

Application of Polyhydroxyalkanoates for Development of Targeted Forms of Pesticides

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Abstract — Polyhydroxyalkanoates (PHAs) – biodegradable biocompatible polyesters of microbial origin – are among the most promising materials, which can be used in various areas. The authors' collection of highly productive strains and original technologies were used to synthesize PHAs. Biodegradation behavior of two PHAs, poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate), was studied in natural environments (soils, freshwater ecosystems, marine environments) of different climatic zones. PHAs are degraded in all studied environments but their biodegradation is influenced by the chemical structure of the polymer, its geometry and the technique used to process it, climate, weather, and the type of the natural ecosystem. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) has been used to design experimental sustained-release formulations of different pesticides in the form of pellets, films and microgranules. The kinetics of polymer degradation and the dynamics of model pesticide (insecticides α -hexachlorocyclohexane and lindane, and herbicide Zellek Super) release show that the rate and extent of pesticide release from the polymer matrix into the soil depends on the geometry of the carrier and the proportion of the pesticide loaded into it (polymer/pesticide mass ratio). Experiments with the creeping bentgrass (*Agrostis stolonifera* L.) show that the formulations of the herbicide constructed as microgranules and films can be successfully used to suppress the growth of grasses. This study demonstrates that biodegradable polyhydroxyalkanoates can be used effectively to construct environmentally friendly sustained-release PHA-pesticide systems that can be placed into the soil together with seeds.

Keywords—polyhydroxyalkanoates, biodegradable polymers, biodegradation, pesticides, slow release forms

I. Introduction

Development of agrochemistry and agricultural technologies led to the appearance and application of a variety of chemicals, which can dissipate and accumulate in the biosphere [1]. A new direction aimed at reducing the uncontrolled distribution of xenobiotics in the environment is the development of environmentally friendly formulations

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with a targeted and controlled release of the active component owing to the use of specific coats and/or carriers made of biodegradable materials. Several examples of polymeric carrier usage have been described, including ethyl cellulose [2,3], polyurethane [4], sodium alginate [5] for delivery of a number of weed and pest killing chemicals.

Polyhydroxyalkanoates, (PHAs) are biodegradable polyesters of microbial origin. These polymers can be processed by a number of conventional techniques yielding products with many different geometries. PHAs are degraded by microorganisms in the environment [6] and can be potentially used as polymeric carriers.

The purposes of this study were to optimize PHA production using highly effective strain *Cupriavidus eutrophus* B-10646, to investigate PHA degradation behavior under different environmental conditions, and to study PHAs as a carrier in the construction of biodegradable, environmentally friendly, sustained-release herbicide formulations.

II. Materials and Methods

A. Cultivation of Microorganisms and Obtaining Biopolymers

The strains used in this study for PHA production were *Cupriavidus eutrophus* B-10646 and *Ralstonia eutropha* B5786, registered in the Russian Collection of Industrial Producers (RCIP). Schlegel's mineral medium was used as a basic solution for growing cells [7]. The main carbon substrate was glucose. A solution of iron citrate (5 g/L), which was used as a source of iron, was added to reach a concentration of 5 ml/L. Hoagland's trace element solution was used: 3 ml of standard solution per 1 L of the medium. Cells were grown in batch culture, as developed previously for PHA synthesis [8]. A two-stage process was used. In the first stage, cells were grown under nitrogen deficiency. In the second stage, cells were cultured in nitrogen-free medium; the other parameters were the same as in the first stage. Dynamics of accumulation of cell biomass and PHA by strain B-10646 was studied in a BioFlo 115 automated laboratory fermentor ("New Brunswick Scientific", U.S.), with a 12-L fermentation vessel. For synthesis of poly-3-hydroxybutyrate-co-3-hydroxyvalerate copolymer (P(3HB-co-3HV)), after 4-6 h of cultivation, when nitrogen supply was discontinued, the culture medium was supplemented with valeric acid as a precursor substrate.

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 [9]. The total polymer content of the biomass and also

monomer composition of polymer 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, U.S.) on a HP-FFAP capillary column, with benzoic acid as the internal standard. 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.

B. PHA Biodegradation Studies

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. Compact pellets were prepared by cold pressing of finely powdered polymer, using an AutoPellet 3887 laboratory press (Carver, USA) at 120 kgs/cm² (diameter 10 mm, height 2.5 mm, mass about 350 mg).

Determination of PHA biodegradation. Experiments were performed in different soils in tropical and moderate regions. PHA specimens (3 of each type) were weighed and placed in close-meshed gauze jackets, which were then buried in test environment. After defined periods they were taken out of the jackets, mechanically cleaned to remove residual soil, washed with distilled water, dried for 48 h at room temperature, and weighed. A decrease in the mass of the specimens, and the percent of the crystalline phase (crystallinity) were measured.

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.

C. Preparation of PHA Based Pesticide Carriers

Herbicides Zellek Super (active ingredient, haloxyfop-P-methyl, or (RS)- 2- {4- [3- chloro- 5- (trifluoromethyl)- 2-pyridyloxy] phenoxy}propanoic acid methyl ester) (Dow AgroSciences, Austria), Zenkor Ultra (metribuzin) (Bayer CropScience) and Magnum Super (metsulfuron-methyl and tribenuron-methyl) (August Ltd., Russia), insecticides α -hexachlorocyclohexane (HCCH) and lindane (Sigma-Aldrich), and fungicide Vial TrasT (thiabendazole and tebuconazole) (August Ltd., Russia) were used as model pesticides.

For preparing pellets loaded with pesticides polymer was ground in a laboratory mill. Ground polymer was then mixed with the pesticides. Thus homogenous powders were cold-compacted under a pressure of 120 kgs/cm² with a AutoPellet 3887 laboratory press. Polymer microgranules loaded with pesticides were prepared from a solution of the pesticide and the copolymer (7%) in dichloromethane. A peristaltic pump was used to pour the solution drop by drop into the settling bath with isopropanol or hexane (precipitate), where the polymer precipitated in the form of microgranules. Fig. 1a shows the resulting microcapsules. Polymer films loaded with

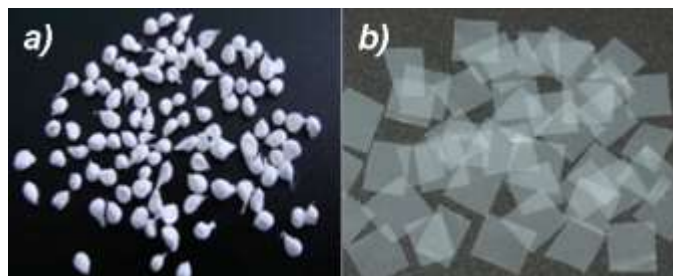


Figure 1. Examples of polymer microgranules and films loaded with pesticide (Zellek Super).

pesticides were prepared by polymer solution casting followed by evaporation of the solvent. A dichloromethane solution containing 2 % (w/v) of the P(3HB/3HV) copolymer was mixed with the pesticide solutions, stirred with an overhead stirrer at a speed of 300 rpm, cast onto degreased glass and dried at room temperature (Fig. 1b).

D. Estimation of Effectiveness of PHA Based Pesticide Carriers

Degradation of the polymer matrix and time course of action of the constructed xenobiotic-polymer systems were studied in laboratory scale experiments. Pesticide loaded polymeric forms were placed in fine mesh synthetic fabric (mill gauze) covers and inserted into the containers, each of them containing 100 g of wet environmental garden soil. The containers were incubated in a constant-temperature cabinet at 25°C at constant soil moisture (50 %). All values presented were calculated for dry soil. Throughout the course of the experiment, the covers with polymer/pesticide samples ($n = 3$ of each type) were removed from the containers at intermittent time points; the samples were removed, mechanically cleaned, washed with distilled water, and dried at 40°C for 24 h. The residual polymer content and the degree of crystallinity (C_x), were evaluated.

For defining pesticide release in soil a model pesticide was extracted with ethanol from the total mass of the soil, after the specimens were removed. Then, after a series of standard procedures (concentration, washing, pH stabilization, and dehydration) [10], the pesticide concentration was determined using gas chromatograph–mass spectrometer Agilent 7890A/5975C with a HP5SM capillary column. Analytical standards of pesticides were used to plot the calibration curves.

The effectiveness of the pesticide loaded in microgranules and films was evaluated using Zellek Super herbicide as a model pesticide and a model plant (weed), creeping bentgrass (*Agrostis stolonifera* L.), which is an annual grass. *A. stolonifera* L. seeds were planted in garden soil (100 g) placed in 500 cm³ containers (30 g of seeds per 1 m²). The specimens prepared and described above were placed into the treatment containers. The positive control plants were sprayed with an aqueous solution of the herbicide at day 19 of the experiment (in the beginning of the tillering phase): 1 ml of the herbicide per 0.1 m². Plants grown in the soil without herbicide application served as the negative control. The experiment was

conducted in the laboratory, under natural photoperiod, at room temperature. Soil moisture content was maintained at 50 %. Plant productivity was estimated as a biomass increase. Vegetative parts of plant specimens were cut off at 10, 20 and 30 days after starting the experiment, dried at 105°C, and dry biomass weight was measured.

III. Results and Discussion

A. Optimizing PHA Production

In order to achieve efficient PHA synthesis, experiments were performed to determine the limits of physiological effect of glucose on the cells. It was shown that for *C. eutrophus* B-10646 strain, the range of tolerable glucose concentrations in the culture medium was 5 to 35 g/L. Glucose concentrations outside this range adversely affected cell yields. Thus, during fermentation, glucose should be added to the culture medium portion-wise or continuously, with a peristaltic pump, and its concentration should be carefully monitored.

Analysis of conditions influencing PHA accumulation by *C. eutrophus* B-10646 cells was performed in the experiment with a two-stage batch culture; in the first stage, cells were grown under nitrogen limitation and in the second – in nitrogen-free medium. In the first stage (20–25 h), under nitrogen deficiency (50% of the cell's physiological requirements, 60 mg/g cells), total cell concentration increased. Intracellular polymer content reached 45% and cell concentration (27 ± 2) g/L. In the second stage, cells were grown in nitrogen-free medium, with glucose supply regulated to maintain its concentration at 5 g/L. At the end of the experiment (50 h), intracellular polymer concentration reached 80–85% and the total cell concentration (42 ± 2) g/L.

For synthesis of P(3HB-co-3HV) copolymer, in the early phase of the first stage, with nitrogen fed to the culture medium and cells cultured in complete nutrient medium, when intracellular polymer concentration was rather low (about 15–20%), potassium valerate was added as a precursor substrate. The highest molar fraction of 3HV was achieved 20–25 h after the addition of precursor substrates to the culture medium.

B. Studying PHA biodegradation

The studies in Siberian soils addressed biodegradation of PHAs with different chemical structures in the form of film discs by soil microorganisms inhabiting the rhizosphere of coniferous and broadleaved trees under varying soil temperature conditions. Experiments were performed under natural conditions, in the arboretum at the V.N. Sukachev Institute of Forest SB RAS (Krasnoyarsk) during two field seasons, which differed in temperature conditions.

In 2007, in the soil under the larch, which was moister and housed more microorganisms, PHA degradation rates were higher than those recorded under the birch. By the end of the experiment, the residual mass of P(3HB) specimens had decreased to 45% of their initial mass, and the residual mass of P(3HB-co-3HV) specimens – to 22%. (Fig. 1). In the soil of the birch rhizosphere, degradation rates of both PHA types

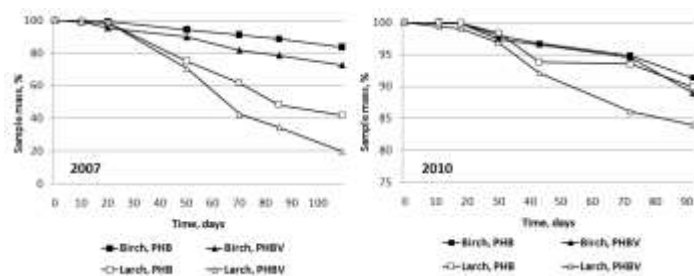


Figure 2. Dynamics of the mass of polymer specimens in Siberian soil

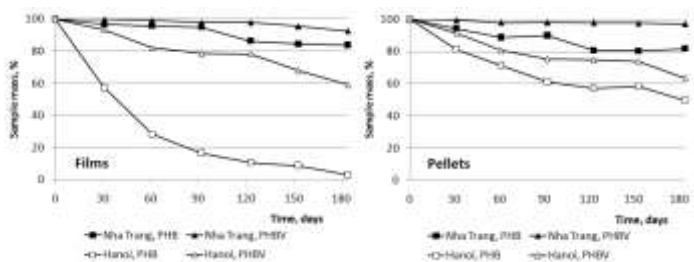


Figure 3. Dynamics of the mass of polymer specimens in Vietnamese soil

were lower, in spite of the great variety of the fungi present in this soil. At day 109 of the exposure, the residual masses of P(3HB) and P(3HB-co-3HV) specimens amounted to 84% and 74% of their initial masses, respectively. In 2010, PHA degradation rates were lower than during the 2007 field season. At the end of the field experiment, the residual mass of the specimens amounted to 89.9% and 74% for P(3PHB) and P(3HB-co-3HV) specimens buried under the larch and to 91.4% and 89% for the specimens buried under the birch. As in 2010 the mass loss was so small, we failed to find any reliable differences in the degradation of the two PHAs used in this study.

Thus, at temperate latitudes (Siberia, Krasnoyarsk) with markedly continental climate, in the soddy-carbonate soil of the arboretum, during the warmer summer season, P(3HB-co-3HV) copolymer films were degraded faster than the higher-crystallinity P(3HB) specimens. These results are in good agreement with the data reported by other authors [11,12], but contradict the data reported by Rosa and coauthors [13]. Degradation rates of P(3HB) recorded by these authors were higher than P(3HB-co-3HV) degradation rates, and they explained their results as being due to specific surface structure and properties of their specimens.

X-ray structure analysis performed at the end of the field experiment showed increases in the degrees of crystallinity of both PHAs, suggesting preferential disintegration of the amorphous phase of PHAs in the soil. This result is consistent with the data reported by a number of authors [14,15].

Another study of PHA degradation in the soil was performed in the tropics. Biodegradation of PHAs of two types – P(3HB) and P(3HB-co-3HV) – was analyzed in soils at field laboratories in the environs of Hanoi (Vietnam) and Nha Trang (Vietnam). The air and soil temperatures and humidity in both study sites were similar throughout the study season. Precipitation at Hanoi was, however, almost an order of magnitude higher than in Nha Trang.

In Nha Trang PHAs were degraded at lower rates because of lower precipitation amounts in this area in summer. PHAs of all types were degraded at higher rates in the soil of the study site at Hanoi. PHA films were more prone to degradation than pressed pellets. At the end of the experiment (after 184 days of soil exposure), degradation of P(3HB) films reached more than 97%, and P(3HB-co-3HV) films were 33% degraded, while the pressed pellets were 42 and 23% degraded, respectively. In the more arid area (Nha Trang), the mass loss of P(3HB) and P(3HB-co-3HV) films was 16 and 7% and that of the pressed pellets – 18 and 3%. (Fig. 2). Therefore, in these conditions P(3HB) specimens were degraded faster than P(3HB-co-3HV) films and pellets. It can be explained by different microbial communities in these environments with different substrate specificity of PHA depolymerases.

So, the results obtained show that PHAs are degraded in all studied environments but their biodegradation is influenced by the chemical structure of the polymer, its geometry and the technique used to process it, climate, weather, and the type of the natural ecosystem.

C. *Estimating Pesticide Release from Targeted Forms of Pesticides*

Using proposed techniques, a set of polymer forms with embedded pesticides – Zellek Super, Zenkor Ultra, Magnum Super, HCCH, lindane and Vial TrasT – was developed. Samples with embedded Zellek Super, HCCH and lindane were further investigated.

An experiment with pressed pellets containing HCCH and lindane continued for 84 days. The experimental forms of pesticides embedded in the polymeric matrix were periodically taken from the containers. Changing weight of samples, the residual content of polymers in pellets, and the dynamics of pesticide release from the polymeric matrix were determined. The polymer containing the pesticides was actively degraded in the course of the experiment. The main mass of the polymer in both variants was degraded 40–50 days after the beginning of the experiment. At the end of observation, the residual content of the polymer in compacted pellets was approximately 5–10% of the initial content.

The release of the active ingredient from the polymeric matrix largely depends on the polymer type and the initial agent–polymer ratio [2]. Our studies showed that, the lower the content of pesticides in the P(3HB-co-3HV) pelleted form, the lower their release rate. This finding is consistent with the data obtained by the authors of [3], who studied the use of ethyl cellulose based microspheres as a polymeric carrier for norfluazon delivery.

Thus, using HCCH and lindane as examples, we showed that pesticides embedded in a degradable PHA based polymeric carrier are slowly and gradually (without surges) released to the environment as the polymer is degraded by the soil microflora (the mean rate of pesticide release was 0.49 and 0.77 g/day per gram soil for HCCH and lindane, respectively). The rate of pesticide release to the soil can be regulated by varying the polymer–pesticide ratio.

PHA microgranules containing the herbicide Zellek Super were placed into the soil for examination of polymer degradation and concomitant herbicide release. Upon degradation of the samples in soil, the weight loss for the microgranules varied depending upon the proportion of the herbicide in the granules. At day 19 of the experiment, the residual mass of the copolymer in microgranules with a polymer to herbicide ratio of 60:40 was 87 %, while in the granules with a polymer to herbicide ratio 90:10, a lower residual mass was observed, amounting to 56 %. At day 42 of the experiment, however, the residual masses of the granules were almost equal to each other, amounting to 28% of their initial masses. The mean specific rate of polymer matrix degradation over the 42 day experiment was about 0.03 mg polymer/day. Analysis of X-ray spectra of the PHA specimens that had been buried in the soil exhibited an increase in their degree of crystallinity (from 58% to 69%), suggesting preferential disintegration of amorphous phases of the copolymer.

The concentration of the herbicide in soil, measured at the conclusion of the experiment, from microgranules that contained the greater proportion of Zellek Super (40 mass%) was twice as high as that of soil containing microgranules that initially contained 10 mass% of Zellek Super, amounting to 30 $\mu\text{g/g}$ dry soil compared to 15 $\mu\text{g/g}$ dry soil. The mean release rate of the herbicide from the granules with the copolymer to herbicide ratio 60:40 was 0.7 $\mu\text{g/day/g}$ soil. Degradation of polymer films with the polymer/herbicide ratio 75:25 occurred at a higher rate than that of the granules. At day 19 of the experiment, the residual mass of the copolymer in these films was 69 %. At the end of the experiment, the residual mass of the polymer was about 30 % of its initial mass, and the specific biodegradation rate was 0.08 mg/day, which was more than twice as high as that of the microgranules. The herbicide release rate was comparable in microgranules. For the first 19 days, 18 $\mu\text{g/g}$ soil of the herbicide, or 90% of the amount loaded into the films, was released.

Analysis of the available literature revealed that research on this concept is in its infancy. In previous studies, P(3HB-co-3HV) microspheres were prepared as a delivery system for the herbicides atrazine [16] and ametryne [17]. The effect of the herbicides on the morphology and size of the microspheres was characterized and the herbicide release profiles in aqueous medium were examined. The genotoxicity of atrazine-loaded P(3HB/3HV) microspheres was decreased in relation to atrazine alone [16].

D. *Estimating the Effectiveness of Targeted Forms of Pesticides*

To the best of our knowledge, no group has examined effectiveness of weed killing using the PHA/herbicide matrices. Thus, we examined the timed release of Zellek Super, from microgranules and films, on the model plant (weed), the creeping bentgrass *Agrostis stolonifera* L. The form of the herbicide carrier significantly affected the response of *Agrostis stolonifera* L. However, regardless of whether the herbicide Zellek Super was loaded in microgranules or films prepared from P(3HB-co-3HV), the

herbicide loaded into biodegradable polymer carriers was more effective than the herbicide when traditionally applied as a spray to plants during the tillering phase. Moreover, the effect of the herbicide applied as a spray had not been observed until day 19 after the treatment.

In the experiment with using polymer microgranules, we observed a few weed shoots at day 10 (about 15-20 % of those in the positive control). At later observation times, as the polymer matrix was gradually degraded and the herbicide was released to the soil and delivered to the plant roots, the number of the shoots decreased: they wilted and became dry. Spraying of the plants with standard doses of Zellek Super was not as effective, as the herbicide was applied to the plants during the tillering phase, and the plants stopped growing 3 to 5 days after spraying, i.e. 23-25 days after the weed was planted. The sprayed plants were measured to be 1.5 – 2 cm in height.

Application of the herbicide loaded into polymer films produced even more effective results than those obtained in the experiment with herbicide-loaded microgranules. No weed growth was observed in the experiment with the herbicide-loaded films applied to the soil. In this experiment, plant growth was demonstrated to be completely suppressed.

iv. Conclusion

The material used to prepare carriers for pesticide formulations must have such properties as biodegradability, environmental safety, long-term (for weeks and months) retention in nature, controlled degradation to nontoxic products, processability by available techniques, and compatibility with the substances loaded into it.

Using highly effective producing strains (like *C. eutrophus* B-10646) and optimization of their production parameters can provide high cell concentrations and PHA yields of necessary chemical composition under batch culture conditions and controlled carbon substrate supply. Such approach can decrease PHA production cost and therefore made environmentally friendly pesticide application systems more readily available.

PHAs are degraded in all studied environments and their chemical structure, geometry and the technique used for preparing PHA specimens can influence their biodegradation in natural ecosystems.

The P(3HB-co-3HV) carriers enable a sustained release of the pesticides into soil, with carrier geometry being the major factor for the rate of release. Also, the rate of pesticide release into the soil can be regulated by varying the polymer/ pesticide ratio. Experiments with the creeping bentgrass (*Agrostis stolonifera* L.) demonstrated that the formulations of the herbicide Zellek Super constructed as either microgranules or films can be successfully used to suppress the growth of grasses. These experiments demonstrates the possibility of using degradable polyhydroxyalkanoates as a matrix for embedding pesticides to ensure their targeted and controlled release to the environment.

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