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A Search for Effective Microorganisms in Garbage Treatment

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This study intends to develop a new method to detect the microorganisms which exist and function actively in the garbage treatment systems, because truly effective microorganisms have not yet been identified although it is undoubtedly certain that microbial activity is the main cause of the decomposition of organic materials in garbage treatment systems. Direct observation of all living bacteria using fluorescence methods was attempted as a first step in order to investigate whether or not non-culturable bacteria really exist and play a role in the process. It was apparent that direct observation of living bacteria on real samples, such as the residue of a garbage treatment system, presented some difficulties in using fluorescence observation methods, probably due to the existence of water soluble and/or suspended solid constituents, although good results could be obtained using clear samples without impurities. Methanol-sonication treatment was not an effective way to avoid the difficulties.

1. Introduction

Since the social significance of garbage treatment at an individual emission source (household) is very large, microbial garbage treatment has been studied in our laboratory for a long time. One valuable result has been the development of microbial static-type small scale garbage treatment systems, which show much better performance than most conventional electric garbage treatment systems [1]. The difference between "static-type" and the conventional systems is related to the use or disuse of continuous stirring and aeration; there is no forced aeration nor mechanical mixing device in the "static-type" system. In the "static-type" system, mixing occurs only once a day, when garbage is input. But no essential difference in the microbial community has been detected between our "static type" and conventional systems. Also, there is little correlation observed between total colony number on the culture media and garbage decomposition rate in either systems. This fact may indicate that the methods we have adopted to analyze the microbial ecosystem have some defects. For instance, the DGGE method is often used to detect present species in a mixed microbial community. However this method can not distinguish between dead and living microbes because DNAs is recovered from all microorganisms present[2,3]. Thus, it is suggested that the most effective microorganisms have been neither clarified nor identified in the garbage decomposition process. Another piece of experimental evidence was that in natural soil or environmental samples only about 1 percent or less of the microorganisms present could be detected using the normal culture method, meaning that there are many kinds of non-culturable microorganisms[4,5,6,7]. So far two major methods have been adopted to analyze non-culturable microorganisms; direct observation and molecular biological techniques such as the DGGE method. However, since the methods which detect total DNA have the defect stated above, it is difficult to determine that microbes are actually alive and function in the garbage decomposition process. Thus, the purpose of this study is to count the number of non-culturable microorganisms using fluorescence observation and examine the percentage of bacteria that live in the garbage system by comparing the numbers obtained from normal colony counting with those from three different methods of Sybr-Gold[8,9,10], CFDA[11], and CTC[12,13].

2. Material and Method

2.1 Common procedure

1) Fluorescence observation: Three methods were used as shown in Table I. The sample solution mounted on a glass slide was subjected to fluorescence observation. A "fining sample" was prepared as follows: colonies of microbes in the garbage residue grown on solid T medium were collected and cultivated in liquid T medium at 60 ℃ for 4 hours. Thus, this sample consists essentially of culturable microorganisms without any suspended solids. For the "suspension sample" three grams of garbage residue were added to sterile water and homogenized for 10 minutes. Thus, this sample contains all kinds of microorganisms living in the garbage residue and suspended solid impurities.

Table I. The methods of fluorescence observation and their conditions.

Method	drip	incubation
Sybr-gold	10μl	37℃,5min
CFDA	15μl	37℃,10min
CTC	20μl	37℃,30min

2) Colony counting: A normal method using nutrient-rich T and LB media and water extracted from garbage residue was adopted to count the number of colonies grown on the culture plate.

2.2 Measurement of the number of bacteria in the fining sample

The purpose of this measurement was to confirm that bacteria in the fining suspension sample can be observed clearly and precisely by the fluorescence methods in Table I in order to count the number of bacteria in the sample. The result was compared with that of the number usually found by colony counting on a culture plate. The measurement values should be expected to be the same due to the preparation procedure of the fining sample.

2.3 Measurement of the number of bacteria in the suspension sample

Since non-culturable microorganisms (mainly bacteria) by definition can not be counted by the usual cultivation method, we tried to observe them directly by using fluorescence methods. At first, it should be confirmed that the number of living bacteria in the suspension sample can be counted clearly and exactly by the fluorescence methods in Table I, because the suspension sample contains various kinds of suspended solid impurities which might affect the measurement result. The usual colony counting method was performed to measure the number of cultural bacteria in the suspension sample, and the difference between the direct observation result and the result of colony counting would be the number of non-culturable microorganisms.

2.4 Improvement of the fluorescence method for the suspension sample

In order to improve the sensitivity and accuracy of the fluorescence method for the suspension sample, sonication with methanol addition was attempted. This operation aimed mainly at tearing bacteria off the surface of suspended solid particles. A methanol-sonication sample was prepared as follows; 0.1 mL of methanol was added to 0.9 mL of suspension sample, and this mixture was subjected to ultrasonic treatment for 5 to 30 min. to separate the microorganisms from suspended solid particles. After this treatment, the fluorescence method for direct observation of bacteria was applied.

3. Results and Discussion

3.1 Fining sample

The total numbers measured by fluorescence observation methods and the colony counting for fining sample were shown in Fig. 1 and Table Ⅱ. Although the kind of colony varied according to the kind of medium, the total number of bacteria in the fining sample was nearly consistent among three different fluorescence observation methods as well as colony counting. Since the fining sample contained all kinds of microbes that can grow in liquid T medium, the result of colony counting on solid T medium and also the fluorescence observation must reflect the exact number in the sample. Thus, the result shown in Fig. 1 indicated that the total number detected on the solid medium was the same as that of fluorescence observation, meaning that both methods could detect all microbes accurately.

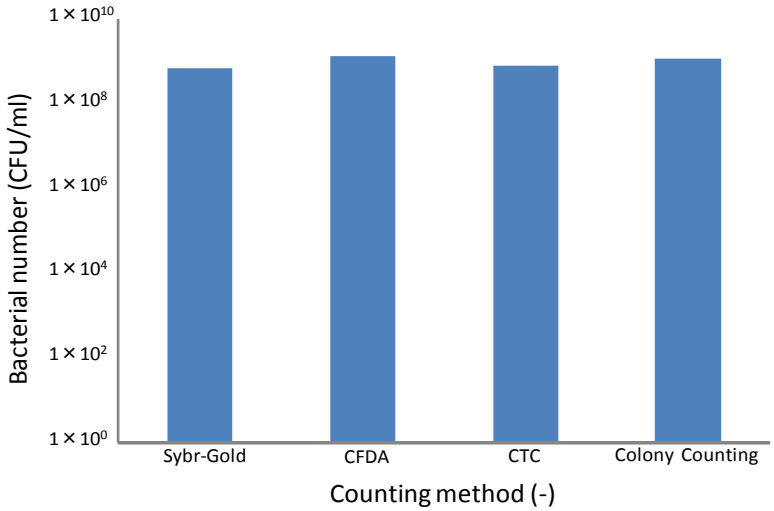
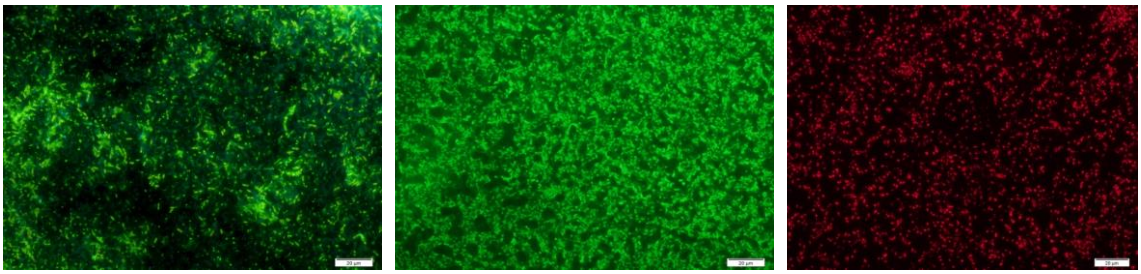


Fig. 1. The total number of bacteria in the fining sample (day 13).

Table II. The exact total number of bacteria in the fining sample (day 13).

Counting method	Bacterial number (× 10^8CFU/ml)
Sybr-Gold	7.5
CFDA	14.4
CTC	8.2
Colony Counting	11.9



(a) Sybr-Gold (day 3) (b) CFDA (day 6) (c) CTC (day 10)

Fig. 2. The images of bacteria strained by three different fluorescence methods for the fining sample.

The images of bacteria stained by three different fluorescence methods were shown in Fig. 2. Although the principle of measurement is different among each of these methods, all the images were equally clear, meaning that each method could be applied to the fining sample. However, there was a slight difference in the exact measured total number between these methods as shown in Table II.

3.2 Suspension sample

The time course of the total number of bacteria in the suspension sample was shown in Fig. 3 and Table III, which was different from the result obtained using the fining sample. The total number detected by fluorescence observation was generally less than that detected by normal colony counting, although the tendency of number change was the same. This result might suggest that there were some unknown factors which decrease the total number detected because direct observation generally must result in a much greater total number of microbes than can grow in the medium, i.e. non-culturable microbes can be detected by direct observation. The possible reasons of this result were determined to be as follows; microorganisms might be adsorbed to the surface or inside the suspended solid particles in the suspension sample, or the suspended solid impurities might affect the sensitivity of the fluorescence observation, or water soluble impurities might have a harmful effect on the chemical reaction of the fluorescence methods. The images of fluorescence were shown in Fig. 4, which reflected the sensitivity of each method as the detected total number results in Fig. 3.

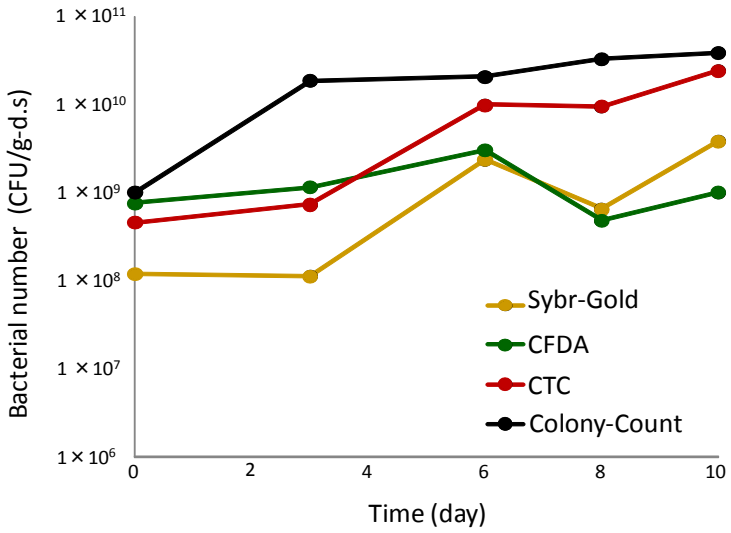
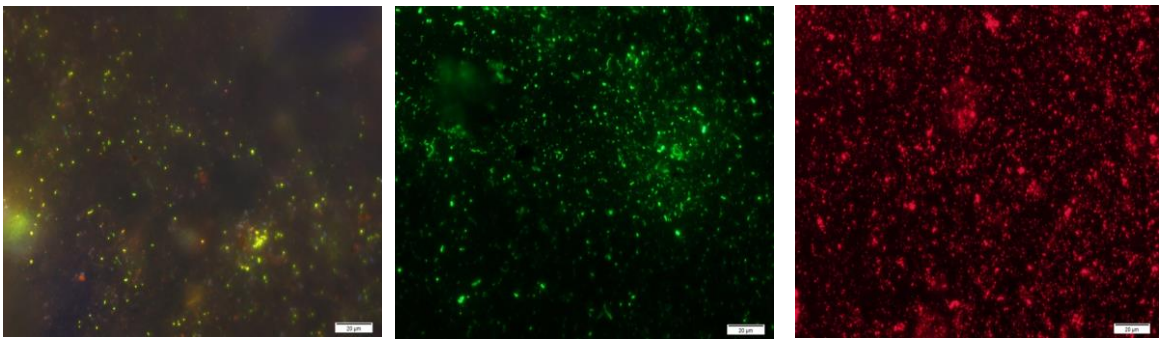


Fig. 3. The total number of bacteria in the suspension sample.

Table III. The exact total number of bacteria in the suspension sample.

Time(day)	SybrGold(× 10^8)	CFDA(× 10^8)	CTC(× 10^8)	Colony Counting(× 10^8)
0	1.2	7.7	4.7	10.2
3	1.1	11.7	7.4	189.1
6	23.9	30.8	100.0	210.2
8	6.6	4.9	96.2	330.2
10	38.8	10.2	245.8	395.7

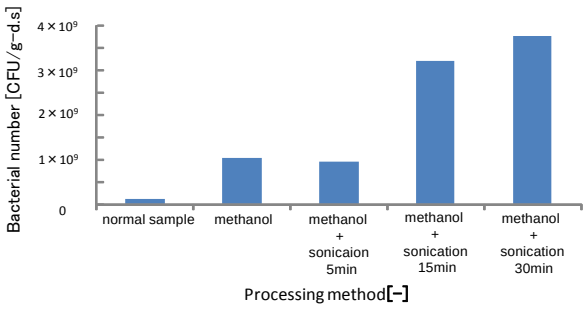


(a) Sybr-Gold (day 3) (b) CFDA (day 6) (c) CTC (day 10)

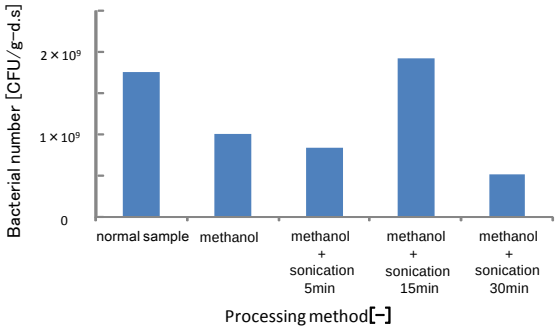
Fig. 4. The images of bacteria strained by three different fluorescence methods for the suspension sample.

3.3 Methanol-sonication treatment

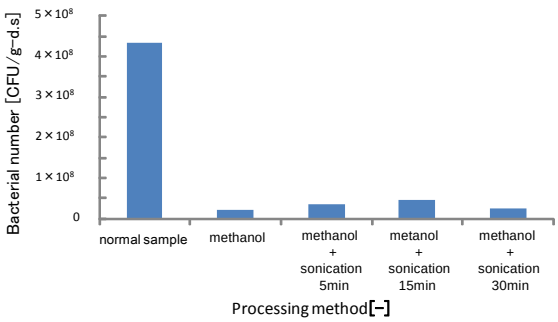
The effects of methanol-sonication treatment on the total number of bacteria measured among three different fluorescence methods were shown in Fig. 5. A positive effect was observed in the case of the Sybr-Gold method as shown in (a) of Fig. 5, but the absolute value of the total number



(a) Sybr-Gold



(b) CFDA



(c) CTC

Fig. 5. The effect of methanol-sonication treatment on the measurement of total number of bacteria among three different fluorescence methods for the suspension sample.

for the normal sample was very small, and the absolute value was not greatly improved even in the best case of methanol plus 30 min. of sonication. In other cases there were some negative effects on the observed results. In particular, the CTC reaction was seriously inhibited by the addition of methanol regardless of sonication time as shown in (c) of Fig. 5. In the case of CFDA shown in (b) of Fig. 5, no consistent tendency was observed in the measurement results, probably because there might be both positive and negative effects of the methanol-sonication treatment on the behavior of the bacteria and/or on the reaction to fluorescence observation. These results suggested that the methanol-sonication treatment had few positive effects on the enhancement of the sensitivity and accuracy of the fluorescence observation of the suspension sample. As a next step, other approaches should be pursued.

3.4 For the future work

This study intends to develop a new method to detect the microorganisms which exist and function in the garbage treating system. Direct observation of all living bacteria using fluorescence methods was attempted as a first step in order to investigate whether or not non-culturable bacteria really exist and play a role in the process. But it was apparent that direct observation of living bacteria on real samples, such as the residue of a garbage treating system, presented some difficulties in using fluorescence observation methods, probably due to the existence of water soluble and/or suspended solid constituents. Methanol-sonication treatment was not an effective way to avoid the difficulties. Thus, other direct observation methods and molecular biological methods such as DGGE should be used together in the future work.

4. Conclusion

1. The total number of microbes in the fining sample counted by normal culture was almost the same as that detected with fluorescence observation, but the result of the suspension sample was different, suggesting that there may be many inhibitory factors in the garbage treating process on the fluorescence observation of living bacteria.
2. So far, the exact detection of all living microbes is difficult, even so for clarifying functional microbes.
3. Methanol-sonication treatment showed few positive effects on the direct observation of living bacteria.

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