S U M M A R Y

BIOSYNTHESIS OF POLYHYDROXYALKANOATES AND THEIR USE IN BIOMEDICAL ENGINEERING

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In recent years, pollution and the accumulation of plastic products that, unrecycled, end up polluting the environment and harming health are just some of the current topics on the global agenda. A major consequence of plastic pollution is the ubiquity of microplastics, which affect the balance of ecosystems around the world. Microplastics have been discovered in the waters of oceans, lakes and rivers, as well as in forests, in the heights of mountains or even in the atmosphere (Bhat et al., 2023). Its presence affects the biodiversity of aquatic and terrestrial organisms (Bordós et al., 2019), through physical injuries, hormonal disruptions and increased mortality rates (through suffocation, wounds that become infected, etc.).

With an estimated quantity of 25 billion metric tons of plastic by 2050 (Xu et al., 2024), the need to identify new materials to replace conventional plastic has brought polihydroxyalcanoates into the focus of research. With these results, many of the major companies have set as their goal by 2025 the use of recycled plastics, 100% biodegradable or reusable (Tumu et al., 2023).

Polyhydroxyalcanoates are biodegradable, non-toxic biopolymers for the environment. They are preferred to be used in different industries, because through the processes of obtaining it offers the opportunity to reduce pollutant emissions and stop dependency on non-renewable resources, used for classic plastics. PHAs are mainly produced by bacteria as intragranular deposits under certain conditions of nutritional stress (Philip et al., 2007). Initially at a high cost, the production of PHA becomes convenient when renewable nutrient substrats such as starch, glucose, sucrose, animal fats or food waste (e.g. peach peels, onion peel, potato peelings, melase, whey, etc.) are used.

The thesis entitled "*Biosynthesis of polihydroxialcanoates and their use in biomedical engineering*" aims mainly to model and optimize the bioprocesses of synthesis for polihidroxyalcanoats using *Pseudomonas putida ICCF 391*, using different carbon substrates (fat acids, glucose, vegetable oils) and to test the biopolymers thus obtained. The thesis consists of two parts: Bibliographic study and own research.

The first chapter "*Chapter I. Biomedical Engineering and Polymers*" summarizes information about biomedical engineering, its applications and the main types of biopolymers (PLA, PLGA/PLG, PCl and PHBV). Biomedical engineering integrates knowledge of engineering sciences with biomedical sciences (biomedicinal electronics, biomaterials, computational biology, cellular, tissue and genetic engineering, medical imaging, orthopedic bioengineering, bionanotechnology) and clinical practice, for the development of revolutionary concepts, such as surgical robots (Edwards et al., 2018), biocompatible prostheses (Shepherd R.K, 2016), new drug therapeutic systems, various diagnostic and therapy medical devices (Aruna et al, 2022), ordinary imaging equipment (RMNs and EKG/ECGs) (Thompson et al. 2016). At the same time, its composition includes the following branches:

- Bioinformatics;
- Biomechanics;

- Tissue engineering;
- Genetic engineering;
- Neural engineering;
- Pharmaceutical engineering;
- Medical devices (Class I, Class II and Class III).

Biopolymers are polymeric substances synthesized, for the most part, by living organisms, especially bacteria, or are chemically synthetized from basic biological systems, which are of particular interest due to their very varied properties and fields of application. Biopolymers are chain-type molecules, formed from repeated blocks of monomers. Being materials that can be used for biomedical applications (injury healing, drug administration and tissue engineering), biopolymers must meet certain standards: be biocompatible, biodegradable, have low antigenicity, high bioactivity, processability to complicated forms with appropriate porosity and ability to support cell growth and proliferation and appropriate mechanical properties (Yousefi and Wnek, 2024). The main types of biopolymers are: polynucleotide, polypeptide, polysaccharides (the latter may be of bacterial, fungal, plant/algae or animal origin), proteins and polyesters of microbial origin. Among the biopolymers of microbial origin, the most used in biomedical engineering are: polylactic acid (PLA), poly-lactic-co-glycolic acid (PLGA/PLG), policaprolactone (PCL) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV).

„Chapter II. Polihydroxyalcanoates (PHA)" encompasses information from the literature about their formation, structure, classification, physical and chemical properties, micro-organisms producing PHA, with emphasis on the *Pseudomonas putida* strain, important parameters influencing the production of PHA as well as the metabolic pathways of biosynthesis, PHA applications, biocompatibility and biodegradability of biopolymers. Polyhydroxyalcanoates are polyesters naturally produced by many species of microorganisms, in the form of intracellular granules that constitute energy and carbon reserves when cells are subjected to imbalances (producing PHA as a precautionary measure against the limitation of nutrients and extreme conditions) (Koller et al., 2017). PHA accumulates when the nutrient contains an excess of carbon, while nitrogen, phosphorus, oxygen or magnesium limit cell growth. Structurally, PHA molecules have between 600 and 35,000 monomeric units of hydroxyalcanoic acids and approximately 150 types of polymers have been identified.

Depending on the number of carbon atoms in the monomeric main chain, PHA may have the short lateral chain (*scl*-PHA), the medium lateral Chain (*mcl*- PHA) and the long side chain (*lcl*-PHA). The general properties considered common to polyhydroxyalcanoates (materials with a high degree of polymerization) are as follows:

- lack of toxicity;
- biodegradability;
- biocompatibility;
- crystallinity;
- optical activity;
- isotactic properties (with stereochemical regularity in repeating units);
- piezoelectric properties;
- hydrophobicity.

Among the most used species producing PHA we mention *Alcaligenes latus*, *Bacillus megaterium*, Cu*priavidus necator*, *Pseudomonas oleovorans* and *Pseudomonas putida*, these being able to use various sources of carbon, including vegetable oils or waste. Thus, PHAs are produced from a wide variety of substrats, from renewable resources (amidon, cellulose, glycerol or by-products - melase, whey, glycerol) or from conventional exhaustible resources (methane, mineral oil, organic acids).

The genus *Pseudomonas* is one of the most versatile genus producing medium side chain PHA (*mcl*-PHA), with great medical importance because some species can produce nosocomial and opportunistic infections. The first strain characterized as producing medium lateral chain PHA was *Pseudomonas putida* (Solaiman et al.,

2000), followed by other species/subspecies to be identified and characterized: *Pseudomonas spp.* (*P. aeruginosa*, *P. mendocina*, *P. campisalis*, *P. stutzeri*, *P. chlororaphis*, *P. citronellolis*), *Halomonas spp.* (*H. campisalis*, *H. elongata*), etc.

Pseudomonas putida is a highly studied species with major importance in the degradation of some pollutants in the environment (e.g. oil, in studies through cleaning the soil or groundwater polluted with it, the bioabsorption of copper and zinc, in the case of soils contaminated with heavy metals). The main advantage of using this species is the low cost that the process itself requires. PHA depends on the source of carbon and the metabolic routes involved. The most important metabolic pathways of PHA synthesis are: *Pathway I*, from Acetyl-CoA to 3-hydroxibutiryl- CoA, *Pathway II*, the degradation of fatty acids and *Pathway III*, the biosynthesis *de novo* of fat acids.

The source of carbon plays a crucial role in the production of polyhydroxyalcanoates (PHA), as it is the raw material necessary for the synthesis of these biodegradable polymers, while at the same time representing one of the parameters that influence the production and accumulation of PHA.

Approximately half of the total production costs of PHA are associated with the raw material used. The choice of carbon source is extremely important from the point of view of the economic efficiency of the PHA production process; the pre-treatment of the substrate influences the increase in the availability of carbon sources, and the supplementation of the growing environment complements the nutritional needs of the growth environments and changes the yield and composition of PHA.

Due to their remarkable mechanical qualities, biodegradability and potential to be used as raw materials for various chiral compounds, polyhydroxyalcanoates (PHAs) are used in various industries (a.Koller and Mukherjee, 2022). Biodegradability is the major advantage of PHA over conventional plastics (which degrade very hard, in decades or hundreds of years). This allows microorganisms to break down the polymer into simple components. Thus, due to its biodegradable nature, PHA does not contribute to the increase of waste deposits and, unlike conventional plastics, the PHA can be composted after use (Dilkes-Hoffman et al., 2019). Therefore, PHA of microbial origin forms a valuable industrial chain, from industrial fermentation, the formation of materials, medicines and biofuel, to chemicals.

The biocompatibility and biodegradability of PHA are two vital characteristics for their use in the biomedical industry. These materials must be biocompatible, i.e. do not cause an immune response and allow cell proliferation and adhesion, respectively biodegradable, ensuring non-toxicity by their degradation into carbon dioxide and water.

The processing of polyhydroxyalcanoates is an essential step for their analysis. In *„Chapter III. Methods of processing PHA. Quantitative and qualitative methods of analysis"* is described the method of solvent extraction, which is based on the modification of the permeability of the cell membrane by the solvent and the release of PHA. After this step, centrifugation is necessary, to remove the unwanted content and to leave only the cellular biomass. Another type of extraction can be achieved by the non-PHA cell mass digestion method (NPCM), using physical treatments (heat mechanical disintegration, ultrasound) chemical treatments such as surface agents, alkali or acids and enzymatic treatments. Following the chosen treatment, the PHA granules are separated from the cellular components by filtration or centrifugation. Biopolymer purification is done to avoid contaminants and immunological reactions and can be carried out by washing the biomass with methanol and ethanol. The estimation of the biomass is made by reading to the spectrophotometer at the absorption of 550 nm and by measuring the weight of the dried cells by freezing. Next, the processing and characterization of PHA can be achieved by high performance liquid chromatography (HPLC), gas chromatology (GC), liquid chrumatography (LC) or nuclear magnetic resonance (RMN).

Part II of the thesis, in which *„Own Research"* is presented, summarizes the experimental chapters (Chapters IV, V and VI) in which three types of experiments were carried out:

1. Chapter IV: Synthesis of PHA by bioprocesses made with conventional carbon sources, of the type of fatty acids: octanoic acid, nonanoic, heptanoic and hexanoic acids; interpretation of the dynamics of the development parameters; discussion of the PHA productivity.

2. Chapter V: Bioprocesses made with conventional carbon sources, using different types of oil (sunflower oil, olive oil, used sunflower Oil, Coffee straw, coffee straw extracted oil, glycerol) as the only source of carbon.
3. Chapter VI: Biodegradability of the biopolymers obtained and their rate of degradation.

For the experimental variants of *„Chapter IV. The optimization of bioprocesses and processing of polyhydroxyalcanoates obtained by supplementing the environment with conventional sources of carbon"* has been used as a strain of bacteria *Pseudomonas putida* (*ICCF 391*), part of the collection of microorganisms of the *National Institute of Chemical and Pharmaceutical Research and Development-ICFF Bucharest*. In order to choose the right inoculate environment, comparison of microbial development on three environments was done: IPS, LB and TSB environments. The best results of bacterial development (optical density measured at the spectrophotometer) were obtained for the IPS environment, which is the medium chosen for the rest of the bioprocesses. A preliminary test for the fermentation medium was carried out between the mediums: E2, BSM, MRM and MSM, noting that the most appropriate was E2. In the experimental studies carried out in this chapter, conventional sources of carbon - precursors of the type of fatty carboxylic acids: octanoic acid, nonanoic, heptanoic and hexanoic.

The fatty acids (octanoic acid, nonanoic, heptanoic and hexanoic acids) are metabolized by *Pseudomonas putida* through the metabolic path III, β-oxidation. Evidence of their metabolism dates back to 1967, when Chapman and Duggleby observed this mechanism in *P. aeruginosa* (Chapman and Douggleby, 1967). It is further generally accepted that β-oxidation is the way through which the strains of P*seudomonas sp*. degrade the dicarboxylic acids. The evolution of experimental bioprocesses was monitored by analysing information on the following experimental parameters: temperature, pH and optical density – correlated with bacterial development.

For this step of optimizing the fermentation bioprocess, it was intended to test different carbon sources, by carrying out 11 experimental variants:

1. Octanoic acid (18 ml as sodium octanoate at 0 hours and at 24 hours);
2. Octanoic acid (18 ml as sodium octanoate at 0 hours and 9 ml at 24 hours);
3. Nonanoic acid (17 ml as sodium nonanoate at 0 hours and 17 ml at 24 hours);
4. Nonanoic acid (17 ml as sodium nonanoate at 0 hours and 17 ml as sodium nonanoate at 24 hours);
5. Nonanoic acid (17 ml as sodium nonanoate at 0 hours);
6. Nonanoic acid and octanoic acid (17 ml as sodium nonanoate at 0 hours and 17 ml as sodium octanoate at 24 hours);
7. Nonanoic acid and heptanoic acid (17 ml as sodium nonanoate at 0 hours and 17 ml as sodium heptanoate at 24 hours);
8. Heptanoic acid and nonanoic acid (17 ml of sodium heptanoate at 0 hours and 17 ml of sodium nonanoate at 24 ours);
9. Octanoic acid and hexanoic acid (17 ml as sodium octanoate at 0 hours and 17 ml as sodium hexanoate at 24 hours);
10. Octanoic acid and hexanoic acid (17 ml as sodium octanoate at 0 hours and 14 ml as sodium hexanoate at 24 hours);
11. Hexanoic acid and octanoic acid (14 ml as sodium hexanoate at 0 hours and 14 ml as sodium octanoate at 24 hours).

The best results were obtained for experimental variant 9, where octanoic acid and hexanoic acid were used as precursors, respectively (17 ml as sodium octanoate at 0 hours and 17 ml as sodium hexanoate at 24 hours). Bacterial development was the most significant and the highest amount of dry biomass was obtained, as well as the highest amount of PHA, respectively 1.95 g/L of bioprocess medium and a foil weighing 4.6931 g.

The following experimental variants with a very good result are experimental variant 3, for which nonanoic acid was used (17 ml as sodium nonanoate at 0 hours and 17 ml at 24 hours), the PHA foil weighing

4.05 g, experimental variant 10, with the acid octanoic and hexanoic acid (17 ml as sodium octanoate at 0 hours and 14 ml as sodium hexanoate at 24 hours), PHA film weighing 3,882 g, and experimental variant 11, with hexanoic acid and octanoic acid (14 ml as sodium hexanoate sodium at 0 hours and 14 ml as sodium octanoate at 24 hours), the PHA film having 3.1031 g. Among the 4 structurally related substrate types, the strain *Pseudomonas putida ICCF 391* prefers the substrate C8 (octanoic acid) for the biosynthesis of an elastomeric PHA with a composition in which the C8 monomer predominates over C6 and C10.

Next „*Chapter V. Optimization of the bioprocesses of production of PHA by supplementing the environment with renewable sources of carbon"* presents the biprocesses made with the same strain, but with regenerable substrates, which ensures a low cost for the production of the PHA. The media used were: for inocul – IPS medium, for fermentation, E2 medium. The experimental variants were made mainly with different types of vegetable oils, but also other renewable materials, with different concentrations, as follows:

1. Coffee grounds (4% at 0 hours);
2. Coffee grounds (4% at 0 hours) and octanoic acid (17 ml at 24 hours);
3. Glucose (2% at 0 hours);
4. Sunflower oil (1% at 0 hours);
5. Sunflower oil (1.5% at 0 hours);
6. Sunflower oil (2% at 0 hours);
7. Olive oil (1% at 0 hours);
8. Olive oil (1.5% at 0 hours);
9. Olive oil (2% at 0 hours);
10. Used vegetable oil (1% at 0 hours);
11. Used vegetable oil (1.5% at 0 hours);
12. Used vegetable oil (2% at 0 hours);
13. Coffee grounds oil (1% at 0 hours);
14. Coffee grounds oil (1.5% at 0 hours);
15. Coffee grounds oil (2% at 0 hours);
16. Engine oil (Castrol – 1% at 0 hours);
17. Engine oil (Castrol – 1.5% at 0 hours);
18. Engine oil (Castrol - 2% at 0 hours).

By analyzing the results obtained, it can be concluded that some oils are easier to use in the bioprocess than others that probably require additional pre-treatment steps for carbon biodisponibilization and to ensure the possibility of being consumed by the bacterial strain. The highest amount of biomass was obtained for experimental variants using olive oil (experimental version 8), followed by experimental version 11, in which the environment was supplemented with used sunflower oil with a biomass of 2.3 g/L. The highest optical densities were recorded for experiments with oil extracted from coffee cane, and the weakest results were for experimental variants using engine oil (experimental versions 16, 17 and 18). The results obtained are consistent with the literature, as there are numerous studies in which *P. putida* uses vegetable oils to synthesize PHA (Song et al., 2008; Solaiman et al., 2006).

Since pure fatty acids have a very high price, their replacement with vegetable oils of different types has been researched for the production of low-cost *mcl*-PHA. In their production, the same behavior isined as in carboxylic acids; the composition of the substrate influences the PHA monomer composition.

Within „*Chapter VI. Biodegradability of the biopolymers obtained"*, biodegradability of the PHA received was checked and the degree of their weight loss was analysed. The experiment was carried out over 24 months, weighing samples after one month, three months, 6 months, 12 months and 24 months. For all four samples submitted to the experiment, a clear degradation of the polymer was observed. According to the calculation formula in *sub-chapter 2.8.2. Biodegradability of PHA*, the weight loss rates were:

- Sample 1: 76.76%;
- Sample 2: 54.67%;

- Sample 3: 45.97%;
- Sample 4: 87.43%;

and the average rate of reduction in the weight of the biopolymers obtained from this experiment was 66.21%, proving the PHA degradation achieved by the microbiome.

This thesis achieved its objectives of biosynthesis of medium side chain polyhydroxyalcanoates (*mcl*-PHA) by microbial biosynthetic, using the strain *Pseudomonas putida ICCF 391*. Following the analysis of their structure and by carrying out tests of biodegradability, the following were found:

- The strain used in experiments is part of the collection of microorganisms of ICCF and is a bacterium capable of accumulating intra-cellular PHA polyhydroxyalcanoates, under conditions of limitation of certain nutrients and excess source of carbon (nutritional imbalance).
- The results obtained during this study facilitate the understanding of the process of controlled biosynthesis of mcl-PHA by the microorganism in different experimental conditions, by using precursors that influence the yield of the bioprocess and the quality of the obtained polymer.
- Also, different concentrations of lipid substrates (unconventional carbon sources) were tested and it was possible to evaluate which is the most suitable concentration for the bioprocess.
- Biopolymers were degraded in soil (at a constant temperature of 28-30°C), with different rates of biodegradability, being suitable materials to be used in the biomedical industry (vascular prostheses, ligaments, sutures, stents, etc.).

The study of PHA production remains a current topic, with many diverse substrates that can be used as a carbon source. Obtaining PHA from renewable substrates (waste oil, coffee grounds, plant peels, etc.) contributes significantly to reducing PHA costs. PHA biopolymers continue to be researched and improved, playing an important role in various industrial sectors. The properties of this material allow its use as a biodegradable material in the cosmetic industry (for packaging), in the production of biodegradable plastic materials, or in biomedical engineering as various prostheses, heart valves or implantable scaffolds.