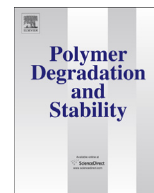
Polymer Degradation and Stability 131 (2016) 9–19

Contents lists available at ScienceDirect

Polymer Degradation and Stability

journal homepage: www.elsevier.com/locate/polydegstab



Effects of polyhydroxyalkanoate degradation on soil microbial community

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
13 June 2016
Accepted 26 June 2016
Available online 27 June 2016

Keywords:
Polyhydroxyalkanoate (PHA)
Biodegradation
Forest soil
16S rDNA metagenomic sequencing
Denaturing gradient gel electrophoresis
(DGGE)

ABSTRACT

Polyhydroxyalkanoate (PHA) serves as an alternative to some petroleum-based materials due to its biodegradability nature. Nevertheless, no study has been conducted before to evaluate the impact of PHA degradation to the environment. The purpose of this research was to investigate the degradation of PHA and its effects towards the soil microbial community. Four types of PHAs with three different thickness were used in this study; poly(3-hydroxybutyrate) [P(3HB)], poly(3HB-*co*-8 mol% 3-hydroxyhexanoate) [P(3HB-*co*-8 mol% 3HHx)], P(3HB-*co*-12 mol% 3HHx) and P(3HB-*co*-21 mol% 3HHx). These films were buried at a secondary forest for a duration of 8 weeks. P(3HB-*co*-21 mol% 3HHx) showed the highest degradation than the rest of the films. 16S rDNA metagenomic analysis revealed that some of the major phyla that were found at the sampling sites included *Actinobacteria*, *Firmicutes* and *Proteobacteria* which had the ability to degrade PHA. 16S rDNA-Denaturing Gradient Gel Electrophoresis (DGGE) profiling successfully showed that the diversity and population of the soil microbial community were correlated with the degradation of PHA. There was a significant change in the soil microbial abundance before and after the PHA was degraded.

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1. Introduction

The dependency on petroleum-based plastics have increased significantly throughout the years. These plastics are known to play an important role in our daily lives due to their desirable stability, versatility, mechanical properties and affordability. One major drawback of plastics is its persistence in the natural environment. Bio-based and biodegradable plastics may be able to solve or at least minimize such problems. Polyhydroxyalkanoate (PHA) is one of the interesting type of bio-based polymers. The building blocks of PHA are hydroxyalkanoic acid (HA) monomers that are biosynthesized by many Gram-positive and Gram-negative bacteria as a form of intracellular carbon and energy storage [1,2] by using sugars [3], plant oils [4], beet molasses [5], alphechin wastes [6] and starch [7]. PHA shares some similar properties to synthetic plastics such as its thermo-plasticity as well as mechanical properties.

Due to its useful properties, the range of potential applications of PHAs have grown wider, thus examining the degradation of these polymers in natural environments has acquired increasing significance. PHA degradation is performed by microorganisms that secrete intra- or extracellular PHA depolymerases, which differ in their molecular organization and substrate specificity [8]. Besides that, it is also known that the rate of biodegradation is significantly influenced by several environmental factors such as microbial population, temperature, moisture level, pH, nutrient supply as well as the composition, crystallinity, additives and surface area of the PHA itself [9]. Several studies have been carried out to investigate the degradation of PHA in the environment; such as in the mangrove area [10], lake [11], soil [12,13], compost [14], natural waters [15] and eutrophic reservoir [16]. However, none of these studies reported on the changes in the soil microbial populations due to the degradation of PHA.

In most environments, it is stated that almost 99% of the microorganisms cannot be cultured by standard techniques and thus metagenomics analysis is essential to understand the genetic diversity, population structure and ecological roles of the microorganisms [17]. In addition, denaturing gradient gel electrophoresis (DGGE) can also be used to detect changes in the composition of soil microflora [18]. Many studies have shown that the functional and taxonomic diversity of soil microbial communities are strongly impacted by environmental factors and thus soil microbial diversity

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http://dx.doi.org/10.1016/j.polymdegradstab.2016.06.024
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may be considered as good indicator of how the ecosystem is functioning [19]. To date, no studies have been conducted to evaluate the changes in microbial community as a result of PHA degradation with the combination of 16S ribosomal DNA (rDNA) metagenomic sequencing and PCR-DGGE approaches. Therefore, the aim of this study was to determine the degradation of PHA and its potential impact on the soil microbial community especially on the bacterial population of secondary forest using culture independent techniques.

2. Materials and methods

2.1. PHA formulation

Four different PHA formulation with three different thickness were used for the biodegradation study. These include P(3HB), P(3HB-*co*-8 mol% 3HHx), P(3HB-*co*-12 mol%-3HHx) and P(3HB-*co*-21 mol% 3HHx). The P(3HB-*co*-8 mol% 3HHx) and P(3HB-*co*-12 mol %-3HHx) were provided by KANEKA Corporation, Japan while P(3HB-*co*-21 mol% 3HHx) was synthesized in the laboratory using crude palm kernel oil (CPKO) by *Cupriavidus necator* Re2058/pCB113 (kindly provided by Prof. Anthony Sinskey, MIT) while *C. necator* H16 was used to produce P(3HB) using palm olein in a 13 L fermentor.

2.2. Preparation of PHA films

Pure polymers of P(3HB-*co*-21 mol% 3HHx) and P(3HB) were obtained using chloroform extraction followed by methanol precipitation method. All PHA films were prepared by conventional solvent-cast technique. Three different concentration of polymer solution (0.01 g/mL, 0.03 g/mL, and 0.05 g/mL) were prepared by dissolving the polymer in chloroform in order to yield different thickness of PHA films. The solution was poured into a Petri dish which was used as the casting surface. The Petri dishes were left overnight for evaporation of chloroform at room temperature. The resulting PHA films were aged for 2 weeks before being used for the experiments.

2.3. Experimental designs and sampling gears

The PHA films were cut into small pieces with the dimension of 1.25 cm × 1.25 cm. Only films with the even surface area were selected for the studies. A nylon net with the pore size of 3 mm was sewed into a pouch with the size of 10 cm × 8 cm to keep the films. Each square film was weighed and placed carefully into the pouch which was further sewn to form different compartment according to its type of polymer and thickness. The pouch and the label for each pouch were tied to a wire mesh. The workflow is as shown in Fig. 1. The wire mesh was placed inside the soil at a depth of 15 cm so that the PHA films were fully buried.

2.4. Sampling sites

The sampling location was at a secondary forest nearby Universiti Sains Malaysia, in Penang Island. The characteristics of the study locations are as listed in Table 1.

2.5. Determination of biodegradation

Three pouches (triplicates of each formulation) were retrieved from the buried conditions at each sampling location every week. Each pouch was soaked and rinsed with distilled water to remove the soil debris attached to it. The sample (PHA film) was removed carefully from the pouch, rinsed with distilled water two to three times to remove the remaining sediments attached to it. The samples were left overnight to dry at room temperature. The dried samples were placed in a desiccator until a constant weight was reached. The weight of each film was weighed and recorded. The degradation of the films was determined in terms of percentage of weight loss by using the following formula

$$\left(\frac{\text{Weight of initial film} - \text{weight of film after degradation}}{\text{Weight of intial film}}\right) \times 100$$

2.6. Scanning electron microscopy (SEM) and digital photography

The SEM micrograph of the PHA films were taken using Leo Supra 50 VP Field Emission SEM (Carl-Zeiss SMT, Oberkochen, Germany) at the magnification of 500–2500 times. Images of the PHA films were also taken using a digital camera (Canon PowerShot A3000 IS) to record the physical changes of the films.

2.7. Molecular weight and crystallinity determination

The molecular weights for each tested film were determined using Agilent 1200 Series GPC with Shodex K-806M and Shodex K-802 column at 40 °C. Chloroform was used as the eluent. The injection volume was 50 µL with the flow rate of 1.00 mL/min. The crystalline structure of the film was determined using Wide-Angle X-ray Diffraction (WAXD) Bruker D8 Advance.

2.8. Genomic DNA extraction, purification and amplification

Soil sample was subjected to total DNA extraction using PowerSoil® DNA Isolation Kit (MOBIO, USA). Extracted DNA was purified using PowerClean® DNA Clean-Up Kit (MOBIO, USA). PCR amplification was carried out to amplify the bacterial variable V2-V5 region of 16S rRNA gene. The primers used were GC*357f (5′-GC*CCT ACG GGA GGC AGC AG-3′) and 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′) [20]. The PCR reaction contained 1× GoTaq® Flexi buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.25 µM of each primer, 1U GoTaq® DNA polymerase and 1 ng of DNA. The conditions of the PCR reaction are shown in Table 2.

2.9. 16S rDNA metagenomic sequencing and analysis

Extracted and purified DNA was sent for metagenomic sequencing at Microsynth, Switzerland. PCR amplification of 16S V4 rDNA fragment was carried out, followed by preparation of individually tagged sequencing libraries. The sequencing was done on Illumina MiSeq Platform (2 × 250 bp paired-end reads). Input sequence reads were prepared (quality filtering and identification of Chimeras) using QIIME [21]. Operational Taxonomic Units (OTUs) were determined by clustering based on 97% (species) level sequence similarity within reads. A representative sequence from each OTU was picked. OTUs were assigned to taxonomic identities using SILVA databases [22]. OTU table was built to relate samples, OTUs and lineage to each other. Diversity metrics for communities of organisms within each sample (intra sample/alpha diversity) was calculated using Chao1 and Shannon indices. The sequence reads were also analyzed using MG-RAST [23].

2.10. Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was performed using DCode™ Universal Mutation Detection System (Bio-Rad, USA). PCR amplicons (~400 ng) for Week 0, 2, 4, 6 and 8 from all the locations were applied onto a 9%

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