Glycosphingolipids as a Possible Signature of Microbial Communities in Activated Sludge and the Potential Contribution of Fungi to Wastewater Treatment under Cold Conditions

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Seven strains of fungi were isolated from activated sludge and identified as Mucor sp., Geotrichum sp., Trichosporon sp., Candida sp., and Trichoderma sp. by 28S rDNA D2 region sequences analysis. The structures of the main ceramide monosaccharides (CMSs) from these fungi were identified as glucosylceramide (GlcCer) consisting of ceramide moieties of 9-methyl-octadecasphingadienine (9-Me d18:2), with 2-hydroxyhexadecanoate (h16:0) (Mucor sp. and Geotrichum sp.), 2-hydroxyoctadecanoate (h18:0) (Trichosporon sp. and Candida sp.), and 2-hydroxyoctadecenoate (h18:1) (Trichoderma sp.). Seasonal changes in glycosphingolipids in activated sludge suggest the possibility that microbial flora in activated sludge changes with the seasons, and that fungi adaptable to low temperatures dominate in the cold period, resulting in stable effluent quality throughout the year. This flexibility is maintained mainly by a seasonal adjustment in microbiological organisms, which is dependent on the quality and/or temperature of the influent. To maintain the ability to treat pollutants, characterization of microbial communities, especially during the cold season, is necessary.

Several techniques are available to obtain information about the dynamic nature of microbial communities. These include analyses of fluorescent in situ hybridization (FISH), used in biofilms, activated sludge, and constructed wetlands; fatty acids, used in activated sludge, biofilms, and sediments of bays; and quinone profiles, used in activated sludge, composting systems, lake sediments, and rivers, but no studies on glycosphingolipids (GSLs) as a signature characterizing the ecology of microbial community structures have been reported. GSLs are ubiquitous as components of eukaryotic cell plasma membranes, but are rare in prokaryotic cells. They serve vital functions in cell biology, as in cell-cell interactions, endocytosis, intracellular protein trafficking, and the adhesion of pathogens to mammalian cells. Because fungi in activated sludge are a concern as to wastewater treatment with bacteria, and can grow at lower temperatures than bacteria, monitoring the seasonal prevalence of fungi, especially in the cold season, is important. If GSLs are available as a signature of fungi, that might yield useful information for this purpose. Hence this

Key words: activated sludge; fungi; glycosphingolipids; wastewater treatment

The activated sludge process is widely used in urban and industrial wastewater treatment plants. During wastewater treatment, organic pollutants in sewage are adsorbed by floc and assimilated by microorganisms. In activated sludge, bacteria, fungi, protozoa, and metazoa associate to form a complex microbial community. In the wastewater treatment process, bacteria and fungi are assumed mainly to degrade the organic compounds, while protozoa and metazoa regulate the community as predators in the ecosystem. Activated sludge is highly adaptable to the environment, and maintains the ability to treat wastewater under harsh conditions, resulting in stable effluent quality throughout the year. This flexibility is maintained mainly by a seasonal adjustment in microbiological organisms, which is dependent on the quality and/or temperature of the influent. To maintain the ability to treat pollutants, characterization of microbial communities, especially during the cold season, is necessary.

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Abbreviations: GSL, glycosphingolipid; CMS, ceramide monosaccharide; GlcCer, glucosylceramide; 9-Me d18:2, 9-methyl-octadeca-sphingadienine; d18:2, octadeca-sphingadienine; h16:0, 2-hydroxyhexadecanoate; h18:0, 2-hydroxyoctadecanoate; h18:1, 2-hydroxyoctadecenoate; h20:0, 2-hydroxyicosanoate
study focused on a possible connection between seasonal changes in GSLs and behavior of fungi in activated sludge. We studied seasonal variations in GSLs profiles in activated sludge and possible correlations with the fungal community. In addition, the ability of fungi in activated sludge to treat wastewater to increase understanding of the contribution of fungi to wastewater treatment was investigated.

Materials and Methods

Activated sludge. Samples of activated sludge (return sludge) were obtained monthly from July 2006 through June 2007 from the Sousei River domestic water treatment plant in Sapporo, Japan. After temperature determination, the samples were transferred to a container and returned to the laboratory within 30 min. After determination of pH, microbial cells were collected by centrifugation at 3,000 rpm for 30 min, and were stored at −30 °C for subsequent lipid extraction.

Isolation and identification of fungi from activated sludge. To isolate fungi from activated sludge, 0.1 ml of fresh sample was spread onto plates of GP agar medium (2% glucose, 0.5% casein peptone, 0.2% yeast extracts, 0.05% MgSO$_4$, 0.1% KH$_2$PO$_4$, and 1.5% agar) and cultivated at 26 °C for 3 d. The cultivation steps were repeated until pure cultures were obtained on the plates. Isolated fungi were identified by determining approximately 300 bp of their 28S rDNA D2 region sequences, and comparing the sequences with the database (GenBank/DDBJ/EMBL).

Growth conditions of fungi isolated from activated sludge. Cultivation was carried out in GP medium, pH 6.8. A loopful of these fungus cells was taken from a slant, inoculated onto a plate containing 1.5% agar, and then pre-cultivated at 26 °C for 3 d. A loopful of these pre-cultivated fungi was inoculated to 2 ml of medium in a 10-ml screw-capped test tube, and then cultivated at 26 °C for 12 h. This culture was then transferred to a 100-ml flask containing 20 ml of the medium and cultivated at 26 °C for 12 h. The same procedure was repeated once more in a 500-ml flask containing 100 ml of the medium. Then the seed culture was transferred to a 3-liters flask containing 1 liter of the medium, and cultivated at 26 °C for subsequent lipid extraction.

Extraction and purification of GSLs. Harvested fungal cells were washed 3 times with 1.5% NaCl before the extraction procedure. Total lipids were extracted from wet cells with chloroform-methanol (1:2 and 2:1, v/v), and the combined extracts were washed with Folch’s partition. The total lipid extracts were evaporated to dryness, followed by precipitation with acetone. The acetone-precipitated fraction was hydrolyzed with 0.5 N NaOH in methanol at 50 °C for 1 h. The hydrolyzate was neutralized with acetate and dialyzed against water for 2 d. The alkali-stable fraction (200 mg) was dissolved in 4 ml of chloroform-methanol-water (30:60:8, v/v/v) and applied to a column (10 × 150 mm) of DEAE-Sephadex A-25 (acetate form, GE Healthcare, Uppsala, Sweden). Elution was performed with 60 ml of the same solvent to elute neutral GSLs, and then with 100 ml of 0.45 M ammonium acetate in methanol to elute the acidic compounds. The neutral GSL fraction was fractionated by silica gel column chromatography. Five grams of silica gel (Wakogel C-200, Wako, Osaka, Japan) was washed 3 times with methanol, and then activated at 125 °C for 12 h. The activated silica gel was suspended in 20 ml of chloroform and packed into a glass column (10 × 300 mm). The column was washed with 150 ml of chloroform before the sample was applied. The neutral GSL fraction (150 mg) dissolved in 2 ml of chloroform was applied to the column. The elution was performed with 60 ml of chloroform, chloroform-methanol, 9:1, v/v, 8:2, v/v, 6:4, v/v, and 2:8, v/v, in that order. The ceramide monosaccharide (CMS) eluted with chloroform-methanol (9:1) was purified by thin layer chromatography (TLC) with a solvent system of chloroform-methanol-water (65:25:4, v/v/v).

Analyses of saccharides, fatty acids, and sphingoid bases. To determine the composition of constituent saccharides and fatty acids in CMS, 0.2 mg of purified CMS was methanolyzed with 0.5 ml of 5% anhydrous methanolic HCl in a 10-ml screw-capped test tube at 100 °C for 3 h. After the reaction, the fatty acid methyl esters were extracted from the reaction mixture with 1 ml of n-hexane, followed by analysis by gas-liquid chromatography/mass spectrometry (GC/MS). The remaining methanol layer (containing methyl glycosides) was neutralized with 2N KOH, converted to TMS derivatives, and applied to GC. To determine sphingoid bases in CMS, 0.2 mg of purified CMS was methanolyzed with 0.5 ml of 1 M aqueous methanolic HCl at 70 °C for 18 h. After the reaction, fatty acid methyl esters were extracted from the reaction mixture with 1 ml of n-hexane. The remaining phase was neutralized with 2 N KOH and evaporated under N2 gas. The residue was dissolved in 1 ml of methanol and 0.2 ml of sodium metaperiodate. After it stood for 2 h under darkness, sphingoid aldehyde was extracted with 1 ml of n-hexane and analyzed by GC/MS.

MS data for fatty acids gave the following results: m/z AS-1, 286 (M$^+$), 268 (M$^+$-H2O), 254 (M$^+$-CH2O), 227 (M$^+$-C2H2O2), 103 (C4H7O2); AS-2, 286 (M$^+$), 268 (M$^+$-H2O), 254 (M$^+$-CH2O), 227 (M$^+$-C2H2O2), 103 (C4H7O2); AS-3, 286 (M$^+$), 268 (M$^+$-H2O), 254 (M$^+$-CH2O), 227 (M$^+$-C2H2O2), 103 (C4H7O2); AS-4, 314 (M$^+$), 296 (M$^+$-H2O), 282 (M$^+$-CH2O), 255 (M$^+$-C2H2O2), 103 (C4H7O2); AS-4, 314 (M$^+$), 296 (M$^+$-
using synthetic sewage (0.015% peptone, 0.01% meat sludge to serve in wastewater treatment was estimated from activated sludge. The ability of fungi isolated from activated sludge was also examined. During the cultivation period, activated sludge was aerated in synthetic sewage for 14 h in 1 d, and a half volume of the sewage was exchanged with fresh sewage every day. The BOD removal test was performed in triplicate.

Matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS). MALDI-TOF/MS analysis of purified CMS was performed with a Bruker Daltomics Autoflex instrument (Bruker Daltonics, Billerica, USA) operating in positive ion reflection mode. Ions were formed with a pulsed ultraviolet laser beam (N2 laser, 337 nm; 3-ns wide pulses/s). The matrix used was 2,5-dihydroxybenzoic acid.

Gas-liquid chromatography/mass spectrometry (GC/MS). A Shimadzu GC-QP2010 instrument (Shimadzu, Kyoto, Japan) with a ULBON HR-1 column (0.25 mm × 50 m, Shinwa Chemical, Kyoto, Japan) was used in the determination of fatty acids and saccharides, and a Cp-Sil88 column (0.25 mm × 50 m, Chrompack, Middelburg, The Netherlands) was used in the determination of sphingoid bases. The following temperature programs were used: 15 °C/min from 80 to 150 °C, then 3 °C/min to 290 °C, followed by a 10-min hold at 290 °C for fatty acids and sugars; 10 °C/min from 80 to 160 °C, then 2 °C/min to 220 °C, followed by a 10-min hold for sphingoid bases. Injection port temperature and the interface (detector) temperature were 230 °C and 240 °C respectively. The voltage of the detector was 1.08 kV, and the flow rate of the carrier gas (He2) was 30 ml/min.

Thin-layer chromatography (TLC). TLC (Silica gel 60 precoated TLC plates, Merck, Darmstadt, Germany) was performed with a solvent system of chloroform-methanol-water (65:25:4, v/v/v). After development, glycolipids were visualized by spraying with an orcinol-H2SO4 reagent. Before the CMSs were isolated from TLC, a preparative TLC plate was pre-developed with the same solvent system as above.

Wastewater treatment with fungi isolated from activated sludge. The ability of fungi isolated from activated sludge to serve in wastewater treatment was estimated using synthetic sewage (0.015% peptone, 0.01% meat extract, 0.035% glucose, 0.001% NaCl, 0.0005% KCl, 0.00066% CaCl2·2H2O, 0.00072% MgSO4·7H2O, 0.00025% FeCl3·6H2O, 0.0025% Na2HPO4·12H2O, and 0.0004% KH2PO4, pH 6.7). The biochemical oxygen demand (BOD) of this synthetic sewage was approximately 500 mg/l. Cultivation of fungi was carried out in GP medium, pH 6.8. The pre-cultivation procedure was same as described above. The seed culture (2 ml) was transferred to a 50-ml flask containing 15 ml of the medium and cultivated at 26 °C for 12 h. The culture was transferred to a 100-ml flask containing 30 ml of the medium and cultivated at 26 °C for 12 h. The cells, harvested by centrifugation at 3,500 rpm for 30 min, were washed thoroughly with sterilized water and then acclimated in a 50-ml flask containing 15 ml of synthetic sewage at 10 °C or 15 °C for 8 h (the same temperature as the test). The test culture was incubated in a 500-ml flask containing 150 ml of synthetic sewage at 10 °C or 15 °C for 24 h on a reciprocal shaker at 60 rpm. After incubation, the culture was chilled in an ice bath, and the BOD of the supernatant, obtained by centrifugation at 4,000 rpm for 15 min, was determined. The amount of wastewater treatment was expressed by the BOD removal rate (%). For comparison, activated sludge cultivated in synthetic sewage at 15 °C for 3 months in our laboratory was also examined. During the cultivation period, activated sludge was aerated in synthetic sewage for 14 h in 1 d, and a half volume of the sewage was exchanged with fresh sewage every day. The BOD removal test was performed in triplicate. Chemicals. GluCer isolated from Saccharomyces kluveri was the kind gift of Dr. Mikio Kinoshita (Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine). The other chemicals used were reagent grade.

Results

Seasonal changes in the characteristics of activated sludge and glycolipid patterns

Activated sludge was collected monthly for 1 year to determine seasonal changes in their characteristics and GSL patterns. The sludge temperatures ranged from 10.5 °C (March, due to snow melt inflows to the sewage) to 25.0 °C (August). The pH values were stable, between 6.5 and 6.8. The sludge volume index (SVI) values were also stable, between 82 and 132, indicating generally good settlement properties. The weights of the recovered cells (110.8–249.8 g) and total lipids (0.9–1.5 g) fluctuated widely by sampling date, and no correlations with season were observed. Alkali-stable lipids from aceton- insoluble lipids in each sample were separated on TLC using chloroform-methanol-water (65:25:4, v/v/v). As shown in Fig. 1, clear spots corresponding to ceramide monosaccharide (CMS) appeared in all the samples, suggesting that CMS is a major component of GSL in activated sludge throughout the year.

Fungi isolated from activated sludge

Seven strains of fungi (AS-1 through AS-7) were isolated from activated sludge (in January, four strains, AS-1 through AS-4; in May, three strains, AS-5 through AS-7) on a GP agar plate. They were identified to the

<table>
<thead>
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<th>Characteristics of Fungi in Activated Sludge</th>
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<tr>
<td><strong>H2O</strong>, 282 (M⁺-CH₂O), 255 (M⁺-C₂H₂O₂), 103 (C₃H₅O₇), AS-6, 312 (M⁺), 281 (M⁺-CH₂O), 253 (M⁺-C₂H₂O₂), 103 (C₄H₅O₇), 314 (M⁺), 296 (M⁺-H₂O), 282 (M⁺-CH₂O), 255 (M⁺-C₂H₂O₂), 103 (C₃H₅O₇). MS data for sphingoid bases gave the following results: m/z AS-1, 236 (M⁺), 192 (M⁺-C₂H₂O), 250 (M⁺), 232 (M⁺-H₂O), 206 (M⁺-C₂H₂O), AS-2, 250 (M⁺), 232 (M⁺-H₂O), 206 (M⁺-C₂H₂O), AS-3, 236 (M⁺), 192 (M⁺-C₂H₂O), 250 (M⁺), 232 (M⁺-H₂O), 206 (M⁺-C₂H₂O), AS-4, 236 (M⁺), 192 (M⁺-C₂H₂O), 250 (M⁺), 232 (M⁺-H₂O), 206 (M⁺-C₂H₂O), AS-6, 250 (M⁺), 206 (M⁺-C₂H₂O).</td>
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genus level as *Mucor* sp. (AS-1), *Geotrichum* sp. (AS-2 and AS-7), *Trichosporon* sp. (AS-3), *Candida* sp. (AS-4), and *Trichoderma* sp. (AS-5 and AS-6) by partial 28S rDNA sequence analysis. Although AS-1 had 99% homology with both *Mucor* sp. and *Penicillium* sp., the morphological characteristics (photographs of AS-1 are shown in Fig. 2) were apparently different from *Penicillium* sp., and hence AS-1 was identified as *Mucor* sp. These fungi (also known as mycopathogens) are widely distributed in the environment, in rivers, soil, plants, silage, compost, house dust, and so on.

Glycosphingolipids of fungi isolated from activated sludge

The TLC of the chloroform-methanol (9:1) fractions eluted by silica gel column chromatography from each fungus (AS-1 through AS-4 and AS-6) neutral GSL fraction is shown in Fig. 3. The CMSs were purified from this fraction on preparative TLC, and their chemical structures were analyzed. The chemical components of the CMSs as determined by fatty acid, sphingoid base, and saccharide analyses are summarized in Table 1. Although the molecular ion peaks of hexadecadienal (m/z 236) for AS-2, AS-3, and AS-6 were not detected by MS, their retention times by GC agreed with that of the standard (data were not shown), and hence we identified octadeca-sphingadienine (d18:2) as the corresponding sphingoid base. All CMSs from the fungi were GlcCer (glucosylceramide). In *Mucor* sp. and *Geotrichum* sp., 2-hydroxyhexadecanoate (h16:0) was the...
sole fatty acid. In contrast, in Candida sp., 2-hydroxy-octadecanoate (h18:0) was determined to be the sole fatty acid, while h18:0 and 2-hydroxyoctadecenoate (h18:1) were dominant in Trichosporon sp. and Trichoderma sp. respectively. The sphingoid base components included d18:2 and 9-methyl-octadeca-sphingadienine (9-Me d18:2) in all the fungi, and 9-Me d18:2 was dominant in all the fungi. The MALDI-TOF/MS spectra for CMS are shown in Fig. 4. The results indicate that

Table 1. Chemical Compositions and Possible Structures of Purified CMS from Isolated Fungi

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Sugar</th>
<th>Fatty acidsa (%)</th>
<th>Sphingoid basesb (%)</th>
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<tr>
<td></td>
<td></td>
<td>h16:0 h18:0 h18:1 h20:0</td>
<td>d18:2 9Me-d18:2</td>
</tr>
<tr>
<td>Mucor sp. (AS-1)</td>
<td>Glc</td>
<td>100</td>
<td>21.5 78.5</td>
</tr>
<tr>
<td>Geotrichum sp. (AS-2)</td>
<td>Glc</td>
<td>100</td>
<td>19.2 80.8</td>
</tr>
<tr>
<td>Trichosporon sp. (AS-3)</td>
<td>Glc</td>
<td>7.9 90.0 2.1</td>
<td>1.3 98.7</td>
</tr>
<tr>
<td>Candida sp. (AS-4)</td>
<td>Glc</td>
<td>100</td>
<td>2.5 97.5</td>
</tr>
<tr>
<td>Trichoderma sp. (AS-6)</td>
<td>Glc</td>
<td>34.6 65.4</td>
<td>43.6 56.4</td>
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</table>

 Possible CMS structures and m/z of the corresponding [M + Na]+ ions from MALDI-TOF/MS are as follows: Mucor sp., GlcCer (d18:2-h16:0), 736.5, GlcCer (9-Me-d18:2-h16:0), 750.5; Geotrichum sp., GlcCer (d18:2-h16:0), 736.5, GlcCer (9-Me-d18:2-h16:0), 750.5; Trichosporon sp., GlcCer(9-Me-d18:2-h18:0), 778.5; Candida sp., GlcCer (d18:2-h18:0), 764.5, GlcCer (9-Me-d18:2-h18:0), 778.6; Trichoderma sp., GlcCer (9-Me-d18:2-h18:1), 776.4.

Fig. 4. MALDI-TOF/MS Spectra of CMS Isolated from Fungi.
Values indicate m/z of sodium-adduced molecular ions, [M + Na]+, in nominal mass. MALDI-TOF/MS of CMS from: A, Mucor sp.; B, Geotrichum sp.; C, Trichosporon sp.; D, Candida sp.; E, Trichoderma sp.; in positive ion reflection mode; matrix: 2,5-dihydroxybenzoic acid.

sole fatty acid. In contrast, in Candida sp., 2-hydroxy-octadecanoate (h18:0) was determined to be the sole fatty acid, while h18:0 and 2-hydroxyoctadecenoate (h18:1) were dominant in Trichosporon sp. and Trichoderma sp. respectively. The sphingoid base components included d18:2 and 9-methyl-octadeca-sphingadienine (9-Me d18:2) in all the fungi, and 9-Me d18:2 was dominant in all the fungi. The MALDI-TOF/MS spectra for CMS are shown in Fig. 4. The results indicate that

the [M + Na]+ ions for CMS were 736 and 750 for Mucor sp. and Geotrichum sp., 778 for Trichosporon sp., 764 and 778 for Candida sp., and 776 for Trichoderma sp. From these results, the ceramide structures of the main CMS from the fungi were identified as 9-Me- d18:2-h16:0 (Mucor sp. and Geotrichum sp.), 9-Me-d18:2-h18:0 (Trichosporon sp. and Candida sp.), and 9-Me-d18:2-h18:1 (Trichoderma sp.).
The ability of fungi isolated from activated sludge to serve in the treatment of wastewater

To elucidate the potential contribution of fungi isolated from activated sludge to wastewater treatment under cold conditions, the BOD removal test using synthetic sewage was conducted at 10°C and 15°C, and the results were compared with activated sludge that had been cultivated in synthetic sewage at 15°C for 3 months. The results of the removal test are summarized in Table 2. At 10°C, the lowest water temperature found at the wastewater treatment plant in Sapporo in 1 year, *Mucor* sp., *Geotrichum* sp., and *Candida* sp. were able to treat synthetic sewage, with *Mucor* sp. especially able to lower BOD (76.0 ± 2.42%) among the tested fungi. Surprisingly, BOD reduction by these three fungal species showed little change between 10°C and 15°C, indicating that they exhibited high growth at low temperatures. In contrast, BOD reduction by *Trichosporon* sp. at 10°C was low (32.3 ± 6.28%), although it significantly reduced BOD at 15°C (80.3 ± 1.07%). *Trichoderma* sp. (isolated in May but not in January) was least able to lower BOD among the fungi tested (less than 50% at 15°C), which suggests a positive association between the seasonal prevalence of fungi in activated sludge and wastewater treatment capacity.

### Discussion

The activated sludge process is one of the most common sewage treatment methods in the world. Bacteria, fungi, protozoa, and metazoa cohabit in the microbial ecosystem and possess great ability to adapt to harsh environments. The balance of microbes in activated sludge can change depending on conditions, resulting in a constant capacity to lower BOD. In the present study, we attempted to use GSL patterns as a signature of microbial community. In addition, we isolated fungi from activated sludge to determine the contribution of fungi to wastewater treatment during the cold season.

The observation of GSL patterns through 1 year led to the discovery that CMS is a major component of GSL in activated sludge. Some Gram-negative bacteria (*e.g.*, *Sphingomonas* sp.) produce GSLs instead of lipopolysaccharides, and have been isolated from activated sludge. Recent reports have shown that these bacteria can degrade refractory contaminants and produce gellan exopolysaccharides. However, since the components of GSLs produced by sphingomonomes are characteristic of galacturonosylceramide, and of amino sugar-containing saccharide chains, and GSLs generally are produced by eukaryotic cells, CMSs must be the products of constituent fungi. To examine the seasonal prevalence of fungi, seven strains of fungi were isolated from activated sludge in January and in May 2007, and their genera were identified as *Mucor, Geotrichum, Trichosporon, Candida*, and *Trichoderma*. These fungi were isolated in January or in May exclusively, except for *Geotrichum*, which was isolated in both months. This phenomenon may be due to seasonal prevalence of fungi in activated sludge. The CMSs isolated from these fungi were typical of those widely found in fungi. These include 9-Me d18:2-h16:0 and 9-Me d18:2-h18:0, which have been reported to be a major ceramide moiety of CMS from fungi, and 9-Me d18:2-h18:1, which was found in *Sporothrix schenckii* and *Histoplasma capsulatum* by Tolendo et al. Aoki et al. reported that *Zygomycetes* species produce a new group of neutral GSLs with α-1-β-1 galactoside linked to phytoceramides instead of glycosylinositolphosphoceramide, which was found in all fungi, although the CMS detected was GlcCer (9-Me d18:2-h16:0).

By observation of glycolipid patterns in activated sludge monthly through 1 year, we noticed that high polar GSLs assumed to possess the oligosaccharide chain tended to increase in the cold period. Such GSLs are not prevalent in fungi, except for *Zygomycetes*, as reported by Aoki et al., and *Mucor* sp. was isolated from activated sludge collected in January, but not from sludge in May in the present study. These results suggest the possibility that microbial flora in activated sludge change with the seasons, and that fungi (especially *Zygomycetes* species) increase in the cold season. The quality of the effluent from the water treatment plants was maintained in the cold season when the influent temperature dropped to 10°C. Although bacteria in activated sludge are thought to adapt lower temperatures, *e.g.*, *Comamonas badia* isolated from activated sludge can grow at 20°C, the dominance of fungi in the cold season might contribute to water treatment during cold periods. The results as to the effects of fungi on BOD reduction generally confirmed this supposition, because high BOD reduction was produced by fungi isolated in January, and *Trichoderma* sp., isolated only in May, had the least capacity to lower BOD among the fungi isolated.

These results indicate that the fungus species (especially *Mucor* sp. in the present study) with the ability to grow well under cold conditions may become dominant in the microbial community during the cold season, resulting in the maintenance of stable effluent quality. However, the detailed behavior of *Mucor* sp. in the
wastewater treatment plant and interactions among fungi species remain unknown. Further work is underway to confirm that the high quality of effluent under cold conditions (the BOD reduction at 10 °C by activated sludge was approximately 90%) was promoted by fungi, not bacteria.

Acknowledgments

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