Control of marine biodegradation of an aliphatic polyester using endospores

Miwa Suzuki[a], Yuya Tachibana[a, b], Phouvilay Soulenthone[b], Tomoya Suzuki[b], Hiroyuki Takeno[a,b], and Ken-ichi Kasuya[a, b]*

[a]Gunma University Center for Food Science and Wellness, 4-2 Aramaki, Maebashi, Gunma 371-8510, Japan
[b]Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin, Kiryu, Gunma 371-8510, Japan

*Corresponding author: Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin, Kiryu, Gunma376-8515, Japan; E-mail: kkasuya@gunma-u.ac.jp

Running title: Marine biodegradation of plastics using endospores

Abstract

Currently, commercially available biodegradable plastics are not well controlled in terms of the rate and timing of biodegradation. To overcome these challenges, several methods have been proposed. Herein, we propose a new system for controlling the onset and degradation rate of plastic biodegradation by encapsulating endospores of degrading microorganisms into plastics with typically slow biodegradation rates. Endospores of bacterial strain YKCMOAS1 germinated and initiated biodegradation when triggered by surface wear of an aliphatic biodegradable polyester, poly(ethylene succinate) (PESu). The weight loss rate of endospore-containing PESu film immersed in seawater was approximately 7-fold faster than that of PESu film without endospores. The YKCMOAS1 strain used in this study grew in seawater and showed PESu film-degrading activity in a marine mineral medium. Based on the weight loss rate of the endospore-containing PESu films, they were estimated to have completely degraded into water-soluble compounds within approximately 400 d. Meanwhile, the biochemical oxygen demand degradation test revealed that the water-soluble products from PESu, which were produced by the vegetative cells were completely biodegraded within 10 d. Taken together, these results indicate that material hydrolysis by bacterial strain YKCMOAS1 was triggered by material wear, followed by rapid biodegradation. This is the first report of the application of bioaugmentation to the stable expression of environmental degradation of biodegradable plastics. These results might provide insight into the design concept of novel biodegradable plastics that not only exhibit environmentally independent biodegradation, but also allow control of the onset time.

Keywords: Marine biodegradation; Endospore; *Bacillus*; Poly(ethylene succinate) (PESu); Biodegradable polymer; Biochemical oxygen demand (BOD)

1. Introduction

Biodegradable plastics are attracting attention as a solution to environmental pollution caused by plastic waste. Microorganisms in the environment can reduce biodegradable plastics to lower molecular mass products through enzymatic and other catalytic substance activity, eventually mineralizing the plastics. Poly(3-hydroxyalkanoic acid) (PHA)s, which are biodegradable plastics produced by some bacteria, are known to biodegrade in any environment, including marine environments, because the microorganisms that degrade PHAs are widely distributed in various environments[1]. In contrast, the environmental degradability of known synthetic biodegradable plastics, such as poly(ethylene succinate) (PESu; Fig. 1), poly(butylene succinate) (PBSu), poly(butylene succinate-*co*-adipate) (PBSA), poly(butylene adipate-*co*-terephthalate) (PBAT), and poly(lactic acid) (PLA), varies depending on environments[2]. The environmental dependence of the biodegradability of PESu is of particular interest because it is known to be highly biodegradable in terrestrial environments but not in marine environments [3–5].

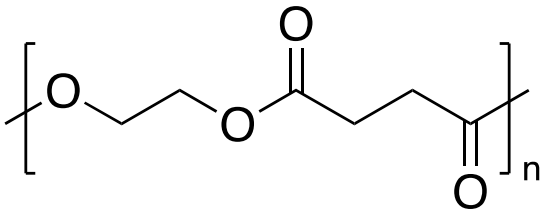

Fig. 1. Structure of poly(ethylene succinate) (PESu).

Thus, most chemically synthesized biodegradable plastics, including PESu, could exhibit little to very low biodegradability in a specific environment in which they are used. In many cases, biodegradable plastics are molecularly designed for environmental runoff; however, the fact that there are environments in which they do not degrade could severely limit their use. Considering that one of the final destinations of plastic waste spilling into the environment is the ocean, it is important to improve the marine biodegradability of such materials.

Herein, we propose the use of endospores to accelerate the marine biodegradation rate of PESu. Endospore-forming bacteria of the genus *Bacillus* have been isolated and characterized as degraders of chemically synthesized biodegradable plastics, including PESu[5–7]. Endospores, a metabolically dormant cell form, exhibit a high degree of resistance to various stresses, including thermostability above the melting point (around 140 °C) of typical chemically synthesized biodegradable plastics[8]. The thermostability of endospores enables their encapsulation in chemically synthesized plastics using melt blending. When the capsular materials are subjected to wear, the endospores are exposed and germinate in the presence of water, releasing stimulants and plastic-degrading enzymes. The resulting enzymatic hydrolysis of materials [9] is expected to lead to biodegradation.

In this study, we screened *Bacillus* endospores to determine their PESu hydrolytic activity in seawater. We then prepared endospore-encapsulated PESu films and evaluated their biodegradability in seawater. The results could be applied to improve and control the timely degradation of biodegradable plastics in different environments, promoting the use of plastics that can be more widely degraded.

2. Materials and methods

2.1. Chemicals

Poly(ethylene succinate) (PESu; number-average molecular mass [Mn]=2.8×10^4, weight-average molecular weight [Mw]/Mn=2.5, melting temperature [Tm]=104 °C); poly(3-hydroxybutyrate) (P[3HB]; Mn=6.8×10^4, Mw/Mn=2.6, Tm=178 °C); poly(ε-caprolactone) (PCL; Mn=1.7×10^4, Mw/Mn=1.6, Tm=62 °C); poly(butylene succinate) (PBSu; Mn=2.1×10^4, Mw/Mn=1.9, Tm=113 °C), and poly(butylene adipate-*co*-terephthalate) (PBAT; Mn=1.9×10^4, Mw/Mn=1.7) were supplied by Nippon Shokubai Co., Ltd., Mitsubishi Gas Chemical Co., Inc., Daicel Co., Showa Denko K.K., and BASF Japan Ltd., respectively. The polymers were purified via precipitation[10]. Differential scanning calorimetry (DSC) (DSC-4000; PerkinElmer Co., Ltd., Yokohama, Japan) was conducted under a nitrogen gas atmosphere over a temperature range of 30–200 °C, at a heating rate of 10 °C/min. The degree of crystallinity was determined using DSC[11]. PESu was compressed into a film (thickness: 0.1 mm) at 15 MPa using a mini Test Press-10 (Toyo Seiki Seisaku-sho, Ltd.) at 110 °C for 1 min. After compression, the films were cooled to about 20 °C, washed with methanol and distilled water, and then dried *in vacuo*. Other chemicals were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. and Sigma-Aldrich Co., LLC. *Bacillus pumilus* NBRC 12092^T (type strain), which was used as a reference strain in this study, was provided by the National Institute of Technology and Evaluation. Seawater and sea sand samples used in this study were collected from Oarai, Ibaraki (36˚22'01.8"N, 140˚37'21.8"E), Japan.

2.2. Isolation and identification of PESu–degrading endospore-forming bacteria

Sea sand was heated to 120 °C for 30 min, cooled to room temperature, and then 1 g was suspended in distilled water (10 mL). The suspension was allowed to stand for 10 min, and then plated onto PESu-containing solid marine mineral (MM) medium (4.6 g/L KH_2PO_4, 11.6 g/L Na_2HPO_4, 1.0 g/L NH_4Cl, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $FeCl_3{\cdot}6H_2O$, 0.5 g/L dried yeast extract [YE; Nihon Pharmaceutical], 0.1 g/L Plysurf, 0.2% [w/v] emulsified PESu, 30 g/L NaCl, and 1.5% [w/v] agar, pH 7.0), and incubated at 25 °C for one week. The bacteria that formed a clear zone on the plate were isolated as presumptive PESu-degrading endospore-forming bacteria. For further purification, solid marine Luria-Bertani (MLB) medium (30 g/L NaCl, 10 g/L polypeptone, 5 g/L dried YE, and 1.5% [w/v] agar, pH 7.5) was used.

Genomic DNA (gDNA) was extracted from the isolate using a PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc.), according to the manufacturer's instructions. The gDNA was sequenced using a DNBSEQ-G400 (MGI Tech.) with 2 × 200 paired-end reads by the Bioengineering Laboratory. Co., Ltd., Kanagawa, Japan). Genome analysis was performed using the DOE Systems Biology Knowledge Base (http://kbase.us)[12]. The quality of the raw reads was analyzed using FastQC software [13], and trimming of the raw reads was performed using the program Trim Reads with Trimmomatic – v.0.36 software[14]. High-quality reads were assembled using Assembly Reads with the program SPAdes v.3.13.0[15]. The annotation of contigs was carried out using the program Rapid Annotations Subsystems Technology (RAST) toolkit v.0.1.1[16]. The average nucleotide identity (ANI) for the whole genome was used to delineate the species using Compute ANI with the program FastANI v.0.1.2[17]. The DNA sequence of the isolate was compared with GenBank sequences using the program translated BLAST (tBLASTn prot-nuc Search – v.2.9.0)[18,19]. A phylogenetic tree was constructed using the program Inserted Genome of the Species Tree v.2.2.0 [20].

2.3. YKCMOAS1 isolate degradation test of PESu films

The PESu-degrading endospore-forming bacterial isolate, strain YKCMOAS1, was cultured in liquid MM medium, artificial seawater (AS; 26.29 g/L NaCl, 0.74 g/L KCl, 0.99 g/L $CaCl_2$, 6.09 g/L $MgCl_3 \cdot 6H_2O$, and 3.94 g/L $MgSO_4 \cdot 7H_2O$, pH 7.8[21]), or marine broth 2216 (MB; Difco) (3 mL), each of which were supplemented with PESu film (1 cm × 1 cm) at 30 °C for 10 d under aerobic conditions (120 strokes/min) (n=3). Control experiments were performed without bacteria. The film was subsequently recovered from each medium and washed with methanol and distilled water. The weight loss of the film was evaluated by subtracting the weight after degradation from the initial weight.

The water soluble products produced by the strain culture were measured by total organic carbon (TOC) using the following procedure. Strain YKCMOAS1 was cultured in liquid MM medium supplemented with PESu film (1 cm × 1 cm) at 30 °C for 1, 3, 5, and 10 d under aerobic conditions (120 strokes/min) (n=3). Control experiments were performed as cultures on the only strain, or only PESu film. The films were then recovered from the media, washed with methanol and distilled water, and weight loss determined as described above. The culture media were centrifuged at 2290 *g* for 10 min at 4 °C, and the dissolved organic carbon (DOC) concentration in the culture supernatants was measured using a TOC-LCPH/CPN instrument (SHIMADZU Corp.).

2.4. Characterization of PESu-degrading endospore-forming isolate strain YKCMOAS1

Strain YKCMOAS1 was plated on five polyester-containing plates (P(3HB)), PCL, PBSu, PBSA, and PBAT) and cultured for one week at 25 °C. The hydrolytic activities of the strain were evaluated using the clear zone method[22]. Strain YKCMOAS1 was cultured in liquid MM medium, which was supplemented with PESu film (1 cm × 1 cm) at 30 °C for 10 d under aerobic conditions (120 strokes/min) (n=3). A control experiment was performed in the absence of bacteria. The film was then recovered from the medium, washed with methanol and distilled water, and weight loss determined as described in 2.3.

To examine the effect of NaCl concentration on growth, the following experiment was performed. YKCMOAS1 was cultivated in MLB medium (100 mL) without NaCl at 30 °C for 24 h under shaking culture conditions (120 strokes/min). An aliquot of the culture (10 mL) was then inoculated into MLB medium (90 mL) containing different concentrations of NaCl (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.8 M). Growth was evaluated by measuring the optical density at 600 nm (OD600) after incubation at 30 °C for 1-5 h. Growth rates were calculated using the linear differential equation: $dN/dt = \mu N$, where N is the number of bacterial cells as measured by OD600, t is the time, and μ is the specific growth rate. Next, the effect of NaCl concentration on PESu-degradation ability of strain YKCMOAS1 was also evaluated after incubation for one week at 30 °C. Specifically, the distance between the center of each colony and the edge of the clear zone was measured on PESu-containing MM medium plates supplemented with different concentrations of NaCl (0, 0.08, 0.17, 0.35, 0.50, 0.70, 1.00, 1.35, and 1.70 M).

2.5. Morphological observations

PESu films incubated with strain YKCMOAS1 were gold-coated after fixation, as previously described [23] and observed using a scanning electron microscope (SEM; JCM-7000 NeoScope™, JEOL Ltd., Tokyo, Japan). Strain YKCMOAS1 and a spore-containing PESu film were also platinum-coated after fixation, as previously described [23], and observed using a

field-emission SEM (FE-SEM; JSM-6700F, JEOL Ltd.) and an SEM (JSM-IT500HR, JEOL Ltd.), respectively. Optical photographs were taken using an EOS Kiss X2 EF-S 18-55 IS (Canon Inc., Tokyo, Japan) equipped with a Canon ZOOM LENS EF-S 18–55 mm 1:3.5–5.6 IS.

2.6. Endospore preparation

Strain YKCMOAS1 was incubated in 1L of Schaeffer's sporulation liquid medium (8 g nutrient broth, 10 mL of 10% KCl, 10 mL of 1.2% $MgSO_4 \cdot 7H_2O$, 0.5 mL of 1 M NaOH, 1 mL of 1 M $Ca(NO_3)_2$, 1 mL of 0.01 M $MnCl_2$, and 1 mL of 1 mM $FeSO_4$ [24]) at 30 °C for 4 d under aerobic conditions (120 stroke/min). The culture medium was then centrifuged (5000 rpm, 10 min, 4 °C) to recover the endospores. After washing three times with sterilized distilled water, the endospores were lyophilized using a FREEZE DRYER FRD-mini (AGC TECHNO GLASS Co., Ltd., Shizuoka, Japan), and stored at 4 °C before use.

2.7. Decimal reduction time (D value) of the endospores

As a thermostability index, the D value, which is defined as the time required at each temperature to sterilize 90% of the microbes, was used[25]. The endospores were heated at each temperature (100, 110, and 120 °C) for 5, 10, 15, 30, and 60 min, and then suspended in sterile purified water. Diluted suspensions (10^0 to 10^{-6}) were then plated on solid MLB medium and incubated at 25 °C for 2 d. The number of colonies on each plate was then counted, and the logarithm of each number was plotted against the corresponding heating time to calculate each D value.

2.8. Substances affecting endospore germination

The efficacy of three substances (YE, L-alanine, and a mixture of L-asparagine and glucose) as candidates for germination-inducing substances of strain YKCMOAS1 endospores was investigated. An endospore suspension in sterile distilled water (0.01 g/mL) was added to AS solid medium containing each test substance (0.5 g/L), and incubation was carried out at 25 °C for 5 d. Spore germination was confirmed by growth of the strain on each medium; colonies were counted and expressed as colony formation/g of endospores.

2.9. Preparation of endospore-containing PESu (esPESu) and sandwiched esPESu (SDesPESu) films

The procedure for preparing esPESu and SDesPESu films is shown in Fig. S1. A mixture of strain YKCMOAS1 endospores (0.01 g) and YE (0.05 g) was placed between two PESu films (5 cm × 5 cm), and then subjected to hot-pressing at 5 MPa and 110 °C for 15 s to produce esPESu. After slow cooling of the esPESu film, degradation tests were performed in media (see 2.10). The esPESu film was cut into rectangular pieces (5 cm × 1 cm). One strip each was placed on two opposite ends of a PESu film (8 × 8 cm) and then overlaid (sandwiched between) with another PESu film. The final sandwiched film was formed by pressing the sandwich at 5 MPa and 110 °C for 15 s. After slow cooling, the SDesPESu films were cut into rectangular SDesPESu specimen strips (8 cm × 1 cm). The SDesPESu specimens were used in degradation tests in natural seawater (see 2.11). To analyze the molecular mass, tensile strength, and surface morphology, PESu specimens (6 × 1 cm) were prepared by cutting off both ends of the SDesPESu specimen, which contained endospores, leaving the center area of the SDesPESu (Fig. S1).

2.10. Degradation of esPESu film in media

To expose the endospores on the esPESu film, the surface of the film (2 cm × 2 cm) was scratched (0.1 cm × 0.1 cm) using a box cutter. Liquid MM and MB media (100 mL) supplemented with the scratched films were incubated at 30 °C for 10 d under aerobic conditions (120 strokes/min) (n=3). Control experiments were performed using the same media as the PESu films, but without endospores. After the films were recovered, they were washed with methanol and water. The degradation of the films was calculated by subtracting the weight after incubation from the initial weight.

2.11. Degradation of SDesPESu films in natural seawater

To expose the endospores in the SDesPESu films, both ends of the SDesPESu specimen strip were scratched using a cutter (0.1 cm × 0.1 cm). Seawater (100 mL), collected from Oarai, Ibaraki, Japan (36˚22’01.8"N, 140˚37'21.8"E), was supplemented with SDesPESu film and NH_4Cl (0.05 g/L) and incubated at 30 °C for 5, 10, and 15 d under aerobic conditions (n=3). Control experiments were performed using the same seawater that was used for the PESu film, which did not contain endospores. After the films were recovered, they were washed with distilled water, and the degradation of the films was calculated as described in 2.10. The degradation rate was calculated from the slopes of the plots between days 5 and 15 (Fig. 7(a)).

2.12. Molecular mass determination

The molecular mass of PESu specimens was analyzed using gel permeation chromatography (GPC) and an LC-2000 Plus Series system (JASCO Co., Ltd.) with three columns (TSKgelGMHXL, TSKgelG1000HXL, and TSKgelG200HXL; TOSOH Co.) in a column oven (JASCO CO-4060); incubation was at 40 °C with a flow rate of 1.0 mL/min, as described previously [26]. Chloroform was used as the mobile phase and polystyrene, with a narrow molecular mass distribution, was used as the molecular mass standard (TSKgel Standard Polystyrene, TOSOH Corp., Japan).

2.13. Tensile testing

The SDesPESu and PESu specimens were dried *in vacuo* at 20 ˚C. Mechanical properties of SDesPESu specimens were investigated after incubation at 30 °C for 0, 5, 10, and 15 d in natural seawater. Tensile testing of a section of the PESu specimen (3 cm × 0.5 cm, without the part of the PESu film containing spores, n=3) was performed using an ORIENTEC TENSILE TESTER STM-20 at a stretching speed of 10 mm/min.

2.14. Biochemical oxygen demand (BOD) biodegradation testing

The BOD biodegradability of PESu, PESu hydrolysates, ethylene glycol, succinic acid, and cellulose in seawater was determined using BOD measurement devices (OxiTop IDS[/B) measuring head with a 300 mL BOD reactor, WTW GmbH, Weilheim, Germany), according to the ASTM D6691-17 standard[27]. The YKCMOAS1 strain was cultured in MM medium with PESu film for 10 d at 30 °C; the film disappeared completely and only a water-soluble product was obtained. The culture was centrifuged and the resulting culture supernatant was used as the PESu hydrolysate. The DOC concentration in the culture supernatants was measured using a TOC-LCPH/CPN instrument (SHIMADZU Corp.). A sample (less than 5 mg as carbon) and seawater with inorganic nutrients (0.1 g/L KH_2PO_4, 0.5 g/L NH_4Cl, and 0.005 g/L N-allylthiourea, 200 mL) was added to the BOD reactor. The OxiTop IDS(/B) measuring head was attached to the head of the BOD reactor, and

the reactor was incubated at 30 ˚C. The BOD-biodegradability of the sample was defined as follows: BOD-biodegradability (%) = [(BOD-sample − BOD-blank)/theoretical oxygen demand (ThOD)] × 100. BOD-sample and BOD-blank are the experimentally observed values of the oxygen demand of the sample and blank media, respectively. The ThOD is a theoretically calculated value for the oxygen demand of a sample, which is obtained by assuming that the sample is completely degraded to CO_2 and H_2O.

2.15. Nucleotide sequence accession numbers

The nucleotide sequence of the 16S rRNA gene of strain YKCMOAS1 was deposited in the DDBJ nucleotide sequence database under accession number LC278954.1. The contigs and annotated data, based on the whole-genome shotgun sequencing of strain YKCMOAS1, were deposited in the DDBJ Whole Genome Shotgun (WGS) under accession numbers BSEI01000001–BSEI01000022.

3. Results

3.1. Characterization of an endospore-forming bacterium that degrades PESu, isolated from sea sand

In an attempt to isolate endospore-forming bacteria from sea sand, the sand was heated at 120 °C for 30 min and used as an inoculum source on a PESu-containing MM medium plate. A bacterium that formed a clear zone around the colony was isolated and designated strain YKCMOAS1. The isolated bacterium was rod-shaped (Fig. 2), and its genome exhibited an ANI of 95.9% and 91.2% against the endospore forming bacteria *Bacillus safensis* (GCF_000691165.1) and *B. pumilus* (GCF_001578205.1), respectively. A species tree was constructed using a set of 49 core universal genes defined by the clusters of orthologous group (COG) gene families (Fig. 3). Genome analysis revealed the presence of a gene encoding homologous protein OAS1_26140 lipase EstA, with high homology to PBAT hydrolase from *B. pumilus* NKCM3201 ($PBATH_{Bp}$, BAV72205.1, identity level 99.1%) in YKCMOAS1.

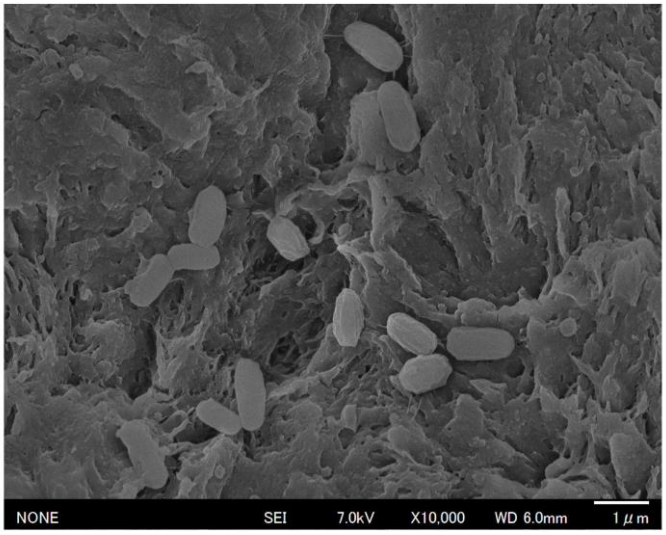

Fig. 2. Field emission scanning electronic micrograph of strain YKCMOAS1. Scale bar indicates 1 μm.

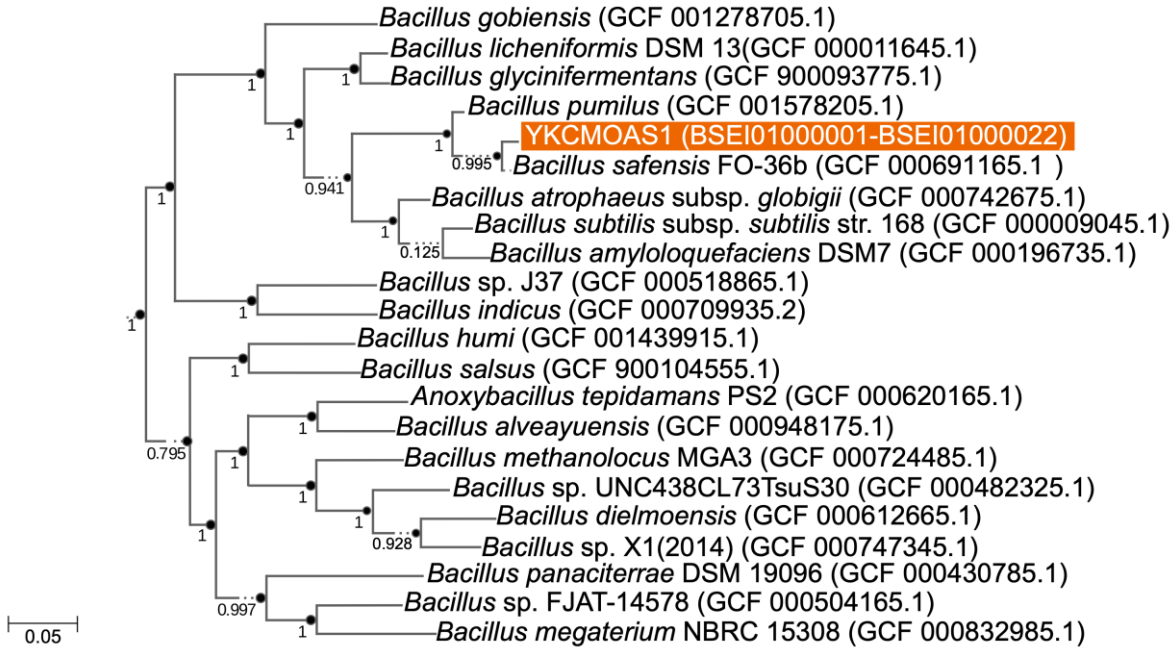

Fig. 3. Species tree using a set of 49 core, universal genes defined by clusters of orthologous group (COG) gene families. GCF identifies are given in parentheses. The bar indicates 5% estimated difference in the sequence.

Strain YKCMOA1 grew in NaCl concentrations ranging from 0.0 to 1.4 M (Fig. S2). The strain formed a clear zone on MM medium plates containing PESu and PBSA emulsions, whereas it did not form clear zones on plates containing P(3HB), PCL, PBSu, or PBAT. Incubation of the strain for 10 d in liquid MM medium with PESu film resulted in a weight loss of 1.03 mg/cm^2 PESu film (Fig. 4A). The surface of the film appeared rougher as incubation time increased (Fig. 4B). In contrast, the surface appearance of the PESu film changed little after incubation for 10 d without the strain compared with that before incubation (Fig. 4B). The concentration of DOC in the MM medium in which the YKCMOAS1 strain was cultured with the PESu film decreased on day 1 of incubation and then increased over time (Fig. 4C). The concentration of DOC in the culture with the PESu film alone increased slightly over time, whereas that in the culture with the YKCMOAS1 strain alone did not change after the first day of incubation. The theoretical organic carbon content calculated from the weight loss of the PESu films incubated with YKCMOAS1 for 10 d was approximately 1.27 mg, whereas the average total DOC in the culture medium (3 mL) was 1.30 mg. This indicates that strain YKCMOAS1 could hydrolyze PESu to water-soluble products, but it could not further metabolize the PESu hydrolysates.

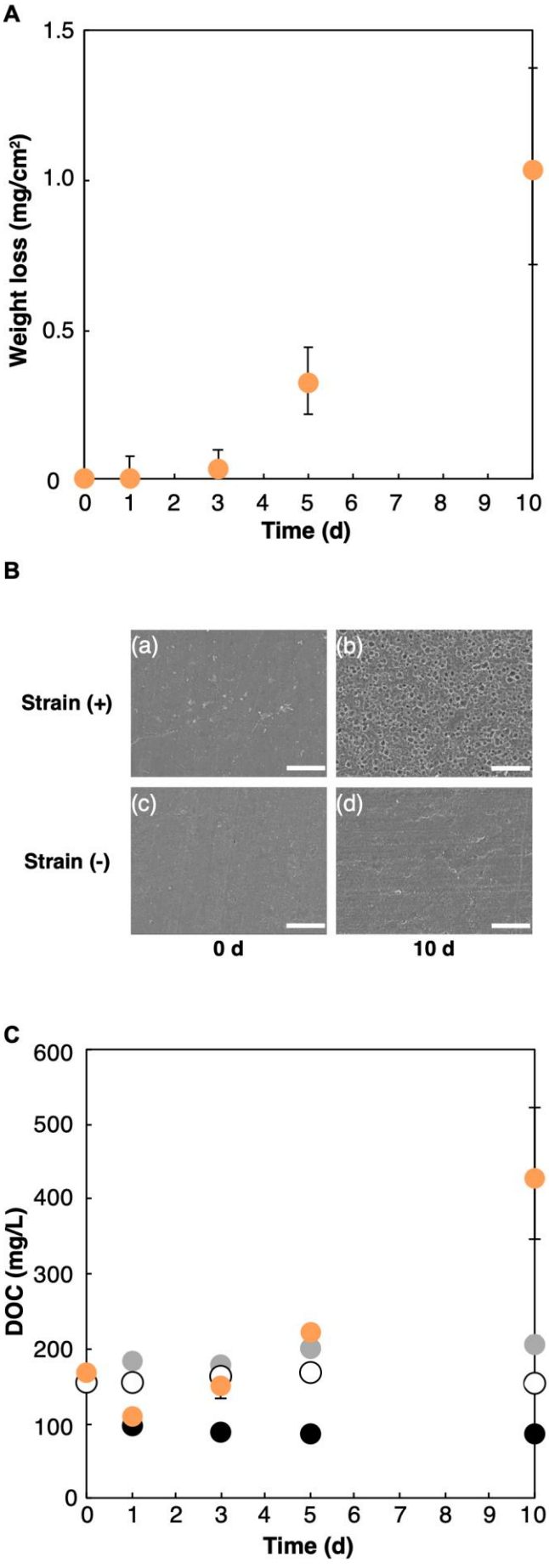
A
Weight loss (mg/cm²)
1.5
1.0
0.5
0
0 1 2 3 4 5 6 7 8 9 10
Time (d)
B
Strain (+)
Strain (-)
(a)
(b)
(c)
(d)
0 d
10 d
C
DOC (mg/L)
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Time (d)

Fig. 4. Biodegradation of PESu film by strain YKCMOAS1. (A) Weight loss of PESu films incubated with strain YKCMOAS1 in marine mineral (MM) medium at 30 °C. Error bars indicate minimum and maximum values of experimental data (n=3). (B) Scanning electron microscope (SEM) images of PESu film surfaces incubated with strain YKCMOAS1 (a and b) or without the strain (c and d) in MM medium at 30 °C for 1 d (a and c) or 10 d (b and d). White bars indicate 50 μm. (C) Concentration of dissolved organic carbon (DOC) in culture supernatants of MM media. Orange circles, PESu film cultured with strain YKCMOAS1; gray circles, PESu film cultured without strain YKCMOAS1; black circles, strain YKCMOAS1 cultured in MM medium without PESu film; white circles, MM medium alone. Error bars indicate maximum and minimum values (n=3).

The D value of YKCMOAS1 endospores, which reflected their thermostability, was 209, 27.8, and 8.3 min at 100, 110, and 120 °C, respectively. Germination-inducing substances were screened for endospore formation. The number of colonies formed per gram of endospores in AS containing each of the following substances was as follows: YE, 4.3×10^{11}; L-alanine, 0; mixture of glucose and L-asparagine, 1.4×10^{9}; and no added substance, 0 cfu/g. Among these, YE was the most effective and thus used as the germination-inducing substance.

3.2. BOD biodegradability of PESu and its degradation products by strain YKCMOAS1 in seawater

The BOD biodegradability in seawater of PESu, PESu with endospores, PESu hydrolysates produced by strain YKCMOAS1, PESu constituents ethylene glycol and succinic acid, and cellulose, was analyzed over a 15 d incubation at 30 °C. The respective BOD values at 15 d were 0, 0, 88.3, 82.7, 61.4, and 67.2 % (Fig. 5).

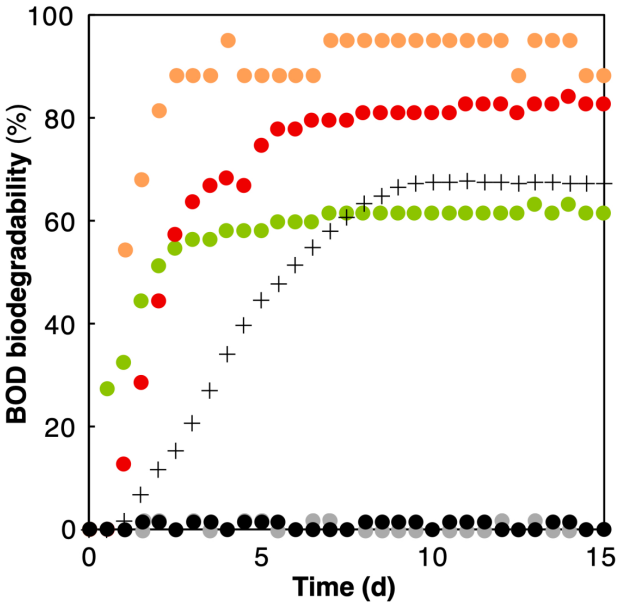

Fig. 5. Biochemical oxygen demand (BOD) biodegradation curves of PESu and various constituents in seawater. Gray circles, PESu; black circles, PESu with endospores; orange circles, PESu hydrolysates; red circles, ethylene glycol; green circles, succinic acid; and plus signs, cellulose.

3.3. Degradability of esPESu film in MM medium

Photographic observation of the esPESu film revealed that some of the endospores and YE aggregated, as evidence by white spots (Fig. 6a). SEM images showed the presence of endospores in sections of the esPESu film (Fig. 6b and c). The partially scratched esPESu film lost weight at a rate of 55.8 and 26.3 μg/cm^2/d when incubated for 10 d at 30 °C in the MM medium and AS, respectively.

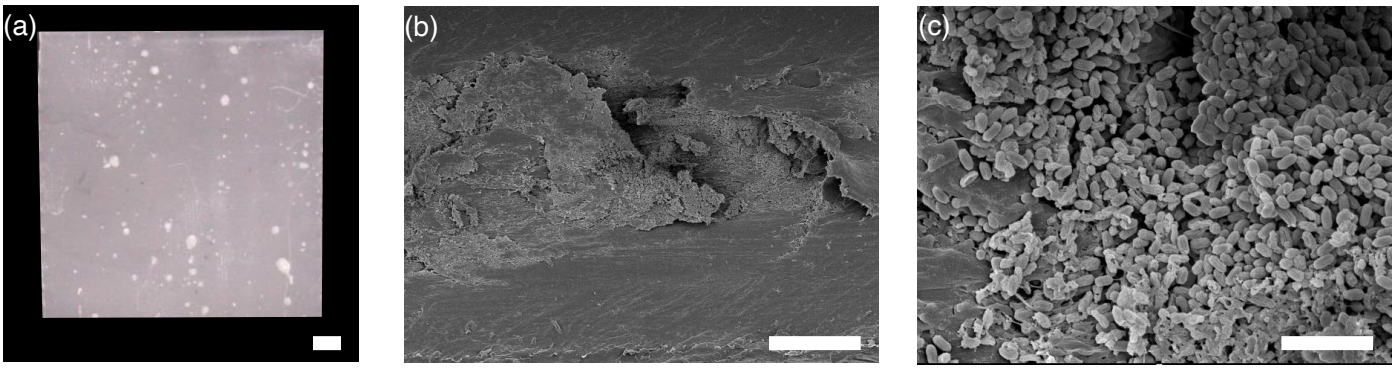

Fig. 6. Appearance of endospores-containing PESu (esPESu) film and morphology of the film section. (a) Photo image of esPESu film. Scale bar indicates 1mm. (b) SEM image shows the sections of esPESu films. (c) The image shows the magnified view of endospore-containing sections in (b). (b and c) SEM images of the film section were prepared by freeze-fracturing of esPESu film. Scale bars indicate (b) 50 μm and (c) 5 μm.

3.4. Degradation of SDesPESu film in seawater

Although tensile strength tests were performed on the PESu film containing endospores and yeast extracts (esPESu), the film tore immediately and no properties could be measured. Therefore, SDesPESu films containing esPESu at both ends were prepared (Fig. S1). To measure the mechanical properties, a specimen with parts of the esPESu film removed from the SDesPESu strip was used. The rate of weight loss in seawater of SDesPESu was 25.9 μg/cm^2/d, whereas that of PESu was 3.5 μg/cm^2/d (Fig. 7A). The surface roughness of the center area (6 cm × 1 cm) of the SDesPESu film increased with time of exposure to seawater, whereas the surface morphology of the PESu film without endospores changed little over time. In addition, microorganisms adhered to the surfaces of the SDesPESu samples (Fig. 7B, panels b and c). The tensile strength of SDesPESu decreased with increasing seawater immersion time, whereas that of PESu remained unchanged after seawater exposure (Fig. 7C). The elongation at break of the PESu specimens with and without endospores decreased with incubation time (Table S1, Fig. S3); the average molecular masses and crystallinities of PESu and SDesPESu changed little during incubation (Tables S2 and S3).

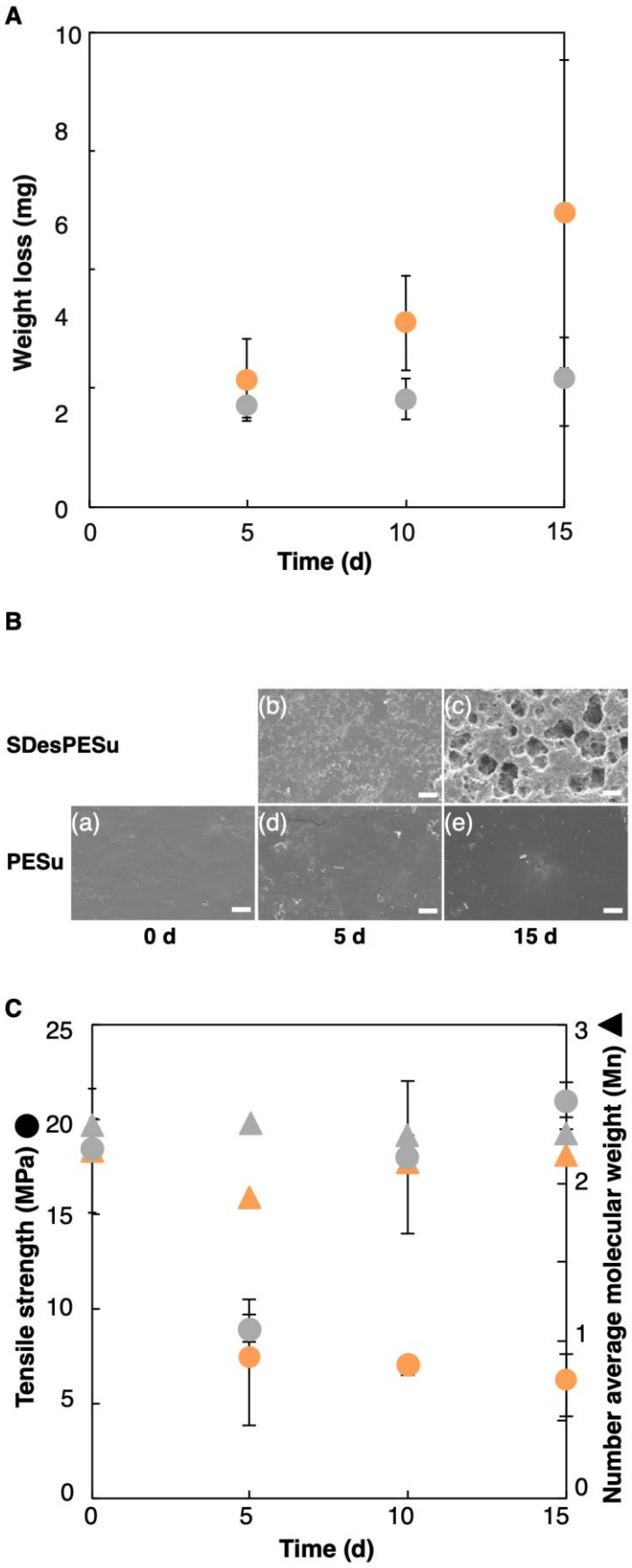
A
Weight loss (mg)
Time (d)
B
SDesPESu
PESu
(a)
(b)
(c)
(d)
(e)
0 d
5 d
15 d
C
Tensile strength (MPa)
Number average molecular weight (Mn)
Time (d)

Fig. 7. Degradation of the sandwiched esPESu (SDesPESu) and PESu films in natural seawater. (A) Weight loss of SDesPESu (orange circles) and PESu (gray circles) films in natural seawater at 30 °C over 15 d. Error bars indicate the standard deviation (n=3). (B) SEM images of SDesPESu film (b and c) and PESu film (a, d, and e) surfaces after immersion for 0 (a), 5 (b and d), and 15 d (c and e) in natural seawater. White bars indicate 10 μm. (C) Changes in tensile strengths and Mn of the center part of SDesPESu (orange circles and triangles, respectively) and PESu (gray circles and triangles, respectively) films during incubation in natural seawater. Error bars indicate the standard deviation for the tensile strength measurements (n=3).

4. Discussion

Currently, commercially available biodegradable plastics are not well-controlled in terms of their biodegradation rate and initiation of degradation. Therefore, there is a concern that they may degrade during use or storage or, conversely, remain in the environment without degradation. The addition of natural polysaccharides, such as cellulose[28], starch [29] calcium oxide[30], and plasticizers [31] to resins has been devised as a technology to control the environmental degradation rate of biodegradable plastics. Although these additions can control the environmental degradation rate of biodegradable plastics, they cannot control the timing of the initiation of biodegradation.

Several methods have been proposed for controlling the timing of biodegradation initiation triggered by environmental factors. One of these is the use of redox potential to cleave disulfide bonds; degradation is initiated when the material reaches a submarine or lake sediment with a low redox potential[32]. A pH switch that uses the weak alkalinity of the ocean as a trigger to initiate biodegradation of materials has also been proposed[33]. In addition to these environmental-factor-triggered methods for controlling the onset of degradation, a wear switch in which proteinase K is encapsulated in PLA triggers enzymatic degradation when the material wears out [34].

In this study, we constructed a new system for controlling the degradation rate and initiating the degradation of biodegradable plastics by encapsulating the endospores of degrading microorganisms into biodegradable plastics with a slow biodegradation rate. In this method, surface wear of the material triggers contact between dormant bacteria, i.e., endospores in the material, and water, causing the spores to germinate and initiate biodegradation of the plastic. As a type of bioremediation, bioaugmentation, in which microorganisms are added to the environment to promote the biodegradation of contaminants, is widely known[35–37]. The strategy used in this study to control the degradation of biodegradable plastics could be classified as an application of bioaugmentation to materials. To the best of our knowledge, this is the first report of such an application of endospores to control material biodegradability.

When PESu (esPESu and SDesPESu) film containing the endospores prepared in this study was immersed in seawater or artificial marine environments, the weight loss of the film was significantly higher than that of film without endospores. Microscopic observation of the SDesPESu film revealed an apparent increase in surface roughness and deteriorated mechanical properties. Despite the fact that the molecular masses changed little, the decrease in mechanical strength was presumed to be due to the roughening of the film surface. The endospore-forming PESu-degrading bacterial strain YKCMOAS1 used in this study grows in 0.5 M NaCl, a salinity similar to that of seawater. In addition, YKCMOAS1 exhibited PESu film-degrading activity in MM medium, suggesting that this strain may contribute to the hydrolysis of PESu in marine environments. Finally, a putative amino acid sequence (GLJ03365) encoded by the genome of YKCMOAS1 shows more than 99% homology to the polyester hydrolyzing enzyme *Bacillus pumilus* NKCM3201($PBATH_{Bp}$, BAV72205.1)[7], suggesting that the deduced protein is directly involved in the hydrolysis of PESu.

Biodegradation tests using PESu films indicated that strain YKCMOAS1 alone could not mineralize PESu hydrolysates, despite the fact that it hydrolyzed PESu into water-soluble compounds. In contrast, a BOD biodegradation test using seawater revealed that the hydrolysates were completely and rapidly mineralized. Taken together, these results suggest that the endospore-encapsulated PESu created in this study is completely mineralized in a real marine environment by a combination of strain YKCMOAS1 germinating and hydrolyzing PESu and other marine microorganisms subsequently fully mineralizing the degradation products (Fig. 8). Extrapolating from the weight loss rate of the SDesPESu film in seawater (25.9 μg/cm^2/d), it is estimated that the SDesPESu film used in this experiment (10.7 mg/cm^2) would take about

400 d to completely degrade to water soluble compounds. In addition, the SDesPESu degradation compounds generated by strain YKCMOAS1 did not go through an induction phase and showed 95% BOD biodegradation in 3 d, suggesting that SDesPESu is quickly and eventually mineralized after hydrolysis by the strain. This implies that the hydrolysis of the specimen by the strain was the rate-limiting step for biodegradation in seawater (Fig. 8, step 3). On the other hand, the hydrolysis rate of PESu film without endospores in seawater was about 1/7 (3.5 μg/cm^2/d) that of endospore-containing PESu film; thus, it is estimated that it would take about 7.7 years to completely biodegrade the PESu film.

PESu biodegrades at a relatively fast rate in freshwater and soil, but only slowly in the ocean[3, 5]. Therefore, a plastic with a very low biodegradation rate in a specific environment is defined as a potentially biodegradable plastic. Such biodegradable plastics are unlikely to lose their physical properties due to biodegradation during use, unlike PHA and PCL, which are readily biodegraded in any environment. Embedding endospores in such potentially biodegradable plastics would allow control over the time of initiation and the rate of degradation of the material. This technology could provide timely degradability to biodegradable plastics, i.e., it could guarantee the robustness of the material in use and promise rapid biodegradation after use. This improvement in the functionality of biodegradable plastics is expected to lead to their widespread use.

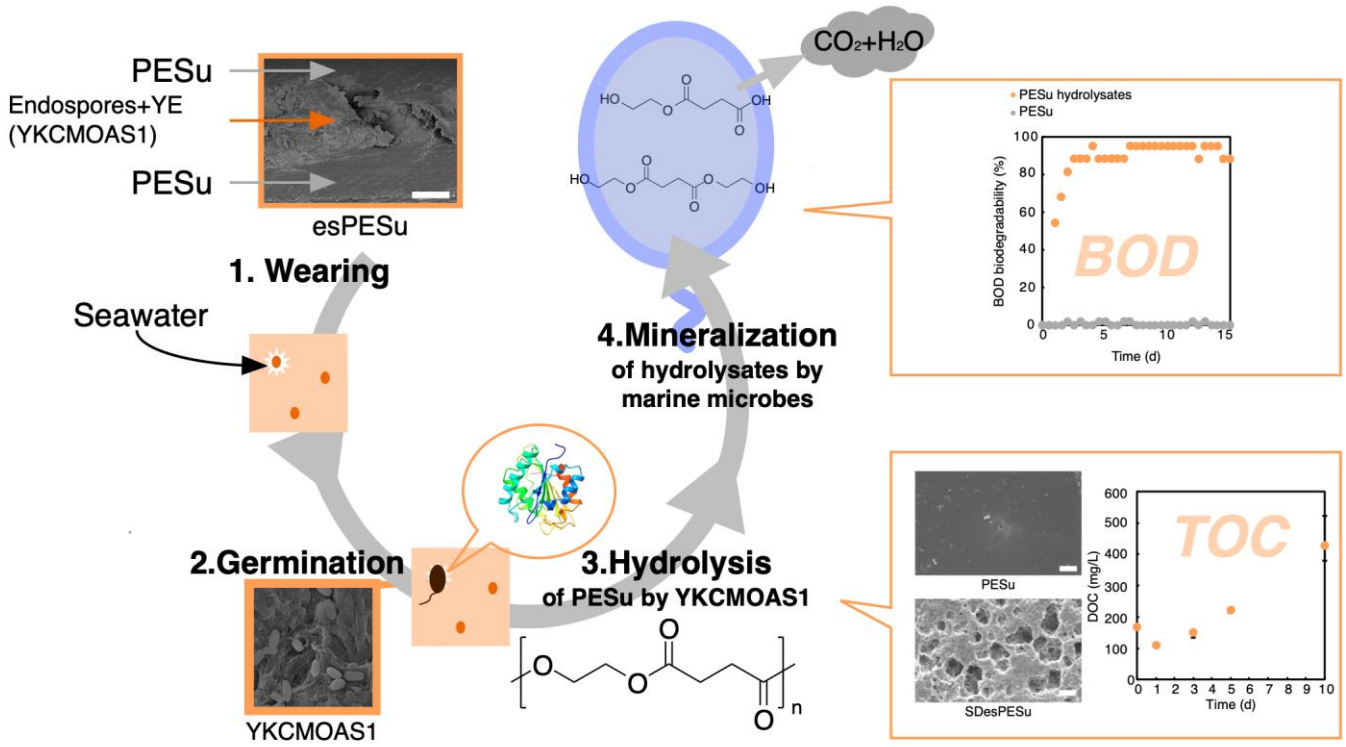

Fig. 8. Proposed mechanism of biodegradation by bacterial strain YKCMOAS1 of sandwiched esPESu (SDesPESu) in seawater. Endospore-containing PESu is abbreviated as esPESu.

Acknowledgement

We would like to thank Dr. Onda of the Gunma Industrial Technology Center for his help in taking the electron micrographs of the microbes.

Abbreviations

AS, artificial seawater; ANI, average nucleotide identity; BOD, biochemical oxygen demand; COG, clusters of orthologous group; DOC, dissolved organic carbon; DSC, differential scanning calorimetry; esPESu, endospore-containing PESu**;** gDNA, genomic DNA; GPC, gel permeation chromatography; MB, marine broth; MLB, marine Luria-Bertani; MM, marine mineral; Mn, number-average molecular mass; Mw, weight-average molecular mass; P(3HB), poly(3-hydroxybutyrate); PBAT, poly(butylene adipate-*co*-terephthalate); PBSA, poly(butylene succinate-*co*-adipate); PBSu, poly(butylene succinate); PESu, poly(ethylene succinate); PHA, poly(3-hydroxyalkanoic acid); PLA, poly(lactic acid); RAST, Rapid Annotations Subsystems Technology; SDesPESu, sandwiched esPESu; SEM, scanning electron microscope; Tm, melting temperature; YE, yeast extract

Author contributions

K.K., Y.T., and H.T. designed the study. M.S. and K.K. wrote the manuscript. M.S., S.P., and T.S. conducted the experiments. K.K. acquired funds and administered the projects. All authors participated in the analysis and discussion of the results.

Funding

This article is based on the results obtained from project JPNP18016 that was managed by K. K. and commissioned by the New Energy and Industrial Technology Development Organization (NEDO). This work is also supported by the JSPS KAKENHI Grant-in-Aid for Scientific Research (B) [grant number 19H04311].

Declaration of competing interest

The authors declare no competing financial interests.

References

[1] Sudesh K, Abe H, Doi Y. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Prog Polym Sci 2000;25:1503–1555. https://doi.org/10.1016/S0079-6700(00)00035-6.

[2] Suzuki M, Tachibana Y, Kasuya K. Biodegradability of poly(3-hydroxyalkanoate) and poly(ε-caprolactone) via biological carbon cycles in marine environments. Polym J 2021;53:47–66. https://doi.org/10.1038/s41428-020-00396-5.

[3] Kasuya K, Takagi K, Ishiwatari S, Yoshida Y, Doi Y. Biodegradabilities of various aliphatic polyesters in natural waters. Polym Degrad Stab 1998;59:327–332. https://doi.org/10.1016/S0141-3910(97)00155-9.

[4] Fujimaki T. Processability and properties of aliphatic polyesters, 'BIONOLLE', synthesized by polycondensation reaction. Polym Degrad Stab 1998;59:209–214. https://doi.org/10.1016/S0141-3910(97)00220-6.

[5] Suzuki M, Tachibana Y, Kazahaya J, Takizawa R, Fumihiro M, Kasuya K. Difference in environmental degradability between poly(ethylene succinate) and poly(3-hydroxybutyrate). J Polym Res 2017;24:217. https://doi.org/10.1007/s10965-017-1383-4.

[6] Tezuka Y, Ishii N, Kasuya K, Mitomo H. Degradation of poly(ethylene succinate) by mesophilic bacteria. Polym Degrad Stab 2004;84:115–121. https://doi.org/10.1016/j.polymdegradstab.2003.09.018.

[7] Muroi F, Tachibana Y, Soulenthone P, Yamamoto K, Mizuno T, Sakurai T, Kobayashi Y, Kasuya K-i. Characterization of a poly(butylene adipate-co-terephthalate) hydrolase from the aerobic mesophilic bacterium *Bacillus pumilus*. Polym Degrad Stab 2017;137:11–22. https://doi.org/10.1016/j.polymdegradstab.2017.01.006.

[8] Gopal N, Hill C, Ross PR, Beresford TP, Fenelon MA, Cotter PD. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. Front Microbiol 2015;6:2015. https://doi.org/10.3389/fmicb.2015.01418.

[9] Kasuya K, Tachibana Y, Yamahata M, Teshima T. WO-A1-2013/180124, 2013.

[10] Sung CC, Tachibana Y, Suzuki M, Hsieh WC, Kasuya K. Identification of a poly(3-hydroxybutyrate)-degrading bacterium isolated from coastal seawater in Japan as *Shewanella* sp. Polym Degrad Stab 2016;129:268–274. https://doi.org/10.1016/j.polymdegradstab.2016.05.008.

[11] Papageorgiou GZ, Bikiaris DN. Crystallization and melting behavior of three biodegradable poly(alkylene succinates). A comparative study. Polymer (Guildf) 2005;46:12081–12092. https://doi.org/10.1016/j.polymer.2005.10.073.

[12] Arkin AP, Cottingham RW, Henry CS, Harris NL, Stevens RL, Maslov S, Dehal P, Ware D, Perez F, Canon S, et al. KBase: The United States Department of Energy Systems Biology Knowledgebase. Nat Biotechnol 2018;36:566–569. https://doi.org/10.1038/nbt.4163.

[13] Andrews S. FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

[14] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014;30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

[15] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. SPAdes: A new genome

assembly algorithm and its applications to single-cell sequencing, J Comput Biol 2012;19:455–477. https://doi.org/10.1089/cmb.2012.0021.

[16] Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R. The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). Nucleic Acids Res 2014;42(D1):D206–D214. https://doi.org/10.1093/nar/gkt1226.

[17] Michael R, Ramon R. Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 2009;106:19126–19131. https://doi.org/10.1073/pnas.0906412106.

[18] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Oxford University Press, 1997. https://academic.oup.com/nar/article/25/17/3389/1061651.

[19] Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. BLAST+: architecture and applications. BMC Bioinform 2009;10:1–9. https://doi.org/10.1186/1471-2105-10-421/FIGURES/4.

[20] Price MN, Dehal PS, Arkin AP. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One 2010;5:e9490–e9490. https://doi.org/10.1371/journal.pone.0009490.

[21] Artificial Seawater. Cold Spring Harbor Protocols. Cold Spring Harbor Laboratory Press. 2012. https:// doi:10.1101/pdb.rec068270.

[22] Nishida H, Suzuki, S, Tokiwa Y. Distribution of poly(p-hydroxybutyrate) and poly(ε-caprolactone) aerobic degrading microorganisms in different environments. J Environ Polym Degrad 1993;1:227–233. https://doi.org/10.1023/A:1022878512743.

[23] Kasuya K, Mitomo H, Nakahara M, Akiba A, Kudo T, Doi Y. Identification of a marine benthic P(3HB)-degrading bacterium isolate and characterization of its P(3HB) depolymerase. Biomacromolecules 2000;1:194–201. https://doi.org/10.1021/bm9900186.

[24] Schaeffer P, Millet J, Aubert JP. Catabolic repression of bacterial sporulation. Proc Natl Acad Sci USA 1965;54:704–711. https://doi.org/10.1073/pnas.54.3.704.

[25] Lewis JC. The estimation of decimal reduction times. Appl Microbiol 1956;4:211–221. https://doi.org/10.1128/am.4.4.211-221.1956.

[26] Muroi F, Tachibana Y, Kobayashi Y, Sakurai T, Kasuya K. Influences of poly(butylene adipate-*co*-terephthalate) on soil microbiota and plant growth. Polym Degrad Stab 2016;129:338–346. https://doi.org/10.1016/j.polymdegradstab.2016.05.018.

[27] ASTM International, ASTM D6691-17

[28] Pinheiro IF, Ferreira FV, Souza DHS, Gouveia RF, Lona LMF, Morales AR, Mei LHI. Mechanical, rheological and degradation properties of PBAT nanocomposites reinforced by functionalized cellulose nanocrystals. Eur Polym J 2017;97:356–365. https://doi.org/10.1016/j.eurpolymj.2017.10.026.

[29] Ratto JA, Stenhouse PJ, Auerbach M, Mitchell J, Farrell R. Processing, performance and biodegradability of a thermoplastic aliphatic polyester/starch system. Polymer (Guildf) 1999;40:6777–6788. https://doi.org/10.1016/S0032-3861(99)00014-2.

[30] He H, Yoshida S, Kawasaki M, Karikomi M, Kimura T, Maruo S. Control of biodegradability of triple-mixed aliphatic polyester films with calcined calcium oxide fine powder. Polym Test 2016;56:180–184. https://doi.org/10.1016/j.polymertesting.2016.10.014.

[31] Yoshie N, Nakasato K, Fujiwara M, Kasuya K, Abe H, Doi Y, Inoue Y. Effect of low molecular weight additives on enzymatic degradation of poly(3-hydroxybutyrate). Polymer (Guildf) 2000;41:3227–3234. https://doi.org/10.1016/S0032-3861(99)00547-9.

[32] Tachibana Y, Baba T, Kasuya K. Environmental biodegradation control of polymers by cleavage of disulfide bonds. Polym Degrad Stab 2017;137:67–74. https://doi.org/10.1016/j.polymdegradstab.2017.01.003.

[33] Tateiwa J, Kimura S, Kasuya K, Iwata T. Multilayer biodegradable films with a degradation initiation function triggered by weakly alkaline seawater. Polym Degrad Stab 2022;200:109942. https://doi.org/10.1016/j.polymdegradstab.2022.109942.

[34] Huang Q, Hiyama M, Kabe T, Kimura S, Iwata T. Enzymatic self-biodegradation of poly(L-lactic acid) films by embedded heat-treated and immobilized proteinase K. Biomacromolecules 2020;21:3301–3307. https://doi.org/10.1021/acs.biomac.0c00759.

[35] Pande V, Pandey SC, Sati D, Pande V, Samant M. Bioremediation: an emerging effective approach towards environment restoration, J Environ Sustain 2020;3:91–103. https://doi.org/10.1007/s42398-020-00099-w.

[36] Roy A, Dutta A, Pal S, Gupta A, Sarkar J, Chatterjee A, Saha A, Sarkar P, Sar P, Kazy S K. Biostimulation and bioaugmentation of native microbial community accelerated bioremediation of oil refinery sludge, Bioresour Technol. 2018;253:22–32. https://doi.org/10.1016/j.biortech.2018.01.004.

[37] Bento FM, Camargo FAO, Okeke BC, Frankenberger WT, Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation, Bioresour Technol. 2005;96:1049–1055. https://doi.org/10.1016/j.biortech.2004.09.008.

Supplemental Information

Control of marine biodegradation of an aliphatic polyester using endospores

Miwa Suzuki[a], Yuya Tachibana[a, b], Phouvilay Soulenthone[b], Tomoya Suzuki[b], Hiroiki Takeno[a,b], and Ken-ichi Kasuya[a, b]*

[a]Gunma University Center for Food Science and Wellness, 4-2 Aramaki, Maebashi, Gunma 371-8510, Japan
[b]Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin, Kiryu, Gunma 371-8510, Japan

*Corresponding author: Division of Molecular Science, Graduate School of Science and Technology, Gunma University, 1-5-1 Tenjin, Kiryu, Gunma376-8515, Japan; E-mail: kkasuya@gunma-u.ac.jp

Running title: Marine biodegradation of plastics using endospores

Supplementary Tables

Table S1. Tensile strength, Young's modulus, and elongation at break of the center part of SDesPESu and PESu films

Samples	Tensile strength (MPa)	Young's modulus (MPa)	Elongation at break (%)
Before degradation	18±2.7	300±82	320±53
5 d PESu	8.9±0.6	77±26	78±28
10 d PESu	18±3.3	267±47	115±107
15 d PESu	21±0.8	233±47	31±13
5 d SDesPESu	7.1±0.3	100±0	25±8
10 d SDesPESu	6.8±1.3	67±25	26±15
15 d SDesPESu	5.9±0.3	63±26	19±5

PESu, poly(ethylene succinate); SDesPESu, sandwiched endospore-containing PESu.

Table S2. Number average molecular weight and polydispersity of the center part of SDesPESu films

Samples	Mn ($x10^4$)	Polydispersity (Mw/Mn)
Before degradation	2.1	2.0
5 d SDesPESu	1.9	2.5
10 d SDesPESu	2.1	2.1
15 d SDesPESu	2.2	2.1

Mn, number average molecular weight; Mw, weight-average molecular weight; SDesPESu, sandwiched endospore-containing poly(ethylene succinate).

Table S3. Crystallinity of the center part of SDesPESu and PESu films

Samples	Crystallinity (%)
Before degradation	30.9
5 d PESu	35.3
10 d PESu	34.3
15 d PESu	33.2
5 d SDesPESu	34.4
10 d SDesPESu	32.7
15 d SDesPESu	33.2

Crystallinity was measured using differential scanning calorimetry. PESu, poly(ethylene succinate); SDesPESu, sandwiched endospore-containing PESu.

Supplementary Figures

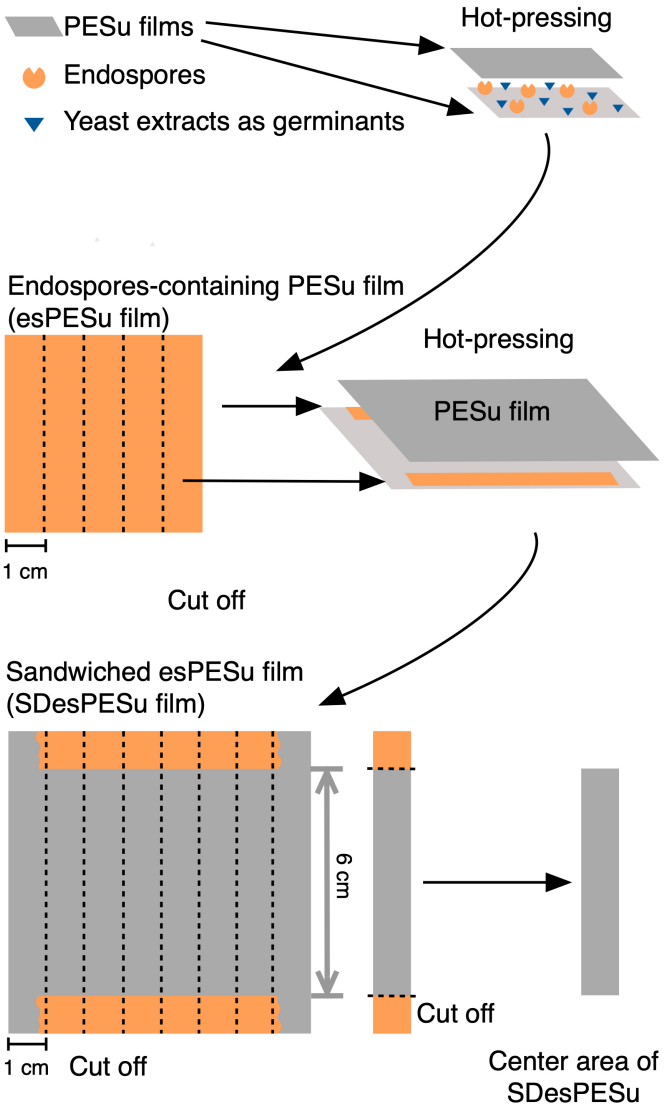

Fig. S1. Procedures for preparing esPESu films and SDesPESu films. esPESu films were prepared by hot pressing, with the endospores and germinants placed between two PESu films. esPESu film strips (5 cm × 1 cm) were placed on the two edges of the PESu film (8 cm × 8 cm), covered with another PESu film and subjected to hot pressing, yielding sandwiched esPESu (SDesPESu) film. For the degradation tests, SDesPESu specimens were prepared by cutting the SDesPESu film into rectangular strips. For morphological observation, molecular mass analysis, and the tensile test, the esPESu part of the SDesPESu film strip was cut off and the center area (6 cm × 1 cm) of the SDesPESu film was used.

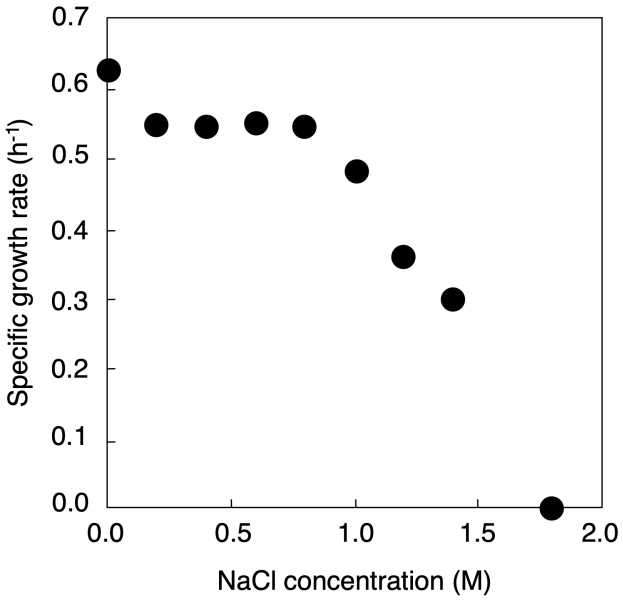

Fig. S2. Specific growth rate of strain YKCMOA1 at different NaCl concentrations at 30 ˚C in Luria-Bertani medium.

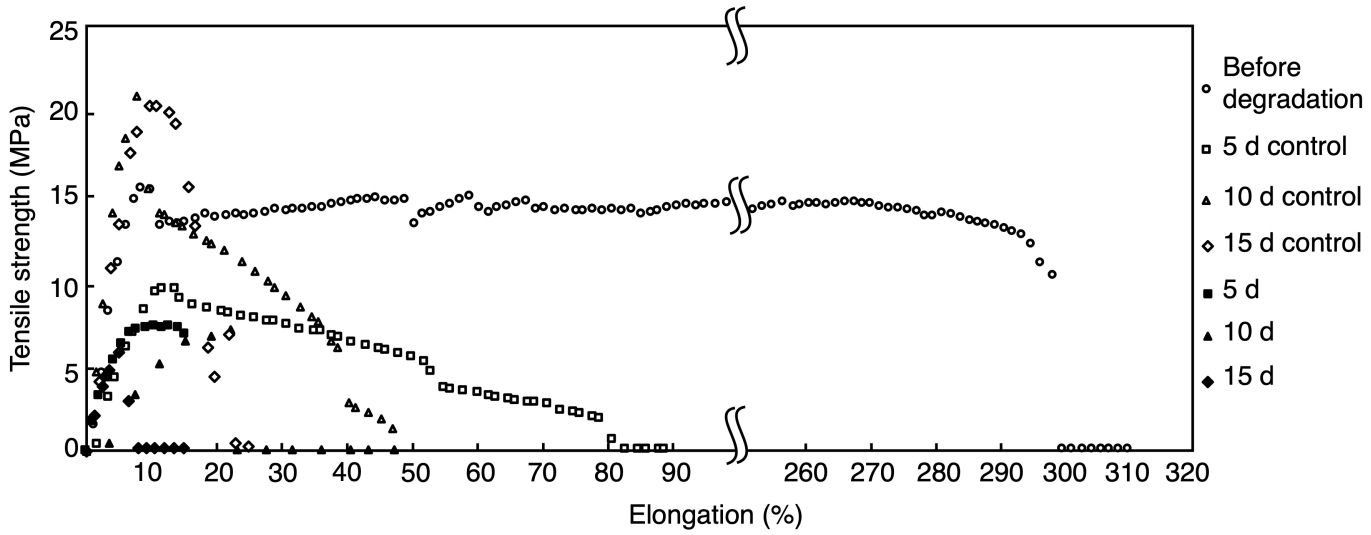

Fig. S3. Stress-strain curves of the center part of SDesPESu film (closed symbols) and PESu (control, open symbols) before (open circle symbols) and after incubation for 5 d (square symbols), 10 d (triangle symbols), and 15 d (diamond symbols) in natural seawater.