Biodegradability and Degrading Microbes of Low-Density Polyethylene

Tomoko Watanabe,[1,2] Yoshito Ohtake,[2] Hitoshi Asabe,[3] Nobunao Murakami,[4] Mutsuhisa Furukawa[1]

[1]*Graduate School of Science and Technology, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[2]*Chemicals Evaluation and Research Institute, Japan, 1600 Shimotakano, Sugito-machi, Kitakatsushika-gun, Saitama 345-0043, Japan*
[3]*Hagihara Industries, Inc., 1-4 Mizushima Nakadori, Kurashiki, Okayama 712-8072, Japan*
[4]*Takenaka Co., 1-5-1 Otsuka, Inzai, Chiba, 270-1395, Japan*

Received 3 March 2008; accepted 23 July 2008
DOI 10.1002/app.29102
Published online 9 October 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: We have isolated and identified three types of low-density polyethylene (LDPE) degrading microbes, for the first time, from soil which was sampled from the field. It has been studied that thermooxidized or UV irradiation LDPE are degraded when incubated with mold (Volke-sepulveda et al., J Appl Polym Sci 2002, 83, 305; Yamada-Onodera et al., Polym Degrad Stab 2001, 72, 323); however, there was no article so far reporting the isolation of these particular microbes from the environment. Here, the mechanism of biodegradation of LDPE by the three microbes is described. We used LDPE film, which was buried in soil and biodegraded, and the surrounding soil, located at Nogi Town, Tochigi Prefecture, Japan as the sources of sample for this study. The sample was added to a liquid medium, together with powder of a photodegraded, antioxidant-free LDPE film, as the sole source of carbon. After enriched cultivation, three strains of microbes were separated from the medium. They were classified on the basis of LDPE degrading capabilities, confirmed by —OH generation and degradation traces. Using "Bergey's Manual of Determinative Bacteriology," the genera of the microbes were identified as *Bacillus circulans*, *Bacillus brevies*, and *Bacillus sphaericus*, which belong among the Natto bacteria, common in soil. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 551–559, 2009

Key words: LDPE (low-density polyethylene); biodegradation; sole source of carbon; microbes

INTRODUCTION

We have been ascertaining that low-density polyethylene (LDPE) fully exhibits its biodegradability when exposure conditions surrounding the LDPE are ready for its degradation, although its development is very slow.[1–15] For example, LDPE mulch films buried and plowed in fields for several years show notably whitened parts over their whole area due to microscopic pores generated by enzymatic degradation by microbes, which is characteristic biodegradation.[10] Also, in case of mayonnaise bottles, made of LDPE by blow molding process and buried in soil for over 32 years, when their surfaces in contact with the soil have been compared with those not in contact with the soil, the decrease of molecular weight and the remarkable tailing of molecular weight distribution to lower molecular weight side have been recognized at the surfaces in contact with the soil.[5] Furthermore, the observation of the degraded and whitened parts in contact with the soil, with a scanning electron microscope (SEM), has revealed the presence of the colonies of LDPE-degrading bacteria.[3,15] When these notably degraded bottles have been observed in detail after they were cleaned with hydrofluoric acid, numerous pores generated by enzyme degradation with $\sim$ 1 to 2 μm in length and 0.5 $\sim$ 1 μm in width have been discerned.[13,15] The configurations of microbes to be displayed on the LDPE film surfaces as body marks, yielded by the enzyme degradation at the time of their cell division in the initial stage of the degradation, have been also recognized.[13] These observation results have come to the evidence for findings that the microbes have been directly degrading LDPE. Considering that the speed of degrading LDPE is extremely slow, it is difficult to identify the bacteria that can degrade LDPE out of at least more than 2000 kinds of soil bacteria, which inhabit under ordinary circumstances. In this article, the microbes being capable to specifically degrade LDPE have been solely isolated and identified by means of using the enrichment shake culture method and

Correspondence to: T. Watanabe (watanabe-tomoko@ceri.jp).

© 2008 Wiley Periodicals, Inc.

taking the time of ~ 6 months. Moreover, we have ascertained the biodegradability of LDPE films, which were photodegraded under outdoor exposure conditions for about 6 weeks, using degrading bacteria that were solely isolated as stated earlier, and then we have identified three kinds of LDPE-degrading bacteria to report in this article.

MATERIALS AND INSTRUMENTS

Specimens for isolating LDPE-degrading bacteria

Picking up soil and LDPE films in process of degradation

The fragments of LDPE mulch films which were scattered in a field of vegetable before dissemination in April when the activities of LDPE-degrading bacteria came to be very active because of the rise of temperature, located at Nogi Town, Tochigi Prefecture, Japan, were collected to make several investigations and analyses; as a result, characteristic microbial degradation phenomena were recognized on them.[10] Appearance of the LDPE mulch film is presented in Figure 1. Then, some portions of soil at three positions adherent and surrounding the LDPE mulch films buried in the field were picked up as soil specimens per patches of field to reproduce LDPE-degrading bacteria for making them into the isolation sources of microbes. The soil at three positions adherent and surrounding LDPE mulch films in process of degradation in a field and a refuse dump were picked up. The soil picked up there were set as the isolation sources D, E, and F, respectively. Also, the two kinds of LDPE mulch films in the process of degradation were selected out of those collected in the same places stated earlier were set as the isolation sources G and H, respectively. The isolation sources list is presented in Table I.

Figure 1 Appearance of the LDPE mulch film.

TABLE I
Isolation Sources List

Code	Isolation source mediums
A[a]	The broth of the soil adherent to LDPE mulch films.
B[a]	The broth of the soil adherent to LDPE mulch films.
C[a]	The broth of the soil adherent to LDPE mulch films.
D	The soil in a field of vegetable.
E	The soil in the refuse dump.
F	The soil in the refuse dump.
G	The LDPE mulch films in a refuse dump. The films in the process of degradation.
H	The LDPE mulch films in a field of vegetable. The films in the process of degradation.

[a] A, B, and C were prepared by cultivating the field soil in Nogi Town, Tochigi Prefecture, Japan for 18 months.

Production of isolation source mediums

The antioxidant-free LDPE- F31N films formed by the inflation process, made by Nippon Petrochemicals Co., Ltd., were treated with outdoor weathering for 3 months (5400 in numerical average molecular weight; 8800 in weight average molecular weight), and the films were pulverized into fine powder by means of freeze shattering. Some portions of soil at three positions adherent and surrounding the LDPE mulch films buried in the field were picked up as soil specimens. Then, 10 g of soil specimens were mixed with the 1 g of fine powder, respectively, and each of the mixtures was cultivated on an ordinary agar medium at the temperature of 30°C for 18 months. The ordinary agar mediums are basic mediums to be used for the cultivation of aerobes (Table II). Each of the cultivated mixtures, which consisted of the soil picked up at the three positions of the field and the fine powder, was set as the isolation source mediums A, B, and C, respectively.

Isolation mediums

As LDPE was preassigned to the sole source of carbon in conjunction with the production of liquid mediums to be used as isolation mediums, the LDPE fine powder made from the photo-degraded LDPE-F31N films was appropriated for one of the ingredients of the liquid mediums. Furthermore, ammonium sulfate and/or sodium chloride, which were the highest quality reagent, were also

TABLE II
Composition of Broth Agar (pH was Adjusted to 7.2)

Ingredient	Amount
Meat extract	10 g
Peptone	10 g
NaCl	5 g
Agar	20 g
Deionized water	1000 mL

TABLE III
Composition of LDPE Agar (pH was Adjusted to 7.5)

Ingredient	Amount
$(NH_4)_2SO_4$	1.0 g
KH_2PO_4	0.2 g
K_2HPO_4	1.6 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.1 g
$CaCl_2 \cdot 2H_2O$	0.02 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.5 mg
$Na_2WO_4 \cdot 2H_2O$	0.5 mg
$MnSO_4$	0.5 mg
Calcium pantothenate	0.4 mg
Inositol	2.0 mg
Niacin	0.4 mg
p-Aminobenzoic acid	0.2 mg
Pyridoxine hydrochloride	0.4 mg
Thiamine	0.4 mg
Riboflavin	0.4 mg
Biotin	2.0 μg
Vitamin B_{12}	0.5 μg
Light-degraded LDPE film (after pulverizing it into fine powder)	1.0 g
Deionized water	1000 mL

appropriated for some of the ingredients of the liquid mediums, as the sources of nitrogen and minerals that were indispensable for growing up microbes in the liquid mediums. The liquid mediums were thus prepared with these integrant ingredients and were set as the isolation mediums (hereinafter, the isolation mediums are referred to as LDPE mediums). The composition of the LDPE mediums is presented in Table III.

LDPE agar mediums used for the pure isolation were prepared by adding 15 g of agar to the LDPE medium shown in Table III. Considering that fungi might be possible to mix in the LDPE agar mediums by any chance even after the enrichment shake culture was completed, the LDPE-N agar mediums were also prepared by adding 100 U/mL of Nystatin (MEIJI SEIKA KAISHA, LTD. polyene macrolide antibiotics preparation) as fungicide to the LDPE agar mediums.

The LDPE films for assimilation tests

The LDPE-F31N films degraded by outdoor exposures for 6 weeks. Several fragments of these LDPE-F31N films added LDPE liquid mediums which were inoculated the harvested bacteria bodies.

Instruments

The evaluation of degradation activity was made by analysis with the Microscopic FTIR (BIO-RAD DIGILAB FTS-60, Infrared Microscope, UMA-300A), using the surface reflectance method with the conditions of 8.0 cm^{-1} resolution and 256 times of scanning, and thereby the existence of —OH stretching vibration to be detected in the vicinity of 1080 cm^{-1} and derived from alcohol was discerned. The presence or absence of the traces (body marks) of the enzymatic reaction was determined with SEM (JEOL JSM T-300) at 10 kV of acceleration voltage, 10,000 times of magnification ratio in high-vacuum mode.

EXPERIMENTAL

Primary screening experiments by enrichment shake culture method

As the enrichment shake culture is a method to solely isolate specific microbes from miscible groups of various microbes after their proliferation, the method was applied to primary screening experiments as follows. The medium presented in Table III, except photo-degraded LDPE fine powder, was filtered and sterilized, and 100 mL of the medium was aseptically put into each of SAKAGUCHI-flasks, sterilized with high pressure, with a volume of 500 mL. Then, 1.0 g of the LDPE fine powder, made by freeze shattering the LDPE-F31N films that were photodegraded by outdoor exposure, was put into each of the flasks to prepare broth. Each of the broth in the flasks was inoculated with 10 mL of broth taken out of the isolation source mediums A, B, and C. The broth in each flask was also added with 3 g of the soil taken out of the isolation sources D, E, and F, respectively, which were the portions of soil adherent to the biodegraded LDPE mulch films buried in the field. The biodegraded LDPE mulch films taken out of the isolation sources G and H were cut into several fragments in the shape of square with 2 ∼ 3 cm side length, respectively, and a piece of fragment was put into each of the flasks, respectively. The enrichment shake culture was carried out on each flask at 25°C, and then 10 mL of the broth was taken out of each flask after 3 ∼ 4 weeks passed to subculture on a fresh medium. From this subculture onward, the subculture was successively conducted until the completion of the fourth passage subculture on each flask to enrich the LDPE-degrading bacteria.

Evaluation of microbial degradation ability to LDPE

We made following evaluation whether the microbes in the process of the enrichment shake culture in the flasks had degradation ability to LDPE. The LDPE fine powder of the broth in each flask was used for the evaluation. A 30 mL of broth was filtered by Whatmann filter paper No.1 to get the remaining LDPE fine powder, and then treated with bacteriolytic enzymes to eliminate protein substances

attached to the powder surfaces. The enzymatic reaction on the powder was brought forward through the following steps:

- 1st step: added the thorough soak quantity of *N*-acetylmuramidase, Achromopeptidase and lysozyme to the powder, and heated at 37°C for 3 h.
- 2nd step: added the thorough soak quantity of protease to the powder, and heated at 37°C for 16 h, and cleaned it with deionized water.
- 3rd step: added sodium lauryl sulfate to the powder, and left it as it was for 10 min.
- 4th step: applied filtration, washed and then dried at 37°C to obtain the powder.

After the earlier treatments were completed, the powder was evaluated. The evaluation of degradation activity was made by analysis with the microscopic FTIR and SEM.

Pure isolation of microbes and identification of genera

Microbes were purely isolated from certain of the isolation source series from A to H, whose microbes were determined to have degradation ability to LDPE by the primary screening experiments described in the Primary screening experiments by enrichment shake culture method section. LDPE agar mediums and the LDPE-N agar mediums were used for the pure isolation. The fourth passage broth in the process of the enriched shake culture, obtained from the isolation sources were diluted by $10^3 \sim 10^7$ fold in five stages, respectively, and they were smeared over the LDPE agar mediums and the LDPE-N agar mediums, respectively. Both the mediums were cultivated at 28°C for 2 weeks, and the pickup of bacteria from their organized colonies were carried out with platinum loops to cultivate bacteria on both mediums, respectively. These operations of the pickup and cultivation were repeatedly conducted to isolate the bacteria. The microbial degradation ability to LDPE was evaluated with the method shown in the Evaluation of microbial degradation ability to LDPE section. Moreover, the bacteria were made to grow up and to discriminate on the ordinary agar mediums shown in Table II, to clearly express their features based on the figurations and natures of the colonies, as the secondary screening experiments. These cultivation were executed at 28°C for 72 h, and then, the emerged colonies were examined by the observation for the discrimination based on their features such as the specific surface natures of the colonies and the color tones of the grown up colonies and for the figurations of bacterial bodies with biological microscope, and thereby the strains believed to be identical with each other were consolidated. The secondary isolated strains obtained through the secondary screening experiments were searched with Bergey's Manual of Determinative Bacteriology (8th ed.).[17]

Acquisition of microbes with degradation ability to LDPE

The strains obtained by the procedures shown in the Pure isolation of microbes and identification of genera section were inoculated by two ways as stated below to make assimilation tests for the purpose of determining the existence or nonexistence of actual degradation of LDPE-F31N films degraded by outdoor exposures for 6 weeks due to the degradation ability of the strains. The first way for the tests is to carry out the pickup of bacteria from one colony out of colonies, which were grown up on plate mediums, to inoculate them on the LDPE liquid mediums. The second one is to apply aseptic centrifugal washing to the bacteria bodies, which were made to cultivate up to a logarithmic growth phase on the ordinary agar mediums, and to inoculate a large quantity of the harvested bacteria bodies on the LDPE liquid mediums. These inoculated bacteria were shaken at 28°C, 120 spm (strokes per a minute) and reacted with the LDPE fine powder for 4 weeks. After these treatments were completed, the degradation ability was evaluated by both ways of detecting —OH with the microscopic FTIR measurement and of discerning the existence or nonexistence of the body marks through observation with SEM.

Identification of *Bacillus* species

Further examination was performed on the strains identified as bacilli, whose mycological natures are described in Pure isolation of microbes and identification of genera section. Their biochemical natures were studied and thus identified with the use of research reagent kits Api50CHB and Api20 (bioMerieux Japan Ltd.). First of all, each bacterium was cultivated with ordinary agar medium, respectively. The colonies of each strain, raised in the medium, were suspended in 1 mL of sterilized physiological saline solution. In this way, enriched suspension of each bacterium was prepared. Each of the suspensions was applied to the Api50CHB and Api20 research kit mediums. After cultivated at 30°C for 24 and 48 h, identification was done by observing the change of the color of the medium. This color change is due to the acid content in the medium, yielded during the fermentation process of the bacterium, and detected by an indicating reagent.

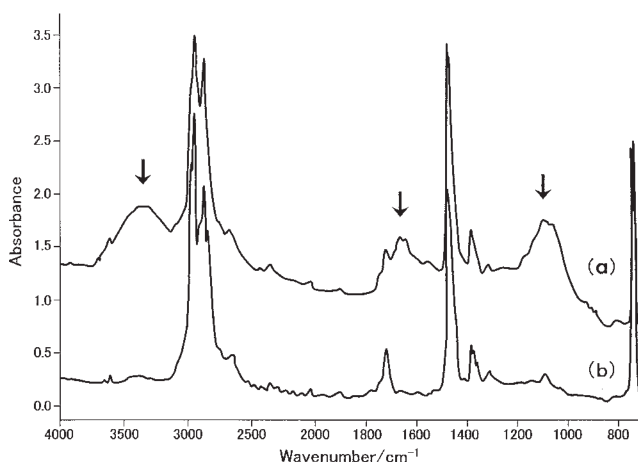

Figure 2 FTIR microscope spectra of the LDPE mulch film. (a) Whitened part. (b) Clear part.

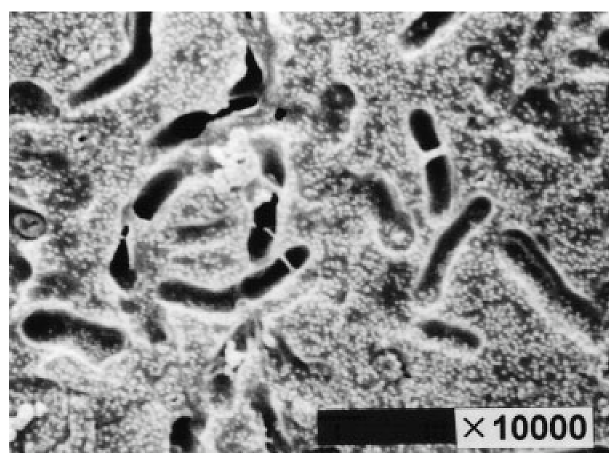

Figure 3 SEM images of body marks on the LDPE mulch film surface.

RESULTS AND DISCUSSION

Specimens for isolating LDPE-degrading bacteria

The fragments of LDPE mulch films which were scattered in a field, located at Nogi Town, Tochigi Prefecture, Japan, were collected.[10] Agricultural mulch films scattered in fields or in soil were observed by optical microscopy after staining with lactphenol cotton blue as well as by SEM and FTIR microscopy. The part of these samples which was concealed in soil was whitened in appearance. Figure 2 presents the whitened part of LDPE mulch film in contact with soil for a long time and of the transparent parts of LDPE mulch films not in contact with soil because of being folded. It has been recognized that —C=C— in the vicinity of 1640 cm^{-1} of the principal chains, —OH deformation vibration in the vicinity of 1080 cm^{-1} derived from alcohol and —OH stretching vibration in the vicinity of 3400 cm^{-1} were notably increased in the whitened parts compared with the transparent parts. The appearance of these peaks of absorption spectra has indicated the occurrence of the cleavage of chains, cleavage which has been accompanied by partial hydrolytic degradation. Furthermore, the observation of the degraded and whitened parts in contact with the soil, with a SEM, has revealed the presence of the colonies of LDPE-degrading bacteria. When these notably degraded bottles have been observed in detail after they were cleaned with hydrofluoric acid, numerous pores generated by enzyme degradation with ~ 1 to 2 μm in length and 0.5 ~ 1 μm in width have been discerned. The configurations of microbes to be displayed on the LDPE film surfaces as body marks, yielded by the enzyme degradation at the time of their cell division in the initial stage of the degradation, have been also recognized. Figure 3 shows SEM image of characteristic body marks on the LDPE mulch film surface. These observation results have come to the evidence for findings that the microbes have been directly degrading LDPE. Then, some portions of soil at three positions adherent to the LDPE mulch films buried in the field were picked up as soil specimens to reproduce LDPE-degrading bacteria for making them into the isolation sources of microbes.

Evaluation of microbial degradation ability to LDPE

The microscopic FTIR spectra of the LDPE powder in the microbe species isolated from the sources C, E, and H showing the existence of —OH in the vicinity of 1080 cm^{-1}, existence which was characteristics in the case of biodegradation was discernible. The remarkable increase of absorption spectra in the vicinity of 1080 cm^{-1} on the LDPE powder in the microbe species isolated from the source E (Fig. 4).

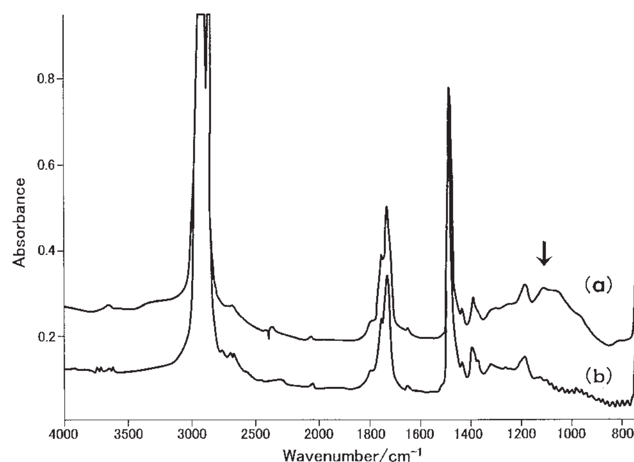

Figure 4 FTIR microscope spectra of LDPE powder after enrichment cultivation. (a) LDPE powder after enrichment cultivation of code E. (b) LDPE film before enrichment cultivation which was degraded by outdoor exposure for 3 months.

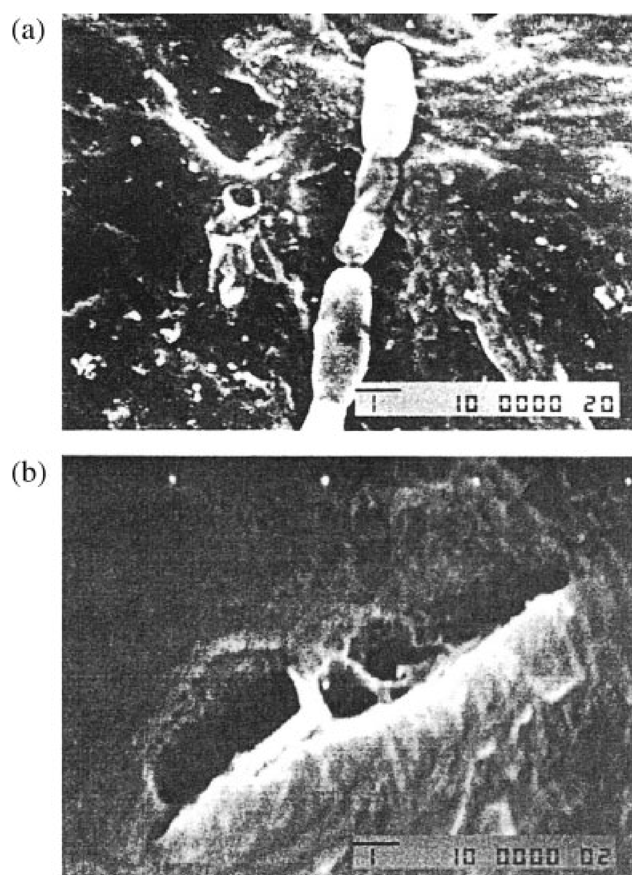

Figure 5 SEM images of the LDPE powder by separated microbes code E. (a) Microbes attached to LDPE powder before cleaning. (b) Body marks on LDPE powder after the cleaning by enzyme.

The microscopic FTIR spectra of the LDPE powder from the sources A, B, D, F, and G were no change. The body marks, which closely resembled figures shown in Figure 3 and were considered as the traces of degradation by the observation with SEM, were discerned in the source series C, E, and H. Body marks which were used as reference marks to discern whether the traces of enzymatic degradation had existed on the surfaces of the LDPE fine powder treated with bacteriolytic enzymes. Figure 5 shows SEM images of the LDPE powder by separated microbes code E. Figure 5(a) exhibits the bacteria bodies discerned on the LDPE fine powder surface of the source E, where the degradation phenomena have remarkably appeared the most, and Figure 5(b) presents the SEM observation photograph of the body marks after the enzyme treatments were finished. The size of body marks was 4 $\sim$ 5 μm in length and 1 μm in width, and their size approximated to the size of bacteria bodies. The appearance order of degradation phenomena by their intensity was E > C > H.

Pure isolation of microbes and identification of genera

Regarding to the source series of C, E, and H, as the primary screening experiments determined their microbes to have the degradation ability to LDPE, the pure isolation of their microbes were carried out. As a result, the following number of strains was sorted out from each isolation sources C, E, and H as the primary isolated strains. The isolated strains of LDPE agar medium were 26 colonies, and those of LDPE-N agar medium 29 colonies from the source C, respectively. Those of LDPE agar medium were 11 colonies, and those of LDPE-N agar medium were 45 colonies from the source E, respectively. Those of LDPE agar medium were 27 colonies, and those of LDPE-N agar medium were 45 colonies from the source H, respectively.

The total number of 175 strains as the primary isolated strains was obtained by the pick- up of bacteria from all the colonies grown up in the LDPE agar mediums. And observing the characteristics of these colonies, it was found that they closely resembled each other from the viewpoints of their surface natures, configurations, and color tones. Considering that the characteristics of the colonies such as their configuration and natures might be possible not to appear distinctly, because the LDPE agar mediums were oligotrophic mediums, it was decided to make

TABLE IV
Quality of Mycology (Morphology)

Test items	Sample code		
	No. 14	No. 19	No. 20
Formation of colony surface	Without gloss and smooth	Without gloss and rough	Gloss and smooth
Tone of maturation colony	Flesh color	Orange color	Yellow brown
Formation of germ	Long and thick rods	Short rods	Rods
Motility[a]	−	±	−
Endospore forming[a]	+	+	+
Form of endospore	Ellipse and sphere	Ellipse and sphere	Sphere
Bulking[a]	+	+	+
Position of endospore	End or semi-end	End or semi-end	End

[a] −, negative; +, positive; ±, both detective.

TABLE V
Quality of Mycology (Growth)

Test items	Sample code		
	No. 14	No. 19	No. 20
Aerobic growth[a]	+	+	+
Anaerobic growth[a]	−	−	−
Growth of broth agar[a]	+	+	+
Growth of Mac Conkey's agar[a]	−	−	−
Gelatin liquefaction test[a]	−	−	−
Litmus milk test[a]	−	−	−

[a] −, negative; +, positive.

them grow up in the ordinary agar mediums to lot them. The secondary screening experiments resulted in obtaining 21 strains as the secondary isolated strains, which were believed to be identical with each other and were consolidated out of 175 strains. The secondary isolated strains obtained through the secondary screening experiments were searched out for the purpose of identifying the genera of the strains, by confirming the quality of mycology with morphology (Table IV), growth (Table V), physiology (Table VI), the resolution of carbohydrate (Table VII), and the existence or nonexistence of the activity of enzyme (Table VIII), in accordance with Bergey's Manual of Determinative Bacteriology (8th ed.). The following number of genera was determined to be identical with each other: Bordetella bacterium was 1 strain, Neisseria bacteria 2 strains, Moraxella bacteria 3 strains, Flavobacterium bacteria 2 strains, Acinetobacter bacteria 1 strain, and Bacillus bacteria 3 strains. Only the chosen results of strains were mentioned in the table, because if all the data were described, they would be vast. The degradation ability of strain Nos.14, 19, and 20 was judged to be high in Acquisition of microbes with degradation ability to LDPE section.

TABLE VI
Quality of Mycology (Physiology)

Test items	Sample code		
	No. 14	No. 19	No. 20
Gram stain[a]	+	+	+
Catalase test[a]	+	+	+
Oxidase test[a]	−	+	−
Glucose (product of acid)[a]	+	+	+
Glucose (product of gas)[a]	−	−	−
Glucose (F/O/−)[b]	−	O	O
Properties of acid–fast[a]	−	−	−
Voges–Proskauer test[a]	+	−	+
Nitrate reduction test[a]	+	−	++
Hydrolysis of starch[a]	+	−	+
Hydrogen sulfide test[a]	−	−	−
Indole test[a]	+	−	+
Deoxyribonuclease test[a]	−	−	−
Urease test[a]	+	++	±

[a] −, negative; +, positive; ±, both detective; ++, positive strongly.
[b] O, glucose oxidative; F, glucose fermentative.

TABLE VII
Quality of Mycology (Resolution of Carbohydrate)

Test items	Sample code		
	No. 14	No. 19	No. 20
Pentose: D-Xylose[a]	−	+	−
Methyl pentose: L-Xylose[a]	−	−	−
Hexose			
Galactose[a]	+	−	−
D-Glucose[a]	+	+	−
Disaccharide			
Maltose[a]	+	−	−
Lactose[a]	+	−	−
Sucrose[a]	+	−	−
Trisaccharide: Raffinose[a]	+	−	−
Polysaccharide soluble starch[a]	+	−	+
Glycoside: Aesculin[a]	+	+	+
Alcohol			
Mannitol[a]	+	+	−
Sorbitol[a]	+	+	−
Inositol[a]	+	+	−

[a] −, negative, +, positive.

Acquisition of microbes with degradation ability to LDPE

The assimilation tests were applied to the 21 strains (strain Nos. 1 ∼ 21) obtained by the procedures shown in the Evaluation of microbial degradation ability to LDPE section to examine whether they were able to actually degrade LDPE films, and as a result, three strains (strain Nos. 14, 19, and 20) believed to have high degradation ability were acquired. The bacteriological natures of the three strain Nos. 14, 19, and 20, whose natures were determined in the Evaluation of microbial degradation ability to LDPE section, were the same as those of aerobe bacilli, which were gram-positive bacteria and were forming endospore, and all the three strains were consequently identified as the strains of Bacilli.

TABLE VIII
Quality of Mycology (Activity of Enzyme)

Test items	Sample code		
	No. 14	No. 19	No. 20
β-Galactosidase[a]	+	−	−
Arginine dehydrodase[a]	−	−	−
Lysine decarboxylase[a]	−	−	−
Ornithine decarboxylase[a]	−	−	−
Urease[a]	+	++	±
Deoxyribonuclease[a]	−	−	−
Tryptophane aminase[a]	−	−	−

[a] −, negative, +, positive, ±, both detective, ++, positive strongly.

TABLE IX
Quality of Mycology (Resolution of Carbohydrate/Oxidation)

Test items	Sample code No. 14	No. 19	No. 20
Glycerol[a]	−	−	−
Erythritol[a]	−	−	−
D-Arabinose[a]	−	−	−
L-Arabinose[a]	−	−	−
Ribose[a]	−	−	−
D-Xylose[a]	−	+	−
L-Xylose[a]	−	−	−
Adonitol[a]	−	−	−
β-Methyl-D-xyloide[a]	−	−	−
Galactose[a]	+	−	−
D-Glucose[a]	+	+	−
D-Fructose[a]	+	+	+
D-Mannose[a]	−	−	−
L-Sorbose[a]	−	−	−
Rhamnose[a]	−	−	−
Dulcitol[a]	−	−	−
Inositol[a]	+	+	−
Mannitol[a]	+	+	−
Sorbitol[a]	+	+	−
α-Methyl-D-mannoside[a]	−	−	−
α-Methyl-D-glucoside[a]	−	−	−
N-Acetylglucosamine[a]	−	−	−
Amigdalin[a]	−	−	−
Arubutin[a]	−	−	−
Aesculin[a]	+	+	+
Salicin[a]	−	−	−
Cellobiose[a]	−	−	−
Maltose[a]	+	−	−
Lactose[a]	+	−	−
Meribiose[a]	+	−	−
Sucrose[a]	+	−	−
Trehalose[a]	−	−	−
Inulin[a]	−	−	−
Melezitose[a]	+	−	−
Raffinose[a]	+	−	−
Amidone[a]	−	−	−
Glycogen[a]	−	−	−
Xylitol[a]	−	−	−
Gentibiose[a]	−	−	−
D-Turanose[a]	+	−	−
D-Lyxose[a]	−	−	−
D-Tagatose[a]	−	−	−
D-Fucose[a]	−	−	−
L-Fucose[a]	−	−	−
D-Arabitol[a]	+	+	+
L- Arabitol[a]	−	−	−
Gluconate[a]	+	+	+
2-Ketogluconate[a]	−	−	−
5-Ketogluconate[a]	+	−	−

[a] −, negative; +, positive.

Identification of *Bacillus* species

As to the three strains of Nos. 14, 19, and 20 identified as bacilli, the determination of identification was carried out with the researching kits on bacillus biochemical natures, available in the markets, to further develop the identification of their species from the viewpoint of biochemical natures (Table IX). As a result, No. 14 was identified as *Bacillus circulans*, No. 19 as *Bacillus brevies*, and No. 20 as *Bacillus sphaericus*. These three strains were deposited to

TABLE X
Identified Microbes Using LDPE as a Sole Source of Carbon

Sample code	Identified microbes	Deposit code
No. 14	*Bacillus circulans*	FERM P-16400
No. 19	*Bacillus brevies*	FERM P-16401
No. 20	*Bacillus sphaericus*	FERM P-16402

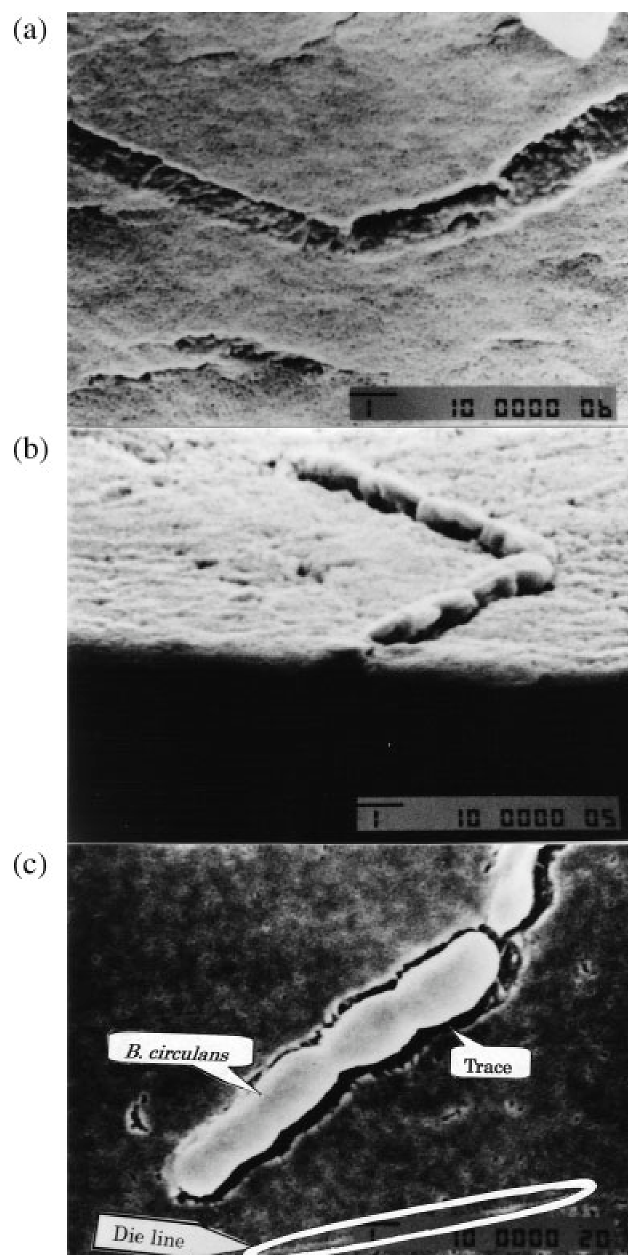

Figure 6 SEM images of the LDPE film degraded by cultivation of *Bacillus circulans* No. 14. (a) Body marks after cleaning LDPE film. (b) Microbes attached to LDPE film before cleaning. (c) Traces of degradation around a microbe and extruder die line.

National Institute of Bioscience and Human of National Institute of Advanced Industrial Science and Technology on the date of September 3, 1997. Each of the acceptance numbers was FERM P-16400, FERM P-16401, and FERM P-16402, respectively.[16] Table X collectively shows the LDPE-degrading bacteria that were identified.

Determination of degradation ability to LDPE

As to the three strains identified by the procedures shown in the Acquisition of microbes with degradation ability to LDPE section, the assimilation tests were applied to them to examine their degradation ability to LDPE films as follows. The bacteria bodies cultivated up to a logarithmic growth phase on the ordinary agar mediums were treated with aseptic centrifugal washing, and a large quantity of the harvested bacteria bodies was inoculated in the LDPE liquid mediums which were added with several fragments of the LDPE-F31N films degraded by outdoor exposures for 6 weeks. These LDPE liquid mediums were vigorously shaken at 28°C to make bacteria react with the fragments. In addition, the half volume of the medium of the LDPE liquid mediums was replaced every 2 weeks to prevent that the degradative reaction of microbes could be weakened by the lack of the inorganic salts. The fragments were taken out of the LDPE liquid mediums after 1 and 3 months, respectively, to evaluate their degradation ability by the same procedures mentioned in the Evaluation of microbial degradation ability to LDPE section. All the fragments taken from the mediums exhibited the body marks of bacteria on their surfaces as a proof of the existence of their degradation, and in particular, the strain of No. 14 yielded the noticeable body marks. Figure 6 presents observation photographs taken with SEM, showing the fragments of the LDPE-F31N films degraded by the strain No. 14 of *Bacillus circulans*. It was discerned that the fragment surfaces of the LDPE-F31N films surrounding the strain No. 14 of *Bacillus circulans* were degraded. Also, fine die marks made at the time of the inflation process of films were discovered on the fragment surfaces of the LDPE-F31N films in the same viewing field of SEM by coincidence. These observations have come to definitive evidence for the findings that the strain No. 14 of *Bacillus circulans* has been assimilating the surfaces of the LDPE-F31N inflation processed films with high-molecular weight that were put into the tests. Hence these observation results of the degradation condition with SEM stated earlier have come to the evidence to fully support the findings that LDPE films are degradable by microbes.

CONCLUSIONS

The isolation and identification of microbes which have degradation ability to LDPE were carried out. As a result, *Bacillus circulans*, *Bacillus brevies*, and *Bacillus sphaericus* were identified as the microbes which specifically degrade LDPE. These microbes are a kind of the Natto bacteria and are habitually present in soil, and hence, it is considered that these microbes are scattered around us and are distributed across the country, particularly in Japan where the culture of rice is popularly conducted. We have thereby considered that LDPE films, which were scrapped on soil after the generation of photodegradation by outdoor exposure to the sunshine, have opportunities to develop their degradation due to the microbes, whenever the conditions of degradation are ready.

References

1. Ohtake, Y.; Gomi, Y.; Kobayashi, T.; Itoh, S.; Hyakutake, K.; Yabuki, M. Nippon Gomu Kyokaishi 1991, 64, 677.
2. Ohtake, Y.; Kobayashi, T.; Gomi, Y.; Itoh, S.; Hyakutake, K.; Yabuki, M. Nippon Gomu Kyokaishi 1991, 64, 688.
3. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Yamamoto, Y.; Yabuki, M.; Asabe, H.; Ono, K. Nippon Gomu Kyokaishi 1993, 66, 266.
4. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Ono, K. Nippon Gomu Kyokaishi 1993, 66, 504.
5. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Ono, K. Nippon Gomu Kyokaishi 1993, 66, 756.
6. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Ono, K. Nippon Gomu Kyokaishi 1994, 67, 448.
7. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Murakami, N.; Ono, K. Nippon Gomu Kyokaishi 1994, 67, 636.
8. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Murakami, N.; Ono, K. Nippon Gomu Kyokaishi 1994, 67, 698.
9. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Murakami, N.; Ono, K. Nippon Gomu Kyokaishi 1995, 68, 551.
10. Ohtake, Y.; Kobayashi, T.; Itoh, S.; Asabe, H.; Yabuki, M.; Murakami, N.; Ono, K. Nippon Gomu Kyokaishi 1995, 68, 808.
11. Ohtake, Y.; Kobayashi, T.; Asabe, H.; Yabuki, M.; Murakami, N.; Ono, K. Nippon Kagakukaishi 1996, 4, 325.
12. Ohtake, Y.; Kobayashi, T.; Asabe, H.; Yabuki, M.; Murakami, N.; Ono, K. Nippon Kagakukaishi 1996, 10, 853.
13. Ohtake, Y.; Kobayashi, T.; Asabe, H.; Murakami, N.; Ono, K. J Appl Polym Sci 1995, 56, 1789.
14. Ohtake, Y.; Kobayashi, T.; Asabe, H.; Murakami, N.; Ono, K. J Appl Polym Sci 1998, 70, 1643.
15. Ohtake, Y.; Kobayashi, T.; Asabe, H.; Murakami, N. Polym Degrad Stab 1998, 60, 79.
16. Ohtake, Y.; Shiraga, K.; Asabe, H.; Koyama, F.; Makino, H.; Ohmura, H.; Nagai, F.; Murakami, N.; Hirose, A.; Okuda, K.; Takagawa, S. Jpn Pat. No. 1999-155562 (1999).
17. Buchanan, R. E.; Gibbons, N. E., Eds. Bergey's Manual of Determinative Bacteriology, 8th ed.; Williams and Wilkins: Baltimore, 1974.