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Assessment of Characteristics of Biofilm Formed on Autotrophic Denitrification

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Abstract A pilot-scale sulfur particle autotrophic denitrification (SPAD) process for the treatment of municipal wastewater was operated for 10 months at Shihwa, Korea, and higher than 90% NO_3^--N removal efficiency was observed. Plate counting showed that the lower part of the denitrifying column reactor had the most autotrophic denitrifiers. The biofilm thickness formed on sulfur particles from the SPAD reactor was approximately 25–30 μm, measured by DAPI (4,6-diamidino-2-phenylindole) staining. The presence of bacteria inside the highly porous sulfur particle was also monitored by SEM observation of the internal surfaces of broken sulfur particles. Biofilm extracellular polymeric substances (EPS) analysis showed that the ratio of carbohydrate to protein decreased with the reactor heights at which biofilm-formed sulfur particles were obtained.

Key words: Autotrophic, biofilm, denitrification, extracellular polymeric substances (EPS), sulfur

When the balance between influx and efflux of nitrogen in the biosphere is broken, discharges containing nitrogen can severely damage a water resource, and the problem is then associated with deterioration of the entire ecosystem. Specifically, nitrite and nitrate nitrogen in drinking water can cause risk to public health [5], which is related primarily to methemoglobinemia (infantile cyanosis) and carcinogenesis. Recently, nitrate contamination of ground water resources has become an increasing problem, therefore, the drinking water standard for nitrate has been set by the U.S. Environmental Protection Agency [13] as 10 NO_3^--N mg/l. Successive nitrification and denitrification are the most effective biological processes to remove nitrogenous chemicals from wastewaters. These two reactions are carried out by two different functional groups of microorganisms. Nitrification involves several nitrifying bacteria that utilize oxygen to oxidize ammonium to nitrite or nitrate ions under aerobic conditions, whereas the denitrification process changes nitrite or nitrate ions to nitrogen gas by bacteria of totally different metabolic groups, i.e. heterotrophs vs. chemolithoautotrophs. Nitrification does not reduce the mass of nitrogen discharged, but it merely changes its state. Consequently, nitrification alone will not alleviate the problem of eutrophication, because that requires a reduction in the availability of nutrients in the aquatic environment. After nitrification, therefore, nitrate nitrogen should be reduced to nitrogen gas by the process called denitrification [11].

Heterotrophic denitrification is very efficient in terms of nitrate removal, provided adequate amounts of organic carbon are available [9]. However, when organic carbon in conventional denitrification processes composed of sequentially aerobic and anoxic reactors is insufficient compared to the nitrogen content, expensive chemicals like methanol or similar organic compounds must be added, or design of the process must be modified. However, the rising cost and increasing scarcity of methanol and similar organic compounds make them increasingly undesirable as chemical additives. Contrary to heterotrophic denitrification, however, autotrophic denitrification can be used to lower the operational cost by substituting the organic addition with the use of a variety of reduced sulfur compounds (S^{2-}, S^{o}, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}) as the electron and energy sources, while reducing nitrate as the final electron acceptor. As elemental sulfur is cheaper than methanol or acetate and is insoluble in water, the autotrophic denitrification process is economically beneficial, and the operation for the treatment of wastewaters becomes easy, especially those with a low organic to nitrate (C/N ratio) ratio. Thus, sulfur-based autotrophic denitrification has recently been receiving more attention [6, 14, 15].

A sulfur particle autotrophic denitrification (SPAD) process has been developed, based on sulfur-based autotrophic denitrification. Comparative experiments between the use

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of thiosulfate and sulfur particles confirmed that sulfur particles were successfully utilized by autotrophic denitrifying organisms as a growth medium within and on the surface of particles [9]. The SPAD process employs a biofilm formed on elemental sulfur particles. The one-year operation of a pilot-scale treatment plant at Shihwa, Korea, proved that the SPAD process had a high nitrate removal capacity and was resistant to the influent of nitrate fluctuation.

Due to the low solubility of elemental sulfur, autotrophic denitrifiers are prone to form biofilm on the sulfur particles. Although the sulfur-based autotrophic denitrification process has been studied for over 30 years, little information on the biofilm formed during the wastewater treatment process is available, partly due to the invisibility of the biofilm formed on the sulfur particles. Autotrophic denitrifiers, such as *T. denitrificans*, are known to be colorless. In this regard, this study employed 4,6-diamidino-2-phenylindole (DAPI) staining to visualize the structure of the biofilm formed on sulfur particles. The extracellular polymeric substances (EPS) in the biofilm were also analyzed.

MATERIALS AND METHODS

Operation of Pilot Plant

As shown in Fig. 1, the pilot plant at the Shihwa municipal wastewater treatment plant was composed of a NaOH tank for pH equilization, a settling tank, a methanol pit, three aerobic rectangular tanks for nitrification, a column reactor for sulfur utilizing denitrification, an effluent pit, a sand filter, and pumps. The system had the capacity to treat a total of 50 m^3 of wastewater per day. It was located in Shihwa, Korea, and was operated for 10 months from February to November 2001. The pH of the influent was controlled to 7–8 by addition of 30% NaOH. Methanol was fed for heterotrophic activity, but far less than the amount required for the theoretical denitrification process.

Nitrification was implemented using three sequential aerobic reactors (24.2 m^3 capacity). Fiber-type media were installed in the reactors to provide a large surface area for the active growth of nitrifiers. Ammonium nitrogen was oxidized to nitrate nitrogen by nitrifiers attached to the fiber media before it was treated by the SPAD column. Total nitrogen levels in the influent wastewater varied with time, with an average of 139 N mg/l. Influent and effluent samples from the nitrifying reactor were taken regularly and analyzed.

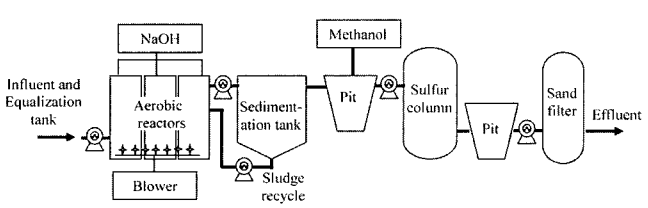

Fig. 1. Schematic of the SPAD process operated at the Shihwa municipal wastewater treatment plant.

The column reactor was packed with sulfur particles (diam. 2–5 mm) and limestone using a three to one ratio by volume and was fed continuously with municipal wastewater oxidized by the aerobic reactors operating in an upflow mode. The nitrate load was increased stepwise from 0.02 to 0.16 kg NO_3^--N/m^3/day by the changing hydraulic retention time (HRT) while maintaining influent nitrate and COD concentrations in the range of 20–70 N mg/l and 60–150 COD mg/l. Sulfur particles were sampled from the three ports of the SPAD column reactor (3.18 m^3 capacity; 1.5 m inner diam., 2.6 m height) at the heights of 0.5, 1, and 1.5 m, respectively, from the bottom of the reactor. The effluent samples were taken regularly and analyzed in the field as well as in the laboratory.

Parameters monitored included pH, temperature, COD, nitrate, nitrite, and sulfate. The pH and temperature were monitored using a standard Fisher Scientific Accumet® pH meter. Nitrate, nitrite, and sulfate were measured by ion chromatography (DX-120, Dionex Corp., CA, U.S.A.) equipped with an IONPACK ® CS12A Analytical Column and a CG12A Guard Column (2×250 mm), a CDM-3 conductivity detector, and an AS40 automatic sampler.

Enumeration and Isolation of Microorganisms

Plate counting was conducted to enumerate viable cells in the column reactor. After serial dilution of the reactor effluent with phosphate-buffered saline (PBS; 0.13 M NaCl, 10 mM Na_2HPO_4, pH 7.2), the sample was spread uniformly on the denitrifying medium [5 g/l $Na_2S_2O_3 \cdot 5H_2O$; 2 g/l KNO_3; 1 g/l NH_4Cl; 2 g/l KH_2PO_4; 0.8 g/l $MgSO_4 \cdot 7H_2O$; 2 g/l $NaHCO_3$; 1 ml trace metal solution containing 50 mg/l disodium EDTA; 11 mg/l NaOH; 7.34 $CaCl_2 \cdot 2H_2O$; 5 mg/l $FeSO_4 \cdot 7H_2O$; 2.5 mg/l $MnCl_2 \cdot 2H_2O$; 2.2 mg/l $ZnSO_4 \cdot 7H_2O$; 0.5 mg/l $CoCl_2 \cdot 6H_2O$; 0.5 mg/l $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 0.2 mg/l $CuSO_4 \cdot 5H_2O$; pH 7.2] and on the nitrate broth medium (5.0 g/l peptone; 3.0 g/l beef extract; 1.0 g/l KNO_3; pH 7.2) plates. Plates were incubated in anaerobic jars at 25°C, nitrifier medium plates for 7 days, and nitrate broth medium plates for 3 days. One g of sulfur particles and 10 ml of PBS were homogenized by vortexing. Supernatant of the homogenized sulfur particles was serially diluted and used for plate counting as described above. Facultative autotrophic denitrifiers were enumerated by replicate plating of colonies on nitrate broth medium plates onto denitrifier medium plates. The number of heterotrophic bacteria was measured by subtracting the number of facultative autotrophic bacteria from the cell number enumerated on the nitrate broth medium plates. Obligate autotrophic denitrifiers were calculated by replicate plating colonies from denitrifier medium plates onto nitrate broth medium plates; obligate autotrophic denitrifiers grew on denitrifier medium plates, but not on nitrate broth medium plates.

EPS Extraction

Sulfur particles obtained from the three sampling ports were used to analyze the composition of EPS, whereas sulfur particles from sampling port 1 were used to visualize the structure of the biofilm formed on the particles. EPS extraction was performed according to the procedure described by Jang *et al.* [3]. Carbohydrate content in extracted EPS was quantified by the phenol-sulfuric acid method, while protein content was analyzed by the modified Bradford method, as described by Horan and Eccles [2]. To evaluate the degree of cell lysis that might be caused by the EPS extraction procedure, DNA concentration in the extracted EPS was measured by a fluorometric method.

Fixation and Sectioning of Biofilm on Sulfur Particles

Sulfur particles were gently washed with PBS, and biofilms on the particles were fixed with 4% paraformaldehyde in PBS for 1 h at 4°C. Fixed biofilm was rinsed with PBS and then embedded in Tissue-Tek OCT compound (Sakura Finetek Inc., U.S.A.) for freezing overnight at −30°C. Sulfur particles were removed from the frozen biofilm that had been subjected to sectioning to a thickness of 25 μm using a Microslicer™ (LEICA CM1800, U.S.A.) at −20°C. Each biofilm section was placed on a gelatin-coated glass slide. The sections were dehydrated by successive passages through ethanol solutions, and then were air-dried before DAPI (4,6-diamidino-2-phenylindole) staining. PBS-rinsed sulfur particles were air-dried and then critical-point-dried with CO_2 for scanning electron microscopy (SEM). After sputter coating with carbon, sulfur particles were examined using a HITACHI-S4500 SEM microscope.

RESULTS AND DISCUSSION

Nitrification Performance

Figure 2 shows the changes of NH_4^+ concentration at the influent of the aerobic tank and the effluent of the settling tank during the experimental period. Nitrification converts ammonium ions to nitrite or nitrate ions. As wastewater was fed in sequence into the three aerobic tanks, NH_4^+ concentration gradually decreased, while NO_3^- concentrations increased, suggesting that nitrification occurred in the aerobic tank.

As shown in Fig. 2, the temperature profile in the aerobic tank showed that the lowest point was about 10°C in winter and the highest point was about 30°C in summer. From February to April, the ammonium was not well oxidized to nitrate, because of the low temperature, even with more than 25 h of long hydraulic retention time (HRT). On the other hand, when the temperature was kept about 30°C, over 90% nitrification efficiency was observed from May to September. It was found that, as temperature increased,

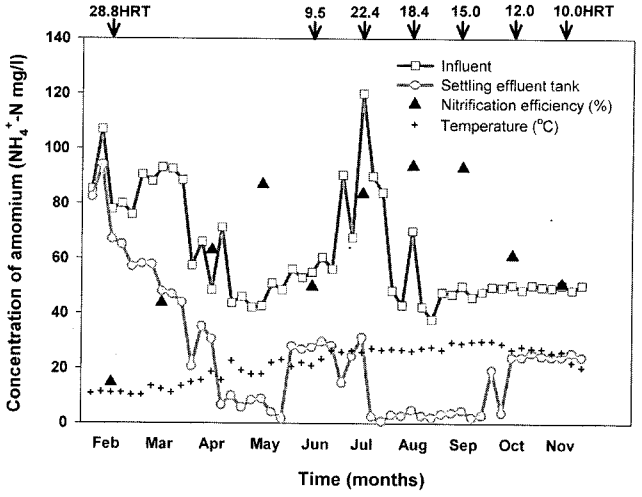

Fig. 2. Ammonium concentration and temperature changes during the operation period.

the nitrification increased; temperature change is one of the most significant factors affecting the reaction rate of nitrification. However, despite the high temperature, in June, the effluent ammonium concentration at the settling tank was measured as 30 mg/l, which was half that of the influent, due to a short HRT of less than 10 h. From October to November, the system could not achieve a high nitrification efficiency due to both the short HRT and the low temperature. It was concluded that, in order to maintain over 90% nitrification efficiency, the aerobic reactor should be kept at 30°C and 15 h HRT of operation.

Performance of the SPAD Process

As shown in Fig. 3(a), denitrification during the experimental period was stable, and the nitrate removal efficiency was over 90% for varying influent levels. The influent and effluent alkalinity ranged from 230–280 and 30–250 mg/l as $CaCO_3$, respectively (data not shown). The denitrification efficiency significantly decreased in June and August due to the short HRT in the nitrification tank, causing high volumetric loading rates in the column. In August, especially, the effluent nitrate ions were significantly increased because of the high volumetric loading rates associated with short HRT and simultaneously to the high concentrations of nitrate. After adding a fourth of the theoretically required dosage of methanol, however, sulfur-utilizing denitrification increased, compared to the case of no organic supplemented sulfur-utilizing denitrification due to the alkalinity saved by organic carbon-utilizing denitrification. The results demonstrate that addition of organic substances accelerates removal of nitrate under mixotrophic conditions. As Oh *et al.* [9] also discussed, simultaneous utilization of heterotrophic denitrification in the sulfur-based autotrophic denitrification column gives several advantages for effluent quality. Production

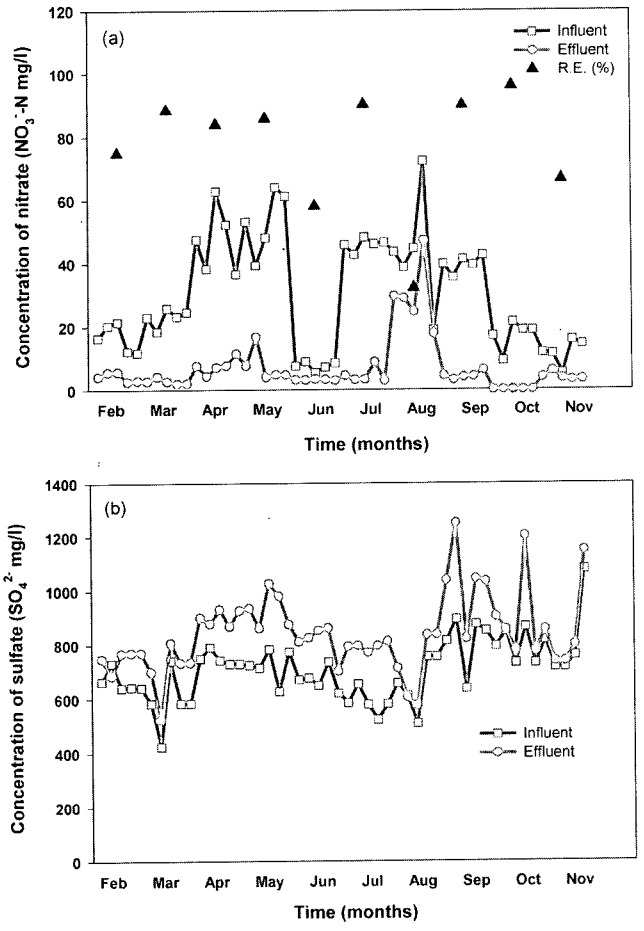

Fig. 3. Nitrate (a) and sulfate (b) concentration changes during the operation period.

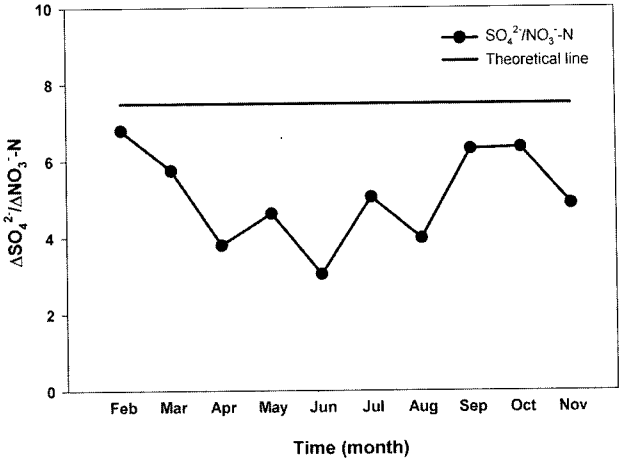

Fig. 4. The ratio of sulfate production and nitrate removal.

of alkalinity from the heterotrophic denitrification provides proper conditions for autotrophic denitrifiers. Therefore, where small amounts of methanol are available for heterotrophic activity, autotrophic denitrification can also be enhanced by using the alkalinity source. The pH of the treated water is also stabilized in neutral ranges.

Sulfate is one of the end products of sulfur oxidizing autotrophic denitrification. It is shown in Fig. 3(b) that denitrification by autotrophic activity was confirmed by the increase of sulfate levels. The influent sulfate concentration varied from 550 to 850 mg/l. In general, the sulfate concentration was continually increasing and reached 700 to 1,200 mg/l in the column. In August, as the HRT was decreased to the critical point, no sulfate production was observed in the column, and the same pattern was seen as nitrate decreased. When the methanol (0.25T) for organic-utilizing denitrification was added after August, sulfate production increased sharply, probably due to heterotrophic denitrification in the column.

Based on the stoichiometric equation for sulfur denitrification, reduction of every 1 mg/l of NO_3^--N should produce approximately 7.5 mg/l of SO_4^{2-}. Other authors observed similar ratios of sulfate production to nitrogen removal, such as 6.39 [10], 7.75 [1], 5.95 [15], and 7.89 [6]. However, as shown in Fig. 4, SO_4^{2-}/NO_3^--N ranged from 3 to 7 with an average of 5.43. This deviation could be due to the irregular NO_3^--N influent loading rate and/or the presence of sulfate-reducing bacteria.

Enumeration and Isolation of Microorganisms

With regard to microbiology, bacteria capable of oxidizing reduced sulfur compounds, such as sulfide, sulfur, or thiosulfate, can be classified physiologically into four types: obligate chemolithoautotrophs, facultative chemolithoautotrophs, chemolithoheterotrophs, and heterotrophs. Obligate chemolithoautotrophs capable of denitrification, like *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*,

Table 1. Bacterial distribution in the SPAD reactor.

		Heterotrophic bacteria	Facultative autotrophic denitrifiers	Obligate autotrophic denitrifiers	H:A[a] (%)
Effluent liquid (cell/ml)		8.5×10^8	4.25×10^7	6.02×10^5	95:5
Cell number/ sulfur particle	P3[b]	1.4×10^8	1.91×10^7	1.04×10^6	87:13
	P2	2.8×10^8	2.40×10^7	5.54×10^6	90:10
	P1	2.4×10^8	3.23×10^7	8.05×10^6	85:15

[a]Heterotrophic bacteria: facultative and obligate autotrophic denitrifiers.
[b]Height of sampling port from the bottom of the reactor: port 1, 0.5 m; port 2, 1 m; port 3, 1.5 m. Values in the table show average results (n=3).

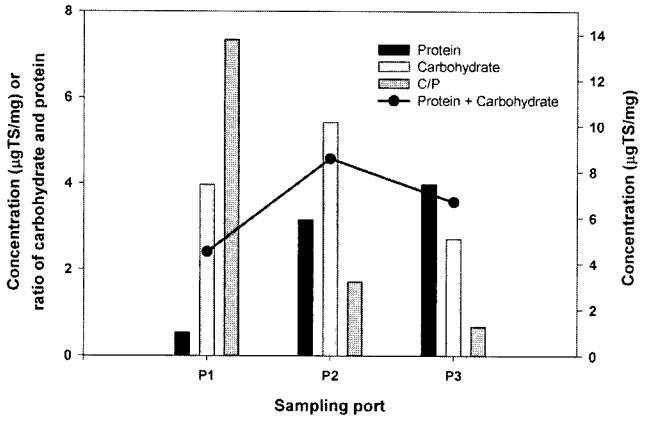

Fig. 5. Comparative analysis of carbohydrate and protein concentrations in EPS extracted from biofilm formed on sulfur particles.
The sulfur particle samples were obtained from three different ports in the sulfur-packed reactor.

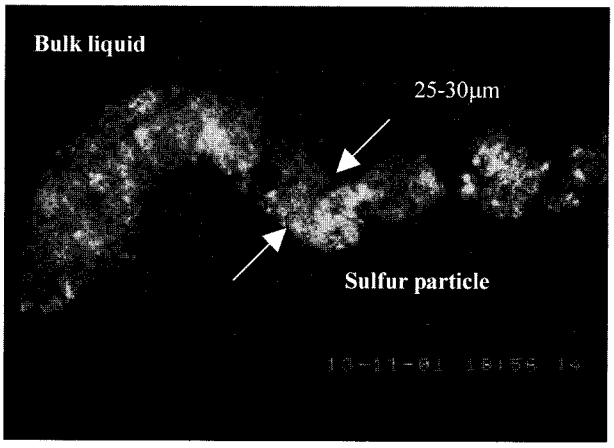

Fig. 6. DAPI-stained biofilm formed on sulfur particle, viewed by epifluorescence microscopy (×400).

are virtually restricted to an autotrophic mode of growth, since they cannot obtain energy from the oxidation of organic compounds; they can only utilize organic compounds to a limited extent [12, 7]. In contrast, facultative chemolithoautotrophic denitrifiers, such as *Thiobacillus versutus, Thiobacillus thyasiris, Thiosphaera pantotropha,* and *Paracoccus denitrificans*, are not only able to grow autotrophically by using reduced sulfur compounds as an energy source, but are also capable of heterotrophic growth. Hence, these bacteria can apparently adapt to different environments (i.e., autotrophic, heterotrophic, or mixotrophic conditions) [8]. Because these bacteria are likely to encounter autotrophic and heterotrophic conditions in nature, it is of considerable interest to determine their nitrate removal characteristics under mixotrophic conditions.

The results (Table 1) of plate counting showed that the lower part of the reactor had the most autotrophic denitrifiers. The fraction of autotrophic denitrifiers in the reactor was generally kept at less than 15%, even though the overall nitrate removal efficiency was maintained above 90%. Thus, it can be concluded that, under mixotrophic conditions, some portion of the nitrate was removed heterotrophically and the remainder was denitrified by sulfur-utilizing autotrophic bacteria without inhibition of organics.

EPS Analysis

Considering the facts that sulfur-utilizing autotrophic denitrifiers are prone to adhere to sulfur particles, because of low solubility of sulfur, and that most of the denitrification activity is found in the lower part of the reactor, biofilm EPS analysis is important. Recent studies have shown that the EPS produced in a capsular form or as free slime in bacteria [4] is generally considered to be important in cementing bacterial cells together in the biofilm structure. Although the chemical compositions of EPS vary, the sum

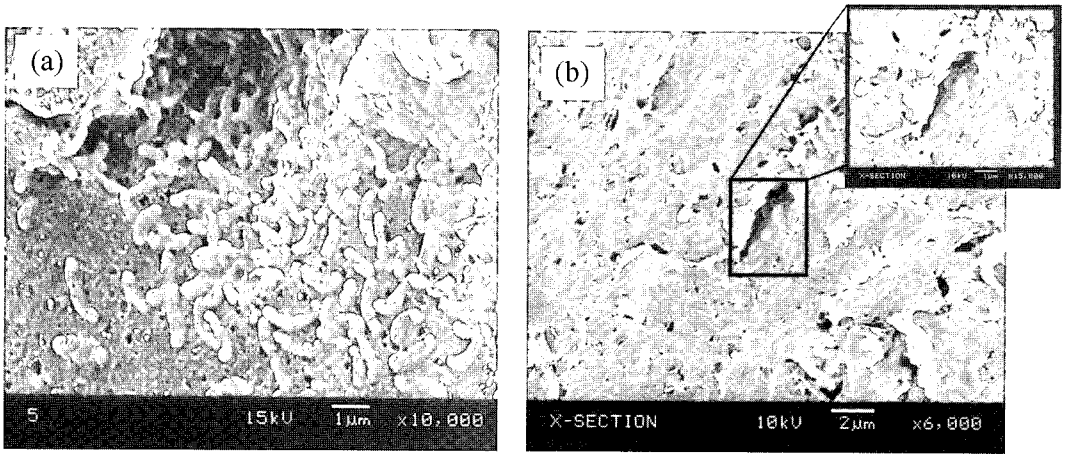

Fig. 7. Surface of intact sulfur particle (a) and internal surface of broken sulfur particle (b), as shown by SEM.

of the amounts of total carbohydrates and proteins represented the total amount of EPS, which are the main components typically found in EPS [2]. The two main components of biofilm EPS showed different concentrations depending on the sampling ports of the reactor employed in this study (Fig. 5): The ratio of carbohydrate to protein (C/P) decreased with the reactor heights at which the biofilm-formed sulfur particles were obtained, suggesting that microbial populations might be diverse depending on reactor height. As shown in Fig. 5, the total amounts of proteins and carbohydrates in the middle of the reactor were relatively high, compared to those at the bottom. It is highly likely that excess biomass accumulation in the bottom had moved to the upper part of the reactor by means of influent flow caused by the high density of biomass in the middle of the reactor.

Monitoring of Biofilm Structure

Biofilm structure was visualized after staining with DAPI (Fig. 6). Biofilm formed on sulfur particles showed 25–30 μm thickness. Some microcolonies were frequently observed throughout the biofilm. Considering the high porosity of the sulfur particles, however, we cannot exclude the possibility of bacterial presence inside the particles, as suggested by SEM observation (Fig. 7).

The reactor located in Shihwa, Korea, was operated for 10 months, showing >90% NO_3^--N removal efficiency. Under mixotrophic conditions, some portion of the nitrate was removed heterotrophically and the remainder was denitrified by sulfur-utilizing autotrophic bacteria without inhibition by methanol. Also, alkalinity consumption was reduced Biofilm EPS analysis showed that the ratio of carbohydrate to protein decreased with the reactor height at which the biofilm-formed sulfur particles were obtained. This suggests that microbial populations might be diverse, depending on the height of the sulfur particles in the reactor. DAPI staining was employed to monitor the structure of the biofilm formed on the sulfur particles obtained from the pilot-scale reactor used for autotrophic denitrification of wastewater. The biofilm was 25–30 μm thick The presence of bacteria inside the particles was suggested by the high porosity of the sulfur particles and by SEM observation of the internal surfaces of broken sulfur particles.

Acknowledgments

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