

Microbial production of poly- β -hydroxybutyrates - an overview

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Abstract. Efficient poly- β -hydroxybutyrate (PHB) production represents an actual preoccupation of fundamental research from current biotechnology. Since PHB production out of renewable sources has a positive environmental impact and particular biotechnological applications, the study of strains with high synthesis capacity for these polymers is required. Poly- β -hydroxybutyrates production presents remarkable importance from many points of view. Firstly these substances are biodegradable, so with no negative impact on the environment. They are obtained out of carbon sources which are available and renewable, not depending on exhaustible deposits. They are biopolymers used in diverse fields: plastic materials, packages, absorbents, biomedical materials and tissue engineering materials. Being substances with a large usability great companies from diverse fields are interested in any progress regarding amelioration of PHB production. Taking into consideration the multiple uses of the biopolymers in modern biotechnologies and the medical field it is needed to deeply understand their production. Thus, this paper is a review of the most recent researches in this field.

Key words: poly- β -hydroxybutyrate (PHB), PHB producing bacterial strains, *phb* gene, biotechnology.

Rezumat. Producerea eficientă de poli- β -hidroxibutirati (PHB) reprezintă o preocupare actuală a cercetării fundamentale din biotehnologia modernă. Deoarece producerea PHB din surse regenerabile are un impact pozitiv de mediu și aplicații biotehnologice deosebite, se impune studiul tulpinilor cu potențial ridicat de sinteză a acestor biopolimeri. Producerea de poli- β -hidroxibutirati (PHB) prezintă importanță remarcabilă din mai multe puncte de vedere. În primul rând aceste substanțe sunt biodegradabile, deci lipsite de impact negativ asupra mediului înconjurător. Se obțin din surse de carbon disponibile și regenerabile, nedepinzând de zăcămintele epuizabile. Sunt biopolimeri utilizați în diverse domenii: materiale plastice, ambalaje, absorbanți, materiale biomedicale și materiale folosite în ingineria tisulară. Fiind substanțe cu o largă aplicabilitate, marile companii din diverse domenii sunt interesate de orice progres privind eficientizarea producției de PHB. Având în vedere multiplele utilizări ale biopolimerilor în biotehnologiile moderne și domeniul medical, se impune cunoașterea aprofundată a producerii lor eficiente. Astfel că această lucrare își propune o trecere în revistă a celor mai noi cercetări în această direcție.

Cuvinte cheie: poli- β -hidroxibutirati (PHB), bacterii producătoare de PHB, gene *phb*, biotehnologie.

Polymers obtained by renewable resources are of world-wide interest for predominantly two major reasons: environmental concerns and the fact that petroleum resources are finite. Poly β -hydroxybutyrate (PHB) was first mentioned in the scientific literature as early as 1901 and detailed studies began in 1925. PHB inclusion bodies were studied primarily as an academic curiosity but beginning with the energy crisis of the 1970s the substitution of synthetic plastics with PHB was attempted. The study of these biopolymers, regarding biosynthesis, microstructure, mechanical and thermal properties and biodegradation, gained momentum in the recent years (Suriyamongkol et al 2007).

Generally, polymers from renewable resources can be classified into three groups: natural polymers, synthetic polymers from bioderived monomers, and polymers from microbial fermentation. In nature, many microorganisms produce polyesters as part of their survival mechanism (Scholz & Gross 2000). Polyhydroxyalkanoates (PHA) are aliphatic natural polyesters produced by microbial processes on sucrose media, representing carbon and energy storage materials in the bacterial cell.

The bacterial origins of PHA make these polyesters a natural material, and therefore many microorganisms have the ability to degrade these macromolecules. It is now well recognized that PHB granules accumulate as energy storage compounds in Gram-negative and Gram-positive bacteria as well as Archaea (Rychter et al 2006). Poly- β -hydroxybutyrates (PHB) and poly-hydroxyvalerates (PHV) belong to poly-hydroxyalkanoates (PHA) family. Aliphatic polyesters as PHA are homopolymers and copolymers of hydroxybutyric and hydroxyvaleric acids and are quickly biodegraded.

These biopolymers are synthesized by microorganisms as cellular accumulation polymers which accumulate during growth in discrete intracellular granules which can constitute up to 90% of cell dry weight (Torabian et al 2004; Verlinden et al 2007). Conditions for optimal production of PHB usually include an excess of carbon source and exhaustion of a single nutrient such as nitrogen, sulphur, iron, magnesium or oxygen (Krallish et al 2009). Under internal or external environmental stress conditions many microorganisms as *Azotobacter* genus (Fig. 1) are able to produce and accumulate intracellularly PHB as energy and carbon storage material (Cabiscol & Tamarit 2000).

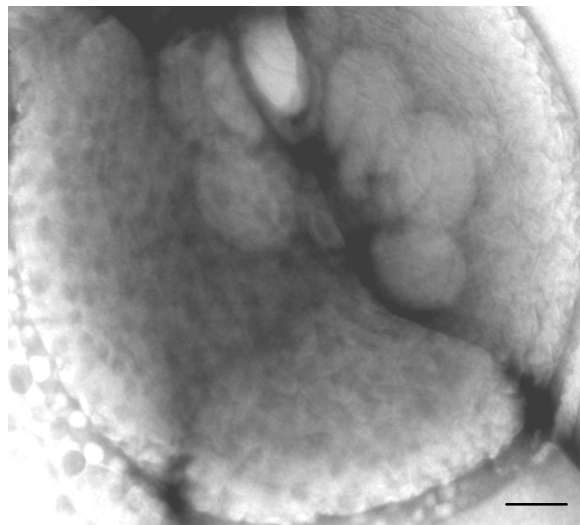


Figure 1. PHB intracellular granule in *Azotobacter* sp. Bar = 500 nm

Biosynthesis and biodegradation of PHB are processes which include a chain of key enzymes as PHB synthetases, PHB depolymerases and degradation involved enzymes which have been investigated by many researchers (Abe et al 2005; Potter & Steinbuechel 2005). The PHB biosynthetic genes (*phbA*, *phbB* and *phbC*) are clustered and organized in one *phbCAB* operon (Reddy et al 2003). Despite considerable progress in understanding the function of individual proteins involved in PHA metabolism, the initiation of PHA synthesis is not understood very well. The literature contains hundreds of images showing PHB granules localized more or less randomly in the bacterial cell, and it has been assumed that PHB granules are cytoplasm-located subcellular structures (Jendrossek 2005; Jendrossek et al 2007).

Mechanical properties of PHAs make them suitable replacements for petrochemically produced bulk plastics (polyethylene, polypropylene etc.), but in contrast to these PHA are completely degradable to carbon dioxide and water through natural microbiological mineralization. PHAs can be produced by biotechnological processes under controlled conditions, obtaining the so called green polymers in contrast to the conventional petrochemical products. Microorganisms are able to incorporate up to 60 different types monomer into their storage polymers including PHA (Bonartsev et al 2007).

PHB plays a key role in the cellular metabolism of many bacterial species. Such bacterial species are found in diverse soil types participating to this living matrix of terrestrial ecosystems. Within this dynamic matrix they actively participate to maintaining the natural balance of the nutrient cycles. Soil is considered a storehouse of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space (Tilak et al 2005; Garbeva et al 2008). Diversity and

structure of microbial consortia found in rhizosphere influences the whole ecosystem contributing to plant nutrition, the welfare of the vegetation cover populating that soil, and also the structure and fertility of that soil. Most studies were performed on the rhizosphere of cultivated plants (Baldani & Baldani 2005; Ahmad et al 2006; Rekosz-Burlaga & Garbolinska 2006) and also on degraded or polluted soils (Qian et al 2006; Chen & Sun 2007). Out of these soils bacterial strains were isolated and at theme PHB producing capacity was assessed. Plants naturally produce numerous polymers, including rubber, starch, cellulose and storage proteins, all of which have been exploited for biodegradable plastic production (Mooney 2009).

PHBs are present in the cytoplasm of bacterial cells as water insoluble granules. Besides playing an important role as a reserve polymer, PHB has been implicated in supporting nitrogen fixation.

Most species of *Rhizobium* synthesize PHB but not all species accumulate it during symbiosis with legumes. The precise roles of PHB in these bacteria during infection, nodulation, and nitrogen fixation are not fully elucidated (Trainer & Charles 2006). Instead, at *A. vinelandii* PHB is related to production of cysts resistant to desiccation with many PHB granules (Galindo et al 2007).

Azotobacter sp. is a Gram negative free, aerobic, diazotroph soil bacterium which populates neutral alkaline soils and under adverse environmental conditions undergoes a differentiation process leading to the formation of PHB cysts (Khanafari & Akhavan Sepahei 2007). Such microorganisms have a vital role in any ecosystem because they convert nitrogen to ammonia which then enters in protein composition. PHB polymers produced by *Azotobacter chroococcum* were tested regarding biodegradability and it was noticed that they degrade in media as soil, water, compost and activated sludge (Manna & Paul 2000).

Another model microorganism for the PHA production and chemolithoautotroph metabolism study is *Ralstonia eutropha* (*Alcaligenes eutrophus*). It is a facultative anaerobe bacteria, non sporogenous which can develop in environment contaminated with heavy metals as Zn, Cd, Co, Hg, Pb, Ni, Cr. It is known that *Ralstonia eutropha* has three genes in one operon, *phbCAB*, responsible for PHB synthesis, which encode the enzymes PHB synthetase (PhbC), β -ketothiolase (PhbA) and acetoacetyl-CoA reductase (PhbB), respectively (Fig. 2) (Liu Quian et al 2007).

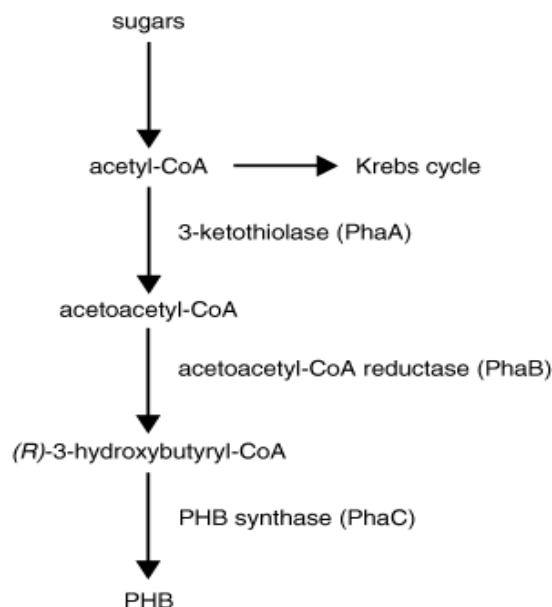


Figure 2. Metabolic pathway to PHB.

In contaminated soils bioremediation *Pseudomonas putida* inocules can be used. This microorganism is capable to convert styrenic oils to PHA, effectively recycling polyesters

otherwise considered non biodegradable (Jiang et al 2006). A new thermophilic microorganism capable of degrading poly-3-hydroxybutyrate (PHB) was isolated from soil. A phylogenetic analysis based on 16S rDNA sequences indicated that it belongs to genus *Streptomyces* (Calabia & Tokiwa 2004).

Another bacteria which synthesize PHB is *Bacillus cereus*. Ma et al (2005) isolated this species from activated sludge and then tested the physical properties of synthesized PHB and PHV copolymers.

It is considered that PHB synthesis by bacterial way is more expensive than synthetic plastics production, and therefore it is attempted to transfer it to eukaryotic cells by using yeasts as model microorganisms in PHB production (Breuer et al 2002; Carlson et al 2002; Terentiev et al 2004). Small amounts of low molecular mass PHBs were described in yeast where they accomplished membrane transport functions (Poirier et al 2001). The genes involved in the synthesis of PHB have been identified and cloned. There are three structural genes which are involved in PHB synthesis and they are clustered in one operon (*phaBAC*). The genes involved in the synthesis of PHB in *Azotobacter sp.* were introduced in plasmids and used for the construction of recombinant *E.coli* strains that accumulate the polymer from different carbon sources (Almeida et al 2007). In other researches *phaBAC* were transferred from bacteria (*Ralstonia eutropha*) to eukaryotic cells like yeasts (Safak et al 2002).

In addition, **yeasts** as hosts for synthesis of PHAs have certain advantages over bacteria. First, yeasts have been studied intensively from physiology, molecular biology and biotechnology points of view. Second, yeasts are physiologically flexible and they are larger than bacteria.

Moreover, yeast like, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Candida utilis* and others, has been considered model microorganisms and they are approved as a GRAS microorganism by Food and Drug Administration (Abd-El-haleem et al 2007).

In cytoplasm of *Saccharomyces cerevisiae* strains *phaCAB* operon, containing *phbA*, *phbB* and *phbC* genes from bacterial strains (*Ralstonia eutropha*), was introduced. These genes codify PHB synthetase (*PhbC*), β -ketothiolase (*PhbA*) and acetoacetyl-CoA reductase (*PhbB*) NADPH dependent. It was noticed that transgenic yeast strains were capable to synthesize PHA, but the efficiency was not very high. After 72 h only 7.06 % of the cell dry weight was obtained. The results obtained suggest that many aspects of its synthesis, metabolism and regulation need to be elucidated before the yeasts can be used for the synthesis of this polymer on a commercial scale (Abd-El-haleem et al 2007).

The importance of poly- β -hydroxybutyrates (PHB) production by microbial way indwells in their advantages; they are produced by renewable sources, are biodegradable and they have multiple applications in modern biotechnologies.

The relevance of these approach must be analyzed from two perspectives: first refers to environmental protection and the second is a practical one, which insists on the utility of biodegradability and biocompatibility properties of these products.

Importance for the environment. A major problem of environmental pollution is that plastics produced by the petrochemical industry are not biodegradable and therefore accumulate in the environment (Juang & Chiou 2007). The importance of this research consists of in the stringent necessity to replace conventional plastics with biodegradable products obtained by renewable sources. The importance of their replacement is given by the exhaustible character of earth oil, the negative impact of plastics manufacturing, their extreme prolonged degradation period and the low recycling rate. The impact of conventional plastics on the environment is significant so there is a need for new methods to obtain alternative materials. Polyesters as PHB or other polyhydroxyalcanoates (PHA) are of commercial and academic interest as new biodegradable materials. The capacity to degrade PHA is highly widespread at bacteria and fungi and depends on specific extracellular PHA-depolymerases secretion and the physical state of the polymer (amorphous or crystalline) (Rychter et al 2006; Shah et al 2008). Polymers as poly- β -hydroxybutyrates are made out of renewable sources which confers them one more advantage, non exerting pressure on exhaustible resources. Polyhydroxyalcanoates present a structural diversity and analogy with plastics which makes them desirable substitutes of them (Harding et al 2007).

Importance for modern biotechnologies. PHB have many technical and medical applications and can play an important role in the future biotechnologies. Based on their biodegradability and biocompatibility PHB present competitiveness on certain market sections. The applicability perspectives include the fields in which the present conventional plastics are used, agriculture (fertilizer slow release systems), medicine (medical devices and drug administration protective systems) (Pötter & Steinbüchel 2005). The potential application of bacterial made PHB as biodegradable, biocompatible, absorbable, injectable material, with controllable degradable rate, attracted a special attention in medicine and tissue engineering (Bonartsev et al 2007). PHBs have a special practical utility because they can constitute conventional medical devices. They can be used to stitches, meniscus devices, consolidations, bone plates, cardiovascular prosthesis, devices for cartilages, tendons, neural conductors, devices for tissue guiding and regeneration (Chen & Wu 2005).

Microbiologically, most of the studies from Romania were done on bacterial strains originating from polluted soils (Stefanescu et al 2007).

The importance of this approach consists in the isolation of strains which are specialized in producing high PHB levels, strains which subsequent will be used to synthesize biopolymers in the lab. Establishing a PHB obtaining protocol represents a compulsory condition for the development of modern biotechnologies. It can be developed a biotechnology based on fermentation process which uses substrate from renewable sources for producing biopolymers with superior properties. It is aimed to transfer the PHB producing responsible gene from bacteria to yeasts, optimise the culture conditions, to quantify the produced PHB as well as to investigate the different fields of these biopolymers. Thus, developing the knowledge about this subject and enlarging the utilization area by obtaining biopolymers will have a strong impact on developing technologies based on materials from renewable sources.

The importance of this approach consists in the connection to environmental issues and also the biotechnological perspectives.

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