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Isolation and characterization of poly(butylene succinate)-degrading fungi

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Abstract

We isolated 12 poly(butylene succinate) (PBSu)-degrading fungi from various soil environments. Among the isolates, the NKCM1706 strain exhibited the fastest degradation rate for the PBSu film (10.5 $\mu g\,cm^{-2}\,h^{-1}$). Phylogenetic analysis revealed that this strain is closely related to *Aspergillus fumigatus* (internal transcribed spacer (ITS) identity, 100%). Further, this strain exhibited PBSu-hydrolytic activity in the presence of poly(ε-caprolactone) (PCL), PBSu, and poly(butylene succinate-*co*-adipate) (PBSA). On adding this strain into the soil sample, the PBSu degradation rate accelerated approximately sixfold, suggesting that this strain plays a crucial role in PBSu degradation in actual soil environments. In addition to PBSu, the NKCM1706 strain could degrade PBSA, poly(ethylene succinate) (PESu), poly(3-hydroxybutyrate) (P(3HB)), and PCL.

Keywords: Poly(butylenes succinate); Fungi; Biodegradation; *Aspergillus fumigatus*; Strain NKCM 1706

1. Introduction

Biodegradable polymers have been developed as a possible solution to certain problems that arise from the disposal of plastic waste [1–3]. Chemosynthetic aliphatic polyesters, such as poly(butylene succinate) (PBSu), have received attention as they are useful biodegradable polymers; this is due to their relatively low production cost and good mechanical properties [4] that are similar to those of polyethylene. Therefore, PBSu is used in diverse fields such as the production of bottles and shopping bags and agricultural materials [4]. In addition, the Japan BioPlastic Association (JBPA) has included PBSu (proprietary name, Bionolle 1000) in the list of biodegradable plastics based on its biodegradability and the nontoxicity of its degradation products.

PBSu is enzymatically degraded by the *Chromobacterium viscosum* lipase [5] and the *Rhizopus delemar* lipase [6,7]. A recent study has indicated that the *Aspergillus oryzae* cutinase (CutL1) forms a complex with an amphipathic protein (RolA or HsbA) for the efficient hydrolysis of PBSu [8,9]. However, the environmental biodegradation rate of PBSu is reported to be considerably lower than that of other readily biodegradable plastics such as poly(ε-caprolactone) (PCL) or poly(3-hydroxybutyrate) (P(3HB)) and also dependant on the ambient environmental conditions [10]. This suggests that PBSu-degrading strains are less common than PCL- or P(3HB)-degrading strains. Thus far, certain actinobacteria [11] and proteobacteria [12,13] have been isolated from natural environments as PBSu-degrading bacterial strains. However, there have been limited reports on the microbial degradation of PBSu by fungal strains, although these strains are known to play a crucial role in the microbial degradation of certain polymeric materials such as P(3HB), PCL, and cellulose [14]. Therefore, investigations on the fungal degradation of PBSu may provide further insights into the mechanism of PBSu degradation in various environments.

In this study, we isolated and identified PBSu-degrading fungi from natural environments. Furthermore, we investigated the biodegradation behavior of these fungal isolates on PBSu.

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2. Experimental

2.1. Chemicals

PBSu was used to isolate fungal strains capable of degrading it. The origin, composition, melting temperature, and average molecular mass of all the polymers used in this study are listed in Table 1. The polymers were used to prepare melt-crystallized film as described previously [15]. Other chemicals were purchased from Wako Pure Chemical Industries and from Sigma Aldrich, Japan.

2.2. Media and culture conditions

Fungi were cultured in LB medium (pH 7.0; tryptone peptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L) or in minimal media (pH 6.0; KH_2PO_4, 1.0 g/L; $NaNO_3$, 2.0 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; KCl, 0.5 g/L; $FeSO_2 \cdot 7H_2O$, 0.01 g/L; NH_4Cl, 1.0 g/L; Plysurf, 0.1 g/L) supplemented with 0.2% (w/v) emulsified polymer; the media were prepared as described previously [18]. P(3HB) granules were directly added to the minimal medium. In the case of solid media, 2% agar was added.

2.3. Isolation of PBSu-degrading fungi

We collected 12 soil samples (dry soil around Gunma University, Gunma, Japan; wet soil around Lake Kasumigaura, Ibaragi, Japan; dry soil from a field in Gunma, Japan; dry soil from a field in Nagano, Japan; soil from Mount Narita, Chiba, Japan; and soil from a mountain in Hiroshima, Japan) as sources of PBSu-degrading fungi. Each soil sample (1 g) was dispersed in 10 mL sterile physiological saline and left standing for 10 min. Further, 50 μL of the soil suspension supernatant was plated onto a minimal medium plate containing PBSu, which has been described in Section 2.2, supplemented with 100 μL of 12 mg/mL tetracycline solution in order to suppress the growth of bacterial colonies. The plates were incubated at 30 °C for several days. The strains were purified on LB medium by performing a streak culture.

2.4. Phylogenetic analysis

The internal transcribed spacer (ITS) sequences of the NKCM1706, NKCM1707, NKCM1708, and NKCM1712 isolates were determined by using a Beckman CEQ 2000 DNA sequencer [16]. After aligning the ITS sequences with other similar ITS sequences by using the ClustalW program, a phylogenetic tree was constructed according to the neighbor-joining method [21] using the DendroMaker program.

2.5. Degradation abilities of the isolates for various substrates

The hydrolytic abilities of the PBSu-degrading fungal isolates for 6 polyesters, namely, PBSu, poly(butylene succinate-*co*-adipate) (PBSA), poly(ethylene succinate) (PESu), P(3HB), PCL, and poly(lactic acid) (PLA), were examined by the clear-zone method, using substrate-containing minimal medium plates, as described previously [15]. Further, the degradation abilities of the strains for solid PBSu, PBSA, PESu, P(3HB), PCL, and PLA films (1 cm × 10 cm × 0.1 mm ca. 15 mg) were evaluated by the weight-loss method [19]. Each strain was incubated at 30 °C for 10 d in minimal medium containing each film. After the residual film was freeze-dried, it was weighed. Weight loss was calculated by subtracting the weight of degraded film from the initial weight of the film.

2.6. Microscopy

An Olympus BX50 light microscope equipped with a phase-contrast apparatus was used to observe the microscopic morphology of the NKCM1706 strain. The samples were prepared as described previously [15,16]. The process of microbial degradation of the PBSu film was observed under a scanning electron microscope (SEM), JSM-6700F. The samples were coated with gold prior to observation under the SEM.

2.7. Analysis of PBSu-film biodegradation in soil

We used 20 g of the soil sample obtained from Gunma University (Kiryu, Gunma Prefecture, Japan) as the soil medium in the degradation analysis. PBSu films (1 cm × 10 cm × 0.1 mm; approximately 15 mg) that were sterilized by soaking in ethanol, followed by rinsing with sterile water, were buried in the soil taken in a glass bottle (internal diameter, 6 cm; height, 3 cm). Further, a 5 mL minimal medium solution containing or lacking the NKCM1706 strain (approximately 1 mg wet cells) was added to the soil, and the bottle was incubated at

Table 1
Properties of polymers used in this study

Polymer	Component(s)	T_m [a]	$M_w \times 10^{-4}$ [b]	Source
PLA	Lactic acid	176	27.5	Shimadzu Co. (Kyoto, Japan)
P(3HB)	3-Hydroxybutyrate	178	70.0	*Ralstonia eutropha* ATCC17699 from fructose [20]
PESu	Ethylene glycol/succinic acid	106	18.1	Nippon Shokubai Co. Ltd. (Osaka, Japan)
PBSu	1,4-Butanediol/succinic acid	117	17.6	Showa High Polymer Inc. (Tokyo, Japan)
PBSA	1,4-Butanediol/succinic acid/adipic acid	92	8.5	Showa High Polymer Inc. (Tokyo, Japan)
PCL	6-Hydroxyhexanoic acid	62	6.4	Daicel Chemical Industries Co. (Osaka, Japan)

[a] T_m, melting temperature.
[b] M_w, weight-average molecular mass.

30 °C. Following incubation for 5, 10, 15, 20, 25, and 30 d, the residual films were recovered, washed with distilled water, and dried to a constant weight. The weight loss of each film was calculated by subtracting its weight following degradation from its initial weight. A negative control experiment was performed by using autoclaved soil. The weight-average molecular mass (M_w) of the residual film was determined by performing gel permeation chromatography (GPC) [15].

2.8. Liquid chromatography electrospray ionization mass spectrometry analysis of water-soluble products

The NKCM1706 strain was cultured in minimal medium containing PBSu film. Following incubation for 10 d, the supernatant was recovered by centrifugation and resolved by performing liquid chromatography (LC; LiChrospher RP-8 column, 4.0 × 250 mm); each fraction was then analyzed by performing electrospray ionization mass spectrometry (ESI-MS), using the Micromass ZQ system (Nihon Waters KK, Tokyo, Japan).

2.9. Enzyme assay

The PBSu hydrolysis activity of the culture supernatant was evaluated by the turbidimetric method. As a substrate, 0.1% (w/v) emulsified PBSu in 50 mM phosphate buffer (pH 7.0) was used; 250 μL of the culture supernatant was added to 750 μL of the PBSu substrate and incubated at 30 °C. We defined 1 unit of PBSu hydrolysis activity as the amount of enzyme required to decrease the turbidity of emulsified PBSu by 1.0 unit/min at a wavelength of 650 nm.

2.10. Accession numbers of nucleotide sequences

The ITS sequences of the NKCM1706, NKCM1707, NKCM1708, and NKCM1712 strains were deposited in the DNA Databank of Japan (DDBJ) under accession numbers AB265145, AB305170, AB305171, and AB305167, respectively.

2.11. Experiment on enzyme induction in the culture supernatant of the NKCM1706 strain

The NKCM1706 strain was cultured in LB medium at 30 °C overnight. The precultured strain (30 μL) was inoculated in 3 mL minimal medium supplemented with a carbon source (0.2% glucose, fructose, starch, olive oil, tributyrin, palmitin, citric acid, succinic acid, 1,4-butanediol, PBSu, PBSA, PCL, PESu, P(3HB), or PLA) and LB medium and cultured at 30 °C for 10 d. The supernatants and cells were separated by centrifugation at 6000 × g for 10 min at 4 °C. The PBSu hydrolytic-activity in the supernatant was determined as described in Section 2.9. After the recovered mycelium was freeze-dried, it was weighed. All experiments were conducted in triplicate.

3. Results and discussion

3.1. Isolation of PBSu-degrading filamentous fungi

The clear-zone method that employs a polymer emulsion is a powerful screening tool for polymer-degrading microorganisms due to its high sensitivity for detecting polymer-degradation activity [19]. From various environments, we isolated 12 fungal strains that formed a clear zone on a plate containing the PBSu emulsion. In addition, these also formed clear zones on the plates containing PBSA-, PCL-, and olive oil-containing emulsions (Table 2).

On the other hand, the hydrolytic activity for the PBSu film in the culture supernatants of the isolates did not significantly correlate with the size of the corresponding clear zone formed on the plates (Table 2). This suggests that not only an emulsion but also a film can be used as the substrate in this method of screening PBSu-degrading strains from various environments; this is useful because plastic products made from PBSu are not in the emulsified state but in the solid state. Further, based on our results, we established that the NKCM1706 isolate that exhibited a high hydrolytic activity toward the PBSu film (10.5 μg cm^{-2} h^{-1}) would likely have a greater impact on

Table 2
The ability of PBSu film degradation and clear zone formation on the plates containing polymer emulsions or olive oil by PBSu-degrading fungal isolates

Strain	PBSu	PESu	PBSA	P(3HB)	PCL	Olive oil	PLA	Degradation rate of PBSu film (μg cm^{-2} h^{-1})	Sources
NKCM1701	+	−	+++	+++	+++	+++	−	1.2 ± 0.2	Dry soil at Gunma university
NKCM1702	++	++	++	+++	+++	+++	−	1.1 ± 0.1	Dry soil of a field at Gunma
NKCM1703	++	++	+++	+++	+++	++	−	1.5 ± 0.3	Wet soil around Lake Kasumigaura
NKCM1704	+	−	++	−	++	++	−	1.4 ± 0.1	Soil at Mount Narita
NKCM1705	++	−	+	+++	+++	+++	−	1.2 ± 0.2	Soil of a mountain at Hiroshima
NKCM1706	++	++	+++	++	+++	+++	−	11 ± 2	Wet soil around Lake Kasumigaura
NKCM1707	++	+++	+++	+++	+++	+++	−	9.0 ± 1	Soil of a mountain at Hiroshima
NKCM1708	++	+	++	++	+++	+++	−	3.1 ± 0.5	Soil at Mount Narita
NKCM1709	+	+	++	+++	++	+++	−	0.38 ± 0.02	Dry soil of a field at Nagano
NKCM1710	++	+	+++	+++	++	++	−	0.08 ± 0.01	Dry soil of a field at Nagano
NKCM1711	+	+	++	+++	+	++	−	0.16 ± 0.02	Dry soil at Gunma university
NKCM1712	++	+++	++	+++	++	++	−	10 ± 1	Soil at Mount Narita

+++, A large clearing zone was formed around the isolate; ++, a medium clearing zone was formed around the isolate; ++, a small clearing zone was formed around the isolate; −, no clearing zone was formed around the isolate.

the environmental degradation of PBSu products compared to the other isolates (Table 2). Therefore, this strain was further characterized in detail.

3.2. Characterization of the PBSu-degrading fungal strain NKCM1706

Light microscopic analysis revealed that the hyphae of the NKCM1706 strain were 5.0 ± 0.5 μm in diameter and possessed some septa. A conidium (20 μm in diameter) was observed at the hyphal terminus. This strain grew at temperatures below 55 °C and exhibited optimal growth at 30 °C. It formed a clear zone on the agar plate containing PBSu in the temperature range of 20–50 °C.

In addition to PBSu, the NKCM1706 strain could degrade 4 polyester films (P(3HB), PESu, PBSA, and PCL); however, it did not degrade PLA (Table 3). Further, this strain grew by using these polyesters as the sole carbon source.

Table 4 shows the effects of various carbon sources on the PBSu-hydrolytic activity of the NKCM1706 strain. This strain grew on all carbon sources tested in this study, except for PLA. PBSu-hydrolytic activity was detected in the supernatants only when this strain was cultured in the minimal media containing PBSu, PBSA, or PCL as the sole carbon source. However, the PBSu-hydrolytic activity of the NKCM1706 strain was not observed toward succinic acid and 1,4-butanediol, which are components of PBSu, although this strain could use these carbon sources to grow. These results suggest that PBSu oligomers induce the activity of PBSu-degrading enzymes. In addition, PCL catabolites may also induce the activity.

3.3. Phylogenetic analysis of the isolates

Phylogenetic analysis was performed based on the ITS-region of the 4 PBSu-degrading strains (NKCM1706, NKCM1707, NKCM1708, and NKCM1712). The GenBank database was used to search for ITS-region sequences that were homologous to those of the isolates. As shown in Fig. 1, the 4 isolates belonged to the phylum Ascomycota in the taxon Fungi. The NKCM1706 and NKCM1708 strains were closely related to *Aspergillus fumigatus* (ITS identity, 100%) and *Aspergillus niger* (99.6%), respectively, which belong to the class Eurotiomycetes. On the other hand, the NKCM1707 and NKCM1712 strains were closely related to *Isaria fumosoroseus* (100%) and *Fusarium solani* (99.6%), respectively, which belong to the class Sordariomycetes. These species related to the isolates were known as PCL-degrading fungi (Fig. 1). Considering the fact that all the PBSu-degrading fungi isolated in this study exhibited PCL-hydrolytic activity (Table 2), PBSu-degrading strains may be a subgroup of the PCL-degrading strains.

Table 3
Degradation of various polyester films by strain NKCM1706

Film	Degradation rate of film (μg cm^{-2} h^{-1})	Dry cell weight (mg)[a]
PBSu	11 ± 2	4.3 ± 0.2
PBSA	22 ± 1	4.7 ± 0.5
PESu	10 ± 2	4.7 ± 0.1
P(3HB)	14 ± 1	4.4 ± 0.3
PCL	4.2 ± 0.5	3.4 ± 0.4
PLA	0	0.1 ± 0.02

All experiments were performed in triplicate. Errors (±) indicate the width of experimental data.
[a] Dry cell weight was determined after incubation at 30 °C for 10 d.

Table 4
Effect of carbon source on the PBSu hydrolysis activity of strain NKCM1706

Carbon source[a]	Dry cell weight (mg)[c]	PBSu hydrolysis activity (U/L)[d]
None	0.3 ± 0.1	0
PBSu	4.3 ± 0.4	23 ± 3.0
PBSA	4.7 ± 0.5	27 ± 1.0
PCL	3.4 ± 0.4	11 ± 3.0
PESu	4.7 ± 0.1	0
P(3HB)	4.4 ± 0.3	0
PLA	0.1 ± 0.02	0
Glucose	5.1 ± 0.2	0
Fructose	4.5 ± 0.1	0
Starch	3.5 ± 0.2	0
Olive oil	4.7 ± 0.1	0
Tributyrin	4.2 ± 0.4	0
Tripalmitin	3.9 ± 0.1	0
Citric acid	4.8 ± 0.8	0
Succinic acid	4.1 ± 0.4	0
1,4-Butanediol	3.7 ± 0.6	0
LB medium[b]	5.2 ± 0.2	0

All experiments were performed in triplicate. Errors (±) indicate the width of experimental data.
[a] To minimal medium 0.2% of each carbon source was added. Strain NKCM 1706 was aerobically cultured in the medium at 30 °C for 10 d.
[b] Strain NKCM1706 was cultured on LB medium.
[c] Dry cell weight was determined after incubation at 30 °C for 10 d.
[d] Enzyme activities(U) per 1 L of culture were indicated.

3.4. Contribution of the NKCM1706 strain to PBSu degradation in soil environments

In order to investigate the contribution of NKCM1706 to PBSu degradation in a field, a soil-buried test of the PBSu film was performed using nonsterile environmental soil containing and lacking this strain (Fig. 2). In the soil containing the strain, the PBSu film degradation rate increased linearly after a 5-d induction period. Following incubation for 20 d, the film began to disintegrate. After 30 d, approximately 80% of the film weight was lost (weight loss, 5.75 ± 0.50 mg cm^{-2}). In the soil wherein the strain was not inoculated, the degradation rate was considerably lower (0.95 ± 0.10 mg cm^{-2}) compared with the soil that contained the strain. On the other hand, no film weight loss was observed following incubation in autoclaved soil at 30 °C for 30 d. Thus, the PBSu degradation rate in soil accelerated approximately sixfold on adding the NKCM1706 strain, suggesting that this strain plays a crucial role in PBSu degradation in actual soil environments.

The surface morphology of the film was observed under the SEM at various time points during incubation with the strain. The virgin film was extremely smooth (Fig. 3A). Following incubation for 5 d, the surface exhibited craters interspersed

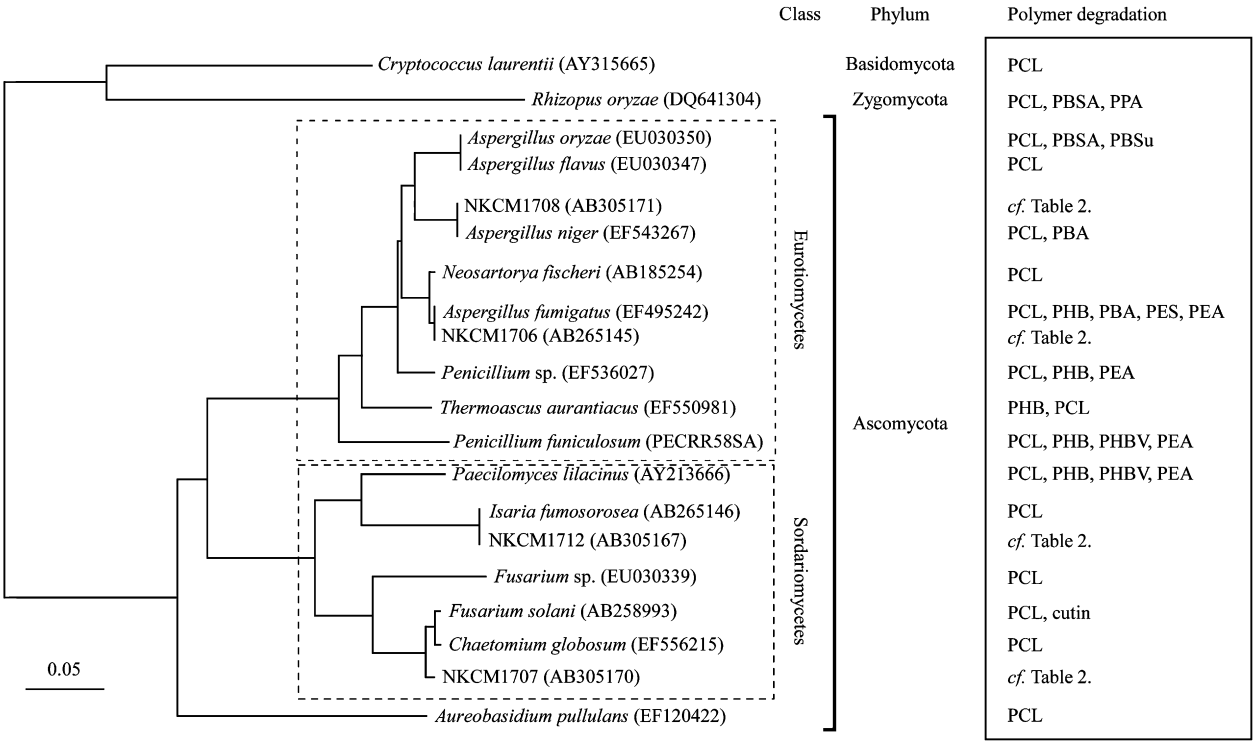

Fig. 1. Phylogenetic tree of the PBSu-degrading isolates based on the ITS and 5.8S rDNA sequence comparisons. The accession numbers are provided in parentheses. The bar indicates 2% estimated sequence divergence.

with smooth areas (Fig. 3B), and after 10 d of culture, the smooth areas disappeared (Fig. 3C). Polygonal craters having a width of 13–22 μm were observed on the surface at 20 d of culture (Fig. 3D). In addition, the M_w of the residual film remained constant (approximately 1.7×10^5) during the degradation, indicating that the degradation began from the surface of the film and not the inside. Similar microbial degradation processes were observed for other semicrystalline biodegradable polyesters such as P(3HB) [18] and PESu [16]. These degradation patterns may be explained by the size and preference of polyester-degrading enzymes, which can hydrolyze ester bonds in amorphous regions at a considerably higher rate than ester bonds in crystalline regions [18].

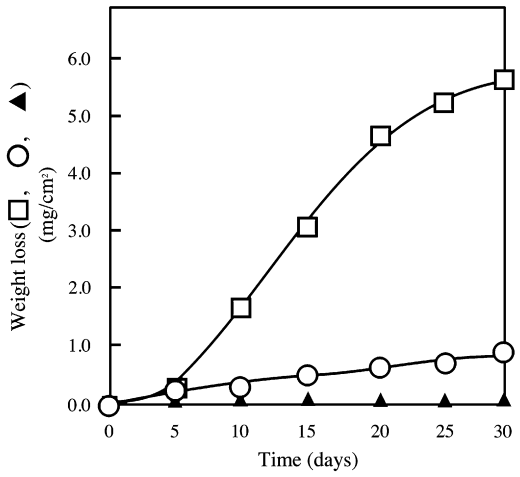

Fig. 2. Degradation of the PBSu film in soil samples. Weight loss of the PBSu film in the presence of the NKCM1706 strain (□), control (unsterilized soil lacking the NKCM1706 strain) (○), and negative control (sterilized) (▲). All experiments were performed in triplicate. The error bars indicate the width of the experimental values.

3.5. Microbial hydrolysis of the PBSu film by the NKCM 1706 strain

Following culture of the strain for 10 d in minimal medium containing PBSu film as the sole carbon source, the water-soluble products released in the supernatant were analyzed by performing LC/ESI-MS. The supernatant contained SB (S: succinic acid unit and B: 1,4-butanediol unit, observed molecular mass: 190), BSB (262), SBS (290), and SBSB (362) as major products and B (90), S (118), BSBSB (434), SBSBS (462), SBSBSB (534), BHB (H, hexamethylene diisocyanate; 348), and SBHB (448) as minor products. Only trace amounts of both S and B, which are the basal components of PBSu, were detected, suggesting that the strain took up both succinic acid and 1,4-butanediol at fast rate for its growth (Table 4). On the other hand, small amounts of 2 oligomers, including hexamethylene diisocyanate (H), were also detected, although they were trace components that were used as joint parts to increase the molecular mass of PBSu. This suggests that these oligomers are resistant to attack by this strain. Kitakuni and coworkers compared the biochemical oxygen demand (BOD) biodegradability of both oligomers containing and lacking the H unit (bis(hydroxybutyl)hexamethylene dicarbamate (BHB) and bis(hydroxybutyl)succinate (BSB)) [5]. They observed that the biodegradability of BHB was

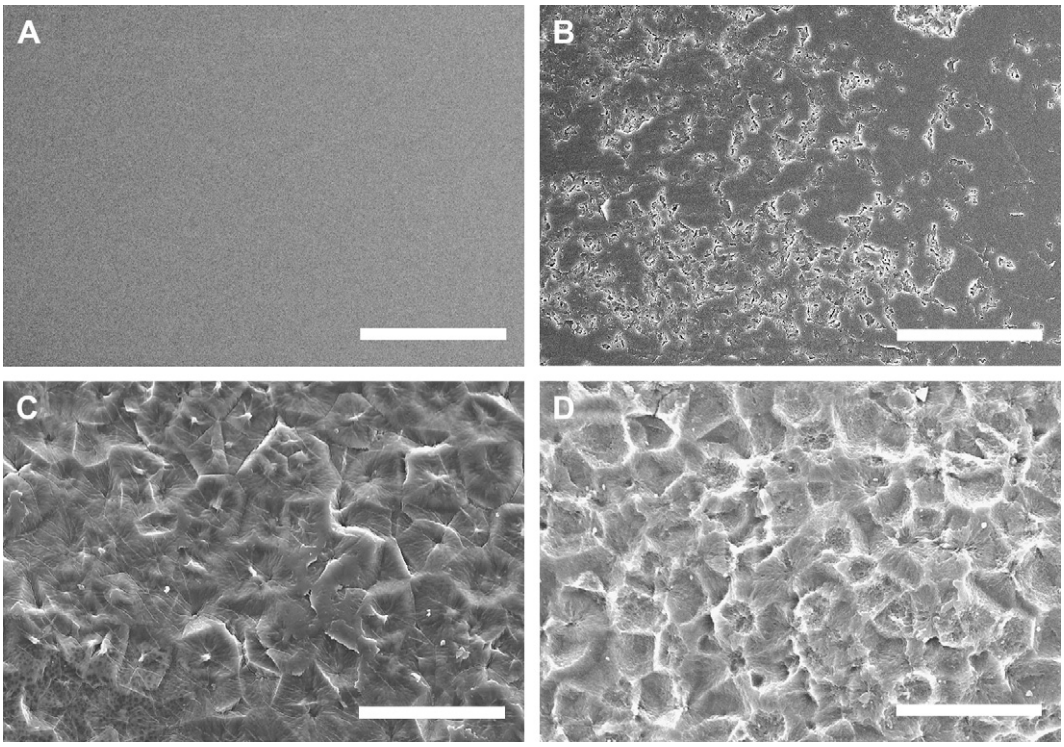

Fig. 3. Scanning electron micrographs of the PBSu film surfaces prior to (A) and following degradation by the NKCM1706 strain for 5 (B), 10 (C), and 20 d (D). The white bars indicate a length of 20 μm.

high (60%) in activated sludge, although its biodegradation rate was considerably lower than that of BSB [5]. Taken together, these results suggest that all the degradation products of PBSu can be almost completely mineralized in actual environments following hydrolysis into oligomers by PBSu-degrading strains such as NKCM1706.

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