(7) Control of Sulfate Reduction under Nitrogen Deficient Condition in a Natural Anaerobic Pond Process for Cultivation of Purple Non-Sulfur Bacteria as Protein Source of a Fishpond

紅色非硫黄細菌を用いた廃水処理・資源化プロセスにおける廃水中の窒素濃度と硫酸濃度の影響

Ryo HONDA*, Kensuke FUKUSHI**, Chart CHIEMCHAISRI*** and Kazuo YAMAMOTO**

本多 了*、福士謙介**、Chart CHIEMCHAISRI***、山本和夫**

ABSTRACT; Influence of nitrogen and sulfate concentrations in wastewater on a purple non-sulfur bacteria (PnSB) pond process were investigated. A laboratory-scale chemostat photobioreactor treating four types of simulated wastewater were tested with different combinations of nitrogen and sulfate concentrations. *Rhodopseudomonas palustris* and sulfate-reducing bacteria were detected by a fluorescent in-situ hybridization (FISH) technique using specific probes, and their population was quantified by pixel counting in fluorescent microscopic observations. As a result, ratios of Rpal686 on EUB338 probe were over 25% throughout the operation period. Average removal of dissolved organic carbon was 94% independent of nitrogen and sulfate concentrations. However, it was found to be difficult to suppress sulfate reduction, even in case that nitrogen-limited wastewater was fed.

KEYWORDS; Fluorescent in-situ hybridization (FISH), purple non-sulfur bacteria, stabilization pond, sulfate reduction, wastewater treatment.

1. INTRODUCTION

A stabilization pond process is popular in various countries, such as USA, tropical Asian countries etc., due to its low capital cost and easy maintenance. The stabilization pond often utilized also as a fishpond. However, a high organic load often drives its oxidation status into anaerobic condition, which might create an odor problem and a treatment failure. Wastewater treatment utilizing purple non-sulfur bacteria (PnSB) have a high efficiency on treatment of wastewater at high organic loadings [1)2)]. An additional benefit of the system is that cell mass of PnSB is reported to be a good alternative of fish feed or supplement because of its richness in proteins and vitamins [3)4)5)]. By introducing PnSB, it might be possible to develop a high-efficiency treatment pond without odorous gas production (**Figure 1**). The proposed system is consisted of two ponds connected in series. In the first pond of this process, it is expected that acidogenic bacteria consume organic matter in wastewater and PnSB grow with their metabolite. Then the following aerobic fishpond is expected to have a high fish yield.

It is known that PnSB are rarely found as mass propagations in nature. Therefore, from the engineering point of view, it is important to clarify the requirements for selective growth of PnSB. Although many researches have investigated on

* Doctoral Student in Department of Urban Engineering, Graduate School of Engineering, The University of Tokyo, Japan (東京大学大学院工学系研究科都市工学専攻博士課程)

** Environmental Science Center, The University of Tokyo, Japan (東京大学環境安全研究センター)

*** Department of Environmental Engineering, Faculty of Engineering, Kasetsart University, Thailand (カセサート大学工学部環境工学科)

application of PnSB to wastewater treatment and single-cell protein production, there have been small amount of reports on mixed culture studies. Sawada *et al.* [6] and Izu *et al.* [7] revealed that microaerobic or anaerobic condition is preferable to keep PnSB predominant under constant light condition. In addition, impacts of timing control of inflow and runoff of wastewater under day and night conditions were investigated in the previous study [9], which concluded that the single-cell production and protein content were higher in case that wastewater was fed in the morning and the treated water was drained in the next morning.

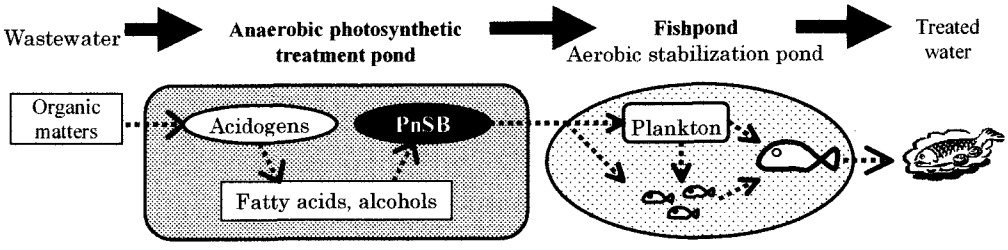

Figure 1. Process flow of photosynthetic pond treatment system

One of the important factors for cultivation of PnSB in mixed culture is to prevent sulfate reduction. Sulfate reduction easily occurs in case sulfate-rich wastewater is treated in anaerobic wastewater treatment processes. It produces toxic hydrogen sulfide as an end product, and it often causes not only an odorous gas but also a treatment failure in anaerobic processes. In a methane fermentation process, it is necessary to give a consideration in design to treat wastewater with COD/SO_4 ratio below 4 [8]. Growth of PnSB is also inhibited with hydrogen sulfide [10]. However, in this PnSB process, it could be possible to treat sulfate-rich wastewater if it is deficient in nitrogen. It is because PnSB can fix nitrogen, while sulfate-reducing bacteria (SRB) don't have that ability. The objective of this study was to investigate influence of nitrogen and sulfate concentration in wastewater in terms of treatment performance, sulfate reduction and growth of PnSB. In this study, four types of simulated wastewater with different combinations of nitrogen and sulfate concentrations was treated in a laboratory-scale chemostat photobioreactor.

2. MATERIALS AND METHODS

2.1. Inoculants

Rhodopseudomonas palustris JCM 2524 was used for the initial inoculant. Pre-cultivation was conducted under illumination of a 60W incandescent lamp (approximately 2000 lux) in a waterbath at 30 °C. A culture medium [11] (per liter) contained 2.0 g of L-malic acid, 2.0 g of sodium glutamate, 2.0 g of yeast extract, 1.0 g of KH_2PO_4, 0.5 g of $NaHCO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$ and 1 mL of vitamin & metal solution for *Rps. rutila*. The vitamin & metal solution for *Rps. rutila* (per liter) contained 2000 mg of $MnSO_4 \cdot 4H_2O$, 1000 mg of thiamine hydrochloride, 1000 mg of nicotinic acid, 500 mg of $FeSO_4 \cdot 7H_2O$, 500 mg of $CoCl_2 \cdot 6H_2O$ and 10 mg of biotin. The pH value of the medium was adjusted to 6.8.

2.2. Operation and monitoring of photobioreactor

Working volume of a photobioreactor was approximately 3 liter, and its diameter and depth were approximately 14 cm and 30 cm, respectively. Each reactor was illuminated by two incandescent lamps (60W) for 12 hours a day. Observed light intensity at the surface of the reactor was 5800 lux. The reactor tanks were covered with infrared transmitting filters in order to prevent algal growth. Heat emitted from the incandescent lamps was cooled by fans. The reactor was a complete mixed type and the hydraulic retention time (equal to sludge retention time) was kept at 6 days. The reactors

were purged with nitrogen gas. Simulated wastewater was fed at the beginning of light condition, and reactor effluent was runoff at the end of dark condition.

Four operations were conducted to treat simulated wastewater with different combinations of nitrogen and sulfate concentrations (**Table 1**); low nitrogen and low sulfate concentrations (Run I), with low nitrogen and high sulfate concentrations (Run II), with high nitrogen and low sulfate concentrations (Run III), with high nitrogen and high sulfate concentrations (Run IV).

Table 1. Glucose, nitrogen and sulfate concentrations in simulated wastewater

Run	Glucose [mgC/L]	NH_4-N [mgN/L]	SO_4^{2-} [mgS/L]	C/N	ThOD[a]/SO_4
Run I	500	26	32	19.2	13.9
Run II	500	26	216	19.2	2.0
Run III	500	183	32	2.7	13.9
Run IV	500	183	216	2.7	2.0

[a] Theoretical oxygen demand for oxidation of glucose.

A glucose medium was used as simulated wastewater, which (per liter) contained 1.25 g (500 mgC/L) of glucose, 0.1 g or 0.7 g of NH_4Cl (depending on operation conditions mentioned above), none or 1.0 g of K_2SO_4, (depending on operation conditions mentioned above) 1.0 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.20 of NaCl, 0.05 g of $CaCl_2 \cdot 2H_2O$, 1 mL of vitamin solution and 1 mL of trace metal solution. The vitamin solution (per liter) contained 500 mg of thiamine hydrochloride, 500 mg of nicotinic acid, 300 mg of p-aminobenzoic acid, 100 mg of pyridoxine hydrochloride, 50 mg of biotin and 50 mg of vitamin B_{12}. The trace metal solution (per liter) contained 1000 mg of EDTA-2Na, 2000 mg of $FeCl_3 \cdot 6H_2O$, 100 mg of $ZnCl_2$, 120 mg of $MnSO_4 \cdot 4H_2O$, 100 mg of H_3BO_3, 100 mg of $CoCl_2 \cdot 6H_2O$, 20 mg of Na_2MoO_4, 10 mg of $CuCl_2 \cdot 2H_2O$, 10 mg of $NiCl_2 \cdot 6H_2O$ and 5 mg of Na_2SeO_3.

Rps. palustris was pre-cultured for two weeks under constant light condition for the purpose of acclimatization to the reactor environment. The simulated wastewater (mentioned above) was also used as a pre-culture medium, though it contained 2.83 g (500 mgC/L) of $CH_3COONa \cdot 3H_2O$ as a carbon source instead of glucose, 0.1 g of NH_4Cl and none of K_2SO_4. After the pre-cultivation, approximately 50 mL of digested sludge (30-50% [sludge mgSS / PnSB mgSS]) from a sludge treatment plant were added to the pure culture reactor as a source of heterotrophic bacteria. Oxidation-reduction potential (ORP), temperature and pH were monitored. pH was automatically adjusted at 7.0 ± 1.0 by pH controllers (SIBATA, Japan) with 1N HCl and 1N NaCl. Bacterial cells attached on the wall of the reactors were detached with magnet equipments once a day.

2.3. Sampling procedures

Samples were taken from the effluent at runoff time every 6-day from day 0 (the first runoff after the sludge were added) to day 42. On day 36, samples were taken from the reactor every 3-hours to investigate fluctuations of water qualities in the reactor throughout a day.

2.4. Runoff water qualities

One mL of sample filtered by a cellulose acetate membrane (pore size: 0.45μm, ADVANTEC) was taken for dissolved organic carbon (DOC) and dissolved nitrogen (DN) analyses by a TOC analyzer (TOC-V, Shimadzu, Japan). TOC and total nitrogen (TN) were analyzed by the TOC analyzer after ultrasonic homogenization. Particulate organic carbon

(POC) was calculated as the balance of DOC and TOC. Particulate nitrogen (PN) was calculated as the balance of DN and TN. Crude protein concentrations were calculated by multiplying PN concentrations by 6.25. Dissolved sulfide ions (HS^-, S^{2-}) concentrations were analyzed by a sulfide test kit (Dr. Bruno Lange GmbH, Denmark). Dissolved free hydrogen sulfide concentrations were determined from an equilibrium state with dissolved sulfide ions and pH.

2.5. Fluorescent in-situ hybridization (FISH) and quantification by image analysis

Four oligonucleotide probes were used: Rpal686 [7], which detected *Rhodopseudomonas palustris* specifically; SRB385 [12], which detected members of *Desulfovibrionaceae* (incomplete oxidizers); SRB385Db [13], which detected members of *Desulfobacteriaceae* (mostly complete oxidizers); and EUB338 [14], which detected most of eubacteria. Probe Rpal686 had the sequence of 5'-CTCACCTCTGCCATACTC-3' (complementary to 16S rRNA positions 703 to 686 [Escherichia coli numbering]) and labeled with X-rhodamine isothiocyanate (XRITC). Probe SRB385 had the sequence of 5'-CGGCGTCGCTGCGTCAGG-3' (complementary to positions 402 to 385 [E. coli numbering]) and labeled with FITC. Probe SRB385Db had the sequence of 5'-CGGCGTTGCTGCGTCAGG -3' (complementary to the same positions as those of probe SRB385) and labeled with XRITC. Probe EUB338 had the sequence of 5'-GCTGCCTCCCGTAG GAGT-3' (complementary to positions 355 to 338 [E.coli numbering]) and labeled with Cy5.

A sample at the volume of 1.8 mL was taken for FISH analysis. The sample was immediately frozen in gas phase of liquid nitrogen and stored at –80 °C. Suspensions of the samples were fixed with 3% paraformaldehyde (PFA) for 2 hours and stored in a 1:1 mixture of 1xPBS and 99% ethanol at –20 °C, as described by Amann [15].

In order to quantify population ratio of *Rps. palustris* against total bacteria, probes Rpal686 and EUB338 were simultaneously used as described by Snaidr *et al.* [16]. In situ hybridization of these probes were performed as described by Amann [15]. The percent formamide in a hybridization buffer was 20%, and a sodium chloride concentration was 0.9 M. In order to quantify population ratio of sulfate reducing bacteria against total bacteria, probes SRB385, SRB385Db and EUB338 were used simultaneously. In situ hybridization of these probes were performed as described by Rabus *et al.* [13]. The percent formamide in a hybridization buffer was 30%, and a sodium chloride concentration was 1.8 mM.

The slides were observed with a confocal laser microscope (TCS-NT, Leica Microsystems, Hidelberg, Germany) equipped with Ar/Kr laser. Images were taken at 630-fold magnification. Ten microscopic fields per sample were randomly chosen when images were taken. The images were saved in TIFF format. Fluorescent areas in the images were quantified with Quantimet 600HR image analyzing system (Leica Microsystems) by pixel counting. Total fluorescent areas were calculated in a threshold by which signal from probe fluorescence and from mechanical noise were clearly separated. Ratio of fluorescent area of probes on that of EUB 338 was calculated for each image. Subsequently the mean and 95%-confidence interval (CI) for 10 images were respectively calculated.

3. RESULTS AND DISCUSSION

3.1. Sulfate reduction

Sulfate reduction occurred in all the runs (**Figure 2**). The maximum concentration was observed at 3 hours after feeding in all the runs. In case that sulfate-limited wastewater was fed (I and III), the total sulfide concentration decreased to less than 1 mgS/L at 6 hours after feeding. However, in case that sulfate-rich wastewater was fed (II and IV), the total sulfide concentration was kept at larger than 2

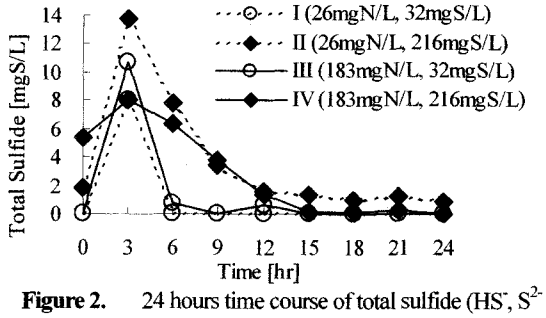

Figure 2. 24 hours time course of total sulfide (HS^-, S^{2-} and dissolved free H_2S) concentration on day 36.

mgS/L until 12 hours after feeding of wastewater. Nitrogen concentrations in wastewater did not result in significant difference in tendencies of sulfide concentrations.

It is considered that C/N ratio of simulated wastewater was sufficient for growth of sulfate reducing bacteria, even in case that the nitrogen concentration was low. Growth yields of sulfate reducing bacteria are reported to be 0.023-0.049 mgVSS/mgCOD [8]. The necessary C/N ratio in wastewater for their growth is 90-130 by calculating based on three assumptions: that they oxidize glucose completely; that C/N ratio of their cells is 4; and that VSS/carbon ratio of their cells is 2.

As we consider that nitrogen is supplied from dead cells as well, it should be quite difficult to suppress growth of sulfate reducing bacteria completely in the presence of sulfate. Therefore, control of sulfide concentrations is important in order to prevent inhibition of PnSB growth. Hansen [10] reported that *Rhodobacter sphaeroides* is able to grow in the presence of total sulfide at 12.8 mgS/L. In this study, growth of PnSB was not inhibited in all the runs (as mentioned below). It is considered that sulfide concentrations were not high enough to inhibit growth of *Rps. palustris*.

The most appropriate approach for sulfide control in a PnSB pond process should be ORP control by aeration. The possible ways of sulfide control are precipitation with metals, such as iron or zinc, and oxidation by aeration or oxidizers. In these approaches, aeration is the most preferable in terms of biomass production in a pond process, because most of the oxidizing reagents are biologically harmful and precipitation treatment requires dredging. In order to oxidize sulfide in anaerobic condition, controlling of ORP is important. Khanal *et al.* [17] reported that ORP control by aeration was effective to control sulfide and to regulate oxygen dosing in an upflow anaerobic filter (UAF) process. It is expected to be effective in a PnSB process as well.

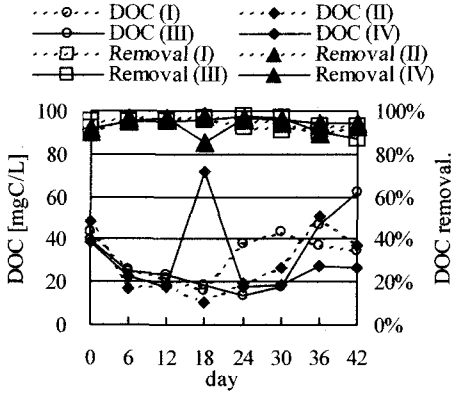

Figure 3. DOC concentration and DOC removal %

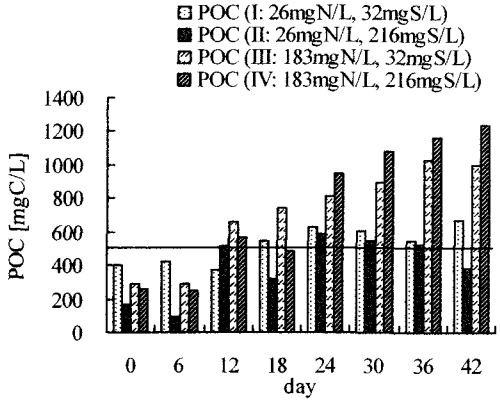

Figure 4. POC concentration in runoff water. TOC of simulated wastewater was 500 mgC/L.

3.2. Treatment performance

Averages of DOC removal ratios during the operation period were 94% in all the runs (**Table 2**). DOC concentrations of runoff water were below 50 mgC/L throughout the operation period except day 42 of Run I, day 36 of Run II and day 18 of Run IV (**Figure 3**). In general, DOC removal ratio of an oxidation pond is 80-95% in an aerobic pond, and 50-85% in an anaerobic pond [18]. Treatment performance was sufficient independent from nitrogen and sulfate concentrations of wastewater.

Table 2. Runoff water qualities (averages during the operation period)

Run		DOC [mgC/L]	DOC removal % (RSD*)	POC [mgC/L]	Crude protein [mg/L]	Crude protein / MLSS
Run I	(26 mgN/L, 32 mgS/L)	32.6	94% (0.02)	525	620	0.59
Run II	(26 mgN/L, 216 mgS/L)	28.0	94% (0.03)	390	464	0.63
Run III	(183 mgN/L, 32 mgS/L)	30.8	94% (0.03)	714	859	0.57
Run IV	(183 mgN/L, 216 mgS/L)	30.2	94% (0.04)	747	879	0.56

*) Relative standard deviation throughout the operation period.

3.3. Single-cell protein production

Crude protein contents were more than 45% throughout the operation period in all the runs. There was no significant difference depending on nitrogen and sulfate concentrations in wastewater. These values of protein contents were comparable to those of past studies on cultivation of PnSB as a fish feed. In those studies, the crude protein contents of pure-cultured *Rps. palustris* strains were reported to be 40% [4] and 72-74% [19]. POC concentrations had exceeded TOC of simulated wastewater, 500 mgC/L, after day 24 except day 42 of Run II (**Figure 4**). Neither microalgae nor purple sulfur bacteria, which are possibly existing autotrophic bacteria in this reactor condition, was found in microscopic observation. The most possible reason is improper sludge drainage, because detaching PnSB cells on the wall was conducted after draining.

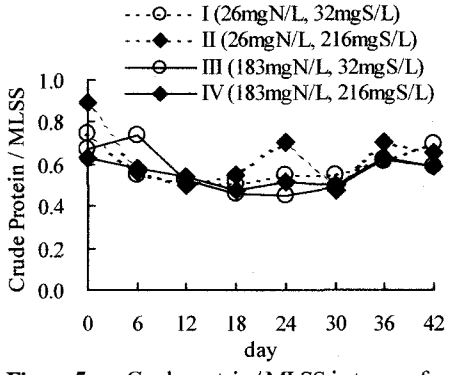

Figure 5. Crude protein / MLSS in terms of protein contents

3.4. Bacterial community

Rpal686/EUB338 ratios were over 0.25 throughout the operation period in all the runs (**Figure 6**). Averages of Rpal686/EUB338 ratio were 0.36-0.44 (**Table 3**). Although these ratios are lower than those of Izu *et al.*[7], which is over 60% as ratio of *Rb. sphaeroides*, it is still comparable to the previous study [9]. *Rps. palustris* is considered to have played an important role in these bacterial communities. It shows that sulfide concentrations were not large enough to inhibit growth of *Rps. palustris* (as mentioned above).

Averages of SRB385/EUB338 and SRB385Db/EUB338 ratios were less than 0.1. Average MLSS corresponding to SRB385/EUB338 and SRB385Db/EUB338 ratios was 50-94 mg/L (**Table 4**). The reason why ratios of sulfate reducing bacteria were much lower than PnSB is related to their growth yield. Growth yields of sulfate reducing bacteria on carbon are calculated as 0.061-0.088 mgVSS/mgC if they are assumed to oxidize glucose completely. Based on these

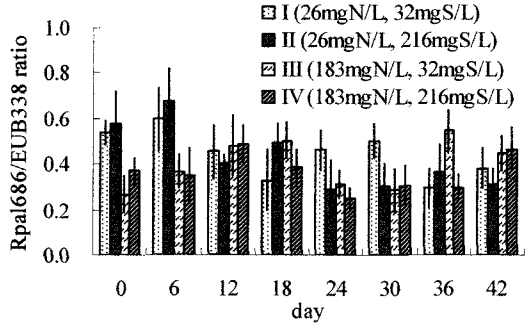

Figure 6. Ratios of detected areas by probe Rpal686 per area by probe EUB338. Vertical lines on the bars stand for 95%-CI.

yields, the maximum VSS of sulfate reducing bacteria in this study is 44 mgVSS/L, because TOC concentration of simulated wastewater was 500 mgC/L.

However, this also means that not a small amount of sulfide might be produced even if sulfate reducing bacteria do not appears to be dominant. Their growth yields on sulfate is calculated to be 0.015-0.033 mgVSS/mgSO_4 by using theoretical COD/SO_4 ratio of sulfate reduction, 0.67. Based on these yields, 10-20 mgS of sulfide is produced by 1 mgVSS growth of sulfate reducing bacteria. This result shows that it is quite important to control sulfide concentrations if growth of sulfate reducing bacteria is difficult to suppress.

Table 3. Average ratios of fluorescent areas of probes Rpal686, SRB385 and SRB385Db on EUB338 during the operation period. The values in parentheses show 95%-confidence interval.

Run		Rpal686	SRB385	SRB385Db
Run I	(26 mgN/L, 32 mgS/L)	0.44 (±0.10)	0.04 (±0.01)	0.05 (±0.02)
Run II	(26 mgN/L, 216 mgS/L)	0.43 (±0.10)	0.08 (±0.05)	0.05 (±0.03)
Run III	(183 mgN/L, 32 mgS/L)	0.40 (±0.09)	0.02 (±0.01)	0.02 (±0.01)
Run IV	(183 mgN/L, 216 mgS/L)	0.36 (±0.08)	0.02 (±0.01)	0.01 (±0.01)

Table 4. Averages of MLSS concentrations corresponding to ratios of Rpal686, SRB385 and SRB385Db on EUB338 during the operation period. The values in parentheses show 95%-confidence interval.

Run		Rpal686	Sulfate reducing bacteria		
			SRB385	SRB385Db	SRB385+SRB385Db
Run I	(26 mgN/L, 32 mgS/L)	453 (±101)	38 (±12)	49 (±24)	87 (±36)
Run II	(26 mgN/L, 216 mgS/L)	287 (±72)	59 (±33)	35 (±26)	94 (±59)
Run III	(183 mgN/L, 32 mgS/L)	653 (±136)	36 (±18)	40 (±28)	76 (±46)
Run IV	(183 mgN/L, 216 mgS/L)	560 (±120)	29 (±21)	21 (±22)	50 (±43)

4. CONCLUSIONS

It was found to be difficult to suppress sulfate reduction even in nitrogen deficient condition. Therefore, sulfide concentration control is important in order to prevent inhibition of PnSB growth. 94% of DOC removal was achieved independent of nitrogen and sulfate concentrations in wastewater. Protein contents were sufficient in comparison with the previous studies. In this study, average ratio of *Rps. palustris* was 36-44% since sulfide concentration did not reached high enough to inhibit growth of PnSB.

At present, a pilot-scale plant of a PnSB pond process is operated in Thailand. Control of algae will be tested in the pilot-scale reactor by using infrared transmitting filters.

ACKNOWLEDGEMENT

Authors acknowledge the Steel Industry Foundation for the Advancement of Environment Protection Technology for their financial assistance to conduct this research.

REFERENCES

1) Kobayashi M., Kobayashi M. and Nakanishi H. (1971). Construction of a purification plant for polluted water using photosynthetic bacteria. *J. Ferment. Technol.*, **49**(9), 817-825.

2) Hiraishi A., Shi J.L. and Kitamura H. (1989). Effects of organic nutrient strength on the purple nonsulfur bacterial content and metabolic activity of photosynthetic sludge for wastewater treatment. *J. Ferment. Bioeng.*, **68**(4), 269-276.
3) Kobayashi M. and Tchan Y.T. (1973). Treatment of industrial waste solutions and production of useful by-products using a photosynthetic bacterial method. *Wat. Res.*, 7(8), 1219-1224.
4) Getha K., Chong V.C. and Vikineswary S. (1998). Potential use of the phototrophic bacteria, *Rhodopseudomonas palustris* as an aquaculture feed. *Asian Fish. Sci.*, **10**, 223-232.
5) Banerjee S., Azad S.A., Vikineswary S., Selvaraj O.S. and Mukherjee T.K. (2000). Phototrophic bacteria as fish feed supplement. *Asian-Aus. J. Anim. Sci.*, **13**(7), 991-994.
6) Sawada H. and Rogers P.L. (1977). Photosynthetic bacteria in waste treatment - mixed culture studies with *Rhodopseudomonas capsulata* -. *J. Ferment. Technol.*, **55**(4), 311-325.
7) Izu K., Nakajima F., Yamamoto K. and Kurisu F. (2001). Aeration conditions affecting growth of purple nonsulfur bacteria in organic wastewater treatment process. *Syst. Appl. Microbiol.*, **24**, 294-302.
8) Speece R.E. (1996). Anaerobic Biotechnology for Industrial Wastewaters, Archae Press, Tennessee, USA.
9) Honda R., Fukushi K. and Yamamoto K. (2003), Optimization of wastewater feeding into a natural anaerobic pond process for cultivation of purple non-sulfur bacteria as protein source of a following fishpond, *Proceedings of IWA Asia-Pacific Regional Conference,* Bangkok, p.151.
10) Hansen T.A. and Van Gemerden H. (1972). Sulfide utilization by purple nonsulfur bacteria. *Arch. Microbiol.*, **86**, 49-56.
11) Hiraishi A., Santos T.S., Sugiyama J. and Komagata K. (1992). *Rhodopseudomonas rutila* is a later subjective synonym of *Rhodopseudomonas palustris*. *Int. J. Syst. Bacteriol.*, **42**(1), 186-188.
12) Amann, R.I., Binder B.J., Olson R.J., Chisholm S.W., Devereux R. and Stahl D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol,*. **56**, 1919 1925.
13) Rabus R., Fukui M., Wilkes H. and Widdel F. (1996). Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. *Appl. Environ. Microbiol.*, **62**(10), 3605-3613.
14) Amann R., Krumholz L. and Stahl D.A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.*, **172**, 762-770.
15) Amann R.I. (1995). In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Molecular Microbial Ecology Manual 3.3.6, Kluwer Academic, Dordrecht, pp. 1–15.
16) Snaidr J., Amann R., Huber I., Ludwig W. and Schlifer K.H. (1997). Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Appl. Environ. Microbiol.*, **63**, 2884-2896.
17) Khanal S.K. and Huang J.-H. (2003). Anaerobic Treatment of High Sulfate Wastewater with Oxygenation to Control Sulfide Toxicity. *J. Environ. Eng.*, **129**(12), 1104-1111.
18) Tchobanoglous G. and Burton F.L. (1993). 水質環境工学：下水の処理・処分・再利用（松尾友矩、大垣眞一郎、浅野孝、宗宮功、丹保憲仁、村上健 監訳）、技報堂出版 、p.474.
19) Kim J.K. and Lee B.K. (2000). Mass production of *Rhodopseudomonas palustris* as diet for aquaculture. *Aquacult. Eng.*, **23**, 281-293.