RESEARCH LETTER

Founder effect uncovers a new axis in polyethylene succinate bioremediation during biostimulation

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Abstract

Biostimulation is a method of *in situ* bioremediation wherein native soil microbes are stimulated by nutrient supplementation. In a previous report, we showed considerable polyethylene succinate (PES) degradation by biostimulation. To gain an insight into this, this study was undertaken to investigate the different facets of the microbial population present in both soil and PES-films during biostimulation-mediated PES degradation. It was observed that addition of PES-films to both nutrient-treated and untreated soil resulted in significant reduction of soil microbial counts compared with the corresponding control. It was observed that a small microbial population containing both PES degraders and non-degraders translocated to PES surface. Over time, the population adhering to PES films changed from having both PES degraders and non-degraders to being mainly PES degraders. This newly developed microbial community on PES-films exhibited low diversity with a distinct cluster of metabolic fingerprinting and higher evenness compared with parent soil microbial population. Thus the establishment of a new community on the PES surface is an exhibition of founder effect, which subsequently resulted in the emergence of a more efficient PES-degrading population and subsequently led to considerable PES degradation.

Introduction

Polyethylene succinate (PES) is an aliphatic polyester with high melting temperature (104 °C) that is widely used in plastic industry for manufacturing shopping bags, agricultural films, and as a coating agent for paper (Byuntae *et al.*, 1991). PES contains ester linkages that make it biodegradable. In addition, PES exhibits mechanical properties similar to non-biodegradable polymers such as polypropylene and polyethylene (Fujimaki *et al.*, 1995). Therefore, PES could be a potential substitute for polypropylene and polyethylene.

PES is degraded under laboratory conditions by various mesophilic and thermophilic bacteria (Tansengco & Tokiwa, 1998; Tezuka *et al.*, 2004; Hoang *et al.*, 2007; Ishii *et al.*, 2007; Tribedi *et al.*, 2012). However, in practical operations, the polymers are degraded *in situ* at landfills. At landfill, bioremediation can be implemented in various ways: (1) natural attenuation, (2) biostimulation and (3) bioaugmentation. With natural attenuation, native soil microbiota degrades the recalcitrant contaminants *in situ*. This method of bioremediation is usually very slow. In bioaugmentation, an organism capable of degrading the pollutant is added to soil; the process has been found useful in remediating several environmental problems (Bento *et al.*, 2005; Teng *et al.*, 2010). However, bioaugmentation is not always successful (Mueller *et al.*, 1992; Bouchez *et al.*, 2000). The introduced organism either may face competition or may damage the preexisting soil ecology as the microbial community in a given habitat is very sensitive to any perturbation of the ecosystem (Margesin *et al.*, 2000). In contrast, biostimulation involves the addition of nutrients to soil to stimulate naturally occurring microbial populations. In this procedure, remediation is mediated by native microorganisms that are well adapted to the respective environment. Since no exogenous organisms are added to the contaminated site, this process minimizes the risk of ecological damage resulting from microbial community modulation by inclusion of foreign isolate(s) into the target site. This process also reduces the effort needed to isolate an efficient organism with the desired degradative capabilities

and thus is a better choice for *in situ* bioremediation. Consistent with this idea, several studies have shown considerable remediation of diesel oil by exploiting biostimulation (Bento *et al.*, 2005; Teng *et al.*, 2010). Previously, we published a report showing considerable degradation of PES by biostimulation (Tribedi & Sil, 2013). However, the underlying mechanism of biostimulation-mediated bioremediation remained unclear. Therefore, in the present study, we have targeted our efforts to understand the underlying mechanism of biostimulation-mediated PES remediation.

Materials and methods

Soil samples

Soil samples were collected from Kolkata municipal solid waste dumping ground. Physicochemical analysis showed that 1 kg soil contained 482 g silt, 410 g sand and 108 g clay. The soil also contained 152 μg microbial biomass C g^{-1} and 21 μg microbial biomass N g^{-1}. The basal respiration of soil was 1.92 μg CO_2–C g^{-1} soil h^{-1}.

Preparation of PES-plates and PES-films

PES-plates and PES-films were prepared as described previously (Tribedi *et al.*, 2012).

Soil microcosm preparation

In this study we used four different microcosms: (1) control soil, (2) soil amended with PES-films, (3) soil supplemented with external nutrients [$(NH_4)_2SO_4$ (250mg kg^{-1} of soil), K_2HPO_4 (100 mg kg^{-1} of soil) and glucose (2 g kg^{-1} of soil)] and (4) nutrient-supplemented soil amended with PES (Table 1). PES-films were prepared by the heat press method and cut into small, rectangular strips (3.5 × 2.5 cm). Nine such strips (about 50 mg each) were added to each individual microcosm. Prior to addition to microcosm, PES-films were made sterile with 70% ethanol. For each microcosm, 450 g of soil was taken in a glass beaker and kept moistened by adding sterile water. Microcosms were incubated at 30 °C for 28 days. Three replicates were used for each type of microcosm.

Table 1. Composition of various treatment strategy used in the study

Microcosm type	Microcosm description
1	Control native soil
2	Soil amended with PES-film
3	Soil stimulated with nutrients
4	Nutrient-supplemented soil amended with PES

PES degradation analysis

After 28 days of incubation, PES-films were picked from differently treated soil microcosm and PES degradation was then analyzed by weight loss of PES-films as described previously (Tribedi *et al.*, 2012).

Microbial count analysis from soil

From each microcosm, 1 g soil was collected at days 15 and 28, and added to 9 mL of 0.85% NaCl in sterile test tubes. Thereafter, a series of dilutions from 10^{-2} to 10^{-5} were prepared in 0.85% NaCl. An aliquot (0.1 mL) of the appropriate dilution from each microcosm was spread onto Luria agar (LA) plates for enumeration of heterotrophic organisms. LA-plates were incubated at 30 °C for 2 days.

Soil enzyme activities

Dehydrogenase activities of soil from each microcosm were examined as described previously (Tribedi & Sil, 2013). Briefly, 5 g soil sample was collected from each microcosm after 28 days of incubation and the sample was then placed in a 50-mL polypropylene tube and mixed with 5 mL of 0.5% 1,3,5-triphenyltetrazolium chloride (TTC)-Tris solution. Tubes were then incubated at 30 °C for 24 h in dark. After the incubation, triphenylformazan produced by reduction of TTC was extracted with 100 mL methanol. The concentration of triphenylformazan was determined spectrophotometrically at 485 nm, and results were expressed as micrograms of triphenylformazan produced per gram of soil.

Estimation of microbial population on PES-films

To estimate the microbial population density on PES-films, PES-films were collected from different microcosms at days 15 and 28, respectively, and adhering microorganisms were extracted in 3.5 mL of sterile Milli Q water from PES-films and serially diluted in sterile 0.85% NaCl. A 0.1-mL aliquot of the appropriate dilution was spread aseptically onto both LA and PES agar plates. Plates were incubated at 30 °C for 2 and 7 days, respectively. After the incubation colonies appearing on the agar plates were counted.

Physiological profiles of soil microbial community

The patterns of potential carbon source utilized by the microbial communities present in either soil or on PES-films in different microcosms were assessed by the BiOLOG-ECO plate system containing triplicates of 31 different

carbon sources (Choi & Dobbs, 1999). To test the physiological profiles of soil microbial community, 1 g soil taken from each microcosm was added to 10 mL sterile distilled water in a test tube and shaken for 10 min. A 150-μL aliquot of 10^{-3} dilution of soil suspension containing *c.* 1000 colony forming units (CFU) from each microcosm was added separately to each well of the ECO-plates. In addition, PES-films were recovered from differently treated microcosms and the adhering microbial population on PES-films was extracted in 3.5 mL sterile Milli Q water by vortexing and sonication. Thereafter, 150 μL of the suspensions containing *c.* 1000 CFU was added to each well of the separate ECO-plates. All plates were incubated at 30 °C for 60 h and absorbance at 590 nm of each well was recorded. The Shannon–Weaver diversity index (*H*) was calculated following the equation: $H = -\sum \text{pi ln pi}$, where pi is the ratio of the activity on each substrate (Absorbance_i) to the sum of activities of all substrates ($\sum \text{Absorbance}_i$), assuming absorbance at 590 nm of 0.25 as the threshold for a positive response (Garland, 1997). The corresponding Lorenz curve was plotted. This curve was used to derive the Gini-coefficient (*G*), which is a measure of inequality, using the formula:

$$G = 1 - 2\int_0^1 L \, dF$$

where *L* is the Lorenz curve and *F* is the standardized cumulative distribution of the standardized population.

Clustering analysis

For clustering analysis, data from the richness tests using ECO-Plates were collected and the similarity matrix was generated by Euclidean distances, which were used to build dendogram with the unweighted pair group mean averages (UPGMA) algorithm where the linkage was single. Cluster analysis was performed using MINITAB 16.

Statistical analysis

Experimental results were subjected to statistical analysis of one-way analysis of variance (ANOVA). Mean values were compared at the 5% level using MINITAB 16.

Results

Variation in abundance of microbial population among different microcosms under different treatment

In our previous study we showed considerable PES degradation in biostimulated microcosm, which was supplemented with glucose, K_2HPO_4 and ammonium sulfate (Tribedi & Sil, 2013). To further explore the underlying mechanism, we investigated the effect of extra nutrients and PES on the abundance of microbial population in microcosms. For this purpose, we set up four different microcosms as described in Table 1, in triplicate, and incubated these microcosms for 28 days at 30 °C. Following this incubation, and prior to assessing microbial abundance, we verified the weight loss of the PES-films in Microcosms 2 and 4. Consistent with our previous report, we observed considerable degradation of PES-films in biostimulated microcosm (Microcosm 4; Supporting Information Fig. S1). Thereafter, 1 g soil was collected from each microcosm to determine the abundance of heterotrophic microorganisms by examining the CFU on LA-plates. The largest numbers of heterotrophic organisms were found in nutrient-stimulated microcosm and the lowest microbial count was observed in the microcosm treated only with PES (Fig. 1a). Interestingly,

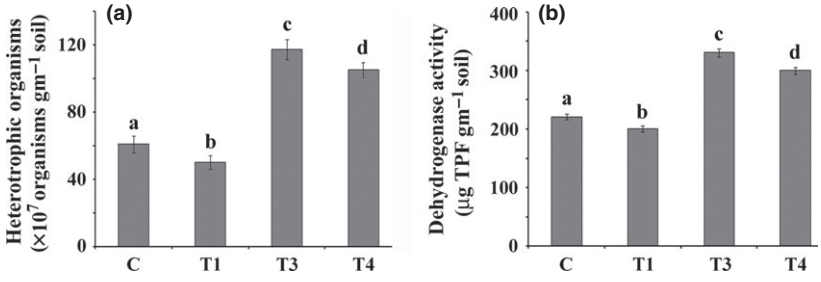

Fig. 1. Effect of nutrient supplementation to soil on soil microbial population of different microcosms. (a) Variation of heterotrophic organisms among different microcosms. After 28 days of incubation at 30 °C, 1 g soil from each microcosm was serially diluted and spread onto LA-plates in triplicate and incubated at 30 °C for 48 h. Colonies appeared on the plates were counted. The result represents the average of three replicates. Error bars indicate standard deviation (± SD). Mean values with different letters are significantly different among treatments. (b) Viable microbial population profile in different microcosms. After 28 days of incubation at 30 °C, a 5-g soil sample was taken from each microcosm and dehydrogenase activities were examined. Three replicates have been used for each type of microcosm and the results represent the average of these three replicates. Error bars indicate standard deviation (± SD). Mean values with different letters are significantly different between treatments. C, control soil (microcosm 1); T1, soil portion of microcosm 2; T3, soil portion of microcosm 3; T4, soil portion of microcosm 4.

addition of PES-films to microcosms resulted in a reduction of the soil microbial population compared with the respective microcosm lacking PES-films (Fig. 1a, compare C with T1; T3 with T4). The result was further confirmed by examining the dehydrogenase activity of microorganisms in each microcosm, as dehydrogenase activity is directly related to microbial viability. The highest level of dehydrogenase activity was observed in microcosm stimulated with nutrients and lowest dehydrogenase activity was observed in microcosm treated only with PES (Fig. 1b). Thus assessment of microbial abundance in different microcosms, using two different approaches, exhibited similar patterns (compare Fig. 1a and b). Thus the addition of PES to soil resulted in a reduction of the soil microbial abundance of the respective microcosms.

Microbial population splits from the source population to the surface of PES-films

The observed reduction of microbial counts in the soil of PES-containing microcosms can be attributed to several factors, including a possible toxic effect of PES. Another likely mechanism is that, in presence of PES, a subpopulation of the soil microorganisms translocates to the PES surface, which results in reduction of the soil microbial population. To verify this, the microbial populations were extracted from the PES-films obtained from Microcosms 2 and 4 at different time points and plated on both LA and agar plates with PES as the sole carbon source. Results showed considerable numbers of CFU for both films (Fig. 2). However, consistent with the previously observed increased population in biostimulated soil (Fig. 1), a larger number of colonies were found for the PES-films that were collected from the nutrient-stimulated soil (Fig. 2). At day 15, in both cases, the result showed more CFU on LA-plates than on PES-plates, whereas at day 28, no significant difference was observed in the number of CFU between LA and PES-plates (Fig. 2). To eliminate the possibility that microbes on the PES plates utilized agar as the C-source for their growth and survival, organisms were allowed to grow on agar plates prepared similarly but lacking PES. Organisms failed to grow on such agar plates lacking PES supporting the idea that these organisms utilize PES for their growth and survival. In addition, we observed clear zones surrounding the colonies on the PES-plates, indicating the degradation of PES by the microbes (data not shown). Taken together, these results indicate that initially microbes, both PES-degraders and non-degraders, are capable of attaching to PES-films translocated from the soil to the polymer. However, in time the microbes that were retained on PES-films were all found to be PES-degraders, as there was no significant difference in number of colonies on LA and PES-plates at day 28. Thus the observed reduction of the soil microbial population after addition of PES-films can be attributed, at least in part, to the translocation of a portion of microbes to PES-films from soil.

The microbial community on PES surface exhibits distinct metabolic finger printing

As microorganisms were transferred from the soil to PES-films, it is important to know whether the microbial community on PES-films develops a distinct microbial community. If the newly developing microbial population becomes adapted to the polymer surface efficiently, it may have undergone physiological changes compared with the original microbial population in the soil of the same microcosm. To verify this hypothesis, the microbial community metabolic fingerprinting was examined on the basis of different carbon source utilization patterns

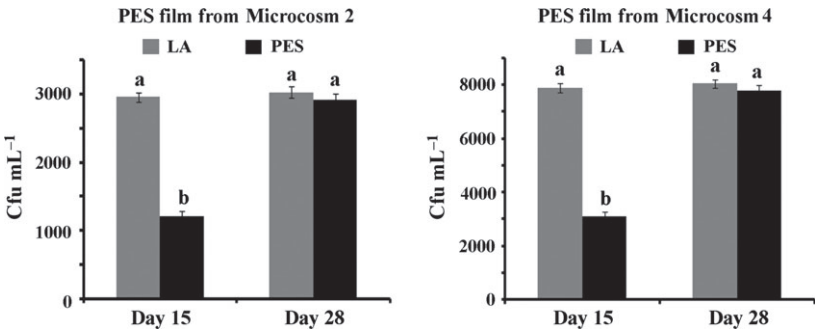

Fig. 2. The microbial population profile on PES-films. Microorganisms adhering to the entire PES-films were extracted with 3.5 mL of sterile Milli Q water and CFU were measured on LA and PES-plates. PES-films from microcosm 2 (soil amended with PES-film) and microcosm 4 (nutrient-supplemented soil amended with PES) were used in this experiment. Three replicates have been used for each type of microcosm and the results represent the average of these three replicates. Error bars indicate standard deviation (± SD). Mean values with different letters are significantly different among treatments. T2, PES-films of microcosm 2; T5, PES-films of microcosm 4.

using BiOLOG-ECO plates. The microbial communities of different origins showed considerable variation in utilization of different important carbon sources in ECO-Plates (Fig. 3a).

To further refine the result, multivariate cluster analysis was performed. The result showed that microbial communities of Microcosms T2 and T5 were significantly similar and formed a distinct cluster away from other microbial communities (Fig. 3b). Thus the newly developed population on the PES surface exhibits a different pattern of metabolic clustering compared with other samples tested. This result suggests that the new community formed on the PES surface undergoes significant alteration of the metabolic pattern, which makes it distinct from its parental soil microbial community.

Directional transfer of microorganisms from soil to PES-films during biostimulation follows 'founder effect'

Founder effect is a form of genetic drift that occurs when a small group breaks off from a larger population and establishes a new colony (Mayr, 1942, 1954). As the small number of founders in new population carries only a fraction of diversity from the original one, the new population exhibits lower diversity, higher evenness and separate metabolic potential compared with the source microbial population.

In the previous sections it has already been shown that only a small population of soil microbes translocated from soil to PES-films (Figs 1 and 2) and this new popu-

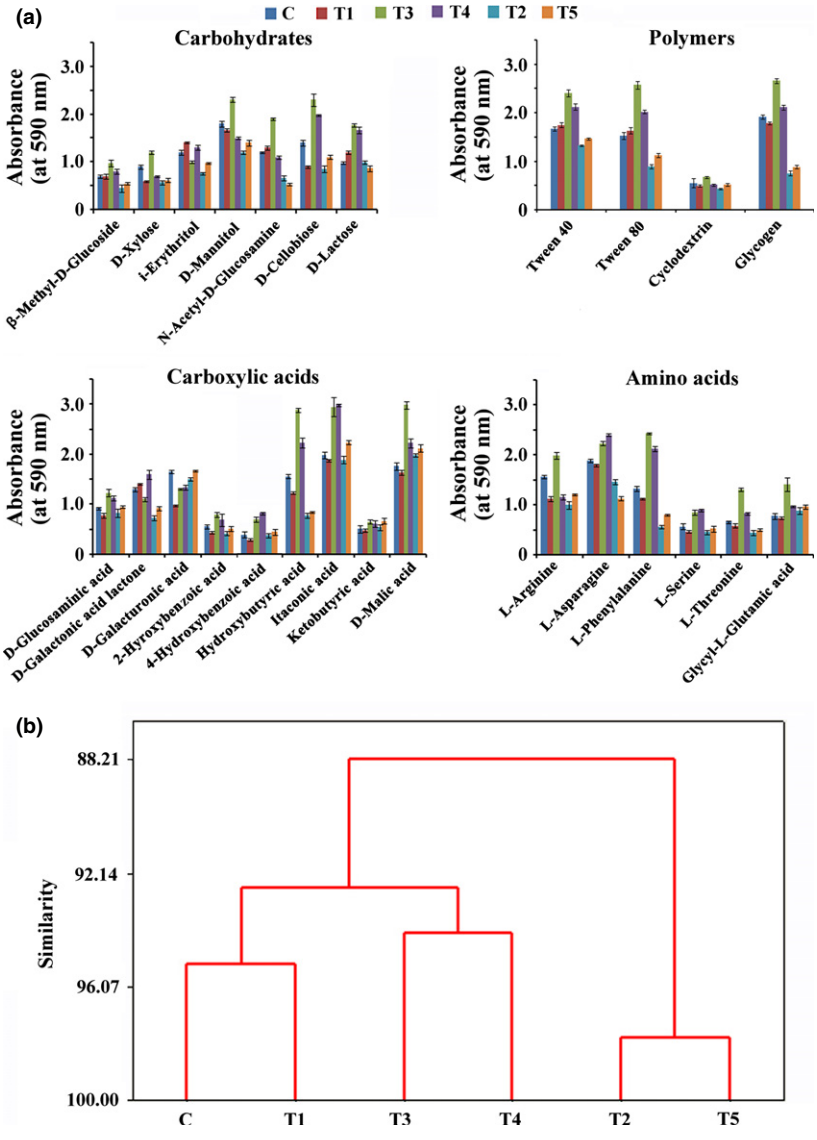

Fig. 3. Metabolic fingerprinting profile. (a) Carbon source utilization patterns of microbial communities from different samples. Serially diluted microbial samples from each sample containing c. 1000 CFU were added separately to wells of BiOLOG-ECO plates and incubated for 60 h at 30 °C. Absorbance at 590 nm of each well was recorded. (b) Metabolic relationship among microbial populations from different microcosms. UPGMA cluster analysis of microbial communities was performed based on results from BiOLOG-Eco Plates. C, control soil (microcosm 1); T1, soil portion of microcosm 2; T3, soil portion of microcosm 3; T4, soil portion of microcosm 4.

lation is metabolically distinct from the soil microbial population (Fig. 3). To understand the diversity, the Shannon-diversity index (Reardon *et al.*, 2004; Teng *et al.*, 2010) of the different microbial communities was determined. The result showed a significant variation in the Shannon-diversity index among different samples (Fig. 4a), with a marked reduction in diversity index being observed for the microbial community on PES-films recovered from biostimulated microcosm compared with the corresponding microbial community of soil sample (Fig. 4a). Thus, there was less variation in the microbial community on PES-films than in its soil counterpart. To verify the evenness of microbial population, Gini-coefficients of different microcosms were determined. Gini-coefficient is a measure of inequality or evenness in a population, ranging from zero, when all individuals are equal and most evenly distributed, to a maximum of one, in which the population exhibits maximum inequality (Harcha *et al.*, 1997; Liu *et al.*, 2011). The Gini-coefficient was found to be reduced for microbial communities extracted from the PES surface recovered from biostimulated microcosm compared with the corresponding soil microbial communities (Fig. 4b). This indicates that the microorganisms anchored to PES-films were more evenly distributed compared with the parent soil microbial community. Thus, analyses of the results obtained from BiOLOG-assay demonstrate that the directional transfer of microorganisms from soil to PES-films, during biostimulation, indicate an instance of 'founder effect'.

It was further observed that, during biostimulation, PES-degraders collected from PES-films at day 28 exhibited significantly higher levels of PES degradation than did the same number of PES-degraders obtained from PES-films that were incubated for 15 days (Fig. 5). Thus the enhancement of PES-degrading ability of the adhered microbial population indicates a genetic drift whereby more efficient PES degraders emerged overtime within a less-diverse microbial community. These results indicate that founder effect promotes PES degradation by aiding the development of a suitable microbial community on PES-films.

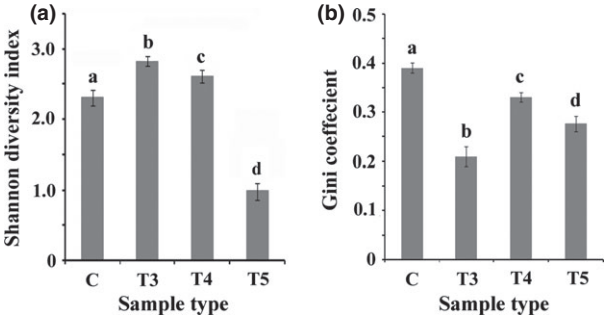

Fig. 4. The diversity and evenness profiles for microbial populations in soil and on PES-films of different microcosms. Serially diluted soil samples from each microcosm containing c. 1000 CFU were added separately to wells of BiOLOG-ECO plates and incubated at 30 °C for 60 h. Absorbance at 590 nm of each well was recorded at different time points. C, control soil (microcosm 1); T1, soil portion of microcosm 2; T3, soil portion of microcosm 3; T4, soil portion of microcosm 4. (a) Shannon–Weaver index of each microcosm was derived from well color absorbance at 590 nm and plotted. The result represents the average of three replicates. Error bars indicate standard deviation (± SD). Mean values with different letters are significantly different among treatments. (b) Variations of Gini-coefficients among microbial populations of different microcosms were examined from Lorenz curves. The results represent the average of three replicates. Error bars indicate standard deviation (± SD). Mean values with different letters are significantly different among treatments.

Discussion

The current study investigated the underlying mechanism of biostimulation mediated considerable degradation of PES in microcosm. It was observed that during biostimulation, a part of the soil microbial population translocated from soil to PES-films. Initially, this population contained both PES-degraders and non-degraders, but with time this community consisted mainly of PES-degraders. The size of the newly formed microbial population on PES-films was very low compared with the original population size present in the soil samples of biostimulated microcosms. The results also showed reduced diversity and higher evenness for this new community on PES-films. As

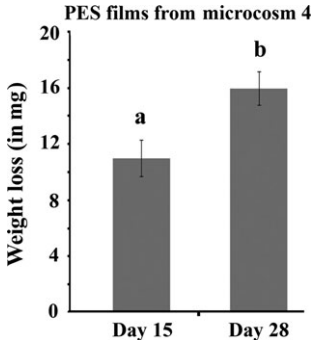

Fig. 5. Comparison of PES degradation by PES-degraders obtained from days 15 and 28 PES-films from biostimulated microcosm. Equal numbers (c. 5000 cells) of PES-degraders were taken from PES-films recovered at days 15 and 28 of biostimulated microcosms. These cells were inoculated separately into media containing PES-films as the sole carbon source. Cultures were incubated at 30 °C for 10 days. After the incubation, dry weights of the PES films were measured. The results represent the average of three replicates. Error bars indicate standard deviation (± SD). Mean values with different letters are significantly different among treatments.

the newly developed microbial community on PES-films utilized PES in a sustained manner, it is conceivable that this new community became adapted to this microenvironment. The adaptation of microorganisms on PES-films necessitates different physiological activities compared with those of the original microbial population. Consistently, metabolic fingerprinting and the dendogram of the C-source utilization patterns demonstrated the development of a distinctly different microbial community on PES-films, which originated from biostimulated microcosms. Thus the new community on PES-films exhibited founder effect, i.e. the establishment of a new population by a few original founders that carries a small fraction of the total genetic variation present in the parent population. It may be regarded as a special case of genetic drift, which is defined as the fixation of a particular genotype. In support of this, we have observed that during biostimulation, founder effect leads to the development of a more efficient microbial network on polymer surface overtime that is capable of better PES degradation. Thus biostimulation not only increases the size of the microbial population on PES-films, but also, over time, results in a community with a better ability to degrade PES. Both these events contribute to considerable PES degradation in biostimulated microcosm.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Weight loss of PES-films exposed to different microcosms.