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Original article

POLYHYDROXYALKANOATES: BIOSYNTHESIS OPTIMIZATION AND DESIGN OF ANTIMICROBIAL COMPOSITES

N.I. Koretska[1*], I.V. Semeniuk[1,2], T.Ya. Pokynbroda[1], N.S. Shcheglova[1], O.V. Karpenko[1], A.R. Kytsya[1], V.I. Lubenets[3], N.V. Polish[3]

[1]Department of Physical Chemistry of Fossil Fuels of the Institute of Physical-Organic Chemistry and Coal Chemistry named after L.M. Lytvynenko, NAS of Ukraine, Lviv, Ukraine
[2]Foshan Tianheng New Material Technology Co., Ltd, Foshan, China
[3]Lviv Polytechnic National University, Lviv, Ukraine

*Corresponding author: natalya.koretska@gmail.com

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Background. The accumulation of plastic waste negatively affects the environment and human health. Currently, one of the strategies to address this global ecological problem involves the utilization of biodegradable plastics instead in place of synthetic ones. Among them, polyhydroxyalkanoates (PHA) – microbial intracellular polymers – hold a significant position. Their advantages are biodegradability, biocompatibility, and favorable thermomechanical properties. Given these attributes, PHA has significant prospects for use in medicine, agriculture, and the food industry, in particular for packaging food products.
Objective. Enchance the efficiency of bacterial synthesis of polyhydroxyalkanoates through nutrient media modification, obtain antimicrobial composites based on PHA, and determine their antimicrobial properties.
Methods. The optimization of PHA biosynthesis involved selecting appropriate cultivation conditions, including carbon and nitrogen sources, cultivation time, and working volume. The isolation of PHA from bacterial biomass was achieved through chloroform extraction (mixing for 10 h at 35 °C, with a biomass-to-chloroform ratio of 1:50); followed by precipitation with double the volume of isopropanol. The resulting polymer was then dried to a constant weight at 60 °C. The hydrophobicity of the biopolymer was assessed using the water contact angle measurement. Composites of biopolymers with antimicrobials in the form of films were obtained using two methods: 1) solution casting method; and 2) layering the biocides onto the polymer film. The antimicrobial activity of the resulting composites was determined using the agar diffusion method.
Results. Through the optimization of the mineral media and the change of cultivation conditions, it was possible to obtain 0.26−1.45 g/l of polyhydroxyalkanoates (5.1−34.0% PHA from biomass). The *R. ruber* UCM Ac-288 strain synthesized the maximum amount of biopolymer (34.0% PHA). This study established the ability of *Gordonia* bacteria to synthesize PHA for the first time. PHA compositions of optimal content were obtained, with hydrophobicity comparable to that of polyethylene packaging films. Antimicrobial properties of biopolymers composites with biocides have been substantiated.
Conclusions. The bacterial synthesis of PHA was increased by modifying nutrient media. Composites based on PHA with biocides were developed. It was determined that these composites exhibit antimicrobial properties and high hydrophobicity. Consequently, they hold promise for use as biofilms for packaging and preserving food products.

Keywords: polyhydroxyalkanoates; *Rhodococcus*; *Azotobacter*; *Gordonia*; antimicrobial composites; packaging biofilms.

Introduction

Over the past 70 years, the production of synthetic plastics in the world has increased more than 50 times. At the beginning of 2022, their production was approximately 450 million tons per year and should double by 2045 [1]. Currently, thousands of types of polymers are produced on an industrial scale, mainly inexpensive thermoplastic polymers with high molecular weight and hydrophobicity, mechanically strong, chemically and biologically inert. These properties make plastics universal in construction, packaging materials, and medicine. However, synthetic plastics pose a significant threat to the environment [2]. In the world, only 18% of plastic waste is recycled, and 24% is incinerated. The remaining 58% is either sent to landfills or enters the natural environment [3]. Since plastics are not biodegradable, their accumulation creates a large-scale environmental problem [4]. Currently, one of the ways to solve this global problem is the widespread use of biodegradable plastics, in particular polyhydroxyalkanoates (PHA).

Despite the high cost, the PHA market is constantly growing. According to the "Global Polyhydroxyalkanoate (PHA) Market Report 2022:

Packaging & Food Services and Biomedical Industries" offer significant opportunities: the PHA market is expected to reach $167 million in 2027, and the average annual growth rate will be 15.3% during the forecast period.

The biocompatibility and diversity of the PHA structure create prospects for use in medicine [5], agriculture [6], in the food industry, in particular, for product packaging [7]. PHA-based films with antimicrobial biologically active substances are a successful alternative to traditional plastic packaging. PHAs are compatible with a wide range of antimicrobial agents: the created films exhibit antimicrobial activity against a wide range of microorganisms [8, 9]. They are safe for the environment and promising for packaging and keeping food products fresh. The most common methods of producing biofilms are solution casting, extrusion, and layer-by-layer technique [10, 11], as well as electrospinning, dip-coating [12]. The use of the biofilms is an alternative to the treatment of the surface of food products with antimicrobial substances.

Nowadays biogenic polymers can be used for food packaging as an alternative to synthetic plastic. These biopolymers include polysaccharides, proteins, lipids, as well as polylactide and polyhydroxyalkanoates [13]. They are biodegradable, biocompatible polymers and can be synthesized from renewable carbon sources. Polysaccharides (starch, chitosan, cellulose, alginate, pectin, etc.), proteins (casein, gelatin, gluten, zein) and lipids (oils, waxes, triglycerides) have poor mechanical properties, so they are added as impurities in the production of polymer films. Polylactide and PHA have similar physico-mechanical and thermoplastic properties since the chemical structures of these polymers are similar. Polylactide is obtained by condensation of lactic acid which is typically made from fermented plant starch. Polylactide may contain catalyst impurities, in particular heavy metals, which are used during its production at the stage of chemical synthesis [14]. At the same time, polyhydroxyalkanoates are bacterial intracellular polymers. PHA producers are more than 300 species of bacteria, including genetically modified ones [15, 16]. Different bacterial species produce polyhydroxyalkanoates with different chain lengths. However, by mixing varieties of PHA, it is possible to obtain polymer films with specified physico-mechanical and thermal properties [17]. The main disadvantage of PHA is the cost of production. One of the options for reducing the price is obtaining this polymer as a co-product of other biotechnologies, where bacterial cells are waste products [18]. In order to reduce the price of raw materials for PHA production, inexpensive substrates and waste are used (unrefined glycerin, sugar cane, molasses, bran). Pilot plants produce PHA from the organic fraction of solid household waste, activated sludge, fruit, and cellulose waste [19].

Although there is now a sufficient amount of scientific research on biopolymers, the industrial use of packaging materials based on polyhydroxyalkanoates still needs further development to improve packaging efficiency. Commercialization of biopolymer films is not cheap and is limited by production and processing costs.

Therefore, the aim of the work was to increase the efficiency of bacterial synthesis of polyhydroxyalkanoates by modifying nutrient media; obtain antimicrobial composites based on PHA, determine their antimicrobial properties.

Materials and Methods

The following bacterial strains were used for the synthesis of polyhydroxyalkanoates: *Azotobacter vinelandii* N-15 from the collection of Daughter enterprise "ENZIM"; actinobacteria *Rhodococcus erythropolis* Au-1 (UCM Ac-603), *Gordonia rubripertincta* UCM Ac-122, *Rhodococcus ruber* UCM Ac-288 from the Ukrainian collection of microorganisms the Institute of Microbiology and Virology named after D.K. Zabolotny, the National Academy of Sciences of Ukraine.

Test microorganisms were used for determination antimicrobial activity: bacteria *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* from the collection of the Department of Technology of Biologically Active Compounds, Pharmacy and Biotechnology, Lviv Polytechnic National University); fungi *Alternaria alternata*, *Aspergillus niger*, *Candida tenuis*, *Fusarium oxysporum* from the collection of Daughter enterprise "ENZIM".

Synthesis of PHA by bacteria. Cultivation of actinobacteria was carried out on the mineral medium (1), g/l: $NaNO_3$ – 3.0; K_2HPO_4 – 2.0; KH_2PO_4 – 2.0; $MgSO_4 \times 7H_2O$ – 0.5; yeast extract – 1.0, carbon sources – sucrose, glycerol – 20 or 30 g/l; nitrogen sources – $NaNO_3$ and yeast extract in different ratios. A mineral medium with fructose and $NaNO_3$ as sources of carbon and nitrogen was also used [20]. Cultivation of PHA-producing bacteria was carried out at 30 °C, 220 rpm for 48, 72, and

120 hours in Erlenmeyer flasks (750 ml, working volumes – 150, 100 ml). A 24-h culture was used as the inoculum; 4% vol, titer 2×10^8 CFU/cm^3. *A. vinelandii* N-15 inoculum was grown on Ashby medium with mannitol [21], actinobacteria *R. erythropolis* Au-1, *R. ruber* UCM Ac-288, *G. rubripertincta* UCM Ac-122 – on medium (1) with sucrose 20 g/l.

Isolation of polyhydroxyalkanoates from bacterial biomass was carried out according to the method [21]. Bacterial biomass was separated from the culture liquid by centrifugation (20 min, 6000 rpm); washed twice with NaCl solution, 0.9% w/w. The precipitate was dried at 60 °C to a constant mass and cooled. PHA was isolated from biomass by extraction with chloroform (biomass – chloroform 1:50), at a temperature of 35 °C and mixing for 10 hours. Chloroform was evaporated from the filtered extract to a viscous consistency; a double volume of isopropanol was added to precipitate PHA. The obtained polymer was separated from the mixture of solvents and dried at 60 °C.

Determination of the contact angles of the obtained biopolymers. The contact angle was determined after 15 s of contact of water drop with the PHA film surface using a Drop Shape Analysis System – DSA100 (KRUSS, GmbH, Germany).

Production of film forms of antimicrobial polymer composites. Biopolymer films were obtained based on polyhydroxybutyrate (PHB) of *A. vinelandii* N-15 and PHA of *R. erythropolis* Au-1 in the ratio of PHB-PHA of 73:27 by weight. Biopolymer films were filled with antimicrobial substances in two ways: I – solution casting method [22]; II – biocide layering on polymer. For the first method, a 3% solution of biopolymers was prepared in chloroform at 40 °C with mixing. A biocide was added to the obtained solution, mixed until complete dissolution; polymer biofilms were formed by casting the obtained solutions into the Petri dishes. Biofilms were air-dried to a constant mass; discs (9 mm) were cut from them to determine antimicrobial activity. According to the second method, an appropriate amount of antimicrobial substance was applied to PHB/PHA films (d = 9 mm). The discs were dried at 40 °C in a vacuum (15 mm Hg) for 60 min and the antimicrobial activity was determined.

The following biocides were used to fill biofilms: ethyl thiosulfanilate – synthetic analogue of garlic and onion phytoncides [23], a naphthoquinone derivative – 2-chloro-3-(3-(3-(p-tolyl)-1H-pyrazol-5-yl) amino) naphthalene-1, 4-dione [24], chloramphenicol, propolis extract, salicylic acid, solutions of silver nanoparticles.

Ethyl thiosulfanilate, 2-chloro-3-(3-(3-(p-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione were synthesized at the Department of Technology of Biologically Active Compounds, Pharmacy and Biotechnology, Lviv Polytechnic National University.

Propolis was extracted with ethyl alcohol 96% vol. with stirring at 25 °C during 3 h. The extract was filtered through a pleated filter and dried (15 mm Hg, 35 °C). The dried extract was used for research.

Solutions of silver nanoparticles were obtained using 1) rhamnolipids; 2) humic acids.

Rhamnolipids obtaining was described in [25]. Silver nanoparticles with rhamnolipids were prepared via method described in [26]

To obtain humic acids [27], biohumus was treated with 1% potassium hydroxide with stirring (60 min, 50 °C). To precipitate humic acids, 5% HCl (pH < 2) was added to the filtrate. The precipitate was centrifuged (6000 rpm, 20 min), washed from sodium and chlorine ions with distilled water, and then dried at 60–70 °C to a constant mass. The total content of acid groups in humic acids was 11.6 mmol/g (26.3 wt. %).

To obtain silver nanoparticles stabilized using humic acids, 1.4 ml of a 4% sodium hydroxide solution was added to 65 ml of a 0.043% aqueous solution of humic acids; the resulting solution was heated to 60 °C and 3.5 ml of 3.4% silver nitrate was added with mixing.

Solutions of silver nanoparticles were centrifuged (10,000 rpm, 20 min), sediments were washed with distilled water, centrifuged again and 0.1% solutions were obtained, which were applied to biopolymer discs.

Antimicrobial activity was determined on test cultures of bacteria and fungi by the agar diffusion method [28]. Discs (9 mm) were cut from biofilms – antimicrobial polymer composites.

Statistical analysis. All the experiments were performed in triplicate. Experimental data were statistically processed using the Microsoft Excel 2010 software package. The results are presented as the meaning ± standard error ($x \pm SE$). In addition, the differences between the experimental data were statistically analyzed using the Statistica software package version 12.0 (StatSoft Inc., Tulsa, OK, USA). Differences were considered as statistically significant at $p < 0.05$.

Results

Since PHA is the intracellular biopolymer, the possibility of obtaining it as a co-product of the synthesis of extracellular and cell-bound biosurfactants was studied. Therefore, actinobacteria *R. erythropolis* Au-1 and *G. rubripertincta* UCM Ac-122 were cultivated according to the parameters for obtaining biosurfactants [29, 30]. After 120 hours cultivation on nutrient medium (1) the yield of PHA was 2.6% w/w (*G. rubripertincta* UCM Ac-122) and 4.8 % w/w (*R. erythropolis* Au-1) from biomass (Tables 1, 2). Thus, obtaining PHA from bacterial cells is a possible, but to increase the yield of polymers, it is necessary to change the cultivation conditions of producers. It is shown the influence of various sources of carbon and nitrogen, duration of cultivation, working volume on increasing the yield of biopolymers (Tables 1, 2).

In order to obtain the highest yield of PHA, the expediency of cultivating *R. erythropolis* Au-1 for 72 hours has been established. It was shown that a high yield of biomass (4.92 g/l) and biopolymer (0.32 g/l – 6.5% w/w from biomass) was obtained when using sucrose (30 g/l), sodium nitrate (3.0 g/l) and yeast extract (1.0 g/l), C:N 21. If C:N increased to 80, PHA yield was 8.9% w/w, but the biomass was 3.70 g/l, only 0.33 g/l PHA was obtained.

It was determined that the change in the ratio of carbon to nitrogen, the duration of cultivation, and the working volume did not have a sufficient effect on increasing the PHA yield of the strain *G. rubripertincta* UCM Ac-122. Thus, the yield of PHA was 2.6–4.6% of biomass, not exceeding 0.11 g/l.

It is known from literary sources that the C:N ratio in the nutrient medium is important for the

Table 1: Parametres of biosynthesis of PHA by strain *R. erythropolis* Au-1

Carbon source, g/l	Sources of nitrogen, g/l	C:N	Cultivation time, hours	Biomass, g/l	PHA	
					g/l	% w/w from biomass
Sucrose 20	$NaNO_3$ 3.0; yeast extract 1.0	15	48	2.82 ± 0.01	0.19 ± 0.01	6.9
		15	72	4.29 ± 0.23	0.27 ± 0.01	6.3
		15	120	5.32 ± 0.26	0.26 ± 0.01	4.8
Sucrose 30		21		4.92 ± 0.26	0.32 ± 0.01	6.5
Glycerol 20		18	72	3.50 ± 0.22	0.20 ± 0.01	5.7
Glycerol 30		26		3.82 ± 0.25	0.30 ± 0.01	6.5
Glycerol 20	Yeast extract 8.0	13	72	3.58 ± 0.18	0.22 ± 0.02	6.2
Glycerol 20	$NaNO_3$ 0.5; yeast extract 0.5	80	72	3.70 ± 0.18	0.33 ± 0.02	8.9

Notes. C:N – carbon to nitrogen ratio; working volume – 150 ml; the content of total nitrogen in the yeast extract is ~10%.

Table 2: Parametres of biosynthesis of PHA by strain *G. rubripertincta* UCM Ac-122

Carbon source, g/l	Sources of nitrogen, g/l	C:N	Cultivation time, hours	Working volume, ml	Biomass, g/l	PHA	
						g/l	% w/w from biomass
Sucrose 20	$NaNO_3$ 3.0; yeast extract 1.0	15	48	100	1.41 ± 0.07	0.020 ± 0.001	3.9
				150	1.15 ± 0.06	0.041 ± 0.001	3.7
		15	72	100	2.88 ± 0.09	0.100 ± 0.005	3.3
				150	2.60 ± 0.08	0.052 ± 0.002	3.1
		15	120	100	3.50 ± 0.17	0.081 ± 0.003	2.7
				150	3.70 ± 0.18	0.100 ± 0.004	2.6
Sucrose 30	$NaNO_3$ 3.0; yeast extract 1.0	21	72	150	3.30 ± 0.16	0.110 ± 0.010	3.2
Sucrose 20	Yeast extract 1.0	84	72	150	1.10 ± 0.06	0.051 ± 0.003	4.5
Sucrose 30		126	72	150	1.31 ± 0.06	0.061 ± 0.003	4.6

Notes. C:N – carbon to nitrogen ratio; the content of total nitrogen in the yeast extract is ~10%.

synthesis of PHA by bacteria of the genus *Rhodococcus* [20]. So how bacteria of the genus *Rhodococcus* effectively synthesized PHA on medium with fructose (C:N 150-200) [20], therefore, strains *R. erythropolis* Au-1 and *G. rubripertincta* UCM Ac-122 were cultivated on the mineral medium with fructose (Table 3). The higher PHA yields were obtained than using the medium (1): 11.0% w/w (*R. erythropolis* Au-1) and 5.1% w/w (*G. rubripertincta* UCM Ac-122).

The obtained positive results prompted to use the medium with fructose for testing of other bacteria: biosurfactant producer *R. ruber* UCM Ac-288 [31] and PHB producer *A. vinelandii* N-15 [21] (Table 3).

It was shown all studied strains produced high amounts of biopolymers when cultivated on the medium with fructose. At the used concentrations of fructose and sodium nitrate, the ratio C:N was in the range of 150–750.

At ratio C:N 210, a maximum yield of PHA of *R. ruber* strain UCM Ac-288 – 1.45 g/l (34.0% from biomass) was achieved. Thus, it is shown that this strain is a promising producer of biopolymers. PHA contents of *A. vinelandii* N-15 was 17% of biomass, however, due to low biomass values, biopolymer yield was only 0.37 g/l.

In previous publications, obtained biopolymers were investigated by methods of thin-layer chromatography, IR spectroscopy, and differential thermal analysis. PHA of *A. vinelandii* N-15 was identified as polyhydroxybutyrate (PHB) with a melting point of 189 °C and the onset of destruction at 294 °C [21]. The biopolymer of the *R. erythropolis* Au-1 strain belongs to polyhydroxyalkanoates with a melting point of 42 °C and a destruction temperature of 160 °C [29].

Polyhydroxyalkanoates of *R. erythropolis* Au-1 and *A. vinelandii* N-15 strains were used to construct composites with antimicrobial substances in the form of biofilms. Commercial LDPE packages (LDPE – low density polyethylene, 8 μm) were chosen for comparison of the hydrophobicity of polymers films surface. It was determined the water contact angle (θ°) of PHA was 125.5°; PHB – 71.3°; LDPE package – 90.0°. To improve the physical properties of biofilms, mixtures of polymers (PHA and PHB) were created in different ratios and the hydrophobicity of their surface was investigated. According to the results, the optimal ratio of biopolymers was selected – the best hydrophobic properties were determined in the composition of PHB/PHA – 73:27 ($\theta = 95°$), therefore, it was chosen for the packaging biofilm.

Antimicrobial biofilms – composites of biopolymers with antimicrobial substances of various natures were created in two ways: 1) solution casting method; 2) biocide layering on PHB/PHA film. Their effectiveness against bacteria and fungi was determined (Tables 4, 5).

It was established that the inclusion of ethyl thiosulfanilate or naphthoquinone derivative in the PHB/PHA matrix by solvent casting method showed high fungicidal and antibacterial activity against the test fungi *F. oxysporum*, *A. alternate*, *C. tenuis*, as well as *S. aureus* bacteria. Therefore, these preparations exhibit a wide spectrum of antimicrobial activity. Biofilms with natural antimicrobial substances (propolis extract, salicylic acid) or with Ag nanoparticles had little or minimal effect on the growth of test microorganisms. To compare the biocidal effect of natural compounds, the antibiotic chloramphenicol was used, biogenic films with this drug showed high antibacterial activity.

At the same time, biofilms obtained by layering antimicrobial substances on the surface of PHA/PHB showed stronger biocidal properties (see Table 5).

Table 3: Biosynthesis of PHA using the mineral medium with fructose

Bacteria	Fructose, g/l	$NaNO_3$, g/l	C:N	Biomass, g/l	PHA	
					g/l	% w/w from biomass
R.ruber UCM Ac-288	30	0.1	750	3.30 ± 0.21	0.32 ± 0.03	9.9
		0.3	243	3.99 ± 0.22	0.93 ± 0.04	23.3
		0.5	150	3.41 ± 0.20	0.71 ± 0.04	20.7
	35	0.4	210	4.26 ± 0.32	1.45 ± 0.05	34.0
	40	0.3	324	4.25 ± 0.32	0.73 ± 0.04	17.3
	50	0.3	405	1.80 ± 0.10	0.12 ± 0.02	6.7
R.erythropolis Au-1	30	0.5	150	3.31 ± 0.20	0.36 ± 0.03	11.0
G.rubriperctincta UCM Ac-122	30	0.5	150	5.20 ± 0.35	0.26 ± 0.02	5.1
A. vinelandii N-15	40	0.3	324	2.17 ± 0.10	0.37 ± 0.02	17.0

Notes. C:N – carbon to nitrogen ratio; cultivation time – 72 hours; working volume – 150 ml.

Table 4: Antimicrobial properties of composites obtained by solvent casting method

Antimicrobial substances	Concentration of antimicrobials, % w/w	Test microorganisms						
		E. coli	*M. luteus*	*P. aeruginosa*	*S. aureus*	*A. niger*	*C. tenuis*	*F. oxysporum*
		Diameter of the growth inhibition zone, mm						
Ethyl thiosulfanilate	2.0	–	–	–	11 ± 1	9 ± 1	9 ± 1	10 ± 1
	8.0	–	12 ± 1	–	22 ± 2	15 ± 2	22 ± 2	25 ± 2
Naphthoquinone derivative	2.0	–	10 ± 1	–	9 ± 1	14 ± 1	25 ± 2	14 ± 1
Propolis extract	8.0	–	–	–	–	–	–	9 ± 1
Salicylic acid	8.0	–	–	–	–	–	–	–
Chloramphenicol	8.0	20 ± 1	35 ± 3	18 ± 1	36 ± 2	–	–	–
Ag nanoparticles	8.0*	–	–	–	–	–	–	–
	8.0**	–	–	–	–	–	–	–

Notes. Naphthoquinone derivative – 2-chloro-3-(3-(3-(p-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione; * – Ag nanoparticles were obtained using rhamnolipids; ** – Ag nanoparticles were obtained using humic acids; "–" – no antimicrobial effect.

Table 5: Antimicrobial properties of composites obtained by layering antimicrobial substances on the surface of polymer films

Antimicrobial substances	Concentration of antimicrobials on the PHB/PHA surface, % w/w	Test microorganisms					
		E. coli	*M. luteus*	*S. aureus*	*A. alternate*	*C. tenuis*	*F. oxysporum*
		Diameter of the growth inhibition zone, mm					
Ethyl thiosulfanilate	2.0	–	9 ± 1	20 ± 2	21 ± 1	20 ± 1	25 ± 2
	8.0	–	13 ± 1	28 ± 2	43 ± 2	40 ± 2	40 ± 3
Naphthoquinone derivative	2.0	–	–	10 ± 1	13 ± 1	13 ± 1	13 ± 1
	8.0	–	–	12 ± 1	19 ± 1	25 ± 2	18 ± 1
Propolis extract	8.0	–	–	–	9 ± 1	–	13 ± 1
	50.0	–	17 ± 1	–	13 ± 1	10 ± 1	17 ± 1
Ag nanoparticles	0.8*	–	–	–	–	–	–
	8.0*	–	18 ± 2	–	11 ± 1	14 ± 1	–
	4.0**	–	21 ± 2	–	12 ± 1	15 ± 1	–
	8.0**	–	26 ± 2	–	16 ± 1	20 ± 2	–

Notes. Naphthoquinone derivative – 2-chloro-3-(3-(3-(p-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione; * – Ag nanoparticles were obtained with rhamnolipids; ** – Ag nanoparticles were obtained using humic acids; "–" – no antimicrobial effect.

It was established that all the investigated substances, layered on the PHB/PHA films, showed antimicrobial properties against the test microorganisms. The obtained results showed that the variants with Ag nanoparticles (4.0% w/w and 8.0% w/w) are effective against a number of tested microorganisms, while when obtaining the composite by the method of solvent casting, they did not show antimicrobial activity. Variants with ethyl thiosulfanilate and a naphthoquinone derivative showed the greatest activity. Although the composite with propolis extract showed a low antimicrobial effect, however, due to its natural origin, this antimicrobial food film should be promising for the market. Thus, composites obtained by layering antimicrobial substances on the surface of polymer biofilms exhibit more effective antimicrobial action.

Discussion

In this work, PHA was obtained from the biomass of actinobacteria *R. erythropolis* Au-1 and *G. rubripertincta* UCM Ac-122 under the cultivation parameters necessary for effective biosynthesis

of biosurfactants [30]: nutrient medium (1), carbon source – sucrose or glycerin (20–30 g/l), nitrogen source – $NaNO_3$ 3.0 g/l and yeast extract 1.0 g/l, cultivation time – 120 hours, working volume of Erlenmeyer flasks (750 ml) – 150 ml (Tables 1, 2). Under these conditions, the yields of PHAs were 2.6% w/w (*G. rubripertincta* UCM Ac-122) and 4.8 % w/w (*R. erythropolis* Au-1) from biomass. Thus, obtaining PHA from bacterial cells (waste products of biosurfactant production) is a possible, but to increase the yield of polymers, it is necessary to change the cultivation conditions of producents. The expediency of cultivating experimental strains of actinobacteria for 72 hours with a working volume of 150 ml (750 ml Erlenmeyer flask) was established. The obtained data are consistent with the literature data [20].

Microbial polymers also can be made cheaper by increasing the productivity of the technology via optimizing the composition of mineral medium [15, 20]. A change in the ratio of carbon to nitrogen made it possible to obtain the PHA yield of 8.9% w/w (*R. rythropolis* Au-1) and 4.6% w/w (*G. ubripertincta* UCM Ac-122) (see Tables 1, 2). The literature provides data on obtaining 1.55 g/l PHA (43.1% w/w from biomass) by actinobacteria *Rhodococcus pyridinivorans* BSRT1-1 on mineral medium with fructose [20]. Therefore, actinobacteria (*R. erythropolis* Au-1, *G. rubripertincta* UCM Ac-122, *R. ruber* UCM Ac-288) were cultivated on the mineral medium with fructose. As a result, high yields of PHA were obtained: *R. erythropolis* Au-1 – 11.0 % w/w (0.36 g/l), *R. ruber* UCM Ac-288 – 34.0% w/w (1.45 g/l) (see Table 3). Despite the fact that *G. rubripertincta* UCM Ac-122 showed a low efficiency of PHA synthesis – 5.1% w/w (0.26 g/l), the result is important, since there are no data on the synthesis of PHA by actinobacteria of the generus *Gordonia*. High yield of PHA also was obtained for the strain *A. vinelandii* N-15 – 17.0%. However, due to the low biomass of bacteria it was obtained only 0.37 and 0.18 g/l PHA, respectively. Based on previous results [21], the biosynthesis of biopolymer for *A. vinelandii* N-15 on mineral medium with fructose is not practical.

In this work it was confirmed the possibility of regulating the yield of PHA when changing the ratio of carbon and nitrogen is shown. Thus, at C:N values of 150–324, high yields of PHA *R. ruber* UCM Ac-288 were obtained – 17.3–34.0%, while at other C:N ratios the yield was only 6.7–9.9%. The obtained results agree with literature data [20, 32].

The obtained biopolymers were used to create antimicrobial composites – biopolymer films based on a mixture of PHB/PHA with antimicrobials. This direction has scientific and practical significance, in particular for environmentally safe packaging technologies. The use of antimicrobial food films is promising and has advantages compared to the treatment of products with biocides. Researchers prefer natural antimicrobial compounds as well as silver or zinc oxide nanoparticles within polyhydroxyalkanoates to produce active food-biopackaging systems [8, 33]. In this work, the following biocides were chosen for antimicrobial composites: propolis extract – biocide of natural origin; chemically synthesized analogues of natural compounds – salicylic acid, ethyl thiosulfanilate (a phytoncide of garlic and onions); an antibiotic of microbial origin – chloramphenicol; solutions of silver nanoparticles; a synthetic compound – 2-chloro-3-(3-(3-(p-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione, which has wide spectrum of biological activity. All biocides were used in concentrations highter than minimal inhibition concentration, because they are located in the PHB/PHA matrix or on the surface of the biofilm and binds non-covalently to the polymer base.

The results of experiments showed the promise of developed antimicrobial composites. Both tested methods – 1) solvent casting method; 2) layering antimicrobials on the surface of polymer films – ensured suppression of foreign microflora on the surface in contact with the biogenic film. Preparation of antimicrobial composites by solvent casting method contributes to the distribution of biocides throughout the volume of the PHB/PHA polymer matrix [13]. When the biocide solution is layered on the surface of the biofilm, it binds non-covalently to the polymer base, so the antimicrobial activity of the composite will be higher. This is confirmed by the results of our experiments. It was shown that all studied biocides are more effective on the surface of biopolymers than when located in the PHB/PHA matrix. Biofilms with Ag nanoparticles showed antimicrobial properties against *M. luteus, C. tenuis* and *A. alternate* only when prepared by layering the biocide on the biopolymer film. An important result is the achieved hydrophobic properties of the PHB/PHA biopolymer compositions, which are required for high-quality packaging film [13]. The effectiveness, safety for the environment and the synergism of the components of the antimicrobial biocomposites, indicate the priority of the development of this direction.

Conclusions

By optimizing mineral medium and cultivation conditions, 0.26–1.45 g/l polyhydroxyalkanoates were obtained, which was 5.1–34.0% of the biomass.

The ability of *Gordonia* bacteria to produce polyhydroxyalkanoates was determined for the first time – 5.1% of PHA was obtained from biomass. The ability of *R. ruber* UCM Ac-288 to biosynthesize PHA was shown: its maximum yield was 1.45 g/l (34.0% by mass from biomass) using fructose – 35 g/l, $NaNO_3$ – 0.4 g/l. The expediency of using medium with fructose for efficient biosynthesis of PHA was determined. High PHA yields of all studied strains were obtained: *R. ruber* UCM Ac-288 – 17.3–34.0%, *A. vinelandii* N-15 – 17.0%, *R. erythropolis* Au-1 – 11.0%, *G. rubripertincta* UCM Ac-122 – 5.1%.

Biofilms based on PHA compositions with optimal content were obtained. Their hydrophobicity was comparable to polyethylene packaging films. Biocomposites were produced in the form of films of a polymer mixture (PHB/PHA 73:27) with antimicrobial substances in two ways: 1) solvent casting method; 2) layering of antimicrobial agents on the surface of polymer films. It was shown that biofilms obtained by solvent casting exhibit fungicidal (with ethyl thiosulfanilate, a derivative of naphthoquinone) and antibacterial properties (with chloramphenicol). All biofilms obtained by applying antimicrobial agents to the surface of the polymer had a more effective antimicrobial effect. Thus, obtained polyhydroxyalkanoates have significant prospects for the use of antimicrobial biocomposites, in particular biofilms for packaging food.

Conflict of interests

Olena Karpenko is the member of the Editorial Council of *Innovative Biosystems and Bioengineering* and was not involved in the editorial evaluation or decision to accept this article for publication. The other authors have no conflicts of interest to declare.

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Н.І. Корецька[1], І.В. Семенюк[1,2], Т.Я. Покиньброда[1], Н.С. Щеглова[1], О.В. Карпенко[1], А.Р. Киця[1], В.І. Лубенець[3], Н.В. Поліш[3]

[1]Відділення фізико-хімії горючих копалин Інституту фізико-органічної хімії і вуглехімії ім. Л.М. Литвиненка НАН України, Львів, Україна
[2]Foshan Tianheng New Material Technology Co., Ltd, Фошань, Китай
[3]Національний університет "Львівська політехніка", Львів, Україна

ПОЛІГІДРОКСИАЛКАНОАТИ: ОПТИМІЗАЦІЯ БІОСИНТЕЗУ І СТВОРЕННЯ АНТИМІКРОБНИХ КОМПОЗИТІВ

Проблематика. Накопичення пластикових відходів негативно впливає на довкілля та здоров'я людини. Натепер одним зі шляхів вирішення цієї глобальної екологічної проблеми є використання біодеградабельних пластиків на заміну синтетичних. Серед них важливе місце посідають полігідроксиалканоати (ПГА) – мікробні внутрішньоклітинні полімери. Їх перевагами є здатність до біорозкладання, біосумісність, а також добрі термомеханічні властивості. Завдяки таким властивостям ПГА мають значні перспективи застосування в медицині, у сільському господарстві, харчовій промисловості, зокрема для пакування харчових продуктів.
Мета. Підвищити ефективність бактеріального синтезу ПГА шляхом модифікації живильних середовищ; отримати антимікробні композити на основі ПГА, визначити їхні антимікробні властивості.
Методика реалізації. Біосинтез ПГА оптимізували шляхом вибору умов культивування: джерел вуглецю та азоту, тривалості культивування та робочого об'єму. Виділення ПГА з бактеріальної біомаси здійснювали екстракцією хлороформом (перемішування 10 год, 35 °С, співвідношення біомаси та хлороформу 1:50); далі осаджували подвійним об'ємом ізопропанолу. Отриманий полімер сушили до постійної маси (60 °С). Гідрофобність біополімеру оцінювали за кутом змочування. Композити біополімерів з антимікробними речовинами у формі плівок отримували двома способами: 1) методом лиття з розчину; 2) нанесенням біоциду на полімерну плівку. Антимікробну активність отриманих композитів визначали за зонами затримки росту тестових мікроорганізмів.
Результати. Розроблено умови синтезу біополімерів і отримано 0,26–1,45 г/л ПГА (5,1–34,0 % ПГА з біомаси). Штам *R. ruber* UCM Ac-288 синтезував максимальну кількість біополімеру (34,0 % ПГА). Вперше встановлено здатність бактерій *Gordonia* синтезувати ПГА. Отримано композиції ПГА оптимального вмісту, гідрофобність яких порівнянна з гідрофобністю поліетиленових пакувальних плівок. Доведено антимікробні властивості біополімерних композитів з біоцидами.
Висновки. Підвищено ефективність бактеріального синтезу ПГА модифікацією живильних середовищ. Визначено, що отримані композити на основі ПГА з біоцидами є гідрофобними та мають антимікробні властивості, отже, мають перспективи використання як біоплівки для пакування та зберігання харчових продуктів.

Ключові слова: полігідроксиалканоати; *Rhodococcus*; *Azotobacter*; *Gordonia*; антимікробні композити; пакувальні біоплівки.