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# BIOTECHNOLOGICAL ASPECTS OF POLY-β-HYDROXYBUTYRATE BIOSYNTHESIS

*O. A. VASYLCHENKO, L. S. YASTREMSKAYA, A. M. SALATA, O. S. SALATA, N. O. FEDUN*

National Aviation University, Institute of Ecological Safety, Kiyv

E-mail: *vasoa@ukr.net*

In the review polyhydroxyalkanoates characteristic, including poly-β-hydroxybutyrate (P3HB) which is a polymer produced by microorganisms (*Ralstonia eutropha* — superproducer) is done. Biochemical pathways and conditions of P3HB microbial synthesis have been examined. Thermoplastic features of microbial P3HB in comparison with classic chemical polymers (polyethylene and polypropylene) have been analyzed. It was shown that according to its properties it was the most similar to synthetic polypropylene in comparison to other biopolymers, and it could be its good substitute.

Microbial P3HB ability for biodestruction to $CO_2$ and $H_2O$ has shown as extremely perspective property for this polymer usage in different industry branches and for environment saving against anthropogenous pollution.

***Key words:*** poly-β-hydroxybutyrate, biopolymer, *Ralstonia eutropha*, biosynthesis.

## Polyhydroxyalkanoates Characteristics

Poly-β-hydroxybutyrate (P3HB) is the first type of polyhydroxyalkanoates (PHA) to be identified. In conditions of nutrients excess many microorganisms assimilate and store them for future consumption. Various storage materials have been identified in microorganisms, which include glycogen, sulfur, polyaminoacids, polyphosphate, and lipids. Polyhydroxyalkanoates (PHA) are lipoid material accumulated by a wide variety of microorganisms in the presence of an abundant carbon source [1]. The assimilated carbon sources are biochemically processed into hydroxyalkanoate units, polymerised and stored in the form of water insoluble inclusions in the cell cytoplasm. The ability to carry out this polymerization process is dependent on the presence of a key enzyme known as PHA synthase. The product of this enzyme is high molecular weight (MW) optically active crystalline polyester. Upon isolation, this microbial polyester is a crystalline thermoplastic with properties comparable to that of polypropylene [2].

The research and development concerning PHA can be traced back to the beginning of the 20$^{th}$ century. Tremendous progress has been made recently motivated by the environmentally friendly properties of PHA. Unlike the present commodity plastics, PHAs are produced from renewable resources [2, 3]. Wide used petrochemical-based plastics currently are being regarded as a major threat of pollution. Plastics have found widespread application in our daily life because they are chemically inert and durable. Over the years, these properties gave rise to the accumulation of plastic materials in our environment. Now, these nonbiodegradable polymers contribute to the pollution of the environment and therefore some attempts at recycling have been made [3]. Nevertheless, a considerable amount ends up on beaches, in the oceans or clog landfill sites. Attempts to dispose of them by other means, i.e., incineration, produce different kinds of equally unacceptable pollution. These problems teach us that it is essential for mankind to develop and use materials that are compatible with our natural ecosystem [1, 3]. This has been the primary motivating factor in the research and development of PHA as a potential substitute for petrochemical-based plastics. PHA are biocompatible as well as biodegradable, and its degradation product, 3-hydroxyalkanoate is a normal mammalian metabolite [3].

Much work concerning PHA is in progress in many developed countries such as USA, Germany and Japan where waste disposal is becoming an increasingly serious problem [2–4]. Cost factors will be critical in determining whether in the long term, PHA can enter into widespread use in fields presently dominated by conventional commodity plastics. The final goal is to be able to produce in a cost-effective manner various kinds of PHA from renewable carbon sources [3].

## Poly-β-hydroxybutyrate Structure and Chemical Properties

P3HB (Fig. 1) is a polyhydroxyalkanoate, a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne [5]. P3HB is produced by microorganisms (like *Ralstonia eutropha* or *Bacillus megaterium*) apparently in response to conditions of physiological stress. The polymer is primarily a product of carbon assimilation and is employed by microorganisms as a form of energy storage molecule to be metabolized when other common energy sources are not available.

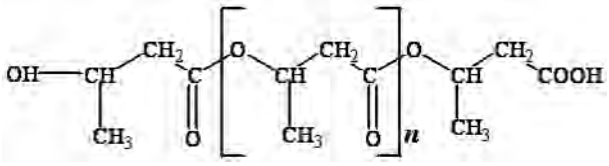


*Fig. 1.* **Chemical structure of poly-β-hydroxybutyrate [3]**

Melting temperature of poly-β-hydroxybutyrate, which is synthesized by various microorganisms, varies from minimum value that is 157 °C to maximal (approximately 188 °C). Specific weight of polymer is 1.23–1.25 g/cm$^3$. Carbon, hydrogen, oxygen are included in composition of poly-b-hydroxybutyrate; their percent correlation is 55.81:7.03:37.16 correspondently [5–7].

Polyhydroxybutyrate dissolves in chloroform, trichlorethylene, ethyl-acetate, dimethyl formaldehyde, phenol, thiaminehydrosine, icy acetic acid, camphor, and NaOH; does not dissolve in methanol, ethanol, acetone, hexane, water, diluted mineral acids [8].

By its thermoplastic properties microbial poly-β-hydroxybutyrate is close to classical chemical polymers of polyethylene and polypropylene types, and it can be pressed into different forms, films, strings, etc. Due to this it has wide prospects of usage nowadays. Comparison of poly-β-hydroxybutyrate and polypropylene properties is given in Table 1.

Today it is known that P3HB is the most common PHA found in nature. Based on the molecular weight (MW) of the biosynthesized P3HB, they can be divided into three distinct groups, i.e., low MW P3HB, high MW P3HB, and ultra-high molecular weight (UHMW) P3HB [5].

*Table 1.* **Comparison of poly-β-hydroxybutyrate (P3HB) and polypropylene (PP) properties [6]**

| Properties | P3HB | PP |
|---|---|---|
| Melting temperature (°C) | 180 | 176 |
| Glass transition temperature (°C) | 15 | –20 |
| Transparency (%) | 80 | 70 |
| Molecular weight (g/mole) | $5 \cdot 10^5$ | $2 \cdot 10^5$ |
| Flexural modulus (GPa) | 4.0 | 1.7 |
| Density (g/cm) | 1.250 | 0.925 |
| Elongation at break (%) | 5 | 150 |
| Strength limit on break (MPa) | 40 | 38 |
| Ultraviolet resistance | high | low |
| Solvents resistance | high | low |

The low MW P3HB which is also known as complexed P3HB (cP3HB) is an ubiquitous cell constituent that exists in Eubacteria, Archaebacteria, and eukaryotes. This cP3HB consists of about 120–200 hydroxybutyrate units and have a MW of about 12,000 Da [6, 7]. Despite having similar physical properties to polypropylene, the P3HB homopolymer produced by microorganisms is rather brittle and thermally unstable. The brittleness is due to the formation of large crystalline domains in the form of spherulites. The formation of large spherulites is a special property of this biologically synthesized P3HB probably because of its exceptional purity. This makes the microbial P3HB an ideal system for the study of spherulites but is definitely a major drawback to the commercial use of this homopolymer. The brittleness can however be reduced to a certain extent by using suitable processing conditions, enabling the production of ductile films [6].

In contrast to the low MW cP3HB, high MW P3HB is synthesized and accumulated in the form of water-insoluble inclusion bodies in microbial cell cytoplasm. They serve as carbon and energy storage compounds for the microorganisms. The MW of this storage P3HB is in the range 200,000 to 3,000,000 Da and the precise value depends on the microorganism and its growth conditions [5, 6].

Recently, the production of UHMW P3HB (MW > 3,000,000) has been achieved by using a

recombinant *Escherichia coli* cultivated under specific fermentation conditions. Unlike the high MW P3HB that is characterized by stiffness and brittleness, the UHMW P3HB seems to show improved characteristics. In addition, it was also found that films prepared from this UHMW P3HB were completely degraded at 25 °C in a natural freshwater river within three weeks [5].

High MW P3HB (MW = 200,000–3,000,000) was the first type of PHA to be identified, and because of its widespread occurrence, much work has been done to determine its physical properties and explore its potential applications. It is well known that P3HB samples obtained from various biological sources were all characterized by exceptional stereochemical regularity. They are linear polyesters and their chiral centres possess only the D absolute configuration. The biosynthesized P3HB is therefore perfectly isotactic (all the repeating units have the same stereochemical configuration) and upon extraction from the microorganisms, have a crystallinity of about 55–80% with a melting point at around 180 °C. The P3HB molecules in the crystalline regions have the conformational structure corresponding to a left-handed helix [7].

## Conditions of Poly-β-hydroxybutyrate Biosynthesis by *Ralstonia eutropha*

In the hydrogen-oxidizing bacterium *Ralstonia eutropha*, P3HB was detected in 1961 and physiologically characterized [4, 9]. The conditions leading to the accumulation of storage lipids are about the same for all organisms: lipids are accumulated when the C/N ratio of the diet is high. In *Ralstonia eutropha* P3HB is synthesized when the nutrient medium contains large amounts of sugar or organic acids and growth limiting amounts of a nitrogen source; the lack of the source of sulfur, phosphorus or magnesium has a similar, less significant effect. Incubation of cells under conditions of restricted oxygen supply also results in the accumulation of P3HB [4]. The growth conditions do not only influence the amount of the lipid stored but also its composition. During growth on fructose, butyrate or acetate a polyester of D(-)-β-hydroxybutyrate is formed. If, however, propionate or valerate (pentanoate) acid is used as a growth substrate-alone or in combination with acetate or butyrate copolyesters of β-hydroxybutyric and β-hydroxyvaleric acids are formed [9]. *Ralstonia eutropha* strains H16 and N9A accumulate very high amounts of P3HB, up to 86% or even 96% (w/w) P3HB [4].

Biochemically P3HB is synthesized from acetyl-coenzyme A via acetoacetyt-CoA and β-hydroxybutyryl-CoA (Fig. 2), and the reactions are catalyzed by the enzymes β-ketothiolase, acetoacetyl-CoA reductase and P3HB synthase [3, 4, 9].

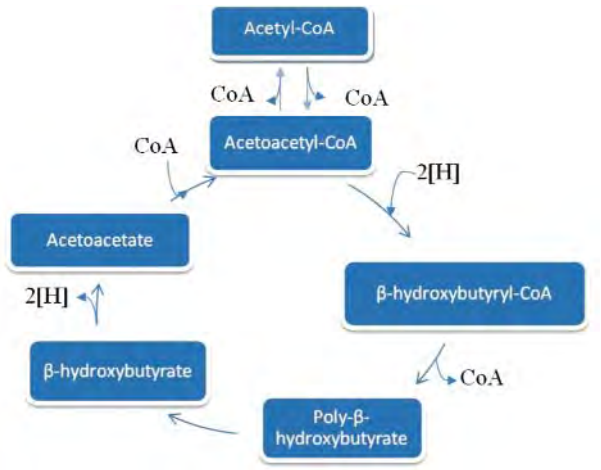


*Fig. 2.* **Synthesis and degradation of poly-β-hydroxybutyrate in *Ralstonia eutropha* [9]**

## Polyhydroxyalkanoates Biosynthesis Process

Unlike other microbial storage materials like glycogen or polyphosphate that have been studied in detail for their physiological importance, only the early studies focused on the physiology of PHA biosynthesis. The objectives of the most recent studies were to produce efficiently various kinds of PHA from simple and renewable carbon sources. With this goal in mind, much effort has been directed to understanding the enzymes, metabolic pathways, and conditions that generate substrates for PHA synthase.

In recent years, recombinant DNA technologies are increasingly used to further understanding of the complex regulatory mechanisms that affect PHA biosynthesis [4].

## Conditions that Promote Biosynthesis and Accumulation of PHA in Microorganisms

Early studies have revealed that the rate of PHA accumulation can be increased by increasing the ratio of carbon source to nitrogen source [3]. Eventually it became evident that PHA accumulation usually occurs when cell growth is impaired due to depletion of an essential nutrient such as sulfate, ammonium, phosphate, potassium, iron, magnesium, or oxygen. Suzuki and co-workers [4] studied 51 methylotrophs for their ability to produce poly-β-hydroxybutyrate (P3HB) from methanol. Similar nutrient limitations were found to stimulate the formation of P3HB. However,

a kinetic study of P3HB production by a fed-batch culture of *Protomonas extorquens* showed that a nitrogen source was necessary even in the P3HB production phase. Feeding with a small quantity of ammonia resulted in a more rapid increase of intracellular P3HB than was the case without ammonia feeding. Excessive feeding of ammonia, however, caused not only degradation of accumulated P3HB but also reduction of microbial P3HB synthetic activity.

PHA accumulation can also take place during active cell growth, but this ability is limited to only a few microorganisms such as *Alcaligenes latus* that can accumulate P3HB up to 80% of the dry cell weight without limitation of any nutrient. This characteristic may be due to a low activity of the β-ketothiolase, which catalyses the cleavage of acetoacetyl-CoA. Besides *A. latus*, *Paracoccus denitrificans* also shows growth-associated PHA accumulation depending on the type of carbon sources available to the bacterium. Kim and co-workers [4, 9], tested linear primary $C_1$-$C_9$ alcohols and linear $C_2$-$C_{10}$ monocarboxylic acids and found that growth-associated synthesis of PHA could be obtained only with the carbon sources with an odd number of carbon, except for methanol.

The advantage of using a bacterium that shows growth-associated PHA accumulation for large-scale production is a shorter fermentation time. In addition, it also avoids the extra operations associated with the two-step fermentation process for PHA-accumulation under nutrient-limited conditions. By using *A. latus* btF-96, Chemie Linz was able to produce more than 1000 kg of P3HB in a week in a 15 $m^3$ fermenter [4]. ICI Company on the other hand chose *R. eutropha* as the production organism although this bacterium accumulates PHA under non-growth conditions. *R. eutropha* was chosen over *Azotobacter* and *Methylobacterium* because of higher polymer content, good molecular mass and also because of easier PHA recovery [9–11].

## Carbon Sources for PHA Production

The plastic materials widely in use today are synthesized from fossil fuels such as petroleum and natural gas. An attractive feature of the microbial PHA is the ability to produce them using renewable carbon sources such as sugars and plant oils, which is an indirect way of utilizing the atmospheric $CO_2$ as the carbon source. Various waste materials are also being considered as potential carbon sources for PHA production. Among them are whey, molasses, and starch. The carbon source available to a microorganism is one of the factors that determine the type of PHA produced (others being the PHA synthase substrate specificity and the types of biochemical pathways available). For industrial scale production, the carbon source significantly contributes to the final cost. This makes the carbon source one of the most important component in the production of PHA and is therefore a prime target for potential cost reduction [2, 10].

A recombinant strain of *R. eutropha* PHB–4 (a PHA-negative mutant), containing the PHA synthase gene from *Aeromonas caviae*, can produce a random copolymer of 3-hydroxybutyrate and 3-hydroxyhexanoate (3HHx) from plant oils such as olive oil, palm oil and corn oil. The P[3HB-*co*-3HHx] content was approximately 80% of the dry cell weight and the 3HHx mole fraction was 4–5 mol% regardless of the structure of the triglycerides fed. The results demonstrate that inexpensive renewable plant oils are excellent carbon sources for the efficient production of PHA [11].

At present plant derivatives such as sugars and oils are the most popular carbon sources for PHA production by microbial fermentation [12].

## Biochemical Pathways Involved in Metabolism of PHA

In order to tap the full potential of microbial systems for PHA production, it is necessary that the existing metabolic pathways in a particular microorganism are modified. This is to ensure that the major portions of the supplied carbon sources are channeled towards PHA biosynthesis. Recent knowledge of the complete genetic makeup of several microorganisms is facilitating the engineering of novel metabolic pathways. New pathways can be constructed by introducing relevant genes into suitable microorganisms. Likewise, unnecessary pathways can be shutdown by inactivating the enzymes involved in a certain reaction. Such manipulations have to be carried out to achieve maximum PHA production in the shortest possible time using cheap and readily available carbon sources, without compromising the cell growth. Another important factor is the stability of the genetically modified strains over many generations. Recombinant strains that do not have this characteristic will not be attractive as an industrial strain for large-scale production of PHA [3,10].

Fig. 3 shows the common metabolic pathways that are frequently encountered in the biosynthesis of PHA in various microorganisms. Along with the type of carbon source

and specificity of the PHA synthase, the metabolic pathways play a crucial role in determining the type of PHA that can be produced by a particular microorganism. Most of the P3HB producing microorganisms possess pathway through which acetyl-CoA is converted into (*R*)-3-hydroxybutyryl-CoA and subsequently polymerized by PHA synthase. Recently it has been shown that a similar pathway also operates in the cyanobacterium *Synechocystis sp.* PCC6803 [9]. In some microorganisms, (*S*)-isomers of 3-hydroxybutyryl-CoA are generated instead of the (*R*)-isomers. Since the PHA synthase is active only towards the (*R*)-isomers, additional reaction steps catalysed by enoyl-CoA hydratases are present in microorganisms such as *Rhodospirillum rubrum* to convert the (*S*)-isomers into the (*R*)-isomers.

Fatty acid β-oxidation pathway is known to generate substrates that can be polymerized by the PHA synthases of pseudomonads. Pseudomonads can synthesize medium chain length polyhydroxyalkanoates ($PHA_{MCL}$) from various alkanes, alkanols, and alkanoates. The monomer composition of the $PHA_{MCL}$ produced is often related to the type of carbon sources. Most of the pseudomonads, except *P. oleovorans*, can also derive (*R*)-3-hydroxyacyl-CoA substrates for PHA biosynthesis from unrelated carbon sources such as carbohydrates. Bastioli C. [17] gave evidence showing that the PHA synthase responsible for PHA synthesis from fatty acids are also involved in PHA synthesis from glucose. It was then presumed that there are at least two distinct substrate supply routes for PHA synthesis in *Pseudomonas putida*, i.e., via the intermediates of fatty acid biosynthesis and via the intermediates of β-oxidation [10, 11].

Although it was known that the intermediates of the β-oxidation cycle are channeled towards PHA biosynthesis, only recently the precursor sources were identified. In *A. caviae*, the β-oxidation intermediate, *trans*-2-enoyl-CoA is converted to (*R*)-3-hydroxyacyl-CoA via (*R*)-specific hydration catalyzed by a (*R*)-specific enoyl-CoA hydratase [13].

Bastioli C. [17] reported the identification of similar enoyl-CoA hydratases in *P. aeruginosa*. In the latter case, two different enoyl-CoA hydratases with different substrate specificities channelled both small chain length (SCL) and medium chain length (MCL) enoyl-CoA towards PHA biosynthesis. In recombinant *E. coli* it was further shown that 3-ketoacyl-CoA intermediates in the β-oxidation cycle can also be channeled towards PHA biosynthesis by a NADPH-dependent 3-ketoacyl carrier protein (ACP) reductase. A similar pathway was also identified in *P. aeruginosa*. In addition, it was also reported that the acetoacetyl-CoA reductase (PhaB) of *R. eutropha* can also carry out the conversion of 3-ketoacyl-CoA intermediates in fatty acid β-oxidation pathway to the corresponding (*R*)-3-hydroxyacyl-CoA in

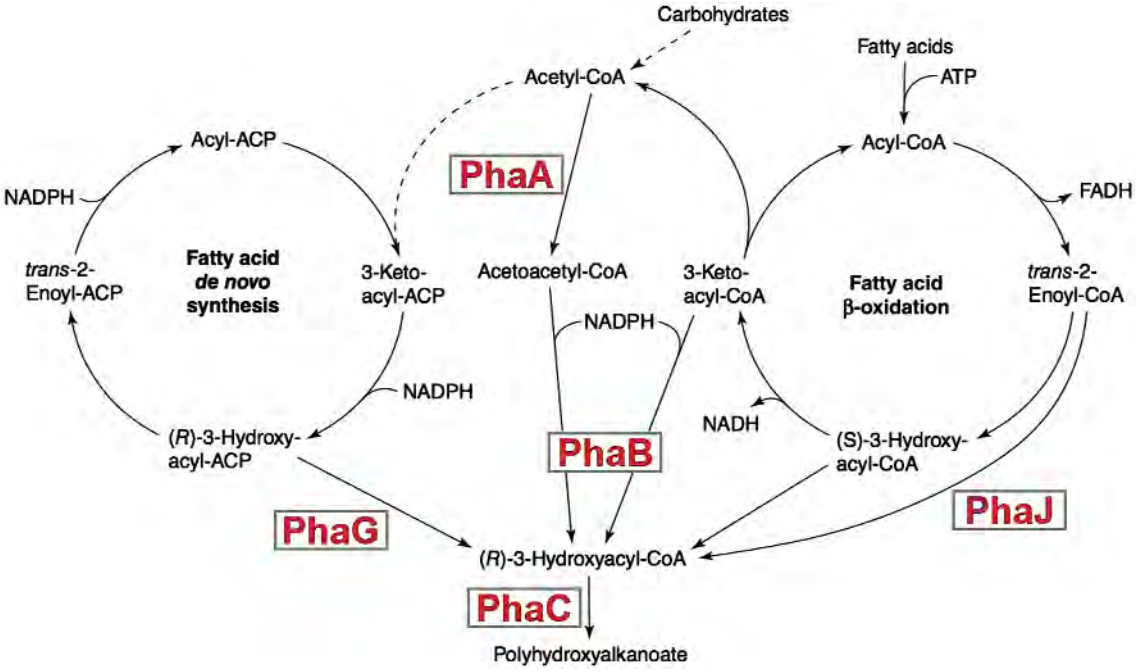


*Fig. 3.* **Common metabolic pathways involved in the biosynthesis of PHA in microorganisms:** PhaA: β-ketothiolase; PhaB: NADH-dependent acetoacetyl-CoA reductase; PhaC: PHA synthase; PhaG: 3-hydroxyacyl-ACP:CoA transferase; PhaJ: (R)-enoyl-CoA hydratase [14]

*E. coli*. The results clearly indicate that several channeling pathways are available to supply substrates from β-oxidation cycle to the PHA synthase. This explains why it was not possible to obtain mutants that completely lack PHA accumulation ability, unless the mutation occurred in the PHA synthase gene [15–17].

Among the various metabolic pathways that are involved in PHA biosynthesis, the fatty acid *de novo* biosynthesis pathway is of particular interest because of its ability to supply various types of hydroxyalkanoate monomers from simple carbon sources such as gluconate, fructose, acetate, glycerol and lactate. It can be envisaged that the potential future production of $PHA_{MCL}$ by using photosynthetic organisms will benefit through the exploitation of such pathways. This is because acetyl-CoA is the starting material (fatty acid *de novo* biosynthesis pathway) that is used to generate hydroxyalkanoate monomers for $PHA_{MCL}$ biosynthesis, and acetyl-CoA is a universal metabolite present in all living organisms. However it must be noted that the intermediates of fatty acid *de novo* biosynthesis pathway are in the form of (*R*)-3-hydroxyacyl-ACP, which is not recognized by the PHA synthase [16].

Studies of $PHA_{MCL}$ biosynthesis in *P. putida* from glucose as the sole carbon source has identified an enzyme that is capable of converting 3-hydroxydecanoyl-ACP to 3-hydroxydecanoyl-CoA. The enzyme was referred to as a 3-hydroxyacyl-ACP:CoA transferase (PhaG). Since then, similar enzymes have been identified in several other pseudomonads. *P. oleovorans* does not have the ability to synthesise $PHA_{MCL}$ from gluconate but shows this ability upon the introduction of the PhaG gene of *P. putida* [17].

The genes for PhaG and PHA synthase from *P. aeruginosa* were expressed together in a non-PHA producing pseudomonad, *P. frugi*. This resulted in the ability to produce $PHA_{MCL}$ by *P. frugi* from gluconate as the sole carbon source. Besides the PhaG protein, overexpressions of transacylating enzymes such as malonyl-CoA-ACP transacylase (FabD) in *E. coli*, also seem to generate monomers for P3HB biosynthesis [13, 15].

Besides the three main pathways mentioned above, there are several other metabolic pathways that can be manipulated to produce substrates for PHA biosynthesis. In recombinant *E. coli*, it has been shown that 4-hydroxybutyryl-CoA can be derived from the intermediates of tricarboxylic acids (TCA) cycle. By providing external precursor substrates such as 4-hydroxybutyric acid, 1,4-butanediol, and butyrolactone to certain wild type and recombinant microorganisms, 4-hydroxybutyrate monomers can be incorporated more efficiently [13, 16, 17].

## The Key Enzyme of PHA Biosynthesis, PHA Synthase

Without doubt, PHA synthase is the key enzyme in the biosynthesis of PHA. Unfortunately, the mechanism of this important enzyme is not yet fully understood. Based on genetic analysis, the primary structures of PHA synthases from a large number of microorganisms are available [18]. The PHA synthases have been classified into three groups based on their primary structures and the types of PHA that they produce [19, 20]. The PHA synthases of *R. eutropha* and *P. oleovorans* represents the groups I and II, respectively, while that of *Chromatium vinosum* represents the group III. The latter differs from the two former groups by the fact that group III synthases consist of two different subunits (PhaC and PhaE) while the members of groups I and II only have one subunit (PhaC). As for the types of PHA produced, PHA synthases of groups I and III are efficient in the synthesis of short chain length polyhydroxyalkanoate ($PHA_{SCL}$), while those of group II are superior in the synthesis of $PHA_{MCL}$. A few exceptions to the above classification are the PHA synthases of *A. caviae*, *Thiocapsa pfennigii*, and *Pseudomonas* sp. 61-3. These PHA synthases are capable of producing PHA copolymers containing both the SCL- and the MCL-PHA. These exceptional PHA synthases are of great interest because they can be used to biosynthesize PHA copolymers containing novel compositions that show promising physical properties [19–21].

### *Ralstonia eutropha* — the Superproducer of Poly-β-hydroxybutyrate

Aerobic heterotrophic bacteria of genus: *Azotobacter, Pseudomonas, Spirillum, Zoogloea, Ralstonia, Rhozobium, Nocardia* (it was showed by Lemming in 1926; Morris, Roberts in 1959; Devis — 1964; Bonarceva and others — in 1989), chemolithotrophic bacteria: *Ralstonia eutropha, Alcaligenes. latus, A. faecalis* (Shlegel — 1962; Shuster, Shlegel — 1967; Shlegel — 1975; Filler, Brand — 1988), anaerobic phototrophic bacteria: *Rodosperillum, Chloreflexus* (Kondratyeva, Krasilnikova — 1989), aerobic photobacteria: *Chloroglaea, Chloreflexus* (Kondratyeva, Krasilnikova — 1988), archaebacteria and oligotrophic bacteria have ability to synthesize PHB biopolymer [7].

*Ralstonia eutropha* (Table 2) can be found in both soil and water. This bacterium has great potential for use in bioremediation as it is able to degrade a great number of chlorinated aromatic (chloroaromatic) compounds and chemically related pollutants [8].

*Table 2.* **Scientific classification of *Ralstonia eutropha* [8]**

| | |
|---|---|
| **Domain** | *Bacteria* |
| **Phylum** | *Proteobacteria* |
| **Class** | *Betaproteobacteria* |
| **Order** | *Burkholderiales* |
| **Family** | *Burkholderiaceae* |
| **Genus** | *Cupriavidus/Ralstonia/Wautersia* |
| **Species** | *R. eutropha* |

*R. eutropha* (Fig. 4) strains can utilize hydrogen, carbon dioxide and organic compounds for development and is a model organism for hydrogen oxidation because it can nurture on hydrogen as the sole energy source. *R. eutropha* H16 was formerly known as *Alcaligenes eutrophus* and was originally isolated from sludge. *R. eutropha* JMP 134 was isolated due to its ability to degrade the herbicide 2,4-dichlorophenoxyacetic acid [22–24].

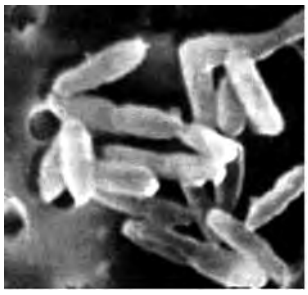

*Fig. 4.* ***Ralstonia eutropha*** **bacterium under the electron microscope [25]**

*R. eutropha* is Gram-negative bacterium and is non-spore forming. Many of the Gram-negative bacteria are pathogenic but this bacterium is not. They have motility and are facultative aerobes which can live in both aerobic and anaerobic environments. *R. eutropha* has two flagella and two membranes and are usually rod shape. *R. eutropha* JMP 134 has multiple habitats and *Ralstonia eutropha* H16 have a specialized habitat but both requires non-halophilic environment. The optimal temperature is 30 °C. *R. eutropha* thrives most successfully in the presence of millimolar concentrations of several heavy metals [8, 26–28].

*R. eutropha* is able to develop with hydrogen and carbon dioxide as its only energy source and carbon source. When oxygen is not present it can use different metabolism to grow.

This bacterium can be used for the production of biodegradable thermoplastic with the characteristics of polyhydroxyalkanoates as it is able to accumulate poly-β-hydroxybutyrate biopolymer up to 80% of cell dry weight (Fig. 5) [28].

*Fig. 5.* ***Ralstonia eutropha*** **with poly-β-hydroxybutyrate inclusions under the electron microscope [29]**

## Technology of Poly-β-hydroxybutyrate Production by the Cultivation of *Ralstonia eutropha*

Technological process of poly-β-hydroxybutyrate production can be divided on three main stages: 1) cultivation of *R. eutropha* H16; 2) concentration of biomass; and 3) polymer extraction [8].

Cultural medium used for poly-b-hydroxybutyrate production has the following composition: 1.14 g/l of $NH_4Cl$; 1.7 g/l of $KH_2PO_4$; 2.18 g/l of $K_2HPO_4$, as well as the trace salts (in mg/l): $MgSO_4 \cdot 7H_2O$ (712); $CaCl_2 \cdot 2H_2O$ (37); $FeSO_4 \cdot 7H_2O$ (50); $CuSO_4 \cdot 5H_2O$ (7.8); $MnSO_4 \cdot H_2O$ (6.1); $ZnSO_4 \cdot 7H_2O$ (4.4); $NaMoO_4 \cdot 2H_2O$ (2.5) [23].

Cultivation proceeds in periodical regime in two stages. In the first one bacterial growth is conducted on medium with nitrogen deficiency. It limits cell growth and stimulates polymer accumulation. This stage lasts 20–24 h. On the second stage polymer accumulation takes place on nitrogen-free medium. Second stage is realized during 34–36 h on nitrogen-free medium. Fermenter volume is 75 l, apparatus is equipped with cooling jacket and turbine mixer, rotation frequency of turbine mixer is 1000 rot/min. Working volume of culture is 40–50 l. Cultivation takes place at the temperature of medium 30±0.3 °C and pH 7.0–7.1. Air supply is increased from 1 to 9 $m^3/h$ depending on the concentration of cells in culture. Specific velocity of air consumption by culture on the first stage is nearly 0.16 kg/(kg·h). Glucose concentration is nearly 5 g/l and nitrogen source addition is limited. Biomass concentration reaches 25–30 g/l, polymer concentration in cells is 45–50%. At the end of cultivation cell concentration is

40–45 g/l and amount of poly-β-hydroxybutyrate reaches 80–85% [16, 17].

Enlargement of biomass concentration in culture on the second stage of bacteria cultivation reflects the polymer amount increasing in culture, synthesis of which requires much less energy than protein synthesis. In result of it glucose supply on the second stage of process is lowered in comparison with the first stage. In average, glucose supply on biomass synthesis is 3.0 kg/kg of biomass and on polymer synthesis — 2.6 kg/kg of biomass [12, 16].

Concentration of bacterial biomass is conducted in vacuum evaporating device at temperature 41–45 °C and absolute pressure 0,008–0,01 MPa during 1.5–2.0 h. In result final volume of culture after evaporation is 2–3 $m^3$ at biomass concentration 80–150 g/l by absolute dry substance. At following centrifugation of concentrated culture (13 500 rot/min during 15 min) bacterial paste with final moist 50–55% is obtained [17].

For poly-β-hydroxybutyrate extraction from bacterial cells different solvents are used (chloroform, chloride methylene, sodium hypochlorite, dichloroethane, tetrachloroethane, dioxane, etc.), and for sedimentation of polymer extracts isopropanol, ethanol, hexane, tetrahydrofuran, dimethyl formamide are used. An important feature of this stage is ability to repeated usage of reactants that are consumed in great amount. For this reason reagents for extraction and separation of polymer must differ by boiling temperature (more than on 40 °C) and not form azeotropic mixtures. Polymer extraction is conducted in device with mixer during 10–12 h. Obtained mass is transferred to separator-clarifier where mixture is separated on shrot of biomass rest that is accumulated in the upper part and polymer extract. Extract is collected into vessel and transferred to evaporator embedded in cubic part of rectification column where solvents evaporation is conducted under the vacuum. Then vapour condensation in heat exchanger of column and their accumulation in collecting vessel takes place. Collected solvents on this stage can be used for new extraction cycles. Recovery percentage of solvents obtained on polymer extract concentration stage is 60–65% [8, 17].

Concentrated extract is transferred into the vessel with mixer (precipitator). Recovered polymer is separated in centrifuge and transferred into desiccator where it is dried at room temperature and air contact to final product with moisture 0.70±0.25%.

Regeneration (separation) of solvents and precipitant is conducted in rectification column. Regeneration level is 80–90%. Completeness of polymer extraction from biomass is 80–90% from its initial content in biomass. For increasing of extraction completeness repeated separation of final polymer can be used. Chemical purity of extracted polymer is 98±1%. Among admixtures there are lipids and hydroxyl derivatives of long-chain fatty acids. Repeated solution and sedimentation of polymer allows to obtain product of 100% purity [8].

Thus the physical and chemical characteristics of poly-β-hydroxybutyrate were analyzed. It was shown that according to its properties it is the most similar to synthetic polypropylene in comparison to other polymers, and it can be its good substitute.

The ability of *Ralstonia eutropha* to synthesize poly-β-hydroxybutyrate was studied and this culture was identified as its superproducer due to ability to accumulate poly-β-hydroxybutyrate granules up to 80% of cell dry weight.

The composition of nutrient media, basic parameters and terms of *Ralstonia eutropha* cultivation for poly-β-hydroxybutyrate production were defined.

A technology of poly-β-hydroxybutyrate production by the cultivation of *Ralstonia eutropha* was investigated and this question requires further researches.

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## БІОТЕХНОЛОГІЧНІ АСПЕКТИ БІОСИНТЕЗУ ПОЛІ-β-ГІДРОКСИБУТИРАТУ

*О. А. Васильченко, Л. С. Ястремська, А. М. Салата, О. С. Салата, Н. О. Федун*

Національний авіаційний університет, Інститут екологічної безпеки, Київ

*E-mail: vasoa@ukr.net*

В огляді наведено характеристику полігідроксіалканоатів, у тому числі полі-β-гідроксибутирату (ПГБ) — полімеру, який продукують мікроорганізми (*Ralstonia eutropha* — суперпродуцент). Розглянуто біохімічні шляхи та умови мікробного синтезу ПГБ. Проаналізовано термопластичні властивості мікробного ПГБ порівняно з класичними хімічними полімерами (поліетиленом і поліпропіленом). Було виявлено, що за своїми властивостями порівняно з іншими полімерами він найбільше схожий на синтетичний поліпропілен і може бути його добрим замінником. Показана здатність мікробного ПГБ до біологічного розпаду до $CO_2$ і $H_2O$, що є надзвичайно перспективним для використання цього біополімеру в різних галузях промисловості та збереження довкілля від антропогенного забруднення.

***Ключові слова:*** полі-β-гідроксибутират, біополімер, *Ralstonia eutropha*, біосинтез.

## БИОТЕХНОЛОГИЧЕСКИЕ АСПЕКТЫ БИОСИНТЕЗА ПОЛИ-β-ГИДРОКСИБУТИРАТА

*О. А. Васильченко, Л. С. Ястремская, А. М. Салата, А. С. Салата, Н. О. Федун*

Национальный авиационный университет, Институт экологической безопасности, Киев

*E-mail: vasoa@ukr.net*

Приведена характеристика полигидроксиалканоатов, в том числе поли-β-гидроксибутирата (ПГБ) — полимера, продуцируемого микроорганизмами (*Ralstonia eutropha* — суперпродуцент). Рассмотрены биохимические пути и условия микробного синтеза ПГБ. Проанализированы термопластические свойства микробного ПГБ в сравнении с классическими химическими полимерами (полиэтиленом и полипропиленом). Было обнаружено, что по своим свойствам в сравнении с другими полимерами он более всего походит на синтетический полипропилен и может быть его хорошим заменителем. Показана способность микробного ПГБ к биологическому разложению до $CO_2$ и $H_2O$, что является чрезвычайно перспективным для использования этого биополимера в различных отраслях промышленности и сохранения окружающей среды от антропогенного загрязнения.

***Ключевые слова***: поли-β-гидроксибутират, биополимер, *Ralstonia eutropha*, биосинтез.