycan structure from its experimentally determined mass.

199 This is done by comparing the mass of glycan to a list of precomputed masses of glycan

compositions (Cooper et al., 2001). Input parameters for calculation were set at the following conditions: mass values, monoisotopic; mass tolerance, 1.0 Da; ion adducts, $[M + Na]^+$; glycan form, O-linked oligosaccharides; form of oligosaccharide, free oligosaccharide. Of the monosaccharide residues that could be used in the calculation by the GlycoMod, hexose (Hex), deoxyhexose (dHex), N-acetyl hexosamine (HexNAc) and hexuronic acid (HexA) were selected because they are common constituents in bacterial polysaccharides (Sutherland, 2007). As described later, a few peaks were repeatedly detected in different samples and were considered to be important in membrane fouling. Therefore, the corresponding plausible compositions were further compared with a database (Bacterial Carbohydrate Structure Database, BCSDB) to determine the producers and specific constituents of such polysaccharides. A menu of “Search for residue composition” in the BCSDB was used in this study.

3. Results and Discussion

3.1 Reproducibility in MALDI-TOF/MS analysis

As a result of the enrichment, which specifically captures oligosaccharides, the obtained MS spectra were fairly clear, although heterogeneous natural samples were dealt with in this study. Analysis of Foulant 1 was carried out repeatedly ($n=3$, Figure 1). As seen in Figure 1, a very high reproducibility of the spectra was confirmed. Although the enrichment process was not specifically developed for the analysis of oligosaccharides in wastewater systems, instead being developed for medical biological samples such as sera,

it can be applied to wastewater oligosaccharides. Most peaks shown in Figure 1 were likely to be of oligosaccharides. Based on the m/z values of major peaks in each MS spectra, structures of the oligosaccharides in the samples were suggested by the GlycoMod tool.

3.2 Analysis of foulants

Table 1 summarizes major peaks found in the MS spectrum measured for Foulant 1-3. By the analysis of MS spectra, many oligosaccharide structures were suggested for each sample, which reflects the diversity of the polysaccharides that cause membrane fouling in MBRs. Blanks in the “plausible structures” columns mean that the GlycoMod tool did not return corresponding structures for the corresponding peaks. Peaks found at m/z of <500 were excluded from interpretation because they were likely to be produced from the matrix used in the analysis. Interestingly, several peaks were repeatedly detected in different samples. Peaks with m/z of 553, 590, 721, 774, 903, 924, 927, 942 and 1132 were detected in the analysis of both Foulant 2 and Foulant 3. Ion peaks of 699, 762, 812, 956 and 1070 were detected for all three of the samples. These likely represent the structures of polysaccharides that are significant in the development of membrane fouling in the MBR. Unfortunately, calculation by the GlycoMod tool could not suggest possible oligosaccharides structures for all of the “important” peaks. Although the peaks with m/z of 699 and 812 were detected in all three of the different samples and were therefore likely important fractions of fouling polysaccharides, plausible structures for the two

peaks could not be suggested. Limited numbers of the suggested structures is partially explained by the fact that the GlycoMod tool did not cover fairly common polysaccharide constituents such as acetate or pyruvate in the calculation, which are likely to be included in bacterial polysaccharides (Sutherland, 2007). However, in a previous study, in which the isolation of polysaccharides in an MBR was not carried out by the glycoblotting, but by lectin affinity chromatography (Kimura et al., 2012), many fewer peaks in the MS spectra could be related to specific structures by the same calculation procedure as that used in this study. Application of the glycoblotting technique substantially increased information that can be obtained from the analysis of polysaccharides in MBRs: several structures of polysaccharides that were responsible for membrane fouling in the MBR were suggested in this study.

Peaks with m/z of 553 and 583 suggested the presence of polysaccharides with the structures of $(\text{HexNAc})_2$ and $(\text{dHex})_3$ in the foulants, respectively. The peak with m/z of 762 suggested the presence of polysaccharides with the structure of $(\text{Hex})_2(\text{dHex})_2$ in the foulants. The peaks with m/z of 924 and 1070 likely reflected the same structure of fouling polysaccharides. The peak with m/z of 774 suggested the presence of $(\text{dHex})_3(\text{HexA})_1$. Information on such structures of polysaccharides in wastewater treatment processes, including MBRs, is totally new. Presence of a simple structure of hexose, $(\text{Hex})_5$, was also suggested by the peak with m/z of 956.

3.3 Analysis of supernatants

266

267 As stated in the experimental section, supernatants of the mixed liquor suspension from
268 the MBR (Supernatants 1-3) were also collected and subjected to MALDI-TOF/MS
269 analysis after enrichment by the glycoblotting method. The results are summarized in
270 Table 2. Detected peaks exhibited a high reproducibility, even though the samples were
271 collected at different days. Particularly, peaks detected in the analysis of Supernatants 1
272 and 2 were considered identical. The data of Supernatant 3 encompasses almost all of the
273 peaks detected in analysis of Supernatants 1 and 2. Several additional peaks were
274 detected in the analysis of Supernatant 3. The data shown in Table 2 implies that
275 properties of polysaccharides in the mixed liquor suspension of the MBR did not
276 significantly change but rather remained stable throughout the operation. A quick
277 comparison between Table 1 and Table 2 reveals that polysaccharides found in foulants
278 and supernatants were not necessarily similar: peaks repeatedly detected in the analysis of
279 foulants were not detected in the analysis of the supernatant, and vice versa. However, a
280 few peaks were repeatedly detected in both foulants and supernatants, as discussed later.
281

282 As was attempted for Foulants 1-3 (Table 1), possible structures of oligosaccharides for
283 the peaks measured for Supernatants 1-3 were calculated and are shown in Table 2. The
284 GlycoMod database could not return possible structures for the all peaks due to the reason
285 suggested for foulant peaks: the lack of consideration of important constituents of
286 bacterial polysaccharides (e.g., acetate) in the calculation. Nevertheless, the GlycoMod
287 tool suggested several structures for polysaccharides in the supernatants.

288

289 Peaks with m/z of 545, 564, 612, 715, 790, 848, 972 and 1013 were always detected in the
290 analysis of the supernatants but never detected in the analysis of the foulants. In contrast,
291 some peaks always detected in the analysis of the foulants were not detected in the
292 analysis of supernatants. As described above, it was considered that the peaks with m/z
293 ratios of 762, 924 and 1070 detected in the analysis of foulants would reflect important
294 fractions of polysaccharides that actually caused membrane fouling. Those peaks were,
295 however, never detected in the analysis of the supernatants. These observations clearly
296 display the limitation of exploring membrane fouling in MBRs by analyzing supernatants.
297 Polysaccharides that dominate in supernatants may not be important in membrane fouling
298 in MBRs. Rather, minor fractions of polysaccharides eventually cause membrane fouling
299 (Kimura et al., 2012). It is thought that the peaks detected only with the foulants (e.g.,
300 $m/z=762$) were present in MALDI-TOF/MS spectra of the supernatant, but intensities of
301 those peaks seemed to be not strong enough to be picked up by the software.

302

303 In contrast, several peaks were always or frequently detected regardless of the types of
304 samples. Peaks with m/z of 590, 699, 721, 774 and 942 seem to be very important, as they
305 were detected in the analysis of both foulants and supernatants. Unfortunately, the
306 GlycoMod tool suggested possible structure for only the peak with m/z of 774.

307

308 3.4 Comparison of the plausible structures with a database

309

By examining the MS spectra obtained for oligosaccharides enriched by the glycoblotting method, some plausible structures for the polysaccharides that would be involved in membrane fouling in the MBR were suggested (see Tables 1 and 2). Of these, two structures were chosen for further investigation using a structure database (BCSDB). This trial demonstrates the potential of the approach suggested in this study for the detailed investigation of polysaccharides causing membrane fouling, leading to the establishment of new methods for the control of membrane fouling. One structure focused on in this section is (dHex)₃(HexA)₁, which is associated with the peak with m/z of 774. Peaks with m/z of 590, 699, 721, 774 and 942 were detected in both Foulants and Supernatants (Tables 1 and 2), and therefore we will focus on polysaccharides with the corresponding structures first. Of these, however, a structure could be suggested only for the peak with m/z of 774. Another structure compared with the database was (hexose)₂(deoxyhexose)₂, which is associated with the peak with m/z of 762. This peak was always detected in the analysis of the foulants but never detected in the analysis of the supernatants. Nevertheless, the structure corresponding to m/z of 762 was considered in this section because the presence of polysaccharides with that structure was strongly supported by the detection of peaks with m/z ratios of 924 and 1070 in the analysis of the foulants. Structures of (Hex)₃(dHex)₂ and (Hex)₃(dHex)₃ were suggested for these peaks, respectively.

Fucose and rhamnose are very common deoxyhexoses in bacterial polysaccharides, whereas glucuronic acid and galacturonic acid are very common hexuronic acids in

bacterial polysaccharides (Sutherland, 2007). Therefore, when searching the database, those four constituents were considered. Namely, for the structure of (dHex)₃(HexA)₁, the following eight patterns of (rhamnose)₃(glucuronic acid)₁, (rhamnose)₂(fucose)₁(glucuronic acid)₁, (rhamnose)₁(fucose)₂(glucuronic acid)₁, (fucose)₃(glucuronic acid)₁, (rhamnose)₃(galacturonic acid)₁, (rhamnose)₂(fucose)₁(galacturonic acid)₁, (rhamnose)₁(fucose)₂(galacturonic acid)₁ and (fucose)₃(galacturonic acid)₁ were examined.

Regarding the structure of (Hex)₂(dHex)₂, fucose and rhamnose were again used as deoxyhexoses in the comparison. The database offers “any hexose” in the search menu, and this parameter was used for hexose. As a result, the following three patterns were examined: (any hexose)₂(fucose)₂, (any hexose)₂(fucose)₁(rhamnose)₁, (anyhexose)₂(rhamnose)₂.

Table 3 summarizes the output from the BCSDB with respect to the reported compositions for the structures of (dHex)₃(HexA)₁ and (Hex)₂(dHex)₂. The supporting information (Table S1) summarizes the ID numbers in the BCSDB of the compositions. By using those ID numbers, specific compositions and literature that reported these compositions are easily displayed in the database. In the tested 11 patterns, 6 patterns were not found in the database. In 4 out of the 5 patterns that were found in the database, rhamnose was a component. In the database, a “compound class” is also shown for a structure of more than 60 classes if it is available. In total, 119 compositions were found

in the database for the two oligosaccharide structures ((dHex)₃(HexA)₁ and (Hex)₂(dHex)₂). Of the 119 compositions, 34 compositions were classified as capsular polysaccharides (CPS), whereas 41 compositions were classified as O-antigen. Some bacteria have a layer outside the cell wall, which is well organized and not easily washed off. This layer is called a capsule and is usually composed of polysaccharides (i.e., CPS) (Prescott et al., 2005; Madigan et al., 2006). O-antigen is a polysaccharide structure in lipopolysaccharides (LPS) found on the outer layer of the cell wall of most gram-negative bacteria (Prescott et al., 2005; Madigan et al., 2006). Endotoxins are a component of the LPS complex known to be released upon cell lysis (Rietschel and Brade, 1992). It is thought that LPS is also released upon cell lysis. Gonçalves et al. (2006) reported that CPS was released by stress. Thus, LPS/CPS that are released from bacteria under stress might be important players in membrane fouling in an MBR. The impact of temperature and temperature shocks on membrane fouling in MBRs has been recently investigated, (Van den Brink et al., 2011; Krzeminski et al., 2012; Ma et al., 2013) and such conditions might explain the release of LPS/CPS due to stress to bacteria. Monitoring LPS/CPS in MBRs is thus highly interesting and is now being investigated.

4. Conclusions

In this study, polysaccharides that cause membrane fouling in an MBR were investigated by the glycoblotting enrichment of oligosaccharides prepared by partial hydrolysis of

crude polysaccharide samples. Enriched oligosaccharides were then analyzed with MALDI-TOF/MS. The quality of MS spectra became fairly high, which enabled the identification of many peaks of oligosaccharides. Several specific structures of oligosaccharides were repeatedly identified in different sampling campaigns in this study, suggesting that polysaccharides that had those oligosaccharides as a major structure are the key players in membrane fouling in MBRs. The composition and origins of such polysaccharides could also be suggested by a database.

Acknowledgements

This study was supported by Scientific Research (B) (General) (24360212) provided by The Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and Core Research of Evolutional Science & Technology (CREST) for “Innovative Technology and System for Sustainable Water Use” from the Japan Science and Technology Agency (JST).

References

1. Al-Halbouni, D., Dott, W., Hollender, J., 2009. Occurrence and composition of extracellular lipids and polysaccharides in a full-scale membrane bioreactor. *Water Res.*, 43, 97-106.

398 2. Amano, M., Yamaguchi, M., Takegawa, Y., Yamashita, T., Terashima, M., Furukawa,
399 J.-I., Miura, Y., Shinohara, Y., Iwasaki, N., Minami, A., Nishimura, S.-I., 2010. Threshold
400 in stage-specific embryonic glycotypes uncovered by a full portrait of dynamic N-glycan
401 expression during cell differentiation. *Mol. Cel. Proteomics*, 9, 523– 537.
402
403 3. Asano, T., Burton, F. L., Leverenz, H. L., Tsuchihashi, R., Tchobanoglous, G., 2006.
404 *Water Reuse: Issues, Technologies, and Application*, McGraw-Hill, New York.
405
406 4. Bacterial Carbohydrate Structure Database,
407 <http://csdb.glycoscience.ru/bacterial/index.html>
408
409 5. Bouhabila, E. H., Ben Aim, R., Buisson, H., 2001. Fouling characterization in
410 membrane bioreactors. *Sep. Pur. Technol.* 22-23, 123-132.
411
412 6. Cooper, C. A., Gasteiger, E., Packer, N. H., 2001. GlycoMod – A software tool for
413 determining glycosylation compositions from mass spectrometric data. *Proteomics* 1,
414 340-349.
415
416 7. Defrance, L., Jaffrin, M. Y., Gupta, B., Paullier, P., Geaugey, V., 2000. Contribution of
417 various constituents of activated sludge to membrane bioreactor fouling. *Bioresource*
418 *Technol.* 73, 105-112.
419

8. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350-356.
9. Fan, F., Zhou, H., Husain, H., 2006. Identification of wastewater characteristics to predict critical flux for membrane bioreactor process. *Water Res.* 40, 205-212.
10. Furukawa, J.-I., Shinohara, Y., Kuramoto, H., Miura, Y., Shimaoka, H., Kuroguchi, M., Nakano, M., Nishimura, S.-I., 2008. Comprehensive approach to structural and functional glycomics based on chemoselective glycoblotting and sequential tag conversion. *Anal. Chem.* 80, 1094– 1101.
11. GlycoMod Website, <http://web.expasy.org/glycomod/>
12. Gonçalves, V. M., Takagi, M., Carneiro, S. M., De Campos Giordano, R., Tanizaki, M. M., 2006. Introduction of air in the anaerobic culture of *Streptococcus pneumoniae* serotype 23F induces the release of capsular polysaccharides from bacterial surface into the cultivation medium. *J. Appl. Microbiol.* 101, 1009-1014.
13. Hirose, K., Amano, M., Hashimoto, R., Lee, Y. C., Nishimura, S.-I., 2011. Insight into glycan diversity and evolutionary lineage based on comparative avio-N-glycomics and sialic acid analysis of 88 egg whites of galloanserae. *Biochemistry*, 50, 4757-4774.

14. 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. Pur. Technol.* 93, 83-91.
15. Kimura, K., Hane, Y., Watanabe, Y., Amy, G., Ohkuma, N., 2004. Irreversible membrane fouling during ultrafiltration of surface water. *Water Res.* 38, 3431-3441.
16. Kimura, K., Yamato, N., Yamamura, H., Watanabe, Y., 2005. Membrane fouling in pilot-scale membrane Bioreactors (MBRs) treating municipal wastewater. *Environ. Sci. Technol.* 39, 6293-6299.
17. Kimura, K., Miyoshi, T., Naruse, T., Yamato, N., Ogyu, R., Watanabe, Y., 2008. The difference in characteristics of foulants in submerged MBRs caused by the difference in the membrane flux. *Desalination*, 231 (1-3), 268-275.
18. 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 Res.* 43, 1033-1039.
19. Kimura, K., Tanaka, I., Nishimura, S.-I., Miyoshi, R., Miyoshi, T., Watanabe, Y., 2012. Further examination of polysaccharides causing membrane fouling in membrane bioreactors (MBRs): Application of lectin affinity chromatography and

464 MALDI-TOF/MS. *Water Res.* 46, 5725-5734.

465

466 20. Kita, Y., Miura, Y., Furukawa, J.-I., Nakano, M., Shinohara, Y., Ohno, M., Takimoto,
 467 A., Nishimura, S.-I., 2007. Quantitative glycomics of human whole serum glycoproteins
 468 based on the standardized protocol for liberating N-glycans. *Mol. Cel. Proteomics*, 6,
 469 1437– 1445.

470

471 21. Krzeminski, P., Iglesias-Obelleiro, A., Madebo, G., Garrido, J. M., van der Graaf, J. H.
 472 J. M., van Lier, J. B., 2012. Impact of temperature on raw wastewater composition and
 473 activated sludge filterability in full-scale MBR systems for municipal sewage treatment. *J.*
 474 *Membr. Sci.* 423, 348-361.

475

476 22. Ma, Z., Wen, X. H., Zhao, F., Xia, Y., Huang, X., Waite, D., Guan, J., 2013. Effect of
 477 temperature variation on membrane fouling and microbial community structure in
 478 membrane bioreactor. *Bioresource Technol.* 133, 462-468.

479

480 23. Madigan, M. T., Martinko, J. M., Parker, J., 2006. *Brock biology of microorganisms.*
 481 11th ed. Prentice Hall, Upper Saddle River.

482

483 24. Malaeb, L., Le-Clech, P., Vrouwenvelder, J. S., Ayoub, G. M., Saikaly, P. E., 2013. Do
 484 biological-based strategies hold promise to biofouling control in MBRs? *Water Res.* 47,
 485 5447-5463.

486

487 25. Miura, Y., Hato, M., Shinohara, Y., Kuramoto, H., Furukawa, J.-I., Kurogochi, M.,
488 Shimaoka, H., Tada, M., Nakanishi, K., Ozaki, M., Todo, S., Nishimura, S.-I., 2008.
489 BlotGlycoABCTM, an integrated glycoblotting technique for rapid and large scale
490 clinical glycomics. *Mol. Cel. Proteomics*, 7, 370– 377.

491

492 26. Miura, Y., Kato, K., Takegawa, Y., Kurogochi, M., Furukawa, J.-I., Shinohara, Y.,
493 Nagahori, N., Amano, M., Hinou, H., Nishimura, S.-I., 2010. Glycoblotting-assisted
494 O-glycomics: Ammonium carbamate allows for highly efficient O-glycan release from
495 glycoproteins. *Anal. Chem.* 82, 10021– 10029.

496

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

500

501 28. Prescott, L. M., Harley, J. P., Klein, D. A., 2005. *Microbiology*. 6th ed. McGraw-Hill,
502 Boston.

503

504 29. Rietschel, E. T., Brade, H., 1992. Bacterial endotoxins. *Sci. Am.* 267 (2), 54-61.

505

506 30. Rosenberger, S., Laabs, C., Lesjean, B., Gnirss, R., Amy, G., Jekel, M., Schrotter,
507 J.-C., 2006. Impact of colloidal and soluble organic material on membrane performance

508 in membrane bioreactors for municipal wastewater treatment. *Water Res.* 40, 710-720.

509

510 31. Siembida, B., Cornel, P., Krause, S., Zimmermann, B., 2010. Effect of mechanical

511 cleaning with granular material on the permeability of submerged membranes in the

512 MBR process. *Water Res.* 44, 4037-4046.

513

514 32. Sutherland, I. W., 2007. Bacterial exopolysaccharides. In *Comprehensive*

515 *glycoscience from chemistry to systems biology* vol. 2, Kamerling J. P., Ed., Elsevier,

516 Oxford.

517

518 33. Teychene, B., Guigui, C., Cabassud, C., Amy, G., 2008. Toward a better identification

519 of foulant species in MBR processes. *Desalination*, 231 (1-3), 27-34.

520

521 34. Van den Brink, P., Satpradit, O.-A., Van Bentem, A., Zwijnenburg, A., Temmink, H.,

522 Van Loosdrecht, M., 2011. Effect of temperature shocks on membrane fouling in

523 membrane bioreactors. *Water Res.* 45, 4491-4500.

524

525 35. Yamato, N., Kimura, K., Miyoshi, T., Watanabe, Y., 2006. Difference in membrane

526 fouling in membrane bioreactors (MBRs) caused by membrane polymer materials. *J.*

527 *Membr. Sci.* 280 (1-2), 911-919.

Table 1. Major peaks in MALDI-TOF/MS analysis performed for Foulant 1, 2 and 3 after partial hydrolysis*.

Foulant 1	Foulant 2	Foulant 3	
m/z	m/z	m/z	Plausible structures**
551.104			
	553.118	553.117	(HexNAc) ₂
		575.092	
583.388			(dHex) ₃
	590.057	590.566	
603.996			
627.06			
636.914			
699.531	699.538	699.556	
	721.208	721.219	
		751.223	
762.483	762.505	762.516	(Hex) ₂ (dHex) ₂
	774.156	774.162	(dHex) ₃ (HexA) ₁
812.043	812.126	812.123	
888.716			(HexNAc) ₂ (dHex) ₁ (HexA) ₁

	903.294	903.305	
	924.306	924.321	(Hex) ₃ (dHex) ₂
	927.276	927.294	
	942.249	942.253	
		943.261	
956.152	956.245	956.256	(Hex) ₅
		958.252	(HexNAc) ₄
1010.099			(Hex) ₂ (HexNAc) ₁ (dHex) ₁ (HexA) ₁
1070.676	1070.732	1070.747	(Hex) ₃ (dHex) ₃
	1132.289	1132.301	
		1156.312	(Hex) ₂ (HexNAc) ₁ (dHex) ₂ (HexA) ₁
1185.097			(Hex) ₃ (HexNAc) ₂ (dHex) ₁
			or
			(Hex) ₁ (dHex) ₆
	1186.261		
1378.876			(Hex) ₄ (dHex) ₄

* Calculated by the GlycoMod tool.

** Hex: hexose, dHex: deoxyhexose, HexNAc: N-acetyl hexosamine, HexA: hexenuronic acid.

Table 2. Major peaks in MALDI-TOF/MS analysis performed for Supernatant 1, 2 and 3 after partial hydrolysis*.

Supernatant 1	Supernatant 2	Supernatant 3	Plausible structures**
m/z	m/z	m/z	
		537.134	
545.103	545.108	545.114	
564.476	564.489	564.48	
578.49	578.497		
590.551	590.56	590.56	
612.498	612.51	612.514	
699.561	699.577	699.244	
		713.256	
715.558	715.57	715.571	
721.234	721.238	721.243	
		735.256	
		744.212	
		758.259	
		767.198	
774.164	774.176	774.188	(dHex) ₃ (HexA) ₁
790.151	790.167	790.173	(Hex) ₁ (dHex) ₂ (HexA) ₁
848.814	848.855	848.843	
		875.346	(Hex) ₂ (HexNAc) ₂
888.76	888.772	888.787	(HexNAc) ₂ (dHex) ₁ (HexA) ₁
942.271	942.278	942.299	
972.261	972.261	972.254	
1013.663	1013.683	1013.684	
		1118.361	(Hex) ₆
		1280.418	(Hex) ₇

* Calculated by the GlycoMod tool.

** Hex: hexose, dHex: deoxyhexose, HexNAc: N-acetyl hexosamine, HexA: hexenuronic acid.

Table 3. Outputs from the BCSDb database for the structures of (dHex)₃(HexA) and (Hex)₂(dHex)₂ for ion peaks of m/z of 774 (detected with both the foulants and the supernatants) and 762 (detected only with the foulants), respectively.

	Numbers of compositions found in the database	CPS	O-antigen or O-polysaccharide
(dHex) ₃ (HexA)			
(rhamnose) ₃ (glucuronic acid)	19	7	5
(rhamnose) ₂ (fucose) (glucuronic acid)	None		
(rhamnose)(fucose) ₂ (glucuronic acid)	None		
(fucose) ₃ (glucuronic acid)	None		
(rhamnose) ₃ (galacturonic acid)	11	1	9
(rhamnose) ₂ (fucose) (galacturonic acid)	None		
(rhamnose)(fucose) ₂ (galacturonic acid)	None		
(fucose) ₃ (galacturonic acid)	None		
(Hex) ₂ (dHex) ₂			
(any hexose) ₂ (fucose) ₂	7	1	5
(any hexose) ₂ (fucose)(rhamnose)	2	1	
(any hexose) ₂ (rhamnose) ₂	80	24	22

Figure 1

Figure 1. MALDI-TOF/MS spectra measured for Foulant 1. Measurements were repeated three times.