ESTIMATION OF THE NUMBER OF DEGRADING MICROORGANISMS FOR BIODEGRADABLE PLASTICS IN NATURAL ENVIRONMENTS

Song, Cunjiang[1], Wang, Shufang[2], Meng, Zeng[1],
Qiao, Mingqiang[1] and Takeuchi, Shigeya[3]

[1]Key Laboratory of Bioactive Materials for the Ministry of Education, Department of Microbiology, Life Science College, Nankai University, Tianjin 300071, China
[2]State Key Laboratory of Functional Polymer Materials for Adsorption and Separation, Nankai University, Tianjin, 300071, China
[3]Faculty of Education, Toyama University, Gofuku, Toyama 930-8555, Japan

ABSTRACT

The proposed film-MPN method was developed to estimate the numbers of degrading microorganisms (degraders) of biodegradable plastics (Bdps) in natural environments. By studying the rule of positive tube appearing, incubation time for the method was determined for aquatic environmental sample. Numbers of aerobic Bdps degraders in natural environments were estimated by the film-MPN method. Results were compared with those estimated by the clear-zone technique. Differences between the numbers of degraders estimated by the two methods ranged from -10.53% to +9.77%. These results showed that the film-MPN method was suitable for estimating the numbers of Bdps degraders in the environments. Visual judgment of the results obtained by using the film-MPN method is easier and only a small amount of Bdps sample (about 0.5-1.0 g) is needed. Even if the Bdps cannot be made into a milky suspension of powder, an emulsifier is not required for the film-MPN method. Also, aerobic Bdps degraders can be easily isolated from a culture solution of positive-growth tubes of a high dilution of an environmental sample.

Keywords: degrading microorganisms, biodegradable plastics, film-MPN method, clear-zone technique, aquatic environments

INTRODUCTION

The relationship between biodegradability and degrading microorganisms (degraders) of various biodegradable plastics (Bdps) has been reported by many groups (Delafield 1965, Mergaert 1993, Schirmer 1993, Brandl 1995). The clear-zone technique is widely used to estimate numbers of Bdps degraders in various environments. In this method, Bdps are dispersed on an agar plate as a milky suspension of powder. When Bdps degraders are inoculated, they secrete Bdps-depolymerases and extracellularly hydrolyze Bdps to water-soluble products. Thus, a transparent clear zone around a depolymerase-producing colony is created on the agar plate. By the technique, many groups cultivated Bdps-decomposing bacteria to isolate depolymerase (Jendrossek 1993), estimated the numbers of Bdps degraders in activated sludge (Briese 1994), and isolated Bdps degraders from soil and compost (Mergaert 1996). Based on the principle of the technique, some groups found that many terrestrial fungal isolates hydrolyzed Bdps in cultured tubes (Matavulj 1992).

However, only a few Bdps can be prepared as milky suspensions of powders. Most other Bdps form large rubber-like aggregations. Hence, the clear-zone technique cannot be used. To solve the problem, some groups proposed a modified clear-zone technique by emulsifying Bdps with a surfactant (Nishida 1993, Nishida 1998, Horowitz 1995), and prepared an artificial granule suspension of Bdps by organic solvent and emulsifier (Ramsay 1994, Marchessault 1995). However, emulsifier or surfactant inhibited microbial growth and Bdps depolymerase activity (Jendrossek 1996).

We previously used Bdps film to estimate the numbers of Bdps degraders in two kinds of soil, which was named as the film-MPN method (Song 2001), and studied the biodegradability of Bdps by the method (Song 2002, Song 2003).

In this study, we studied the film-MPN method as applied to aquatic environmental sample. The numbers of Bdps Poly (3-hydroxybutyrate-co3-hydroxy-valerate) (PHB/V) degraders in natural environments were estimated by the clear-zone technique and the film-MPN method. These results were compared. Aerobic PHB/V degraders were also easily isolated from the culture solution of positive-growth tubes.

MATERIALS AND METHODS

Garden soil

Soil samples were collected from topsoil (0-10 cm in depth) on the campus of Toyama University, Toyama, Japan, which had no history of PHB/V exposure. Some of the soil properties were as follows: pH (H_2O), 7.35; pH (KCl), 6.85; H_2O, 21.5%; C, 3.01%; H, 0.66%; and N, 0.21%.

Paddy field soil

Paddy field soil samples were collected from topsoil (0-10 cm in depth) on a farm near Toyama University, Toyama, Japan, that had no history of PHB/V exposure. Some of the farm soil properties were as follows: pH (H_2O), 5.48; pH (KCl), 4.09; H_2O, 17.03%; C, 2.43%; H, 0.71%; and N, 0.25%.

Farm soil

Farm soil samples were collected from the topsoil (0-10 cm in depth) on a farm in Toyama University. Some of the properties of the brown lowland soil were as follows: H_2O, 20.79%; C, 2.02%; H, 0.56%; N, 0.31%; and pH (H_2O), 7.35.

Infertile garden soil

Infertile garden soil samples were collected from the topsoil (0-10 cm in depth) in the garden of Toyama Industrial Technological Center farm, Takaouka, Toyama, Japan. Some of the properties of the sandy soil were as follows: H_2O, 12.59%; C, 0.42%; H, 0.40%; N, 0.04%; and pH (H_2O), 6.29.

Riverbank soil

Riverbank soil samples were collected from the topsoil (0-10 cm in depth) from the riverbank of Zinzukawa River, near Toyama University, Toyama, Japan, which had no history of PHB/V exposure. Some of the riverbank soil properties were as follows: pH (H_2O), 5.91; pH (KCl), 4.59; H_2O, 25.03%; C, 2.04%; H, 0.61%; and N, 0.23%.

Aerobic activated sludge

Aerobic sewage sludge was obtained from the Hamakurosaki Water Purification Center, Toyama, Japan.

River water and seawater

River water was sampled from the Zinzukawa River, near Toyama University, Toyama, Japan. Seawater was sampled from the Yokata Seacoast in Toyama Bay, Japan.

Preparation of PHB/V films

Based on a previous method (Song 2001), PHB/V-film was prepared. The film was further dried under vacuum at 50°C for 4 h. The thickness of the film was 0.05 mm-0.08 mm (Fig. 1).

Film-MPN method to estimate numbers of PHB/V degraders

A medium was prepared with 0.033 M KH_2PO_4-Na_2HPO_4 buffer (pH 6.8, 1 L) containing 1 g NH_4Cl,

Fig. 1. Preparation of PHB/V-film

0.5 g $MgSO_4$ $7H_2O$, 0.05 g ferric ammonium citrate, 0.005 g $CaCl_2$ $2H_2O$, 0.05 g yeast extract, and 0.1 g casein hydrolysate. Five (5) ml of the medium was poured into a tube containing a piece of PHB/V film. The film stood in the tube using a glass bar, which was sterilized by autoclaving at 110°C for 30 min. The environmental samples were diluted at 10^1-10^{10} with the liquid medium. (The seawater samples were diluted at 2^4-2^{13}). One (1) ml of each diluted solution was inoculated into each of the five tubes. The tubes were incubated at 28°C under aerobic conditions in the dark. The numbers of positive-growth tubes were counted after the fixed time. A MPN statistical table was used to determine the growth code and to calculate the MPNs (Ishikuri 1992). The film-MPN method tubes were shown in Fig. 2.

Clear-zone technique to estimate numbers of PHB/V degraders

A solid medium containing PHB/V powder as the carbon source was prepared by pouring an overlay of 5 ml of hot mineral agar solution containing 0.25% (w/v) polymer powder onto the preheated (37°C) bottom layer of mineral agar (25 ml), resulting in the formation of an opaque top layer. Other components of the medium were the same as those mentioned above. The plates were incubated at 28°C for 4-5 weeks (Briese 1994), and the number of colonies with clear zones were counted after 5 weeks (Fig. 3).

RESULTS AND DISCUSSION

Film-MPN method as applied to aquatic ecosystem

We previously proposed the film-MPN method for the solid environmental sample (Song 2001). To construct the film-MPN method as applied to aquatic environmental sample, we studied the rule of positive tube appearing, and determined the incubation time for the method.

Fig. 4 shows the relationships between the numbers of positive-growth tubes and incubation

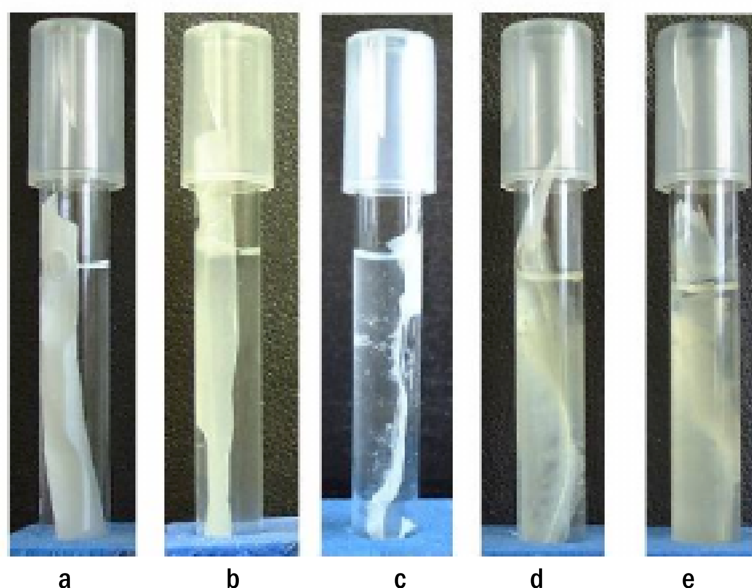

Fig. 2. Tubes for estimation of numbers of PHB/V degraders by the film-MPN method

a. Before inoculation.
b. Diluted soil suspension of 1 ml was inoculated, and tubes were cultivated for 1 week. The solution in the tubes became opaque.
c. After 8 days cultivation, small pieces of film appeared when the tube was shaken lightly.
d. A pigmentary deposit, such as a yellow-colored deposit, began to appear near the solution surface after 2 or 3 weeks.
e. After 4 weeks of cultivation.

time. One (1) ml of 10^{-4}-fold diluted river water and 10^{-6}-fold diluted garden soil, riverbank soil, and activated sludge were inoculated into each of three groups of tubes. Every group included 75 tubes. In every tube, there were 5 ml of growth medium and a piece of PHB/V film, and the tubes were incubated at 28°C in the dark under aerobic conditions.

We used previously the First-Order Reaction (FOR) model to determine the incubation time for soil environmental sample. The expected final numbers were close to the numbers observed after incubating for 9 weeks in soil 1 group and soil 2 group (Song 2001). In fact, the numbers of positive tubes did not change for soil environmental sample after 8 weeks. Fig. 4 shows that the numbers of positive tubes did not change for aquatic environmental sample after 7 weeks. Therefore, we determined that incubation time was 8 weeks for aquatic environmental sample in the film-MPN method.

Fig. 3. The number of PHB/V degraders was estimated by the clear-zone technique

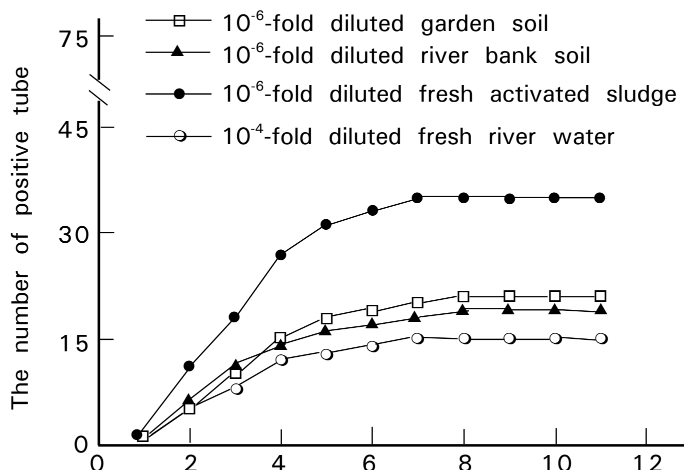

Fig. 4. Relationships between numbers of positive-growth tube and incubation time

Mode of growth and detection of positive-growth tubes

After 1 week, the solutions in some of the tubes inoculated with different environmental samples became opaque. Positive growth was detected by observation of the film pieces in the tube. When each tube was lightly shaken by hand, small pieces of film appeared after 7 days of inoculation with river water and after 6 days with activated sludge. After the tubes were further incubated for 2 or 3 weeks, pigment deposit, such as a yellow color, began to appear on some films near the solution surface in tubes inoculated with the soil samples. Similarly, a green color began to appear in tubes inoculated with the river sample. Some of the films near the surface of the solution in the positive-growth tubes appeared to be broken after incubating for 4 weeks in the garden soil, paddy field soil, infertile garden soil, and riverbank soil groups; after 3.5 weeks in the river water and seawater groups; and after 3 weeks in the activated sludge group.

Obviously, the growth rates of PHB/V degraders from aquatic ecosystems were faster than those from soil samples.

The positive-growth tube was the tube in which PHB/V-film was degraded by PHB/V degraders. Small pieces of PHB/V-film appeared and/or the films near the surface of the solution appeared to be broken in the positive-growth tube.

Sterilization conditions and additional factors in this method

Regarding sterilization of the tubes containing liquid medium and PHB/V-film in the film-MPN method, it was reported that the rate of degradation by heat-treated PHB/V (treated at 121°C for 10 min) was much less than that by untreated PHB/V in soil (Miwa 1996). After sterilization at 121°C, the film resisted the growth of degraders in the soil. Therefore, 110°C and 30 min were chosen as the sterilization temperature and time. Two additional factors affected the growth of PHB/V degraders in the tube. One was the components in the liquid medium. To make PHB/V degraders grow well, small amounts of yeast extract and casein hydrolysate were added to the liquid medium (Mergaert 1993). The other was the material used to support the PHB/V-film. In the experiments, an ultrathin glass bar was chosen as the support material of the PHA film, since an ultrathin wooden bar provided nutrients for cellulose degraders and increased the number of PHA degraders. Also, an ultrathin metal bar became rusted during cultivation and hindered both the growth of PHB/V degraders and the visual judgment of the results.

Numbers of PHB/V degraders in different environments

Through preliminary experiments, we decided to use tenfold dilution for estimating the numbers of PHB/V degraders in soil, activated sludge, and river water. The numbers of PHB/V degraders in natural environments estimated by the film-MPN method are shown in Table 1. The numbers of PHB/V degraders differed greatly depending on differences in natural surroundings. In infertile garden soil, riverbank soil, garden soil, paddy field soil, and farm soil, the numbers ranged from 1.6×10^{4}/g dry soil to 8.71×10^{5}/g dry soil, while the numbers in river water and activated sludge were 2.2×10^{3}/ml and 5.14×10^{5}/ml, respectively. In a preliminary experiment, the number of PHB/V degraders in seawater was between 10^{2} and 10^{3}. Therefore, a twofold dilution was used to estimate the number of degraders in seawater. The number was estimated to be 2.68×10^{2}/ml.

The numbers of PHB/V degraders in different environments estimated by the film-MPN method were compared with those estimated by the clear-zone technique (data not shown). The numbers estimated by the two methods were of the same orders, differing from -10.53% to +9.77%. These results indicated that the film-MPN method was suitable for estimating the numbers of PHA degraders in natural environments. Interestingly, the numbers of PHB/V degraders estimated by the film-MPN method in aquatic environments such as river water, aerobic sewage sludge, and seawater were higher than those estimated by the clear-zone technique. These results showed that use of the film-MPN method in an aqueous system was favorable for estimating the numbers of microorganisms living in a water ecosystem.

Isolation of Bdps degraders from positive tubes

The MPN method is based on the appearance of turbidity due to proliferation or on the recognition of a chemical or biological reaction due to the activity of proliferated cells. This is similar to colony formation in the plate count method (Hattori 1985). Therefore, the film-MPN method can also be used for the isolation of aerobic Bdps degraders in different environments. Specifically, some Bdps degraders, which cannot be prepared as a milky suspension and in which the clear-zone technique cannot be used, can be isolated by the film-MPN method. The culture solution of the positive-growth tube of high dilution was inoculated on a general nutrient solid medium plate and incubated at general temperature. The colonies formed from the culture solution of the positive-growth tubes were chosen for isolation. The author used this method and isolated *Pseudomonas bathycetes* which can synthesize medium-chain-length polyhydroxyalkanoates.

CONCLUSIONS

The film-MPN method can be used to estimate the numbers of PHB/V degraders in different natural environments. Visual judgment of the results obtained by using the film-MPN method is simple and only a small amount of PHB/V sample (about

Table 1. Results to estimate the numbers of PHB/V-degraders in natural environments by the film-MPN method

Dilution rate	Garden soil	Fresh river water	Fresh aerobic sewage sludge	Paddy field soil	River bank soil	Fresh sea water	Farm soil	Infertile garden soil
	Numbers of positive- or negative-growth tubes							
10^{-1}		+ + + + +						
10^{-2}		+ + + + +						
10^{-3}	+ + + + +	+ + + + −	+ + + + +					
	+ + + + +							
10^{-4} (2^{-8})	+ + + + +	+ + − − −	+ + + + +	+ + + + +	+ + + + +	(+ + + + +)	+ + + + +	+ + + − −
10^{-5} (2^{-9})	+ + + + +	− − − − −	+ + + + +	+ + + + −	+ + + + −	(+ + − − −)	+ + + + +	+ + − − −
10^{-6} (2^{-10})	+ − − − −		+ + − − −	+ + − − −	+ − − − −	(+ − − − −)	+ + − − −	− − − − −
10^{-7} (2^{-11})	− − − − −		− − − − −	+ − − − −	+ − − − −	(+ − − − −)	+ − − − −	− − − − −
10^{-8} (2^{-12})				− − − − −	− − − − −	(− − − − −)	− − − − −	
	Average degrader numbers according to the MPN statistical table							
Numbers	3.40×10^5 /g, wet-garden soil	2.20×10^3 /ml, fresh river water	5.14×10^5 /ml, aerobic activated sludge	4.20×10^5 /g, wet-paddy field soil	2.90×10^5 /g, wet-river bank soil	2.68×10^2 /g, fresh seawater	6.9×10^5 /g, wet-farm soil	1.4×10^4 /g, wet-infertile garden soil
Numbers	4.33×10^5 /g, dry-soil			5.06×10^5 /g, dry-soil	3.87×10^5 /g, dry-soil		8.71×10^5 /g, dry-soil	1.6×10^4 /g, dry-soil

Note: 1) Average degrader number was calculated from a series of five tubes of two-fold dilution (the fresh sea water) and ten-fold dilution (other samples) in the MPN statistical table (Japan Society of Soil Microbiology 1992).
2) + : one positive-growth tube, − : one negative-growth tube.

0.75 g-1.0 g) is needed. By using the film-MPN method, even if the Bdps cannot be made into a milky suspension of powder, an emulsifier is not required. The film-MPN method is more suitable than the clear-zone technique for the samples obtained from aquatic ecosystems. Theoretically, provided the cultivation period and the component of medium are determined, the film-MPN method is applicable to all polymers that can be processed into films. Also, aerobic Bdps degraders can be easily isolated from a culture solution of positive-growth tubes of a high dilution of an environmental sample.

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