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Microbial degradation of polyhydroxyalkanoates in tropical soils

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ABSTRACT

The integrated study addressing biodegradation of microbial linear polyesters of hydroxyalkanoic acids (polyhydroxyalkanoates, PHAs) in tropical conditions by microbial communities of Vietnamese soils was performed in locations close to Hanoi and Nha Trang, which differed in their weather conditions and microbial communities. It shows that PHA degradation in tropical soils is influenced by polymer chemical composition, specimen shape, and microbial characteristics. The homopolymer of 3-hydroxybutyric acid is degraded at higher rates than the copolymer of 3-hydroxybutyric and 3-hydroxyvaleric acids. The average rates of mass loss were 0.04–0.33% per day for films and 0.02–0.18% for compact pellets. PHA degradation was accompanied by a decrease in the polymer molecular mass and, usually, an increase in the degree of crystallinity, suggesting preferential degradation of the amorphous phase. Under the study conditions, representatives of the bacterial genera *Burkholderia*, *Bacillus*, *Cupriavidus*, *Mycobacterium*, and *Nocardiopsis* and such micromycetes as *Acremonium*, *Gongronella*, *Paecilomyces*, and *Penicillium*, *Trichoderma* have been identified as major PHA degraders.

1. Introduction

The annual output of synthetic polymers that cannot be degraded in the natural environment has reached 300 million tons (Thompson et al., 2009) and continues to grow, posing a global environmental problem. Polymer materials market is strategically aimed at gradual replacement of the non-degradable polyolefins (mostly polyethylene and polypropylene) by the new generation of degradable polymers (Kijchavengkul and Auras, 2008). Biodegradable linear polyesters of hydroxyalkanoic acids (polyhydroxyalkanoates, PHAs), which are synthesized with good yields (up to 80–90% of the cell mass) (Braunegg et al., 1998; Khanna and Srivastava, 2005), together with polylactides, are good candidates to gradually replace synthetic polymers.

There are a number of pilot and small-scale facilities in different countries, producing PHAs trademarked as Biopol™, Nodax™, DegraPol/btc®, Mirel® (Chen, 2009). The decrease in the cost of PHAs has widened their applications: in addition to medicine and pharmacology, the polymer is used in production of degradable bags and packaging materials, disposable dishes and domestic items (Poliakoff and Noda, 2004; Noda et al., 2005). Poly-3-hydroxybutyrate (poly-3-HB) and poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (poly-3-HB/3-HV) are among the most commonly occurring bacterial PHAs. Poly-3-HB is a highly crystalline and brittle thermoplastic. Poly-3-HB/3-HV has much better properties, including reduced brittleness. This copolymer is more useful commercially because its melting point can be lowered, and its mechanical properties and thermoplastic characteristics can be greatly improved by increasing the ratio of 3-hydroxyvalerate to 3-hydroxybutyrate repeating units (Dufresne et al., 2003).

In the absence of biological agents PHAs are practically not subject to mass lost under normal conditions (Doi et al., 1989). They are degraded in biological media to form products innocuous to the environment: carbon dioxide and water under aerobic conditions or methane and water under anaerobic conditions. PHA biodegradation is performed by microorganisms that secrete intra- or extracellular PHA depolymerases, which differ in their molecular organization and substrate specificity (Knoll et al., 2009). While intra-

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cellular PHA depolymerases are synthesized by PHA producing bacteria and are used by them to hydrolyze their own PHA storages, extra-cellular enzymes are produced by other microorganisms to utilize PHAs usually released into environment after death and cell lysis of PHA accumulating cells (Jendrossek and Handrick, 2002).

The first microorganisms degrading poly-3-HB were isolated over 40 years ago (Chowdhury, 1963). Six hundred PHA-depolymerases from various microorganisms have been identified by now; comparison of their amino-acid sequences provided a basis for uniting them in 8 superfamilies including 38 families (Knoll et al., 2009). The same strain can contain several genes encoding PHA depolymerases with different specificities. The ability to degrade extracellular PHAs is determined by the activity and type of PHA depolymerases, which hydrolyze the polymer by surface erosion to water-soluble monomers and/or oligomers – a substrate for microorganisms. Many microorganisms have extracellular PHA depolymerases.

In PHA degradation studies, the greatest consideration is given to isolation of microorganisms involved in this process. Among PHA degraders described in the literature are bacteria, actinomycetes and micromycetes that degrade PHAs in soil, compost, activated sludge, and river and sea water (Wallen and Rohwedder, 1974; Doi et al., 1992; Imam et al., 1999; Kim and Rhee, 2003; Beleneva and Zhukova, 2009). However, although PHA degrading microorganisms have been studied for more than 40 years, many aspects of the complex process of PHA degradation in natural environments still remain to be understood. They include, e.g., dependence of PHA degradation rate and mechanisms on PHA chemical composition, properties (crystallinity, molecular mass, polydispersity), macro- and microstructure, the shape and size of PHA-based devices, and physicochemical conditions of the environment (temperature, pH, oxygen availability, salinity, etc.), weather and climate in different regions.

Biodegradation of polyhydroxyalkanoates is performed by microorganisms, which inhabit a specific natural environment. Soil is the natural environment with the greatest capacity for PHA degradation. However, most of the studies addressing PHA degradation in soil were carried out in laboratory (Mergaert et al., 1993; Suyama et al., 1998; Bonartseva et al., 2003; Erkske et al., 2006; Woolnough et al., 2008) and some of them used isolated cultures of PHA degrading microorganisms (Nishida and Tokiwa, 1993; Mokeeva et al., 2002; Colak and Güner, 2004). There are very few published data on PHA biodegradation in soil under field conditions. One of the first studies that addressed PHA degradation under natural conditions showed (Mukai and Doi, 1993) that a golf tee made of the polymer was almost completely degraded in soil within four weeks; unfortunately, the authors of this study did not describe either the exact composition of the PHA or the soil characteristics. There are data, however, suggesting that the type of the soil is an essential factor affecting PHA degradation. For instance, in the mangrove soil, the degradation rate of medium-chain-length PHAs was 0.04% mass loss per day, while in the rainforest soil it ranged from 0.03% to 0.15% (Lim et al., 2005). Sridewi et al. (2006) reported that in the mangrove soil, degradation rates of different PHAs (copolymers of 3-hydroxybutyrate, 3-hydroxyvalerate, and 3-hydroxyhexanoate and a homogenous poly-3-hydroxybutyrate) were not equal and that the copolymers were degraded faster than the homopolymer. Yew et al. (2006) showed that PHA degradation rate in the garden soil was influenced by the density of microbial populations. These studies do not give a comprehensive idea of the diverse and complex process of PHA biodegradation, nor do they provide insight into degradation behavior of PHAs consisting of monomers with different carbon chain lengths in various types of soils, with different PHA degrading microorganisms; climate and weather effects are not taken into account either.

Thus, the data on soil degradation of PHAs in natural environments are scant. The purpose of this study was to investigate PHA degradation behavior in the soil under tropical conditions and isolate major PHA degrading microorganisms.

2. Materials and methods

2.1. Preparation and characterization of biopolymers

The polymers were synthesized in *Wautersia eutropha* B5786 microbial culture (the strain is registered in the Russian Collection of Industrial Microorganisms). Cells were batch-cultured in a 14-L New Brunswick Scientific BioFlo 110 fermentor filled to 40% of its volume in standard Schlegel mineral salts medium (Schlegel et al., 1961) with fructose and deficient in NH_4Cl, under strictly aseptic conditions, at 30 °C, aeration, and 1000 rpm agitation. To induce 3-hydroxyvalerate units in polymers, the culture medium was supplemented with potassium salt of valeric acid (Sigma) at concentrations of 0.5–2.0 g/L.

Accumulation of the biomass in the culture was monitored by measuring the dry matter weight and optical density of the culture. Dry biomass samples were subjected to methanolysis (Brandl et al., 1989) and the total polymer content of the biomass and monomer compositions were determined by chromatography of methyl esters of fatty acids on an Agilent 7890A gas chromatography system with an Agilent 5975C VL MSD mass spectrometer (Agilent Technologies, USA) (Volova et al., 1998). Polymer and lipids were extracted from cells with a chloroform-ethanol mixture (2:1 v/v), and then the polymer was separated from lipids by precipitation with hexane. The extracted polymers were re-resolved in chloroform and precipitated again for purification. The chemical purity of the resulting specimens was estimated by conventional biochemical methods. The presence of protein impurities was determined by the Kjeldal micromethod (McKenzie and Wallace, 1954), carbohydrates by the anthrone method (Seifter et al., 1950) and fatty acids by gas chromatography.

2.2. Preparation of PHA samples

Films were prepared by casting chloroform solution (3% w/v) on degreased glass and subsequent drying at room temperature for 2–3 days in a dust-free box. The resulting film discs were 30 mm in diameter and 0.1 mm thick. Segments of equal thickness were selected and discs of diameter 30 mm, thickness 0.1 mm, and mass 73 ± 5 mg were cut out to be further used in the experiments. The specimens were sterilized using a Sterrad NX System hydrogen peroxide gas plasma sterilizer (Jonhson&Johnson, USA). Compact pellets were prepared by cold compaction of finely powdered polymer, using an AutoPellet 3887 laboratory press (Carver, USA) at 120 kg-f/cm^2 (diameter 10 mm, height 2.5 mm, mass about 350 mg).

2.3. Experimental designs

Experiments were performed under natural conditions, in soils at Climate test stations (CTS) of the Joint Russian-Vietnam Tropical Research and Test Center in Vietnam. CTS "Hoa Lac" is located 30 km from Hanoi (22° 45′ N, 105° 48′ E, about 100 km from a sea), and CTS "Dam Bai" – on the Che island in Dam Bai Bay (12° 14′ N, 109° 11′ E, area of the city of Nha Trang, about 50 m away from the sea).

PHA specimens (3 of each type) were weighed and placed in close-meshed gauze jackets, which were then buried 15 cm deep in soil.