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Studies on the Control Index of Activated Sludge. VII.¹⁾ Relation between Bacteria Flora and Dimethyl Disulfide Formation in Activated Sludge

BANICHI TOMITA,^a NORIKATSU HAMAMURA,^a AKIRA NAKAMURA,^a

MASUO YAMADA,^a HIROMASA INOUE^a and YOUKI OSE^b

Aichi Prefectural Institute of Public Health,^a 7-6, Nagare, Tsuji-machi, Kita-ku, Nagoya

462, Japan and Department of Environmental Hygiene, Gifu Pharmaceutical

University,^b 5-6-1, Mitahorahigashi, Gifu 502, Japan

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Dimethyl disulfide (DMDS) formation by bacteria isolated from normal or abnormal activated sludges was examined. Bacteria with high DMDS-forming ability could not be isolated from normal activated sludge. However, when the activated sludge was abnormally conditioned at high biochemical oxygen demand (BOD) loading, bacteria capable of forming DMDS from methionine were isolated.

Keywords—activated sludge; abnormal condition; control index; dimethyl disulfide; BOD loading; formation ability; isolated bacteria; methionine

Introduction

The activated sludge process is one of the most widely used methods for wastewater treatment. However, correct operation and maintenance are essential to obtain good effluent quality. Effluent biochemical oxygen demand (BOD) and sludge volume index (SVI) are commonly used as parameters for evaluation of the state of the activated sludge process, but with these indices, it is difficult to evaluate changes accurately at an early stage. We have proposed that the pattern of nitrite nitrogen content in anaerobically treated activated sludge (NO₂-N pattern)²⁾ and the formation of dimethyl disulfide (DMDS)³⁾ might be useful new indices, and we showed that DMDS can be formed from methionine by activated sludge.⁴⁾

The formation of DMDS by several organisms (stock strains or isolates from soil, wastewater, fish and so on) has been reported.⁵⁾ However, DMDS-forming bacteria have not been isolated from activated sludge, and the relation between these bacteria and the con-

dition of activated sludge has never been investigated.

In the previous paper,¹⁾ we suggested that when activated sludge was abnormally conditioned by high BOD loading, the ability of activated sludge to form DMDS from methionine increased. In this study, in order to clarify whether the content of DMDS-forming bacteria is increased in activated sludge abnormally conditioned by increasing the BOD loading, we isolated bacteria from normal and abnormal activated sludges, and tested them for DMDS-forming ability.

Experimental

Culture of Activated Sludge—Activated sludge was obtained from the aeration tank of Toyoake Domestic Sewage Treatment Plant in Aichi Prefecture, and acclimated to synthetic wastewater for 2–3 years in a batch installation. The synthetic wastewater was prepared as follows;³⁾ glucose 1.50 g, peptone 2.25 g, Na₂HPO₄·12H₂O 0.125 g, KCl 0.100 g and MgCl₂·6H₂O 0.126 g were dissolved in distilled water and made up to 1 l. The pH was

Fig. 1. Experimental Apparatus for Activated Sludge Treatment

adjusted to 7.3 with 20% NaOH. The BOD of this wastewater was 2730 mg/l, and total-N was 308 mg/l. In order to change the BOD loadings, concentrations of components in synthetic wastewater were varied.

Experimental apparatus for the activated sludge treatment is shown in Fig. 1. The temperature of the mixed liquor in the aeration tank was kept at 20–25°C.

In order to obtain normal and abnormal activated sludges, activated sludge was cultured as shown in Fig. 2. For abnormal activated sludge, the BOD loading was increased (experiments II and III).

Experiments I and II were performed at the same time in separate apparatus. Before the experiment, activated sludge was acclimated for two weeks with a load of 0.51 kg-BOD/m³·d as a normal BOD loading. For experiment I, BOD loading was set at 0.51 kg-BOD/m³·d throughout the experimental period to obtain normal activated sludge. For experiment II, the BOD loading was increased to 5.1 kg-BOD/m³·d as a high BOD loading to obtain abnormal activated sludge after the 16 th d.

Experiment III was performed separately. Before

Fig. 2. Time-Course of Volumetric BOD Loading

experiment III, activated sludge was acclimated for two weeks on a load of 0.59 kg-BOD/m³·d. To obtain normal activated sludge, the activated sludge was cultured at the load of 0.59 kg-BOD/m³·d (normal BOD loading) up to 15 th d. After the 16 th d, to obtain abnormal activated sludge, the BOD loading was increased to 5.9 kg-BOD/m³·d (high BOD loading).

In this paper, activated sludge on the 32 nd d

in experiment I is designated as I-N, that on the 32 nd d in experiment II as II-Ab, that on the 15 th d in experiment III as III-N, and that on the 28 th d in experiment III as III-Ab.

Sampling—Mixed liquor from the aeration tank was sampled at the times (d) shown in Fig. 2 during the experiment, and the pH, oxidation-reduction potential (ORP), DO, SVI, mixed liquor suspended solids (MLSS), SVI, $\text{NO}_2\text{-N}$ pattern^{2a)} and DMDS-forming ability¹⁾ were measured. A part of the mixed liquor from the aeration tank was filtered through Toyo filter paper No. 5A; this filtrate (designated as MLF) was used for the measurement of nitrification rate, dissolved BOD, dissolved COD, absorbance at 600 nm, DMDS content³⁾ and free methionine. Nitrification rate was calculated by using the following equation;⁶⁾

Nitrification rate (%)

$$= \frac{(\text{NO}_2\text{-N (mg/l)} + \text{NO}_3\text{-N (mg/l)}) \times 100}{\text{NH}_3\text{-N (mg/l)} + \text{NO}_2\text{-N (mg/l)} + \text{NO}_3\text{-N (mg/l)}}$$

For bacterial studies, four kinds of mixed liquor (I-N, II-Ab, III-N and III-Ab) were used.

Determination Methods—(i) pH, ORP, DO, $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, Dissolved BOD, Dissolved COD, MLSS, SVI and Absorbance at 600 nm: pH, DO, $\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, dissolved BOD, dissolved COD, MLSS, SVI were measured according to the previous papers.^{1, 2a)} ORP was measured with a Central Kagaku UC-2 ORP meter. Absorbance at 600 nm was measured with a Hitachi 102 spectrophotometer in a 10 mm length cell.

(ii) DMDS Content in MLF³⁾: A 20 ml aliquot of MLF was taken into a 40 ml test tube, and 6.0 g of NaCl was added. The tube was plugged with a double rubber stopper covered with a Teflon sheet, and the DMDS content was measured by the head-space gas chromatographic method.

(iii) DMDS-Forming Ability of Mixed Liquor¹⁾: A 100 ml aliquot of mixed liquor was taken into a 350 ml flask, then 2.0 mg of L-methionine for experiment I or II, or DL-methionine for experiment III was added. The flask was plugged with a double rubber stopper covered with a Teflon sheet. The mixture was stirred for 20 min, then DMDS

was measured.

(iv) $\text{NO}_2\text{-N}$ Pattern^{2a)}: Mixed liquor from the aeration tank was taken into four test tubes, and plugged to exclude air. After shaking for 0, 1, 3 and 5 h in a Monodo's shaker at 20°C, the mixed liquor was filtered and the $\text{NO}_2\text{-N}$ content was measured. Four kinds of $\text{NO}_2\text{-N}$ pattern were found and termed types A, B, C and D.^{2a)} In type A, the $\text{NO}_2\text{-N}$ content increased and the $\text{NO}_2\text{-N}$ content after 5 h was higher than the initial value. In type B, $\text{NO}_2\text{-N}$ content increased up to 1 or 3 h, but then decreased, and after 5 h it was lower than the initial value. In type C, $\text{NO}_2\text{-N}$ content decreased gradually and continuously. In type D, $\text{NO}_2\text{-N}$ was not detected for 5 h. When the condition of activated sludge became abnormal, the $\text{NO}_2\text{-N}$ pattern changed successively in order of type A, type B, type C and type D.

(v) Free Methionine in MLF: Changes of free methionine content in the mixed liquor were investigated in experiment II. A 200 ml aliquot of MLF was treated with sulfosalicylic acid for removal of protein and concentrated to 5.0 ml under a vacuum at 40°C in a rotary-evaporator. Free methionine was determined with a Hitachi 835 amino acid analyzer.

(vi) Bacterial Counting and Isolation: For bacterial counting and isolation, activated sludges I-N, II-Ab, III-N and III-Ab were used. Each activated sludge (50 ml) was homogenized for 10 min in a homogenizer (Sakuma, Homo-blender 500). Serial dilution was done for the determination of bacterial counting with synthetic wastewater agar. Bacterial colonies were counted every day during a 7-day incubation period at 20°C. A random sampling method⁷⁾ yielded 38 and 43 bacterial strains from sludges I-N and II-Ab, respectively. Many of them were isolated from colonies formed during 1–3 d of incubation, and some (strains I-N-34–38, II-Ab-39–43) were isolated after 5 d of incubation. Fifty-four and 60 strains were isolated from sludges III-N and III-Ab, respectively, during 1–7 d of incubation.

(vii) DMDS-Forming Ability of Isolated Strains: The 20-fold concentrated synthetic wastewater

was prepared, and 0.01% (w/v) DL-methionine was added. The mixture was sterilized by passing it through a membrane filter (Millipore, Steril-GV, 0.22 μm) and 10.0 ml aliquots were taken into 40 ml test tubes. Each isolated strain was picked up from the slant culture with a platinum wire, and inoculated into a test tube, which was plugged with a double rubber stopper covered with a Teflon sheet. After shaking culture for 48 h at 20°C, 3.0 g of NaCl was added and the DMDS content was determined by the head-space gas chromatographic method.

(viii) Effect of Culture Time on DMDS Formation: In order to clarify the effect of culture time on DMDS formation, strains with high DMDS-forming ability (strains II-Ab-43 and III-Ab-5) and strains with low DMDS-forming ability (strains III-N-1 and III-N-54) were cultured with shaking for 6 d at 20°C, and the changes of DMDS contents were measured daily.

Results and Discussion

1. The Condition of Activated Sludge and Water Quality

In order to obtain normally conditioned

Fig. 3. Time-Courses of pH, ORP, DO and Nitrification Rate
 ↑ : BOD loading increased.

activated sludge and abnormally conditioned activated sludge, the BOD loading was changed as shown in Fig. 2. The time-courses of pH, ORP, DO and nitrification rate are shown in Fig. 3. Those of dissolved BOD, dissolved COD, SVI, DMDS content in MLF and the $\text{NO}_2\text{-N}$ pattern are shown in Fig. 4.

Under the normal BOD loading, ORP, DO, and nitrification rate were stable at high values of +370—+430 mV, 8.0—9.0 mg/l and 75—91 %, respectively. The pH was nearly constant at about 4.5—5, which may be due

to the high nitrification rate.⁶⁾ Dissolved BOD, dissolved COD and SVI were constant at low values of 2.0—13.1 mg/l, 5.0—13.6 mg/l and 44—56, respectively. DMDS was not detected, and the $\text{NO}_2\text{-N}$ pattern was type A. However, under increasing BOD loading, the pH rose to 7.6—7.9, and the ORP, DO and nitrification rate fell. Dissolved BOD, dissolved COD and SVI rose. DMDS was detected and increased gradually, and the $\text{NO}_2\text{-N}$ pattern changed to type C. From the results shown in Figs. 3 and 4, it was confirmed that activated sludges

Fig. 4. Time-Courses of BOD, COD, SVI, DMDS and the $\text{NO}_2\text{-N}$ Pattern

↑ : BOD loading increased, □ : $\text{NO}_2\text{-N}$ pattern.

Fig. 5. Time-Course of DMDS-Forming Ability
 ↑ : BOD loading increased, ○ : 2.0 mg of L-methionine added,
 ● : 2.0 mg of DL-methionine added.

I-N and II-N were "normal," and activated sludges II-Ab and III-Ab were "abnormal."

The time-courses of DMDS-forming ability are shown in Fig. 5.

Under normal BOD loading, the activated sludge was in a normal condition, and the DMDS-forming ability was constant at low values. The values for I-N and III-N were 28.0 $\mu\text{g-DMDS/g-MLSS}$ from L-methionine and 51.6 $\mu\text{g-DMDS/g-MLSS}$ from DL-methionine, respectively. However, under increasing BOD loading, the activated sludge became abnormally conditioned, and the DMDS-forming ability rose gradually. The values for II-Ab and III-Ab were 113 $\mu\text{g-DMDS/g-MLSS}$ from L-methionine and 542 $\mu\text{g-DMDS/g-MLSS}$ from DL-methionine.

In the previous paper,¹⁾ we suggested the possibility of DMDS formation in abnormally conditioned activated sludge under high BOD

Fig. 6. Time-Course of Free Methionine in MLF
 ↑ : BOD loading increased.

loading. In this paper, we confirmed that the DMDS-forming ability is increased in abnormally conditioned activated sludge.

2. Free Methionine in MLF

The time-course of free methionine content in MLF in experiment II is shown in Fig. 6. Free methionine contents were 6–8 $\mu\text{g/l}$ at the 12–16 th d under normal BOD loading, while DMDS was not detected. With increasing BOD loading, free methionine increased to 18–57 $\mu\text{g/l}$ at the 19–27 th d, and DMDS amounted to 1.7–8.5 $\mu\text{g/l}$ at the 22 nd to 27 th d. At the 30 th d when the DMDS value was 64.5 $\mu\text{g/l}$, the free methionine content was as high as 395 $\mu\text{g/l}$, but on the 32 nd d, DMDS content was 24.4 $\mu\text{g/l}$, and free methionine content was 126 $\mu\text{g/l}$.

TABLE I. Comparison of Total Bacterial Counts, MLSS and Absorbance at 600 nm

Experiment	Activated sludge	Condition of activated sludge	Total bacterial counts (n/ml)	MLSS (mg/l)	Absorbance at 600 nm
I and II	I-N	Normal	9.8×10^5	5570	0.005
	II-Ab	Abnormal	8.6×10^8	4740	0.534
III	III-N	Normal	2.6×10^7	4570	0.004
	III-Ab	Abnormal	1.2×10^{10}	5620	0.051

Fig. 7. The DMDS-Forming Ability of Isolated Strains (I)

We have tested DMDS formation from several sulfur-containing amino acids, and have confirmed that methionine is a precursor of DMDS.⁴⁾ In the present experiment, high DMDS contents were detected when the free methionine contents were increased in MLF.

3. Total Bacterial Counts of Activated Sludge

Total bacterial counts, MLSS and absorbance at 600 nm of each activated sludge (I-N, II-Ab, III-N and III-Ab) are summarized in

Table I. The total bacterial counts of abnormally conditioned activated sludges were higher than those of normally conditioned activated sludges. Though the MLSS values of all activated sludges were almost the same, the absorbance values at 600 nm of abnormally conditioned activated sludges (II-Ab, III-Ab) were clearly higher than those of normally conditioned activated sludges (I-N, III-N). Thus, it is considered that counts of suspended bacteria were increased in abnormally con-

Fig. 8. The DMDS-Forming Ability of Isolated Strains (II)

ditioned activated sludge.

4. The DMDS-Forming Ability of Isolated Bacteria

Bacteria were isolated at random from normally conditioned activated sludges (I-N and III-N) and abnormally conditioned activated sludges (II-Ab and III-Ab), and their ability to form DMDS from methionine was tested. The results are shown in Figs. 7 and 8.

Several strains (strain II-Ab-39–43, III-Ab-5 and III-Ab-28) from abnormally conditioned activated sludges II-Ab and III-Ab formed DMDS at concentrations as high as 1590–3510 ng per tube. However, normally

Fig. 9. Effect of Culture Time on DMDS Formation

○ : strain II-Ab-43, ● : strain III-Ab-5, □ : strain III-N-1, ■ : strain III-N-45.
Culture temperature : 20°C $n=2-6$.

conditioned activated sludges I-N and III-N yielded no strains which formed DMDS at a concentration higher than 1000 ng per tube. Thus, high-DMDS-forming bacteria were increased in the abnormal activated sludges.

5. Effect of Culture Time on DMDS Formation

The effect of culture time was tested with strains II-Ab-43 and III-Ab-5, which formed DMDS at high concentrations, and strains III-N-1 and III-N-54, which formed DMDS at extremely low concentrations. The results are shown in Fig. 9. Strains II-Ab-43 and III-Ab-5 showed maximum DMDS levels after 2–3 d of culture. Thus, determination of DMDS-forming ability of isolated strains after 2 d of culture was suitable. In the case of strains III-N-1 and III-N-54, which formed DMDS at extremely low concentration, little DMDS was formed even after 6 d. Thus, it is unlikely that bacteria acquire high DMDS-forming ability by induction of DMDS-forming enzyme ac-

tivity.

The results of these experiments show that the increase of DMDS-forming ability in abnormally conditioned activated sludge is due to the growth of DMDS-forming bacteria. In the next paper, we will report on a suitable environment for the growth of high DMDS-

forming bacteria and identification of these bacteria.

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References

- 1) B. Tomita, N. Hamamura and Y. Ose, *Eisei Kagaku*, **28**, 71 (1982).
- 2) a) B. Tomita, N. Hamamura and Y. Ose, *Eisei Kagaku*, **25**, 13 (1979); b) B. Tomita, T. Shoka, N. Hamamura and Y. Ose, *ibid.*, **25**, 120 (1979).
- 3) B. Tomita, N. Hamamura and Y. Ose, *Eisei Kagaku*, **26**, 218 (1980).
- 4) B. Tomita, N. Hamamura and Y. Ose, *Eisei Kagaku*, **27**, 98 (1981).
- 5) R.E. Kallio and A.D. Larson, "A Symposium on Amino Acid Metabolism," ed. by W.D. McElroy and H.B. Glass, Johns Hopkins Press, Baltimore, 1955, pp. 616—631.; W. Segal and R.L. Starkey, *J. Bacteriol.*, **98**, 908 (1969); J. Ruiz-Herrera and R.L. Starkey, *ibid.*, **99**, 544 (1969); W. Kreis and C. Hession, *Cancer Res.*, **33**, 1862 (1973); A. Miller III, R.A. Scanlan, J.S. Lee and L.M. Libbey, *Appl. Microbiol.*, **26**, 18 (1973); S. Ito, T. Nakamura and Y. Eguchi, *J. Biochem. (Tokyo)*, **79**, 1263 (1976); N.J. Hayward, T.H. Jeavons, A.J.C. Nicholson and A.G. Thornton, *J. Clin. Microbiol.*, **6**, 187 (1977); M. Pohl, E. Bock, M. Rinken, M. Avdin and W.A. König, *Z. Naturforsch.*, **39c**, 240 (1984).
- 6) B. Tomita, N. Hamamura and Y. Ose, *Eisei Kagaku*, **25**, 338 (1979).
- 7) Japanese industrial standards committee, "Random Sampling Methods JIS Z 9031-1956," Japanese Industrial Standards Committee, Tokyo, 1969.