Korean J. Chem. Eng., **24**(1), 93-98 (2007)

Fluorescence *in situ* hybridization and INT-dehydrogenase activity test to assess the effect of DO concentration in aerobic biofilm reactor

Sung Ho Hur, Jeung Jin Park, You Jin Kim, Jae Cheul Yu, Im Gyu Byun*, Tae Ho Lee and Tae Joo Park†**

Department of Environmental Engineering, Pusan National University, Busan 609-735, Korea
*Institute for Environmental Technology and Industry, Pusan National University, Busan 609-735, Korea
**Department of Environmental Engineering, Andong National University, Andong 760-749, Korea
(*Received 11 July 2006 • accepted 6 September 2006*)

Abstract–To assess the effect of DO concentration in aerobic biofilm reactor, we investigated the bacterial communities and their activity utilizing the combination of two methods, fluorescence *in situ* hybridization (FISH) and INT-dehydrogenase activity test. D-1, D-2, D-3 and D-4 reactor with different DO concentrations (1, 3, 5 and 7 mg/L, respectively) were set up in the thermostat and acclimated. The optimal DO concentration with stable nitrification efficiency in aerobic biofilm reactor was above 5.0 mg/L. FISH method showed us that higher DO concentration led to the increase of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria ratios and the decrease of heterotrophs ratio. The INT-DHA activities of each reactor were 203, 153, 36 and 45 mgO_2^*/g VSS/day, respectively. The INT-DHA activities in D-1 and D-2 reactors were higher than those of D-3 and D-4 reactors, which indicates that filamentous microorganisms affected the INT-DHA activity. In the case of D-3 and D-4 reactors, as nitrification efficiency increased, INT-DHA activity also increased. In the relationship between the attached biomass and nitrification efficiency, only active biomass influenced the nitrification efficiency.

Key words: Fluorescence *in situ* Hybridization, INT-dehydrogenase Activity, Dissolved Oxygen, Aerobic Biofilm, Nitrification

INTRODUCTION

It is obvious that total nitrogen can be removed by nitrification and denitrification in a biological wastewater treatment plant. Nitrification is divided to two mechanisms; one is nitritation (from NH_4^+-N to NO_2^--N) by ammonia-oxidizing bacteria (AOB), and the other is nitratation (from NO_2^--N to NO_3^--N) by nitrite-oxidizing bacteria (NOB) [Surmacz-Gorska et al., 1996; Zhang and Bishop, 1996]. Efficiency of nitrification is limited by pH, temperature, and inhibitory chemicals, while denitrification is performed easily in a biological treatment plant and denitrifying bacteria are widely distributed in nature [Sharma and Albert, 1977]. The biofilm process differs from the activated sludge process in that the latter operates with the activated biomass suspended in the system while it attaches to the carriers in the biofilm process, which means biomass grow and adhere to the surface of the carriers [Wang et al., 1991; Park et al., 1996, 1998]. Generally, nitrifying bacteria in aerobic biofilm are more sensitive than heterotrophs in using oxygen. It has often been found that one of the most important factors affecting nitrification performance in biofilm is the limitation of dissolved oxygen (DO) [Zhang and Bishop, 1996; Lazarova et al., 1998; Jang et al., 2002; Kim et al., 2005]. And it was reported that only active biomass is responsible for nitrification and organic material removal, rather than the total biomass present in biofilm [Imat et al., 1993; Lazarova et al., 1994; Liu et al., 1996].

To characterize nitrifying bacterial communities, the routine bacteriological enumeration by spread-plate or most-probable-number count, has been studied. However, these methods are not suitable because some weeks of incubation time are necessary before these bacteria are enumerated. As well, these techniques often detect only a minor portion of biofilm that may increase the statistical uncertainty of the enumerations. Thus, a better monitoring of the content of bacteria in the microbial community, recently, fluorescence *in situ* hybridization with rRNA-targeted oligonucleotide probes has been utilized and proven as an effective method for analysis of microcolonies and biofilm without the need for disruptive or labour consuming procedures [Schramm et al., 1998; Jang et al., 2002].

On the other hand, the biodegradation of an organic compound proceeds through a series of oxidation reactions involving loss of electrons or removal of hydrogen atoms from organic compounds. The process of removal of hydrogen atoms from an organic compound is called dehydrogenation. The enzymes, which catalyze dehydrogenation reactions, are called dehydrogenases. If the number of dehydrogenases in the biodegradation can be measured, microorganism activity can be determined [Yang et al., 2002]. Bitton and Koopman [1982] have developed a tetrazolium reduction assay to determine the physiological activity of filamentous microorganisms in activated sludge, which involves the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan (INTF) by the active bacterial electron transport system and is named the INT-dehydrogenase activity (DHA) test. This method of measuring DHA is quite easy to perform and is very sensitive. A close correlation has been reported between INT-DHA and oxygen consumption [Koopman et al., 1984; Awong et al., 1985]. Therefore, DHA concentration was used to indicate microorgan-

†To whom correspondence should be addressed.
E-mail: taejoo@pusan.ac.kr

ism activity in this study. The method has been reported by Kim et al. [1994].

In this study, we investigated the bacterial communities and their activity by utilizing the combination of two methods, FISH and INT-DHA test, to assess the effect of DO concentration in aerobic biofilm reactor. These methods would be expected to provide the optimal DO concentration for nitrification and the microbial characteristics in aerobic biofilm reactor treating wastewater.

MATERIALS AND METHODS

1. Experimental Apparatus

The experimental apparatus consisted of four aerobic biofilm reactors with fixed-bed, shown in Fig. 1. Each reactor was filled with ceramic media, whose packing ratio was 0.15. The effective volume of each reactor was 5.3 L. All reactors were set up in the thermostat to maintain the constant temperature at 25 °C. The reactors were inoculated with activated sludge obtained from a municipal sewage treatment plant and were acclimated with the raw wastewater, whose concentrations are shown in Table 1. The reactors were operated with the DO concentration at 4 mg/L for 60 days, and then D-1, D-2, D-3 and D-4 reactor were maintained with the DO concentration at 1, 3, 5 and 7 mg/L, respectively. Hydraulic retention time of all reactors was six hours.

2. Oligonucleotide Probes

The following rRNA-targeted oligonucleotides were used: EUB 338, Nso190, Nsv 443, Nsm 156, Ntspa662, Nit3. Oligonucleotides were synthesized and fluorescently labelled with fluorescein isothiocyanate (FITC), a hydrophilic sulfoindocyanine dye (CY3), rhodamine red at the 5' end by CoreBioSystem (Seoul, Korea). All probe sequences, the hybridization conditions and references are given in Table 2.

3. Fluorescence *in situ* Hybridization

The biofilms of each reactor were vortexed strongly with 0.1% sodium pyrophosphate solution and fixed by immersing in freshly prepared paraformaldehyde solution (4% in phosphate buffered saline, PBS) for overnight at 4 °C. Thereafter, the biofilms were rinsed with PBS solution. Each sample was immobilized on a gelatine coated

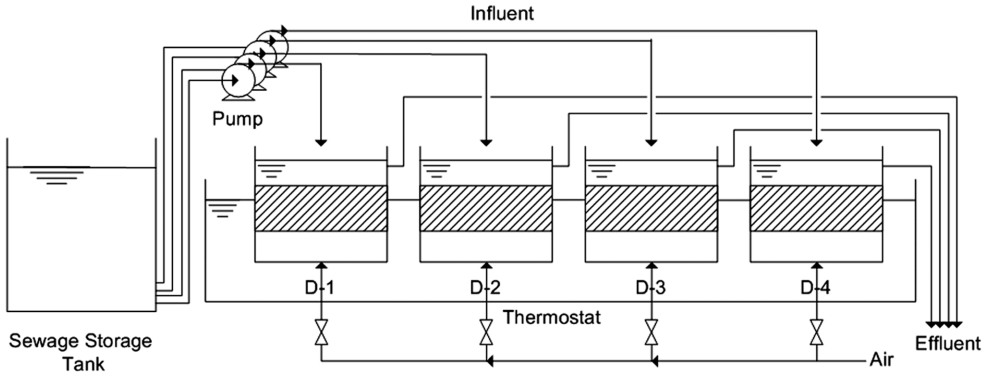

Fig. 1. Schematic diagram of the aerobic biofilm reactors with different DO conditions.

Table 1. Characteristics of the raw wastewater

pH	$TCOD_{Cr}$ (mg/L)	BOD_5 (mg/L)	$SCOD_{Cr}$ (mg/L)	SS (mg/L)	NH_4^+-N (mg/L)	PO_4^{3-}-P (mg/L)	Alkalinity ($mgCaCO_3/L$)
7.12-7.42 (7.28)	106.0-175.5 (129.0)	62.5-70.4 (67.3)	71.0-136.6 (94.2)	53.8-58.9 (55.7)	13.5-33.9 (26.8)	2.3-3.2 (2.8)	230-340 (273)

( ) is mean value during the operating days

Table 2. Oligonucleotide probes applied

Probe	Specificity	Sequence (5'-3')	Target site[a]	% FA[b]	[NaCl] (mM)[c]	Reference
EUB338 (II)	Eubacteria	GCAGCCACCCGTAGGTGGT	338-355	20	215	Daims et al., 1999
Nso190	Ammonia-oxidizing *β-Proteobactria*	CGATCCCCTGCTTTTCTCC	190-208	20	215	Mobarry et al., 1996
Nsv443	*Nitrosospira* spp.	CCGTGACCGTTTCGTTCCG	444-462	30	102	Mobarry et al., 1996
Nsm156	*Nitrosomonas* spp.	TATTAGCACATCTTTCGAT	156-174	5	630	Mobarry et al., 1996
Ntspa662	*Nitrospira genus*	GGAATTCCGCGCTCCTCT	662-679	20	215	Daims et al., 2000
Nit3	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	1035-1048	40	46	Wagner et al., 1996

[a]16S rRNA position according to *Escherichia coli* numbering.
[b]Formamide concentration in the hybridization buffer.
[c]Sodium chloride concentration in the washing buffer.

slide glass. The sample was finally dehydrated by successive passage through ethanol solution, and air dried. The fixed samples were hybridized by spiking sequentially 8 μL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulphate (SDS)) and 2 μL of fluorescent probes and then quickly transferring them to pre-warmed moisture chamber under a temperature condition of 46 °C. Finally, the slide was dipped into the washing solution at 48 °C. After the hybridization, digital images of the aggregates were taken by a fluorescence microscope (Zeiss Axioskop 2plus, Germany) and visualized by using Zeiss Axiovision digital imaging software. Analysis was performed with a standard software package using Carl Zeiss Imaging Solution system (Zeiss, Germany).

4. INT-dehydrogenase Activity Test

The general method of Koopman et al. [1984] was used. A 0.2% (w/v) solution of INT (Sigma-Aldrich Co., USA) was prepared by using deionized, filter-sterilized water. Triplicate, 5 mL samples were amended with 1.0 mL of INT, incubated in the dark at room temperature (20±2 °C) for 30 min and fixed with 1.0 mL of 37% formalin. Treated samples were centrifuged at 2,500 rpm for 10 min and excess water removed. Pallets were extracted with 5 mL of an acetone/dimethyl sulfuroxide solution (2/3 v/v) for 20 min in the dark, after which the extracts were centrifuged for 10 min and optical density of the supernatants determined at 465 nm. INT-dehydrogenase activity was calculated in equivalent oxygen (O_2^*) units by using the following equation:

$$DHA = \frac{905 D_{465} \nu}{VCtF} \quad (1)$$

Where DHA=INT-dehydrogenase activity (mgO_2^*/g VSS/day), D_{465}= optical density through a path length of 1.0 cm, ν=final solvent volume (mL), V=volume of INT-treated sample used in the extraction procedure (mL), C=initial biomass concentration of the sample (g VSS/L), t=incubation time (min) and F=factor to adjust for dilution caused by adding INT and formalin.

Attached biomass [mg/L] was calculated as that biomass [g], vortexed with 0.1% sodium pyrophosphate solution and detached from some ceramic media, divided by effective volume of the reactor [L].

5. Analytical Methods

The influent and effluent samples for each reactor were tested within 3 days of sampling. The pH and dissolved oxygen concentration were measured by using an Orion Research pH meter (Model230A, USA) and YSI DO meter (Model58, USA), respectively. The soluble chemical oxygen demand (SCOD), NH_4^+-N, NO_3^--N and NO_2^--N concentrations were measured after filtering the sample through a 0.45 μm membrane filter with an auto analyzer (AA3, Bran+Luebbe, Germany). The total chemical oxygen demand (TCOD), alkalinity and suspended solids (SS) were measured by using Standard Methods (APHA, 2005). The stored samples were kept refrigerated at 4 °C until tested.

RESULTS AND DISCUSSION

1. COD Removal and Nitrification Efficiencies

Initially, all reactors were acclimated at 4.0 mgO_2/L and 25 °C and acquired almost 90% nitrification efficiencies. Thereafter, the reactors were operated with different DO concentrations. The SCOD removal and nitrification efficiencies of each reactor are given in Fig. 2(a) and (b), respectively.

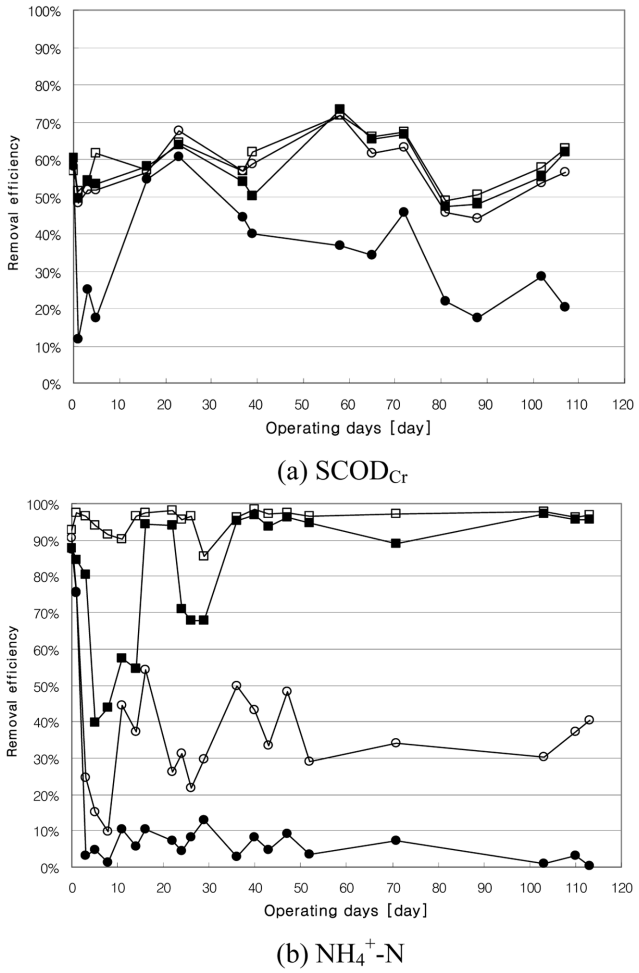

Fig. 2. Removal efficiencies of the each reactor with different DO concentrations; D-1 (●), D-2 (○) D-3 (■), D-4 (□).

Shown in Fig. 2(a), only the D-1 reactor was affected by DO concentration in COD removal efficiency. During the operating days, the SCOD removal efficiencies of D-2, D-3 and D-4 reactor were 56.2, 57.2 and 59.4%, respectively, whereas the D-1 reactor was 32.8%. The effluent SCOD concentrations of D-2, D-3 and D-4 reactors were 40.4, 39.5 and 37.4 mg/L, respectively. In the relationship between BOD_5 and COD (Table 1), residual SCOD matters of D-2, D-3 and D-4 reactor were assumed hardly biodegradable matters.

Fig. 2(b) showed the nitrification efficiencies of each reactor. The nitrification efficiencies of D-1, D-2, D-3 and D-4 reactor were 12.9, 38.4, 80.8 and 95.5%, and effluent concentrations of NH_4^+-N were 23.4, 16.7, 5.3 and 1.3 mg/L, relatively. Lazarova et al. [1998] reported that the maximum removal rate measured for the completely developed biofilm is almost twice that of the partially developed biofilm, but the transition value of the bulk oxygen concentration is higher. The transition values are 3.4 and 5.2 mg/L, respectively, for the case of the partially covered and completely covered by the biofilm carrier. This increase for the bulk oxygen concentration could be related to the higher amount of biomass on the carrier, increasing the resistance of oxygen transfer into the biofilm. Similar phe-

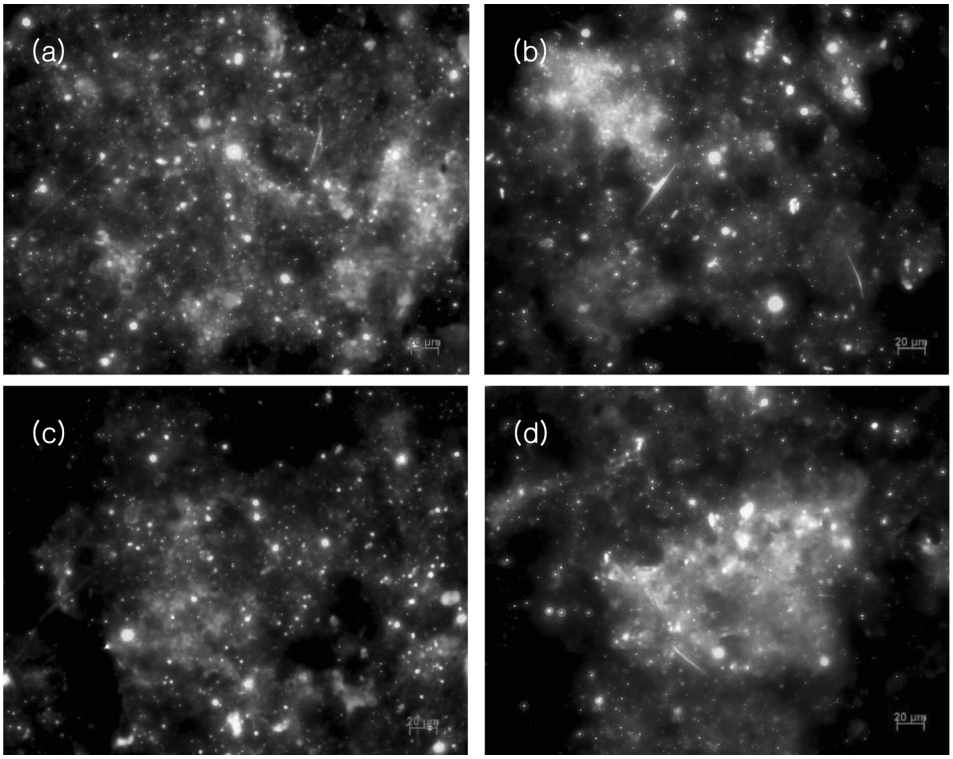

Fig. 3. Simultaneous *in situ* hybridization of nitrifying bacteria in aerobic biofilm: (a) EUB338() (green) and Nso190 (red) (b) Nso190 (green) and Nsm156 (red) (c) EUB338(II) (green) and Ntspa662 (red) (d) EUB338(II) (green) and Nit3 (red), by fluorescence image analysis. Overlapping labels are visualized in yellow.

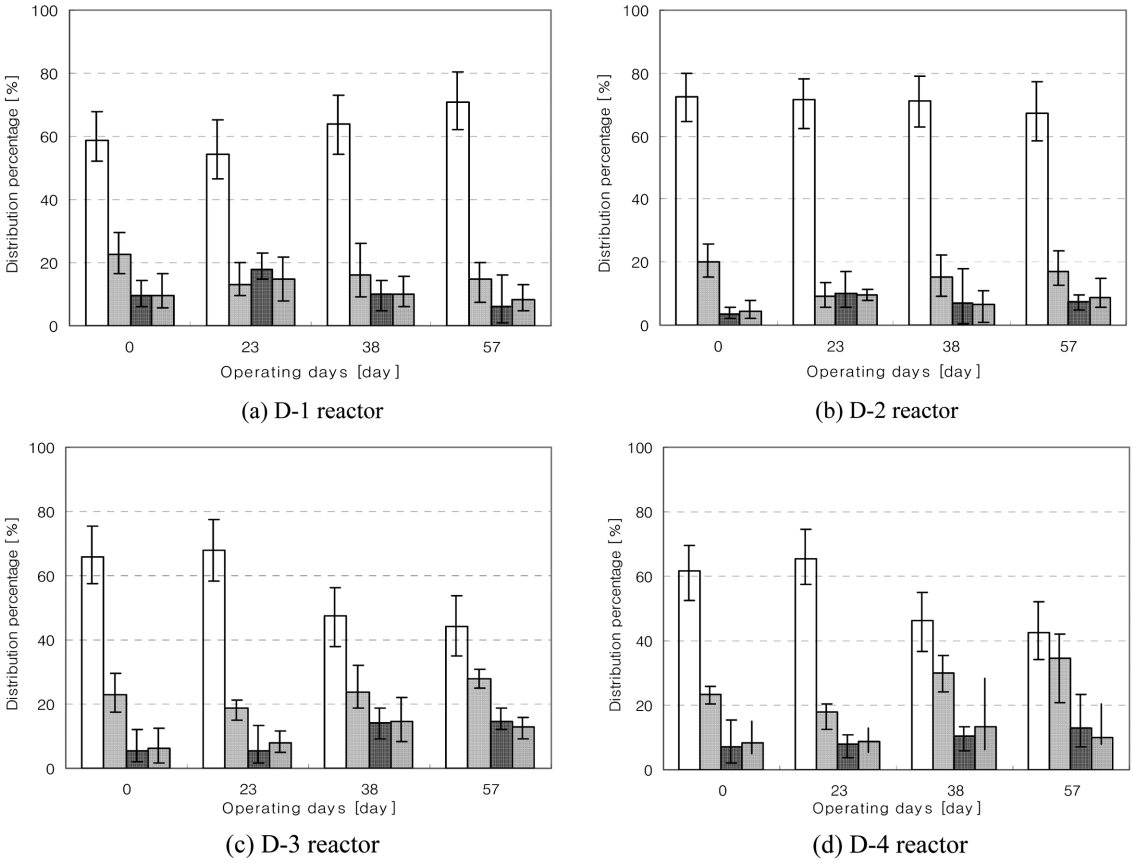

Fig. 4. Distribution of heterotrophs (□), AOB (▨), *Nitrospira* spp. (▥), *Nitrobacter* spp. (▧) in each reactor.

nomena appeared in this study. Considering that nitrification needs higher oxygen concentration than COD removal, the optimal DO concentration of aerated biofilm reactor for stable nitrification was above 5.0 mg/L.

2. Distribution of Microorganisms Using FISH

To investigate the distribution ratios of microorganisms, the FISH method was performed by the combination of two probes. Fig. 3 shows the digital images of aggregates. And Fig. 4 shows the bacterial distribution ratios of each reactor with different DO conditions by using the FISH method.

The distribution ratios of AOB (probe Nso190), *Nitrospira* genus (probe Ntspa662) and *Nitrobactor* spp. (probe Nit3) were displayed by the relative distribution of eubacteria, and that of heterotrophs was calculated with eliminating those ratios.

As shown in Fig. 4, the initial distribution ratios of heterotrophs of each reactor were 58.7, 72.3, 65.7 and 61.6%, respectively. After 57 days, only the distributions of D-1 reactor increased to 71.0%, while D-3 and D-4 were decreased to 44.3 and 42.6%, respectively. In considering that the distribution ratios of the AOB, D-1 and D-2 were decreased to 14.6 and 16.9% in comparison with the initial ratios of 22.5 and 20.1%, while those of D-3 and D-4 increased to 28.0 and 34.6% in comparison with the initial ratios of 22.9 and 23.2%, respectively. To investigate the composition of AOB, probe Nsv443 and probe Nsm156 were used. Initally, the ratios of *Nitrosomonas* spp. of the AOB were 90.9, 87.3, 87.5 and 88.5%, respectively. As time goes on, those of D-1 and D-2 increased to 96.2 and 97.2%, while those of D-3 and D-4 showed 57.8 and 39.0%, respectively. This result means that *Nitrosospira* spp. of the AOB hold a dominant position in higher DO concentration.

In case of NOB consisting of *Nitrospira* genus and *Nitrobacter* spp. with probe Ntspa662 and probe Nit3, as proceeded, higher DO condition led to higher distribution ratio. But higher DO concentration did not lead to dominant position between them because their composition showed similar ratios during the operating days.

3. INT-Dehydrogenase Activity

Fig. 5 shows the relationship between mean INT-DHA activity and mean concentration of attached biomass in each reactor. INT-DHA activity and attached biomass were measured once a week after 60 days. The mean attached biomass of D-1, D-2, D-3 and D-4 was 257, 142, 112 and 137 mg/L, respectively. In consideration of the relationship between the attached biomass and nitrification efficiency, only active biomass concerned the nitrification efficiency. Same results were reported by other researchers [Imat et al., 1993; Lazarova et al., 1994; Liu et al., 1996].

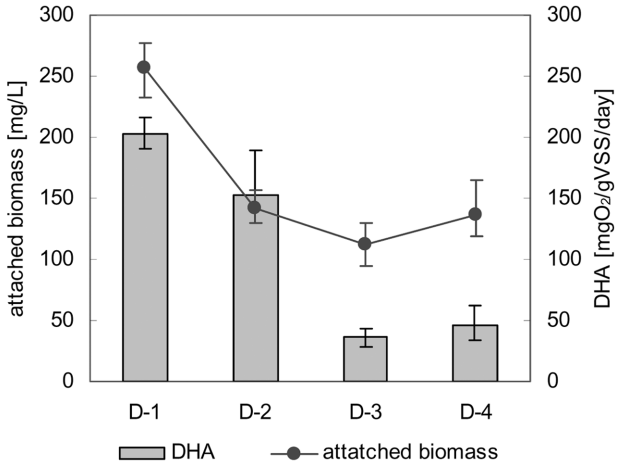

Fig. 5. Relationship between DHA and attached biomass in each reactor with different DO conditions.

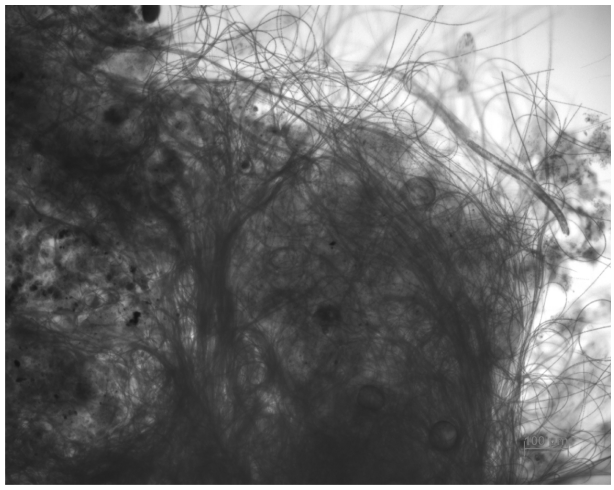

Fig. 6. Optical microscope image of type 021N (*Eikelboom*) in D-1 reactor.

But INT-DHA activities of each reactor were 203, 153, 36 and 45 mgO_2^*/g VSS/day, respectively. The INT-DHA activities in D-1 and D-2 reactors were higher than those of D-3 and D-4 reactors, which indicates that filamentous microorganisms affected the INT-DHA activity. Shown in Fig. 6, filamentous microorganisms overgrew in D-1 and D-2 reactors. It was reported that INT-DHA activity was useful to measure filamentous microorganisms for preventing sludge bulking [Koopman et al., 1984; Awong et al., 1985; Kim et al., 1994]. In cases of D-3 and D-4 reactors, as nitrification efficiency increased, INT-DHA acivity also increased.

CONCLUSIONS

Recently, the concerns surrounding the biofilm process have been increasing because of its high nitrification efficiency and the convenience of operation. So, a greater understanding of biofilm process is needed. To assess the effect of DO concentration in an aerobic biofilm reactor, a combination of FISH and INT-DHA tests was used in this study.

First results were obtained with operating the aerobic biofilm reactors under different DO conditions. The optimal DO concentration with stable nitrification efficiency was above 5.0 mg/L. For the FISH method, higher DO concentration led to an increase of AOB and NOB ratios and the decrease of heterotrophs ratio. The INT-DHA activities of D-1 and D-2 reactors were higher than those of D-3 and D-4 reactors, which indicates that filamentous microorganisms affected the INT-DHA activity. For the D-3 and D-4 reactors, as nitrification efficiency increased, INT-DHA acivity also increased. Lower DO concentration induced the overgrowth of filamentous microorganism, such as type 021 N. In consideration of the relationship between the attached biomass and nitrification efficiency, only active biomass influenced the nitrification efficiency.

Such fundamental knowledge will be useful for design of aerobic biofilm reactors and will help us to understand the nitrification in aerobic biofilm reactors.

ACKNOWLEDGMENT

This work was supported by grant No. R01-2003-000-11640-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

REFERENCES

Awong, J., Bitton, G. and Koopman, B., "ATP oxygen uptake rate and INT-dehydrogenase activity of actinomycete foams," *Wat. Res*., **19**, 917 (1985).

Bitton, G. and Koopman, B., "Tetrazolium reduction-malachite green method for assessing the viability of filamentous bacteria in activated sludge," *Appl. Environ. Microviol.*, **43**, 964 (1982).

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H. and Wagner, M., "The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set," *System Appl. Microbiol.*, **22**, 434 (1999).

Daims, H., Nielsen, P. H., Nielsen, J. L., Juretschko, S. and Wagner, M., "Novel Nitrospira-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology," *Wat. Sci. Tech*., **41**(4/5), 85 (2000).

Imat, T., Kusuda, T. and Furumat, H., *Kinetic study and mathematical modeling of biofilm in an anaerobic fluidized bed*, In Proc. 2nd Int. Specialized Conf. on Biofilm Reactors, Paris, France, 463-470 (1993).

Jang, A., Bishop, P. L., Okabe, S., Lee, S. G. and Kim, I. S., "Effect of dissolved oxygen concentration on the biofilm and *in situ* analysis by fluorescence in situ hybridization (FISH) and microelectrodes," *Wat. Sci. Tech*., **47**(1), 49 (2002).

Kim, C. W., Koopman, B. and Bitton, G., "INT-dehydrogenase activity test for assessing chloine and hydrogen peroxide inhibition of filamentous pure cultures and activated sludge," *Wat. Res*., **28**(5), 1117 (1994).

Kim, D.-J., Ahn, D.-H. and Lee, D.-I., "Effects of free ammonia and dissolved oxygen on nitrification and nitrite accumulation in a biofilm airlift reactor," *Korean J. Chem. Eng*., **22**, 85 (2005).

Koopman, B., Bitton, G., Logue, C., Bossart, J. M. and Lopez, J. M., *Validity of tetrazolium reduction assays for assessing toxic inhibition of filamentous bacteria in activated sludge*, In Toxicity Screening Procedures using Bacterial Systems, 147-162, NY, USA (1984).

Lazarova, N. R., Manem, J. and Melo, L., "Influence of dissolved oxygen on nitrification kinetics in a circulating bed reactor," *Wat. Sci. Tech*., **37**(4), 189 (1998).

Lazarova, V., Pierzo, V., Fontviell, D. and Manem, J., "Integrated approach for biofilm characterization and biomass activity," *Wat. Sci. Tech*., **29**, 345 (1994).

Liu, Y. and Capdeville, B., "Specific activity of nitrifying biofilm in water nitrification process," *Wat. Res*., **30**(7), 1645 (1996).

Mobarry, B. K., Wagner, M., Urbain, V., Rittmann, E. and Stahl, D. A., "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria," *Appl. Environ. Microbiol*., **62**, 2156 (1996).

Park, T. J., Lee, K. H., Kim, D. S. and Kim, C. W., "Petrochemical wastewater treatment with aerated submerged fixed-film reactor (ASFFR) under high organic loading rate," *Wat. Sci. Tech*., **34**(10), 9 (1996).

Park, T. J., Lee, K. H. and Lee, J. H., "Simultaneous organic and nutrient removal from municipal wastewater by BSACNR process," *Korean J. Chem. Eng*., **15**, 9 (1998).

Schramm, A., De Beer, D., Wagner, M. and Amann, R., "Identification and activities *in situ* of *Nitrosospira* and *Nitrospira* spp. as dominant population in a nitrifying fluidized bed reactor," *Appl. Environ. Microbiol*., **64**(9), 3480 (1998).

Sharrma, B. and Ahlert, R. C., "Nitrification and nitrogen removal," *Wat. Res*., **11**, 897 (1977).

Surmacz-Gorska, J., Cichon, A. and Miksch, K., "Nitrogen removal from wastewater with high ammonia nitrogen concentration via shorter nitrification and denitrification," *Proceedings of the Env. Biotech*., **1**, 78 (1996).

Wagner, M., Rath, G., Koops, H.-P., Flood, J. and Amann, R., "*In situ* analysis of nitrifying bacteria in sewage treatment plants," *Wat. Sci. Tech*., **34**(1/2), 237 (1996).

Wang, B., Yang, O., Liu, R., Yuan, J., Ma, F., He, J. and Li, G., "A study of simultaneous organics and nitrogen removal by expended aeration submerged biofilm process," *Wat. Sci. Tech*., **24**(5), 197 (1991).

Yang, H., Jiang, Z., Shi, S. and Tang, W. Z., "INT-dehydrogenase activity test for assessing anaerobic biodegradability of organic compounds," *Ecotoxicology and Environmental Safety*, **53**, 416 (2002).

Zhang, T. C. and Bishop, P. L., "Evaluation of substrate and pH effects in a nitrifying biofilm," *Wat. Environ. Res*., **68**(7), 1107 (1996).