Agar Plate Method for Detection and Enumeration of Alkylbenzenesulfonate-Degrading Microorganisms

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A simple method for detection and enumeration of alkylbenzenesulfonate (ABS)-degrading microorganisms by using agar plates was developed and used in microbiological studies of coastal marine and polluted river waters. The method depends upon the color responses of neutral red in alkaline medium. Neutral red changes from pink, when it enters into ABS micelles, to yellow, when the ABS is degraded, and does not form micelles. When neutral red-tris(hydroxymethyl)-aminomethane buffer solution and then cationic surfactant solution were sprayed onto the agar surface of ABS-nutrient agar cultures, transparent haloes appeared around the colonies of ABS-degrading microorganisms against a pink background. Viable counts of ABS-degrading bacteria isolated from both seawater and freshwater environments were considerably higher in polluted waters than in less polluted areas. Viable counts of ABS-degrading bacteria averaged 1.5 × 10⁴/ml in samples from the surface water of polluted Tokyo Bay and 3.0 × 10⁴/ml in samples from the surface water of polluted Tamagawa River but were fewer in number in samples from less polluted waters.

Various isomers of the surfactant alkylbenzenesulfonate (ABS), a component of domestic and industrial detergents, are generally regarded as molecules refractory to biological degradation. A steady increase in detergent use has been accompanied by pollution problems such as foaming in river waters and sewage systems (5). Furthermore, recent studies (2, 7) have demonstrated that considerable amounts of ABS accumulate in the bottom sediment of coastal marine areas as well as inflowing rivers, even though the "hard" or branched-chain type of ABS has been replaced by "soft" or straight-chained type of ABS in detergents.

Although many microorganisms are known to degrade these surfactants (10, 11), few quantitative data on the distribution of these microorganisms in natural environments are available. The absence of a simple method for the detection and enumeration of ABS-degrading microorganisms in natural water may account for this lack of information.

Supplying ABS as the sole sulfur source, Benarde et al. (3) were able to isolate ABS-metabolizing bacteria from various sources, using ABS-glucose-iron agar. A discoloration of the medium around colonies of ABS-degrading bacteria, as a result of iron sulfide formation, was noted.

Ichikawa and Asaka (4) observed the formation of a halo around the colonies of ABS-decomposing bacteria when methylene blue solution was poured over the surface of ABS-nutrient agar plates. However, the halo was not very clear.

In the current study, a simple method for isolation and clear demonstration of ABS-degrading microorganisms was developed by modifying a method for the volumetric determination of anionic surfactant. Neutral red (NR) was used as an indicator (12). This method was found to be useful in a study of the distribution of ABS-degrading bacteria in both seawater and freshwater.

MATERIALS AND METHODS

Culture media. The following media were used to obtain viable counts of aerobic heterotrophic bacteria, ABS-resistant bacteria, ABS-degrading bacteria, and bacteria using ABS as sole source of carbon in seawater and freshwater samples. To estimate the number of viable aerobic heterotrophic bacteria present in the seawater samples, ZoBell medium 2216E (13) was used. Sakurai medium (9), which consisted of polypeptone, 2.0 g; Bacto-yeast extract, 1.0 g; glucose, 0.5 g; agar, 15.0 g; and tap water to 1,000 ml (pH 7.2), was used for isolation of aerobic heterotrophic bacteria from the freshwater samples. To enumerate ABS-resistant bacteria, ABS was added to seawater or freshwater medium at a concentration of 1 g/liter. Bacteria capable of producing colonies on the ABS-nutrient medium are considered to be ABS-resistant for the purposes of this study. The agar plates upon which ABS-resistant bacterial colonies

40
developed were examined further to enumerate the ABS-degrading bacteria following the procedure described below. ABS inorganic seawater medium [(NH₄)₂SO₄, 1.0 g; K₂HPO₄, 0.01 g; ABS, 1.0 g; washed agar, 15.0 g; and charcoal-treated seawater (8) to 1,000 ml (pH 7.6)] and ABS inorganic freshwater medium (6) [(NH₄)₂SO₄, 2.0 g; K₂HPO₄, 2.0 g; Na₂HPO₄, 3.0 g; MgSO₄ · H₂O, 0.01 g; ABS, 1.0 g; washed agar, 15.0 g; and charcoal-treated tap water to 1,000 ml (pH 7.2)] were employed to enumerate bacteria utilizing ABS as sole carbon source in the seawater and freshwater samples, respectively.

Portions (0.1 ml) of serially diluted water sample were placed on a nutrient medium and spread evenly over the agar surface with a glass rod. The inoculated plates were incubated in air at 20 C for 2 weeks.

Detection and enumeration of ABS-degrading microorganisms. (i) Reagents. For ABS, tetrapropylene-derived sodium dodecylbenzenesulfonate (TBS), obtained from Tokyo Kasei Co., was used throughout the study. For NR solution, 0.3 g of NR was dissolved in distilled water to 100 ml. For tris(hydroxymethyl)aminomethane (Tris) buffer solution, 2.42 g of Tris was dissolved in 100 ml of distilled water. To this solution 24.4 ml of 0.2 M HCl was added, and the volume was increased to 400 ml with distilled water. The pH was adjusted to 9.6. For NR-Tris solution, the NR and Tris buffer solutions were mixed (1:9, vol/vol). The mixture was always prepared on the day of use. For cationic surface active agent (CSAA) solution, 0.64 g of cetyl trimethyl ammonium chloride was dissolved in distilled water to 1,000 ml.

(ii) Assay procedure. Both total colony counts and counts of colonies, revealed by the following procedure, were obtained. NR-Tris solution was evenly sprayed onto the surface of ABS-nutrient agar plates upon which colonies of ABS-resistant microorganisms had developed. After the plates were left standing at room temperature for 5 to 10 min to dry the surface, a small amount of CSAA solution was sprayed evenly onto the agar. Transparent haloes appeared around certain of the colonies.

(iii) Determination of MBAS. Methylene blue active substances (MBAS) in the water samples were measured by the method of Abbott (1) with sodium dodecylbenzenesulfonate as the standard. The net content of ABS in the MBAS present in the water samples was not determined (2).

RESULTS AND DISCUSSION

The typical appearance of ABS-degrading bacterial colonies on a plate inoculated with a seawater sample is shown in Fig. 1. Transparent rings that appeared around colonies of presumed ABS-degrading bacteria were seen against the pink background of the ABS agar. To determine whether the halo-producing bacteria actually degraded ABS, representative halo-producing marine and freshwater bacteria and non-halo-producing strains were examined further. Separate lots of 100 ml of liquid 2216E

or Sakurai medium containing 10 mg of ABS per liter were inoculated with the strain to be assayed and incubated at 20 C with continuous stirring. Portions were taken at periodic intervals and were analyzed for MBAS (1). All of the halo-producing bacteria examined (marine bacteria, 25 strains; freshwater bacteria, 10 strains) diminished the concentration of MBAS by 10 to 85% after 7 days. On the other hand, the decrease in MBAS was found to be less than 7% in the media of representative non-halo-producing bacteria (marine bacteria, five strains; freshwater bacteria, five strains) after 7 days. The ability of bacteria to produce haloes and their capacity for diminishing the concentration of MBAS were thus closely correlated. ABS-degrading bacteria were, therefore, enumerated by counting the number of halo-producing colonies on ABS-nutrient agar plates following the method described above.

Uno and Miyajima (12) developed a volumetric method for the determination of anionic surface active agents (ASAA) which depends upon the difference of the color of NR (yellow) and the NR-ASAA complex (pink) in alkaline medium. When NR is added to a solution of ASAA, complexes of NR with ASAA are produced. However, the complexes are included and solubilized by the ASAA micelles, and alkaline color of NR is not observed, even in
alkaline medium (pH of 8 to 9). If this solution is titrated with the standard solution of cationic surface active agent, the alkaline NR appears at the end point. In the present study, this reaction was modified to detect ABS-degrading microorganisms with an agar medium. That is, NR-Tris solution (yellow) was sprayed onto the ABS-nutrient agar plate and presumably formed complexes with the ABS. By analogy, the complexes could be solubilized into ABS micelles. As a result, the color of the agar surface turned pink (the acid color of NR) even though the pH of the agar surface was 8.6. On the other hand, the alkaline color of NR (yellow) appeared around the colonies of ABS-degrading bacteria because the concentration of the ABS apparently became too low to form NR-ABS complexes as a result of ABS degradation.

ABS-nutrient medium becomes turbid by precipitation of ABS when the medium is prepared with seawater. Halo formation was then observed around colonies of ABS-degrading bacteria even before treatment, probably as a result of their degradation and solubilization of ABS during growth. It may thus be simpler to consider these haloes as indicative of ABS-degrading marine bacteria and enumerate them accordingly. However, the treatment described is necessary for revealing freshwater samples.

To enumerate ABS-degrading bacteria in natural environments, samples of river and coastal waters were assayed. Some of the estimates of numbers of these bacteria in coastal waters of Japan are given in Table 1. It is obvious that not only the viable count of aerobic heterotrophic bacteria (3.5 × 10⁶/ml) but also those of the ABS-resistant bacteria (1.7 × 10⁵/ml), bacteria using ABS as sole carbon source (6.8 × 10⁴/ml), and ABS-degrading bacteria (1.5 × 10⁴/ml) were considerably higher in polluted surface water of Tokyo Bay than in the less polluted waters of Nabeta Bay and Aburatsubo Inlet.

Table 2 shows results of assays of water from Lake Sagami and the Tamagawa River. The latter is one of the rivers carrying municipal sewage and industrial waters from surrounding areas into Tokyo Bay. In contrast to the Tamagawa River samples, the water of Lake Sagami was found to be relatively less polluted. It is evident that the population of ABS-degrading bacteria, as well as other bacterial groups, was much higher in the waters of the Tamagawa River than in those from Lake Sagami.

Thus, from Tables 1 and 2, it can be concluded that ABS-degrading bacteria are widely distributed both in the seawater and freshwater environments, though the population fluctuated with the samples. Furthermore, the populations of ABS-degrading bacteria enumerated by the method employed in this study was found to be much higher than was indicated using the ABS inorganic medium employed by the other investigators (5) for isolation of ABS-degrading bacteria.

Although only a preparation containing branched-chain isomers of sodium dodecylbenzenesulfonate as substrate was used to detect ABS-degrading bacteria in this study, the method can be employed for detection and enumeration of bacteria degrading other anionic surface active agents, viz., linear alkylben-

### Table 1. Viable counts of bacteria and amounts of MBAS in water and sediment samples collected from the coastal waters of Japan in January 1974

| Sampling station       | Depth (m) | Colonies/ml of water or g of sediment | MBAS (mg/liter) |
|------------------------|-----------|--------------------------------------|-----------------|
|                        |           | Heterotrophic bacteria | ABS-resistant bacteria | Bacteria using ABS as sole C source | ABS-degrading bacteria |                   |
| **Nabeta Bay**         |           |                                 |                 |                            |                      |                 |
| Station A              | 0         | 2.8 × 10⁴                 | 2.0 × 10⁵        | 5.5 × 10¹                  | 1.1 × 10⁷              | u               |
| Station B              | 0         | 6.6 × 10⁴                 | 2.8 × 10⁵        | 2.2 × 10²                  | 1.1 × 10⁶              | u               |
| **Aburatsubo Inlet**   |           |                                 |                 |                            |                      |                 |
| Station A              | 0         | 1.8 × 10⁴                 | 2.0 × 10⁵        | 4.0 × 10¹                  | 1.0 × 10⁷              | u               |
| Station B              | 0         | 8.1 × 10⁴                 | 2.0 × 10⁵        | 9.8 × 10²                  | 8.0 × 10⁷              | u               |
| Sediment               |           | 8.0 × 10⁵                 | 2.0 × 10⁶        | 3.3 × 10⁴                  | 1.0 × 10⁷              |                 |
| **Tokyo Bay**          |           |                                 |                 |                            |                      |                 |
| Kawasaki               | 0         | 3.5 × 10⁴                 | 1.7 × 10⁵        | 6.8 × 10³                  | 1.5 × 10⁵              | 0.20            |

* u, Undetectable amounts.
TABLE 2. Viable counts of bacteria and MBAS in surface water samples from Lake Sagami and the Tamagawa River on 13 February 1974

| Sampling station | Colonies/ml | MBAS (mg/liter) |
|------------------|-------------|-----------------|
|                  | Heterotrophic bacteria | ABS-resistant bacteria | Bacteria using ABS as sole C source | ABS-degrading bacteria | |
| Lake Sagami      | $1.8 \times 10^4$ | $1.5 \times 10^4$ | $2.0 \times 10^1$ | $5.0 \times 10^1$ | u* |
| Tamagawa River   | $9.3 \times 10^4$ | $8.0 \times 10^4$ | $4.5 \times 10^1$ | $5.0 \times 10^1$ | 0.50 |
| Sekido Bridge    | $3.0 \times 10^4$ | $1.1 \times 10^4$ | $7.0 \times 10^1$ | $7.0 \times 10^1$ | 0.59 |
| Tamasuido Bridge | $3.4 \times 10^4$ | $3.0 \times 10^4$ | $2.2 \times 10^1$ | $3.0 \times 10^1$ | 1.80 |
| Maruko Bridge    | $4.1 \times 10^4$ | $1.1 \times 10^4$ | $1.8 \times 10^1$ | $9.0 \times 10^1$ | 1.70 |
| Rokugo Bridge    | $2.2 \times 10^4$ | $2.1 \times 10^4$ | $4.0 \times 10^1$ | $5.5 \times 10^4$ | 1.40 |

*u*, Undetectable amounts.

zenesulfonate and alkylsulfate.

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