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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.

2.4. Sampling gears

Investigations were performed during 10–12 months: from 14 May 2010 to 15 March 2011 at CTS Hoa Lac, and from 7 May 2010 to 28 May 2011 at CTS Dam Bai. At time intervals about 30 days, jackets were taken out of the soil. Polymer specimens were taken out of the jackets, cleaned by distilled water using a metal scraper for removing soil remains, dried for 48 h at room temperature, and weighed. Air temperature and humidity and the amount of precipitation were measured over a period of the experiment.

2.5. Determination of PHA biodegradation

Biological degradation of PHAs was evaluated based on the following parameters: a decrease in the mass of the specimens, changes in the molecular weight and molecular-weight distribution of polymers, and a change in fine structure properties such as the ratio between the ordered (crystalline) phase and the disordered (amorphous) phase, measured as crystallinity (C_x, %).

Polymer specimens were weighed using a Mettler balance (USA) of precision class 4. Mass loss was calculated as the difference between the initial and final sample masses.

Molecular weight and molecular-weight distribution of PHAs were examined using a gel permeation chromatograph ("Agilent Technologies" 1260 Infinity, U.S.) with refractive index detector using Agilent PLgel Mixed-C column. Chloroform was the eluent, at a flow rate of 1.0 mL/min at 40 °C. Typical sample volumes were 50 μL at a polymer concentration of 2 mg/mL. Narrow polydispersity polystyrene standards (Agilent, USA) were used to generate a universal calibration curve, from which the molecular weights were determined. The parameters determined were the average molecular weight (M_n), the weight average molecular weight of the polymer (M_w), and polydispersity (PD = M_w/M_n).

X-ray structure analysis and determination of crystallinity of PHAs were performed using a D8 ADVANCE X-ray diffractometer ("Bruker, AXS", Germany). To determine the degree of crystallinity, C_x, spectra were collected with the high-speed detector Vantec, exposure time 300′, to measure intensity at point. The operating mode of the instrument was 40 kV × 40 mA.

2.6. Counting and isolation of microorganisms

Samples from soil and specimen surfaces were taken for microbial analysis after 6 months of specimen exposure in soil. Concentrations of microorganisms (CFU/g soil) were calculated for the control soil and the soil layer on the surface of the specimens. An aliquot of the soil sample was preliminarily suspended in water. The specimens were weighed (aseptically), washed in water (20 mL), dried, and weighed again. The ratio of the difference between specimen masses before and after washing to the volume of the wash water was used to determine the initial concentration of the soil in the suspension. Soil suspensions were inoculated on agar plates (beef peptone agar for bacteria and wort agar for fungi) in 10^4–10^7 dilutions. All platings were performed in triplicate. The inoculated plates were maintained for 3–7 days at a temperature of 30 °C prior to counting bacteria and for 7–10 days at 25 °C prior to counting micromycetes (Zvyagintsev, 1990). A temperature of 30 °C is optimal for PHA degradation and growth of PHA degrading bacteria (Manna et al., 1999; Manna and Paul, 2000). Micromycetes were counted at a lower temperature (25 °C) in order to prevent or slow down growth of spore forming bacteria on wort agar (Zvyagintsev, 1990).

The cultured PHA degraders were isolated using the conventional clear-zone technique (Mergaert et al., 1993; Jendrossek and Handrick, 2002; Sangkharak and Prasertsan, 2012). The suspensions of the control soil and the soil from the surface of polymer specimens prepared at the end of the field experiment as described above were inoculated onto the mineral medium that contained 0.25% powdered poly-β-hydroxybutyrate as sole carbon source (Delafield et al., 1965). Growth of true PHA degraders (i.e. microorganisms with PHA-depolymerase activity) was accompanied by the formation of clear zones around colonies of microorganisms due to hydrolysis of polyhydroxybutyrate dust.

2.7. Identification of microorganisms

About 200 microbial isolates were isolated and analyzed. Bacteria isolated were first identified by conventional methods, based on their cultural and morphological properties and using standard biochemical tests found in identification guides (Weyant et al., 1996; Garrity et al., 2005). Soil micromycetes were identified based on their micro- and macro-morphological traits (structure and color of colonies, structure of mycelium and spore-bearing organs), which enable objective identification of these microorganisms (Egorova, 1986; Sutton et al., 1998; Kurakov et al., 2007; Bogomolova and Kirtsideli, 2010; Kondratyuk, 2010).

In addition to this examination, PHA degrading microorganisms were identified by 16S (for bacteria) and 28S (for fungi) rRNA gene sequence analysis. Extraction of DNA was carried out using a commercial AquaPure Genomic DNA Isolation reagent kit (Bio-Rad, USA), following the manufacturer's protocol. The prepared DNA was used as template for PCR amplification of the 16S rRNA gene using the pair of universal primers 27F (AGAGTTTGATC(AC)TGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Weisburg et al., 1991; Macrae, 2000). For eukaryotes, the 28S rRNA gene was amplified using yeast primers D1/D2 R (TTGGTCCGTGTTTCAAGACG) and D1/D2 U (GCATATCAATAAGCGGAGGA). The PCR product was purified using Qiaex II Gel Purification Kit (Qiagen, USA) and sequenced. Sequences were assembled using Vector NTI (Invitrogen, USA). The 16S and 28S rRNA based species identification was done using data from the Ribosomal Database Project (Wang et al., 2007), the National Center for Biotechnology Information (NCBI), or the EzTaxon server (Chun et al., 2007).

The obtained nucleotide sequences of the 16S and 28S rRNA gene were compared with the sequences in the GenBank, EMBL and DDBJ databases, using the BLAST tool for the search for sequences with high homology, of the NCBI Web site (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed using the Jukes and Cantor one parameter model with the neighbor-joining method implemented in the TREECON v. 1.3b software package. The obtained nucleotide sequences were deposited in the database of GenBank NCBI (Nos. JQ518340–JQ518351). All the strains were deposited in Culture Collection of Biotechnological Department of Siberian Federal University (Krasnoyarsk, Russia).

2.8. Statistical analysis

Statistical analysis of the results was performed using the standard software package of Microsoft Excel 2003. Arithmetic means and standard deviations were found. Significant differences between mean values were tested using Student's t test (significance level: $p = 0.05$) by standard methods.

3. Results and discussion

3.1. Characterization of the field season conditions and PHA biodegradation dynamics

A number of parameters of soil and weather conditions in the study locations were slightly different (Table 1). The air and soil temperatures and humidity in both study locations were similar

Table 1
Climate parameters at the Hoa Lac and Dam Bai test stations in 2010.

Month	Monthly average air temperature, °C		Monthly average relative humidity, %		Precipitation, mm	
	Hoa Lac	Dam Bai	Hoa Lac	Dam Bai	Hoa Lac	Dam Bai
May	29	29.5	81	80.6	150	27.4
June	31	29.4	74	78.2	175	7.2
July	31	29.0	74	78.8	280	40.2
August	29	28.4	82	81.4	274	82.0
September	29	27.9	79	82.9	172	138.8
October	26	26.9	70	84.0	25	325.7

throughout the study season. Levels of precipitation at Hoa Lac during the first months of the experiment were, however, almost an order of magnitude higher than those at Dam Bai. The pH values of the Hoa Lac soil were lower than those of the Dam Bai soil (5.5 and 6.6, respectively).

Degradation rates of all PHA specimens in the Hoa Lac soil were higher, too (Fig. 1). At the end of the incubation period, degradation of homopolymer (poly-3-HB) films reached more than 98%, and poly-3-HB/3-HV films were 61% degraded, while the pressed pellets were 55 and 35% degraded, respectively. At the Dam Bai station, the mass loss of poly-3-HB and poly-3-HB/3-HV films was 47 and 14% and that of the pressed pellets – 28 and 8%, respectively. The daily mass loss of PHA specimens of different compositions amounted to 0.20–0.33% (Hoa Lac) and 0.04–0.13% (Dam Bai) for films; 0.12–0.18% (Hoa Lac) and 0.02–0.08% (Dam Bai) for pellets.

In both locations, all poly-3-HB specimens were degraded faster than poly-3-HB/3-HV films and pellets.

The results obtained are in good agreement with the data reported by Rosa et al. (2003). Degradation rates of poly-3-HB recorded by these authors were higher than poly-3-HB/3-HV degradation rates, and they explained their results as being due to specific surface structure and properties of their specimens. A similar degradation pattern was reported in a number of studies that investigated PHA degradation by isolates of bacteria (Manna and Paul, 2000), actinomycetes (Manna et al., 1999), and fungi (McLellan and Halling, 1988; Sanyal et al., 2006). Kita et al. (1997) reported that in the pure culture of the bacterium *Alcaligenes faecalis*, degradation rates of poly-3-HB were higher than those of poly-3-HB/3-HV specimens. Similar results were obtained by Thellen et al. (2008), who studied degradation behavior of PHA films in the laboratory media containing slurry of marine sediment and found that under certain conditions, the mass loss of poly-3-HB specimens was greater than that of poly-3HB/3-HV ones.

These results, however, contradict the data reported in other studies, showing faster degradation of the less crystalline copolymer specimens (Mergaert et al., 1992, 1993; Volova et al., 1992, 1996). Mergaert et al. (1994) studied biodegradation of poly-3-HB and poly-3-HB/3-HV copolymers with different percentages of 3-hydroxyvalerate (10 and 20 mol%) in household compost heaps and showed that significant mass loss was only recorded in the poly-3-HB/3-HV specimens with a high 3-hydroxyvalerate percent (20 mol%). In our previous study (Boyandin et al., 2012) we showed that at temperate latitudes (Siberia, Krasnoyarsk) with markedly continental climate, in the soddy-carbonate soil of the arboretum, during the warmer summer season, poly-3-HB/3-HV copolymer films were degraded faster than the higher-crystallinity poly-3-HB specimens.

This discrepancy can be accounted for by differences in soil microbial communities and, hence, substrate specificity of extracellular PHA depolymerases secreted by PHA degrading microorganisms (Manna and Paul, 2000). It is noteworthy that the mass of polyethylene films, which were used as the control, remained unchanged throughout the period of exposure.

PHA biodegradability is influenced not only by the chemical composition of the polymer and the temperature of the environment, but also by the polymer stereoconfiguration, crystallinity, and molecular mass (Nishida and Tokiwa, 1993; Abe et al., 1998; Jendrossek and Handrick, 2002). The significant decrease in the molecular weight and increase in polydispersity of PHA specimens suggested that polymer chains were cleaved, resulting in the formation of smaller fragments with different degrees of polymerization.

Structural changes in the PHA specimens during the course of their degradation are shown in Table 2. The weight average molecular weight of all PHA samples dropped considerably, while their polydispersity increased. PHA pellets showed a less significant mass loss (Fig. 1) but a greater molecular weight decrease than PHA films. The reason for this may be that pellets underwent bulk

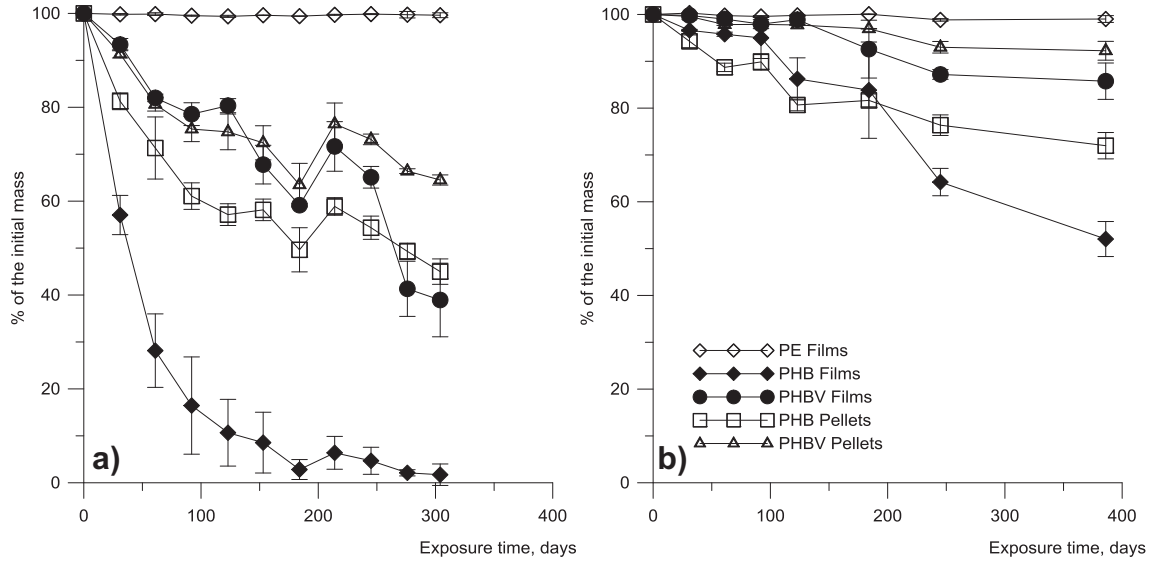

Fig. 1. Mass behavior of polymer specimens (% of the initial mass) buried in the tropical soils: a) Hoa Lac; b) Dam Bai.

Table 2
Degrees of crystallinity and molecular weights of PHA specimens in the course of their biodegradation in tropical soils.

Parameter	Initial value	After 180 d	Initial value	After 180 d
	Homopolymer, poly-3-HB		Copolymer, poly-3-HB/3-HV	
Hoa Lac, films				
C_x, %	60	60	51	58
M_w, kDa	1401 ± 106	1162 ± 37	1318 ± 85	960 ± 6
PD	2.00 ± 0.37	2.16 ± 0.16	2.01 ± 0.23	2.22 ± 0.15
Hoa Lac, pellets				
C_x, %	67	70	68	71
M_w, kDa	1401 ± 106	563 ± 18	1318 ± 85	478 ± 16
PD	2.00 ± 0.37	3.42 ± 0.07	2.01 ± 0.23	3.60 ± 0.08
Dam Bai, films				
C_x, %	60	62	51	59
M_w, kDa	1401 ± 106	1075 ± 60	1318 ± 85	828 ± 17
PD	2.00 ± 0.37	2.54 ± 0.21	2.01 ± 0.23	2.48 ± 0.15
Dam Bai, pellets				
C_x, %	67	70	68	68
M_w, kDa	1401 ± 106	502 ± 6	1318 ± 85	363 ± 6
PD	2.00 ± 0.37	3.71 ± 0.27	2.01 ± 0.23	5.56 ± 1.43

C_x – cristallinity; M_w – weight average molecular weight; PD – polydispersity.

degradation, while films were mainly degraded by surface erosion, and, thus, their bulk retained the properties similar to the initial ones.

X-ray structure analysis showed a slight increase in the degree of crystallinity of all PHA specimens as they were degraded, and the C_x of the copolymer specimens increased more significantly. This effect suggested preferential disintegration of the amorphous phase of PHAs in the course of degradation by the soil microflora under tropical conditions. A similar result was obtained in our study on degradation of PHAs in Siberian soils (Boyandin et al., 2012), but the weight average molecular weight of PHAs (M_w) decreased more significantly in the tropical soils than in the Siberian ones.

PHA film specimens were degraded at a faster rate than polymer pellets due to their larger surface area and, hence, a larger polymer/soil interface area, which allowed better attachment of microorganisms and quicker formation of biofilms. The molecular weight of the slowly degraded pressed pellets, however, decreased more significantly. A possible explanation for this may be that microorganisms and enzymes secreted by them can effectively attack the surface of the films, but extracellular PHA depolymerases cannot penetrate into the dense and smooth specimens, and their effect on the molecular weight of the non-degraded polymer is insignificant. Degradation of PHA films by surface erosion was reported by other authors (Lim et al., 2005). The pressed pellets, on the other hand, consist of PHA microparticles, and the enzymes can penetrate into the specimens through micropores at interparticle boundaries and degrade the material inside the pellets.

3.2. Isolation and identification of PHA degrading microorganisms

Climate parameters (Table 1) and soil properties (such as pH) were the factors that influenced the composition and abundance of soil microbiological communities in the study locations in Vietnam. The total counts of bacteria in the Hoa Lac soils were 16 million CFU/g, while in the Dam Bai soils, they were about 8 million CFU/g. The number of fungi was one order of magnitude higher in the soil at Hoa Lac than in the Dam Bai soil (84 000 and 8000 CFU/g, respectively).

The more rapid degradation of PHA specimens in the soil of the Hoa Lac station is in good agreement with the data reported by Yew et al. (2006), suggesting that PHA degradation is influenced by the number of microorganisms present in the soil.

Table 3
Total counts of bacteria and micromycetes on the surface of specimens and in soil.

	Poly-3-HB, pellets	Poly-3-HB/3-HV, pellets	Poly-3-HB, films	Poly-3-HB/3-HV, films	Soil
Bacteria, Hanoi	6.9×10^8	1.7×10^8	N/A	1.2×10^8	1.6×10^7
Micromycetes, Hanoi	2.2×10^6	6.2×10^5	N/A	2.2×10^6	8.4×10^4
Bacteria, Nha Trang	9.2×10^8	5.5×10^8	3.3×10^8	7.1×10^8	8.0×10^6
Micromycetes, Nha Trang	1.2×10^6	6.8×10^4	3.1×10^5	3.3×10^4	8.0×10^3

In order to gain insight into PHA biodegradation patterns and mechanisms of this process, it is important to isolate and identify PHA degrading microorganisms.

Microbial communities of the soils in the two study areas in Vietnam were significantly different. Microbial populations of the Hoa Lac soil were dominated by *Acinetobacter calcoaceticus*, *Arthrobacter artocyaneus*, *Bacillus aerophilus*, *Bacillus megaterium*, *Bacillus* sp., *Brevibacillus agri*, *Brevibacillus invocatus*, *Chromobacterium violaceum*, *Cupriavidus gilardii*, *Mycobacterium fortuitum*, *Ochrobactrum anthropi*, *Staphylococcus arlettae*, *Staphylococcus haemoliticus*, *Staphylococcus pasteuri*, *Pseudomonas acephalitica*, *Rodococcus equi*; while the major species in the Dam Bai soil were *Bacillus cereus*, *B. megaterium*, *Bacillus mycoides*, *B. agri*, *Gordonia terrari*, *Microbacterium paraoxydans*.

Analysis of total counts of bacteria from the biofilm on the surface of polymer specimens showed that their concentration was one or two orders of magnitude higher than in the control soil (Table 3). The counts of fungi on the surface of all PHA specimens were also higher than those in the control soil; the difference was more pronounced in the experiment in Dam Bai, reaching 2 or 3 orders of magnitude.

It is noteworthy that isolation of PHA degraders is often performed by analyzing the media (soil, compost, water) in which polymer specimens have been maintained and microorganisms isolated from biofilms on the surface of polymers, by inoculating them onto standard microbiological media. Among the isolated microorganisms there may be commensal organisms, which utilize monomers and other degradation products of high-molecular-weight PHAs and which exist in the medium due to the vital activity of primary and true PHA degraders. A reliable way to isolate true PHA degraders is to use the clear-zone technique (Mergaert et al., 1993), which involves inoculation of the isolates onto mineral agar that contains PHA as sole carbon source. Clear zones are formed around colonies of microorganisms with PHA-depolymerase activity on the surface of the agar medium, as a result of polymer degradation.

Microorganisms capable of PHA hydrolysis were isolated from all samples, but their percentages were higher in biofilms (Table 4);

Table 4
Quantities of PHA degrading microorganisms isolated from control soil samples and from the surface of polymer specimens.

Soil samples	Number of isolates of PHA degrading microorganisms					
	Dam Bai			Hoa Lac		
	Bacteria	Actinomycetes	Fungi	Bacteria	Actinomycetes	Fungi
Isolated from the surface of PHA specimens	44	4	14	9	12	48
Control soil	6	1	4	3	6	8

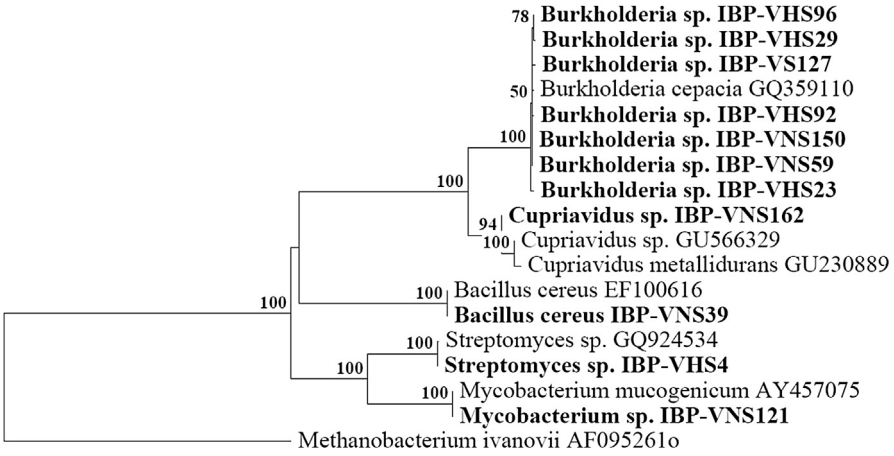

Fig. 2. Phylogenetic positions of PHA degrading bacterial strains (bold type) based on comparison of nucleotide sequences of the 16S rRNA gene using the neighbor-joining method. Numbers show bootstrap indices equal to or higher than 50%.

primary PHA degraders constituted only 7.5–10% of the total number of microorganisms isolated from the surface of the specimens.

Determination of the species composition of true PHA degraders, based on the combination of cultural, morphological, biochemical, and molecular-genetic properties, showed that PHA degrading bacteria were dominated by Gram-negative rods of *Burkholderia* sp.; they were isolated from the samples of both study areas. Actinobacteria of the genus *Streptomyces* were also present in the samples of both areas. Other major PHA degraders isolated from the samples at Dam Bai were *Bacillus*, *Cupriavidus* and *Mycobacterium* spp.; *Nocardiopsis* actinobacteria were isolated from the soil at Hoa Lac.

Inoculation of the samples onto the diagnostic medium confirmed PHA-depolymerase activity of the fungi species belonging to the genera *Gongronella*, *Penicillium*, *Acremonium*, *Paecilomyces*, and *Trichoderma*.

Gongronella butleri and *Penicillium* sp. were the fungi found in both study areas. *Acremonium recifei*, *Paecilomyces lilacinus*, and *Trichoderma pseudokoningii* were only isolated from the soil at Hoa Lac.

Comparison of the gene sequences obtained, 16S rRNA (for bacteria) and 28S rRNA (for fungi) with the sequences in the GenBank revealed high homology with the sequences of some previously identified strains (Figs. 2 and 3).

Among PHA degrading bacteria that have been described in the literature are representatives of various genera: *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Comamonas*, *Rhodococcus*, *Rhodocyclus*, *Syntrophomonas*, *Ilyobacter*, *Terrabacter*, *Terracoccus*, *Brevibacillus*, *Agrobacterium*, *Duganella*, *Ralstonia*, *Matsuebacter*, *Rhodoferax*, *Variovorax*, *Acinetobacter*, *Azospirillum*, *Mycobacterium*, *Streptomyces*, *Nocardiopsis* etc. (Mergaert et al., 1993, 1994; Jendrossek et al., 1996; Suyama et al., 1998; Ghanem et al., 2005; Bonartsev et al., 2009; Volova et al., 2010). Fungi are considered to be more efficient PHA degraders: *Ascomycetes*, *Basidiomycetes*, *Deuteromycetes*, *Zygomycetes* (Matavulj and Molitoris, 1992) and *Mixomycetes*, *Mastigiomycetes*, *Penicillium*, *Fusarium* (Brucato and Wong, 1991; Hocking and Marchessault, 1994; Jendrossek et al., 1996). The higher degradation capacity of fungi is accounted for by the fact that fungal PHA-depolymerases are more mobile than PHA-depolymerases secreted by bacteria (Reddy et al., 2008). Most soil PHA degraders are believed to be capable of degrading short-chain PHAs, i.e. ones that consist of monomers containing not more than 5 carbon atoms. Only few of them can degrade medium-chain PHAs, and this is accounted for by the substrate specificity of extracellular PHA depolymerases (Kim and Rhee, 2003; Kim et al.,

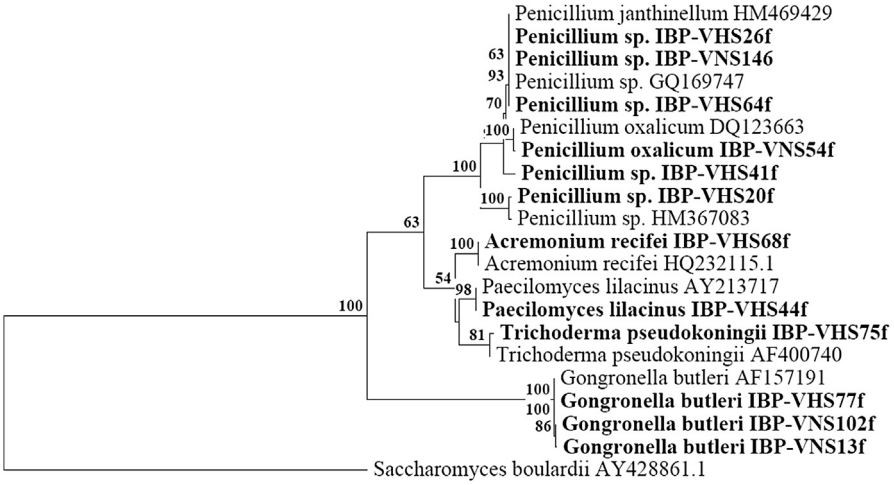

Fig. 3. Phylogenetic positions of PHA degrading fungi (bold type) based on comparison of nucleotide sequences of the 28S rRNA gene using the neighbor-joining method. Numbers show bootstrap indices equal to or higher than 60%.

2007). Bacteria belonging to the genera *Stenotrophomonas* and *Pseudomonas* were reported to be the dominant medium chain length PHA (MCL-PHA) degraders in soil (Kim et al., 2007). The presence of MCL-PHA depolymerase activity has also been found in *Streptomyces* spp. (Klingbeil et al., 1996; Kim et al., 2003) and *R. equi* (Kim et al., 2007).

Mokeeva et al. (2002) in their study described a wider range of fungi degrading PHAs, which included representatives of *Penicillium*, *Aspergillus*, *Paecilomyces*, *Acremonium*, *Verticillium*, *Cephalosporium*, *Trichoderma*, *Chaetomium*, and *Aureobasidium*, but the authors of that study did not inoculate samples on the diagnostic medium, i.e. their data were not based on the use of the clear-zone technique. In experiments performed in Siberian soils, PHA degraders in the soil of the larch rhizosphere were mainly represented by *P. lilacinus*, amounting to 81.5%. The fungi localized on polymer surface in the birch rhizosphere were dominated by *Penicillium* sp. *BP-1* and *Penicillium* sp. *BP-2*, totally amounting to 81% (Boyandin et al., 2012). *P. lilacinus* was also described as a polymer degrader by Sang et al. (2002). Lopez-Llorca et al. (1993) emphasized that *Penicillium* species were the major PHA degraders (up to 88% of the isolates). The ability of *Gongronella* sp. to degrade PHAs has been first shown in this study.

Differences revealed in the kinetics and mechanism of PHA biodegradation are caused by dissimilarities between microbial communities of the soils. Microbial communities formed on the surface of polymer specimens are habitat-specific and differ both qualitatively and quantitatively from the microbial communities of the control soil samples.

In this study, the compositions of microbial communities in the two study sites of Vietnam differed significantly. *Burkholderia*, PHA degrading bacteria, dominated in the soils in Dam Bai, while *Penicillium*, PHA degrading fungi, were major PHA degraders in the soil of Hoa Lac. This may be accounted for by differences in soil parameters such as pH: in Hoa Lac, the soil was weakly acidic (pH = 5.48), which is a favorable condition for the development of fungi, while in Dam Bai, soil pH was close to neutral (6.63).

Microbial communities of Siberian soils also contained such PHA degrading fungi as *Paecilomyces*, *Penicillium*, and *Acremonium* spp. Among Siberian PHA degrading fungi, however, there were representatives of *Verticillium* and *Zygosporium*, but there were no *Gongronella* and *Trichoderma*, which are typical for Vietnamese soils. Of PHA degrading bacteria, only representatives of *Bacillus* have been found in both Siberian and Vietnamese soils.

So, the integrated study addressing PHA biodegradation in Vietnamese soils shows that PHA degradation in tropical conditions is influenced by polymer chemical composition, specimen shape, and microbial characteristics.

4. Conclusion

This is the first study to investigate biodegradation of two types of PHAs by soil microorganisms in Vietnamese soils as influenced by various factors, including differences in soil and climatic conditions of study locations. The hot and humid climate of Vietnam facilitated polymer biodegradation, and the daily mass loss of PHA specimens of different compositions amounted to 0.20–0.33% (Hoa Lac) and 0.04–0.13% (Dam Bai) for films; 0.12–0.18% (Hoa Lac) and 0.02–0.08% (Dam Bai) for pellets. The study revealed faster degradation of the homopolymer, poly-3-HB, compared with the poly-3-HB/3-HV copolymer. Microbial communities formed on the surface of polymer specimens were habitat-specific and differed both qualitatively and quantitatively from the microbial communities of the control soil samples. Polymers were degraded by both bacteria and fungi. The major true PHA degraders identified in the tropical soils were bacteria of the genera *Burkholderia*, *Bacillus*, *Cupriavidus*, *Streptomyces*, *Nocardiopsis*, and *Mycobacterium* and the fungi *G. butleri*, *Penicillium sp.*, *A. recifei*, *P. lilacinus*, and *T. pseudokoningii*.

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