Microbial production of polyhydroxyalkanoates

Iveta Rektoříková

Bakalářská práce
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1. Charakteristika polyhydroxyalkanoátu (PHA).
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Vedoucí bakalářské práce: **Ing. Pavel Pleva, PhD.**
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L.S.

prof. Ing. Roman Čermák, Ph.D.
děkan

doc. Ing. Tomáš Sedláček, Ph.D.
ředitel ústavu

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ABSTRAKT

Cílem této bakalářské práce bylo zpracování literární rešerše, zabývající se polyhydroxyalkanoáty jako potencionální náhradou za plasty vyráběné z fosilních zdrojů, jako jsou polyolefiny, které se ve velkém množství akumulují s nesnadnou možností jejich odbourání. Práce se zabývá obecnou charakteristikou PHA, jeho udržitelným rozvojem a součástí biomasy. Dále se práce zabývá metodou detekce PHA v kmenech bakterií po-mocí metody barvení Nile Blue A, Nile Red, Sudan Black a polymerázové řetězové reakce, které jsou charakterizovány a popsány. V poslední části je probrána metoda izo-lace PHA z biomasy a náročnost jeho výroby.

Klíčová slova: Biopolymery, Polyhydroxyalkanoáty, Nile Blue A, Nile Red, Sudan Black, PCR, Cupriavidus Necator, Pseudomonas Mendocina, izolace, detekce

ABSTRACT

The aim of this bachelor's thesis was to develop a literature search dealing with polyhydroxyalkanoates as a potential substitute for plastics produced from fossil sources, such as polyolefins, which accumulate in large quantities with the difficult possibility of their degradation. The work deals with the general characteristics of PHA, its sustainable development and components of biomass. Furthermore, the work deals with the method of PHA detection in bacterial strains using the method of staining Nile Blue A, Nile Red, Sudan Black and polymerase chain reaction, which are characterized and described. The last part discusses the method of isolation of PHA from biomass and the complexity of its production.

Keywords: Biopolymers, Polyhydroxyalkanoates, Cupriavidus Necator, Pseudomonas Mendocina, Nile Blue A, Sudan Black, isolation, detection

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I hereby declare that the print version of my Bachelor's thesis and the electronic version of my thesis deposited in the IS/STAG system are identical.

CONTENT

1 INTRODUCTION

There is no doubt that we live in a world of plastics that surrounds us from all sides, be it packaging materials, various components, pharmaceuticals, foams and much more. That is why we can no longer imagine today's world without them. These plastics, which come from non-renewable sources, have a number of advantages, such as easy formability, resistance to degradation by microorganisms, moisture or oxygen and much more, so their physical and chemical properties have replaced materials used previously and it is not surprising that they are produced in such massive quantities. In 2002, about 204 million tons of plastics were produced worldwide, and in 2018 world production of these plastics rose to 359 million tons.[1,2,3]

But they have one disadvantage, which is most of these convection plastics end up in landfills (20-40%) or, even worse, in the environment. 10-20 million tonnes of plastics per year are accumulated just in the seas and, unfortunately, plastics decompose over a very long time. [2][3]

The degradation of these fossil fuel-based plastics is time-consuming and can even be dangerous. For example, if we opt for a method of burning plastics, which is also relatively expensive, dangerous toxic substances are created. Recycling is a possible solution, but it is a very long and demanding process by which a minimal amount of plastics are processed in this way. For example, in 2012, 26% of plastic waste was recycled in Europe, and even less in America, where recyclability was only 9%.[1]

The solution to this problem can be bioplastics, biodegradable polymeric materials which, unlike synthetic plastics, come from a renewable source and are biodegradable. Polyhydroxyalkanoates (PHAs), which are polymers produced by a number of bacteria known to be more than 250 today, have great potential in this industry. PHAs serves for bacterium as an intracellular store of carbon and energy, which it forms in excess of carbon sources and lack one of the essential nutrients such as oxygen, nitrogen, phosphorus, etc. PHAs have become a very interesting polymer due to its properties, which are very similar to polyolefins and could eventually replace them. Unfortunately, this is not yet possible due to high costs, so research and development are still being carried out to reduce production costs and make their production more efficient. [3-7]

2 POLYHYDROXYALCANOATES

From the name, it can be deduced that polyhydroxyalkanoates belong to the group of hydroxyalkanoic acid polyesters, which are synthesized by gram-positive and gram-negative bacteria producing PHA in large quantities up to 90% by weight of dry matter if they have an excessive carbon source and other essential elements such as oxygen or nitrogen is limited. PHA then, mainly due to its low solubility and high molecular weight which causes minimal osmotic pressure inside the cell, serves as an energy store if crisis conditions persist for extended periods of time. PHAs are synthesized in the cytoplasm and stored in the form of granules as we can see in figure 1. [2,4]

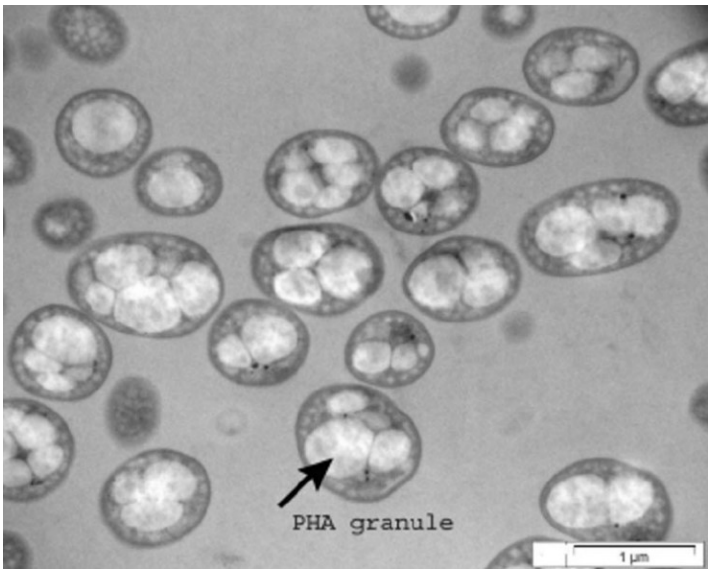

Figure 1: PHA under electron microscope produced by *Pseudomonas putida* [9]

However, they have become attractive to humans due to other abilities. PHAs are either thermoplastic or elastomeric, insoluble in water. They also very promising for the medical and pharmaceutical industries, due to their biocompatibility, biodegradability, nontoxicity, piezoelectricity and biological diversity. Their applications can be utilized in many additional industries as well. PHAs are green plastics and have a positive social and environmental impact compared to conventional plastics in terms of production and recycling. In addition, PHAs have no acute or chronic effects on human health when used in vivo. These bioplastics represent a renewable and sustainable alternative while reducing stress on landfills. [2-5]

It is of significant interest that PHAs can consist of various hydroxyalkanoate monomers. The result is the production of PHA with different physical properties or degradation rates, which can be influenced by a change in bacteria or by a change in fermentation conditions. These polymers attract commercial interest due to their remarkably similar physical properties to synthetic polymers such as polypropylene. [2][4]

2.1 History of polyhydroxyalcanoates

Beijerinck was the first to notice the existence of polyhydroxyalkanoates in 1888, observing PHA granules under a microscope. However, it took another 40 years before polyhydroxyalkanoates were further described by M. Lemoigne in 1923 at the Pasteur Institute, where he proved that sporulating bacteria can form hydroxybutyric acids in anaerobic suspensions. In 1927, the polymer was finally extracted from *Bacillus magaterium* using chloroform and the material was proved to be a 3-hydroxybutyric acid polymer. However, the production of poly-(3-hydroxybutyrate), or P(3HB), was not explored on a commercial scale until the early 1960s. A pioneer in this field was the work of Babtista and Werber in W.R. Grace & Co. (U.S.A.) has issued a number of patents for the production of this polymer. These scientists began to use the polymer for various prosthetic devices. Unfortunately, the production process was stopped very quickly due to the high cost of polymer extraction and isolation. In addition, the polymer was heavily contaminated. [8]

2.2 Chemical structure

The general structure of the most common types of PHA can be seen in Figure 2 and Table 1. Besides the structures depicted in Figure 2, the monomers can have different variations in the form of saturated, unsaturated, straight or branched side chains. The side chains are usually aliphatic. However, aromatic as well as halogenic substituents have also been prepared by in vitro and in vivo synthesis and have become major components of PHA building blocks.

PHAs are primarily linear, optically active water-insoluble polyesters [18]. The monomeric subunits occur in the form of 3-hydroxy-substituted acids. All acids are in the R-configuration due to the stereospecificity of the polymerization enzyme PHA synthase. The S-configuration has never been recorded before. The carboxyl group in one of the monomers

forms an ester bond in these polymers with the hydroxyl group of the adjacent monomer. The position of the hydroxyl group is largely variable and 4-, 5-, and 6-hydroxy acids have already been incorporated.[15]

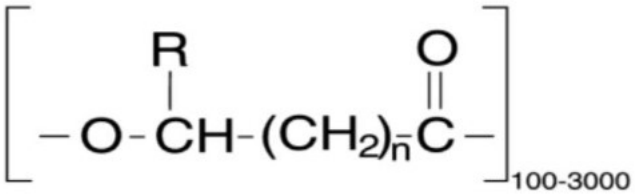

Figure 2: Structure of PHA [21]

Table 1: Representatives of PHA depending on n and R

n	R	polyhydroxyalcanoate	abbreviation
n=1	hydrogen	poly(3-hydroxypropionate)	P(3HP)
	methyl	poly(3-hydroxybutyrate)	P(3HB)
	ethyl	poly(3-hydroxyvalerate)	P(3HV)
	propyl	poly(3-hydroxycaproate)	P(3HC)
	butyl	poly(3-hydroxyheptanoate)	P(3HH)
	pentyl	poly(3-hydroxyoctanoate)	P(3HO)
	hexyl	poly(3-hydroxynonanoate)	P(3HN)
	heptyl	poly(3-hydroxydecanoate)	P(3HD)
	octyl	poly(3-hydroxyundecanote)	P(3HUD)
	nonyl	poly(3-hydroxydodecanote)	P(3HDD)
n=2	hydrogen	poly(4-hydroxybutyrate)	P(4HB)
n=3	hydrogen	poly(5-hydroxyvalerate)	P(5HV)

2.2.1 Division of PHA

Polyhydroxyalkanoates are generally divided into 3 groups according to the number of monomer units capable of producing both homopolymers and heteropolymers.[20]

Scl-PHA - Polyhydroxyalkanoates which contain side chains formed by hydroxyalkanoates such as hydroxybutyrate or hydroxyvalerate belong to the group scl-PHA (short-chain-lenght PHA), which consists of monomers with 3-5 carbon atoms. [20]

Mcl-PHA (medium-chain-lenght) PHA is a polyhydroxyalkanoate which contains hydroxyalkanoates with 6-16 carbons or aliphatic carbon chains as the side chain. The ability to accumulate mcl-PHA is unique to the strain *Pseudomonas*. [20,30,31]

Lcl-PHA or long chain length polyhydroxyalkanoates are formed by hydroxy acids with a carbon chain of more than fifteen carbon atoms. So far, lcl-PHAs have only been prepared in vitro and their presence in nature has not yet been observed. [30,31]

2.2.2 Structure of PHA granules

PHA is found in cells in the form of granules, the size and number of which depend on a number of conditions. Among the most important are the culture conditions and the type of bacterial culture chosen. Most often, the granule size is around 0.5 μm and the density is about 1.05 to 1.24 g.cm^{-3} depending on the type of PHA. [10-13]

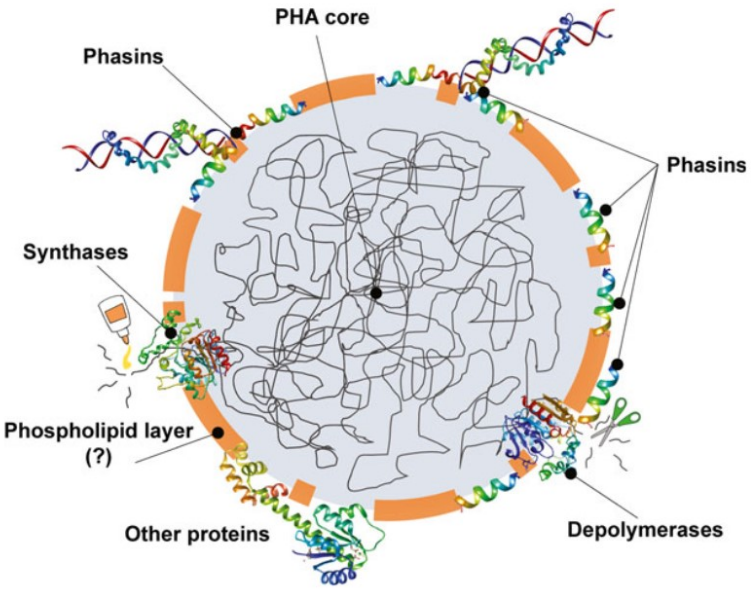

Figure 3: Model of PHA granule [14]

The cover of PHA inclusion consists of lipids, phospholipids, proteins and phasins. On the surface of the granule there is a lipid layer into which proteins (synthases, polymerases, depolymerases) are inserted, which perform various functions (synthesis, degradation, catalysis). PHAs have a hydrophobic characteristic, where the lipid layer separates the hydrophobic core of the granule from the hydrophilic cytoplasmic space and thus forms an

interface between the PHA and the environment. Granules in PHA-rich cells can be stained with Sudan Black, Nile blue A or Nile red [10-13].

2.2.3 Representatives of PHAs

2.2.3.1 Poly(3-hydroxybutyrate), PHB

The best known scl-PHA is a homopolymer of poly(3-hydroxybutyrate), which occurs not only as natural material in amorphous form as water insoluble inclusion, but also as an oligomer (120-200 monomers), produced by variety of microorganism. Isolated PHB from bacteria can contains 55-80% of crystallinity which makes it stiff and brittle. In nature it is found in plant tissues, microorganisms and humans, where it likely performs the function of ion channels as a polyphosphate complex. The general structure of PHB is shown in figure 4.

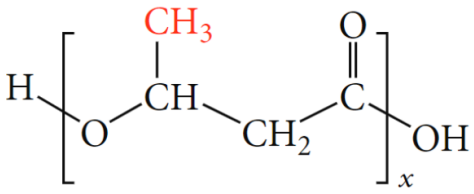

Figure 4: General structure of PHB [21]

Table 2: Molecular weight depending on x [18]

x	M
120-200	low molecular weight
1 000-20 000	high molecular weight
100 000	ultrahigh molecular weight

PHB can be divided into 3 groups depending on the molecular weight as is shown in figure 3. The best mechanical properties have ultra-high molecular PHB, which can be prepared by transgenic *Escherichia coli* with genes from *Cupriavidus necator*. [18]

Compared to PP, PHB is stiffer and more brittle plastic material. The brittleness is due to the formation of large crystalline domains in the form of spherulites. Comparation with PP is shown in table 1. [17]

2.2.3.2 Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

PHA copolymers are generally of great importance because they can significantly improve the mechanical properties of the polymer. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) consists of hydroxyvalerate (3HV) and hydroxybutyrate (3HB) units as seen in Figure 4. One of the possible ways to produce this copolymer is by preparation by the bacterial strain *Cupriavidus necator* by fermentation, where the bacterial strain is cultured on glucose with the addition of propionic acid, which serves as a precursor for the carbon source that allows the incorporation of 3HV groups into the chain.

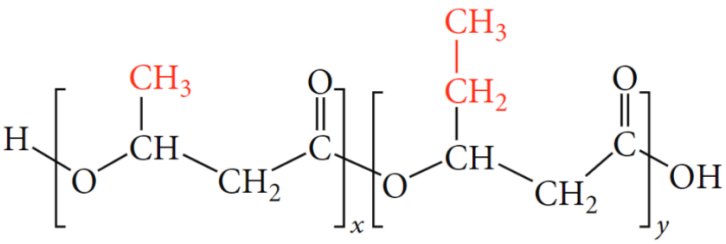

Figure 5: General structure of P(3HB-co-3HV) [21]

By changing the concentration of the carbon source, the content of 3HV groups can be easily controlled, which improves flexibility. Other properties compared to P(3HB) can be seen in Table 1. [15][16]

Table 3: comparation of properties between P(3HB) a P(HB-co-HV)

	P(HB-co-HV)	P(3HB)
Young's modulus [GPa]	0,7	3,5
Tensile strength [MPa]	30	40
Melting temperature [°C]	130	179
Elongation to break	Increses as 3HV content increases	
Temperature of degradation [°C]	180	200

Due to the properties shown in table 1, this copolymer can be melted without decomposition. [15]

2.2.3.3 Special PHA

Just as there is a high variety of PHA properties, so many kinds are known, about 150 different PHAs are discovered. In addition to the most common PHAs mentioned above, it is worth noting to mention the sulfur-containing PHA: Poly(3-hydroxybutyrate-co-3-mercaptopropionate). (P(3HB-co-3MP) was prepared by *Ralstonia eutropha*, in which 3-mercaptopropanoic acid was added to the medium as a precursor. The P(3HB-co-3MP) thus prepared contained a thioester bond instead of the classical ester bond [27].

2.3 Physical and chemical properties

The molecular weight of PHA is generally in the range of 50,000-1,000,000 Da due to their high degree of polymerisation. This value, as well as other physical and chemical properties, depending on the type of producer, the carbon source used and the cultivation conditions. They can be thermoplastics or thermosets, which offer a wide range of uses, including the production of films and fibers. They are optically active, biodegradable, non-toxic, piezoelectric and insoluble in water. [2,4,48]

2.3.1 Scl-PHA, P(3HB)

Historically, polyester poly P(3HB) has been the most extensively studied example. This is the most common type of PHA belonging to the scl-PHA group. The ability of bacteria to accumulate this polyester is often used as a taxonomic characteristic. There is P(3HB) in the cell in a "liquid" amorphous state. After extraction from the cell with organic solvents, the polymer becomes highly crystalline and in this state is solid but very brittle. Due to this fragility, it is not a very resistant material to stress. The melting point P(3HB) is about 180 °C. At 200 °C, the polymer thermally decomposes, limiting the ability to further process the homopolymer. PHB copolymers can lead to the formation of polymers containing 3-hydroxyvalerate or 4-hydroxybutyrate monomers. [19][48]

2.3.2 Mcl-PHA

Mcl-PHA is characterized by better processability, elasticity and durability. Besides having lower crystallinity (20–40%) with much higher extension to break (300–450%), mcl-PHA also exhibit lower melting points and glass transition temperatures. Due to these properties, mcl-PHAs behave like elastomers or adhesives with the potential of having consistency and texture of resins or latex based on the exact mcl-PHA composition. [21] [22]

2.3.3 Comparation of PHB properties with PP

Table 4: PHB properties compared to PP

		PHB	PP
Mechanical properties	Young's modulus [GPa]	3,5	1,7
	Tensile strength [MPa]	40	38
	Melting point [°C]	180	176
	Elongation to break [%]	6	400
Chemical properties	Temperature of degradation [°C]	180	200
	M [10^5 g/mol]	1-8	2,2-7
	ρ [kg.dm^3]	1,25	0,905
	Crystallinity [%]	80	70
	Biodegradability	yes	no
	UV-resistance	good	bad
	Resistance to solvents	bad	good

We can see, that stiffness, which is presented by Young 's modulus is little higher in PHB than in PP. Tensile strength is very similar such as melting point for both of polymers. The biggest differences are in elongation to break, known as fracture strain, which is much higher in PP. The difference is also in biodegradability, UV-resistance and resistance to solvents, where PP is not biodegradable having bad UV-resistance but has good resistance to solvents while PHB is biodegradable having good UV-resistance but bad resistance to solvents.

2.4 Synthesis of PHA

As mentioned above, the synthesis of PHA occurs when there is an excess of carbon source and a lack of essential nutrient. The substrate specificity of PHA synthase, as well as the carbon source, determines what type of PHA (homopolymer, heteropolymer, copolymer,sscl,mmcl) the organism produces. These enzymes are found on the surface of the granule along with the proteins. [52] [53]

If the bacterial strain is not in a crisis stage, then the production of high levels of coenzyme A from the Krebs cycle blocks PHA synthesis by inhibiting 3-ketothiolase (Pha) by direct-

ing acetyl-CoA to the Krebs cycle for energy production and cell growth. Under crisis conditions (e.g., when an essential nutrient such as nitrogen and phosphorus reduces the presence of excess carbon), coenzyme A levels are non-inhibiting, allowing acetyl-CoA to move toward PHA synthetic routes for PHA accumulation. [50.51]

In most cases, acetyl-CoA is formed by catabolism of sugars, pyruvate or acetate, which is converted by condensation of 2 of its units (Krebs cycle) to (R) -3-hydroxybutyryl-CoA by reduction of acetoacetyl-CoA with the enzyme Acetoacetly CoA reductase followed by condensation of PHA synthase, which catalyzes polymerization via esterification from 3-hydroxybutyryl-CoA to poly (3-hydroxybutyrate). Most organisms use this biosynthetic pathway. [51]

When oils are used as the carbon source, β-oxidation of fatty acids occurs first and the substrate for the synthesis of P(3HB) is acetyl-CoA or acetoacetyl-CoA and the reaction takes place in the presence of the enzyme transacylase. [51]

In some organisms, 3-hydroxybutyryl-CoA (S)-isomers are generated instead of (R)-isomers. Since PHA synthase is stereospecific, in particular active only for (R)-isomers, the polymerization must be preceded by reactions leading to the conversion of (S)-isomers to (R)-isomers. These transformations are catalyzed by enoyl-CoA. [51]

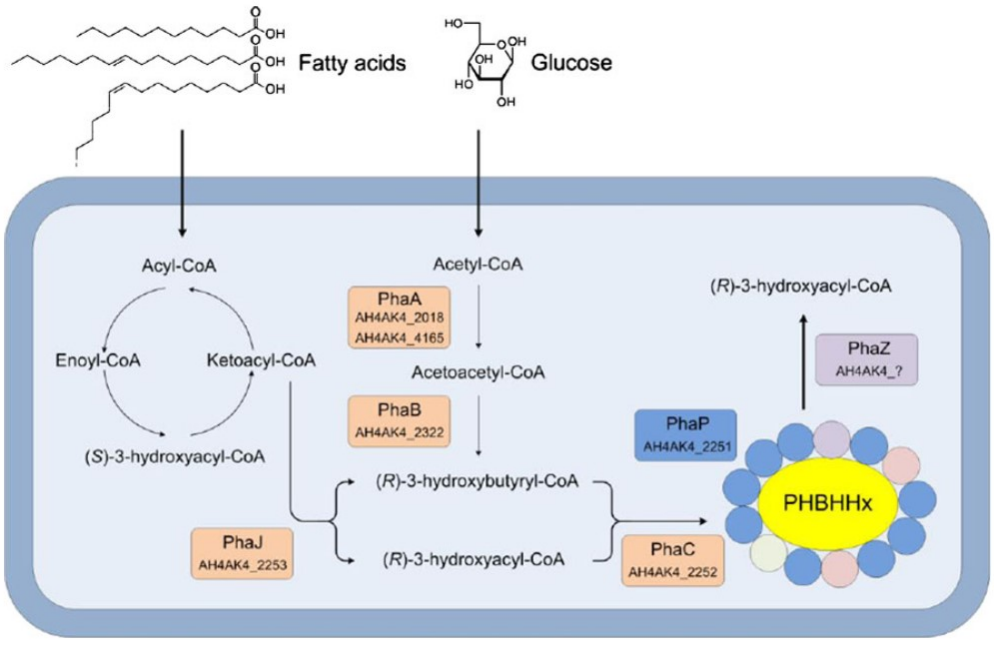

Figure 6: PHB synthesis pathways. Where: PhA β-ketothiolase, PhaB acetoacetyl-CoA reductase, PhaC PHApolymerase, PhaJ(R) enoyl-CoA hydratase, PhaP phasin, PhaZ PHAdepolymerase [54]

2.5 Biodegradability

2.5.1 Characterization of the term „biodegradable“

When one hears the term “biodegradable”, we imagine a material that is 100% degradable. However, this term is not specified in any way. It is not specified in which environment it decomposes, at what rate, under what conditions or whether it decomposes completely, or the decomposition is only partial. The concept of biodegradable has been considered for many years. The direct measure to evaluate the biodegradability of the material has become the rate and amount of microbial decomposition from the carbon-based polymer, which is taken from the ratio of 1 to the amount of CO_2 released in this process, as microorganisms obtain their energy through carbonaceous substrates. This principle has thus become the basis of various international standards. [23-25]

ASTM states that in order for a polymer to be classified as biodegradable, it must be the result of degradation from a process of naturally occurring microorganisms such as bacteria, fungi or algae. [24]

ISO and CEN state that a biodegradable polymer is one in which the resulting degradation products are found with a lower molecular weight than in the polymer by the original action of naturally occurring microorganisms such as fungi, algae or bacteria.[24]

When we compare the individual standards, we find that they are based on the same principle of the action of microorganisms on a given polymer and its conversion into the resulting products of CO_2 and water.[24]

What differs in the standard is the percentage degradability of a given material over a period of time, which is needed to obtain the title of biodegradable material. This parameter is still a very important point of discussion at ISO level. The requirements for the approval of the standard are given in the table.[24]

Table 5: Biodegradation requirements [24]

Norm	biodegradation requirements	
DIN	60%	6 months
ASTM	60%	6 months
CEN	90%	-
OECD (for chemicals)	60%	28 days

2.5.2 Biodegradability of PHA

One of the most important capabilities of PHA is biodegradability. This happens in nature with the help of microorganisms, which break down PHA with the help of PHA hydrolases and PHA depolymerases, which hydrolyze the polymer extracellularly to water-soluble oligomers and monomers. [28]

Biodegradation of PHA can be divided into 3 steps:
-In the first step, when the physical properties of the material change due to the action of a microorganism, we call biodeterioration. This can take place either by physical means, chemical influences or enzymatic biodeterioration. In the case of the physical pathway, the microorganisms grow on the surface of the polymer and form a mucus which penetrates and disrupts the structure of the polymer or penetrates the organism itself and the polymer grows. In the case of chemical influences, the microorganism can produce, for example, organic acids, which disrupt the structure of the polymer and thus contribute to its decomposition. By enzymatic biodeterioration, the microorganism produces enzymes which break down the bonds in the polymer and thus break it down. [26]

-In the second step, biofragmentation takes place, where the polymer is decomposed into small fragments (oligomers and monomers). [26]

- In the third step, assimilation takes place, when the microorganism consumes sufficiently small fragments to produce carbon dioxide and water. [26]

According to the composition of the polymer (crystallinity, additives) and ambient conditions (temperature, humidity, pH) the time of biodegradation may vary. Piece degradation rate PHB is for anaerobic microorganisms, for example, in compost in the order of several months and in marine water is the degradability in years. In mammals, this is a nontoxic effect polymer hydrolyzes slowly, depending on unit composition, about 1 to 6% in 6 months. [28-30]

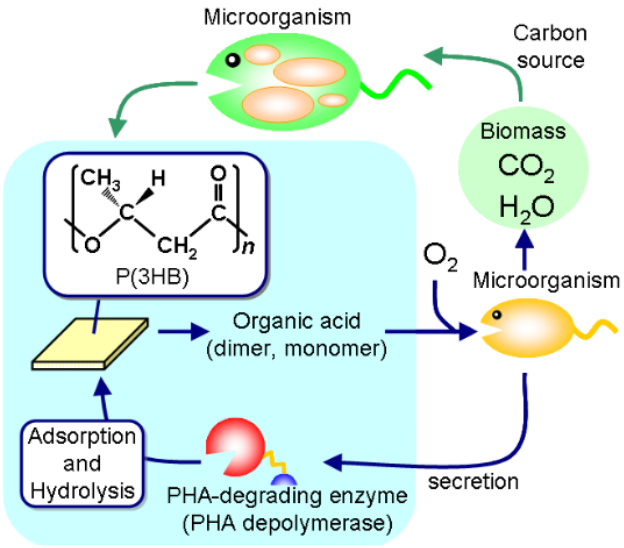

Figure 7: Biosynthesis and biodegradation process of PHA in a natural environment. [49]

2.6 Use of polyhydroxyalkanoates

As was mentioned earlier, the properties of PHA are very similar to polyolefins, for example polyethylene PE and polypropylene PP. Their use will therefore be mainly in packaging plastics. Figure 5 shows the world production of bioplastics in 2018 and their use. We can see that the PHA is still for its high production cost and underused production amounts to only 1.4% of all produced bioplastics. We can use PHA as a biofuel, we can find it in flexible and rigid packaging materials or, for example, in nanomaterials and healthcare. The most widely used biopolymer is polyethylene terephthalate, which is used not only in packaging materials but also in textiles or the automotive industry. [35] [37]

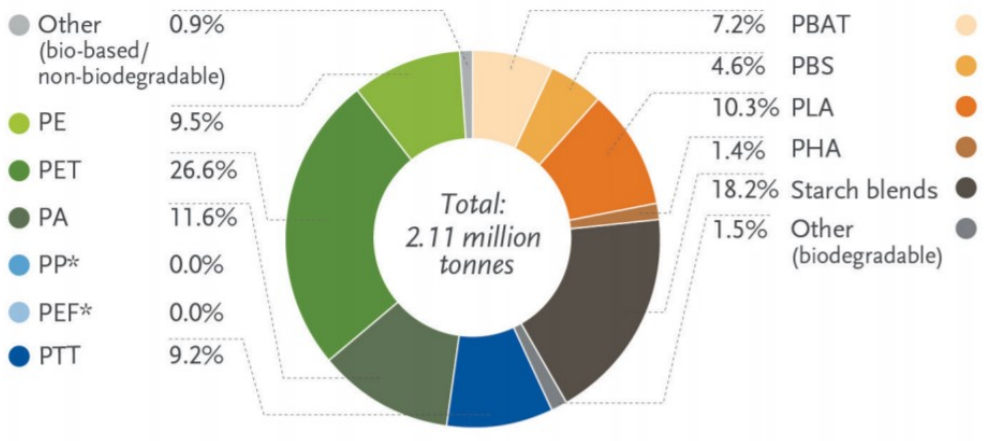

Figure 8: Global production capacities of bioplastics 2018 [36]

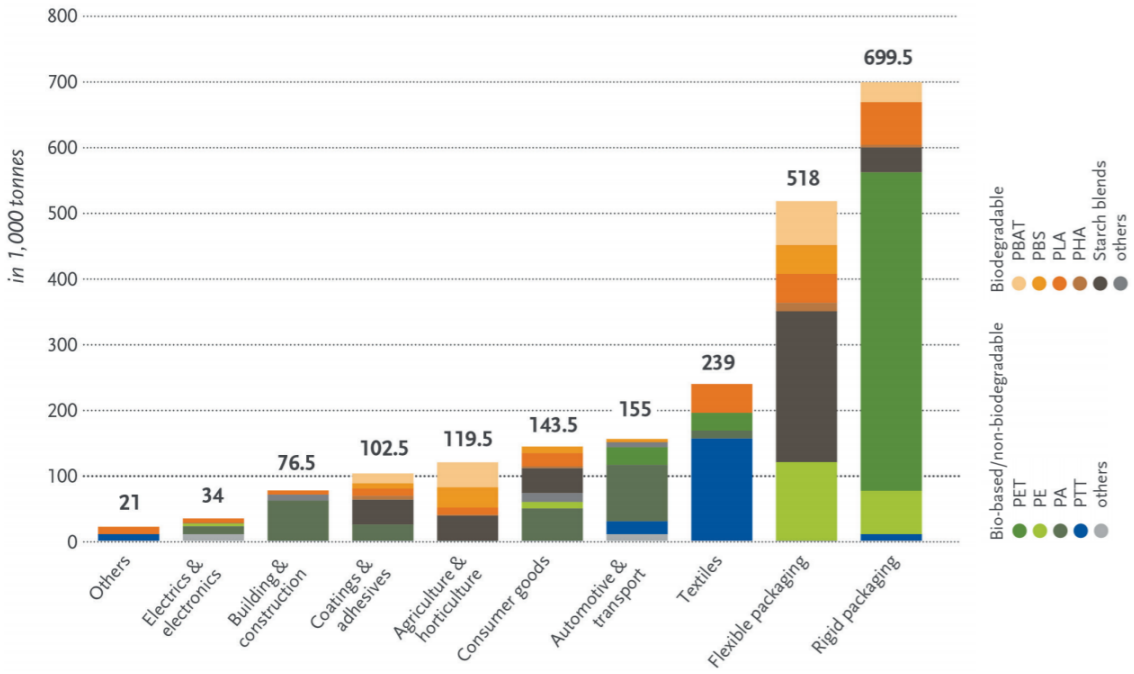

Figure 9: Global production capacities of bioplastics 2018 [36]

We can also notice that the total production of bioplastics is only 1% of all plastics produced. However, the demand for PHA is growing rapidly every year. In Figure 6, we can expect that in 2024 PHA production is expected to increase by up to 1.000%.

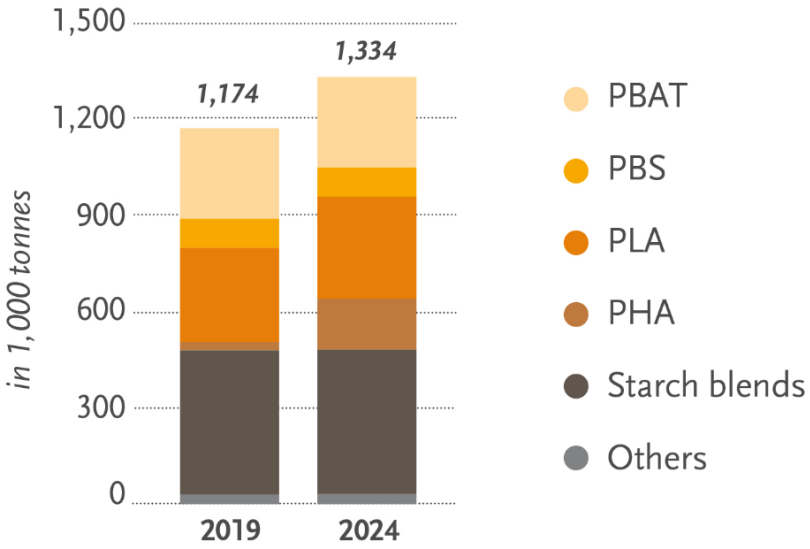

Figure 10: Bioplastic 2019 vs 2024 [36]

2.6.1 Applications in healthcare

Due to its biocompatible nature, low inflammatory response and biodegradability, PHA represents great hope in tissue engineering. In surgery, it can be used to cover wounds or as resorbable sutures. PHB and PHBV copolymer can be used to prepare human tissues. The replacement of the hard buzzard, which may be, for example bone, was tested. Another hope is in valve replacement, tissue engineering of neurons due to their piezoelectric nature or as a carrier for drug delivery, where PHA serves as the outer shell of the drug, which contains the active substance, which is gradually released along with the degradation of PHA in the body. These drugs have already been tested in humans for the treatment of gingivitis. Currently, the use as an anticancer drug carrier is also being investigated, when the anticancer drug ellipticine was encapsulated in PHBV and it was found that the cytotoxic effect of ellipticine was increased. [38-40]

2.7 Bacterial strains

One of the problems why PHA is not produced on a larger scale is that bacterial strains that produce PHA naturally grow very slowly at lower temperatures and so only a few of them can be applied for industrial use. These bacteria are often difficult to lyse and contain metabolic pathway for degradation of intracellular PHA stores.[32]

The applicability of a bacterial strain is affected by a number of factors. Above all, it is stability and safety, growth and accumulation capacities, achievable amount of biomass and amount of PHA. Furthermore, the degree of extractability of PHA, the molecular weight of PHA, the number of usable substrates and the financial demands of the individual components of the medium.[32]

However, this does not apply to genetically modified bacteria, such as Escherichia coli, which, although not capable of degrading PHA, grows rapidly even at higher temperatures and lyses easily. It is capable of producing PHA in extremely large intracellular amounts (up to 80 to 90% of dry matter. Rapid growth is allowing large amounts of polymer to accumulate and easy cell lysis is saving the cost of cleaning PHA granules. This makes genetic engineering very interesting regarding PHA production and hides great potential for the future.[32]

More than 90 PHA-producing bacterial strains have been described to date. These strains are listed in the table below. [45]

Table 6: PHA accumulating microbial strains [45]

Acidovorax	*Corynebacterium*	*Methylobacterium*	*Rhodococcus*
Acinectobacter	*Cupriavidus*	*Methylocystis*	*Rhodopseudomonas*
Actinobacillus	*Cyanobacterium*	*Methylomonas*	*Rhodospirillum*
Actinomycetes	*Defluviicoccus*	*Methylosinus*	*Rubrivivax*
Aeromonas	*Delftia*	*Methylovibrio*	*Saccharophagus*
Alcaligenes	*Derxia*	*Micrococcus*	*Sjinorhizobium*
Allochromatium	*Ectothiorhodospira*	*Microcoleus*	*Sphaerotilus*
Anabaena	*Erwinia*	*Microcystis*	*Spirillum*
Aphanothece	*Escherichia*	*Microlunatus*	*Spirulina*
Aquaspirillum	*Ferrobacillus*	*Microvoleus*	*Staphylococcus*
Asticcaulus	*Gamphospheria*	*Moraxella*	*Stella*
Axobacter	*Gloeocapsa*	*Mycoplana*	*Streptomyces*
Azohydromonas	*Gloeothece*	*Nitrobacter*	*Synechococcus*
Azomonas	*Haemophilus*	*Nitrococcus*	*Syntrophomonas*
Azospirillum	*Halomonas*	*Nocardia*	*Thiobacillus*
Azobacter	*Hydrogenophaga*	*Nostoc*	*Thiocapse*
Bacillus	*Hyphomicrobium*	*Oceanospirillum*	*Thiococcus*
Beggiatoa	*Chloroflexus*	*Oscillatoria*	*Thiocystis*
Beijerinckia	*Chlorogloea*	*Paracoccus*	*Thiodictyon*
Beneckea	*Chromatium*	*Paucispirillum*	*Thiopedia*
Bradyrhyzobium	*Chromobacterium*	*Pedomicrobium*	*Thiosphaera*
Brachymonas	*Klebsiella*	*Photobacterium*	*Variovorax*
Burkholderia	*Lamprocystis*	*Protomonas*	*Vibrio*
Caryophanon	*Lampropedia*	*Pseudomonas*	*Xanthobacter*

Caulobacter	*Legionella*	*Ralstonia*	*Zoogloea*
Clostridium	*Leptothrix*	*Rhizobium*	
Comamonas	*Methanomonas*	*Rhodobacter*	

3 PRODUCTION OF PHA BY DIFFERENT MICROORGANISM

3.1 Scl-PHA producers

3.1.1 Cupriavidus necator

Cupriavidus necator H16, formerly known as *Ralstonia eutropha*, *Wautersia europha* and *Alcaligenes eutrophus*, is one of the organisms that, when there is an excess of carbon source and limit of certain nutrients, form polyhydroxyalkanoates, specifically polyhydroxybutyrate. It is a Gram-negative, facultative chemoautotrophic bacteria belonging to the group of proteobacteria, occurring in soil and fresh water. In the absence of an organic substrate, it uses hydrogen and carbon dioxide as its sole source of energy. If no oxygen is present in the environment, the bacteria can switch to a complete denitrification pathway, using alternative electron donors such as NO_3- and NO_2-. It is therefore an anaerobic respiration. It is the best researched PHB producing bacterium. [19, 20, 21].

3.1.2 Bacillus megaterium

The bacterial strain *Bacillus* is very widespread in the environment, forming gram-positive sporulating rods. But it has played an important role in the history of the PHA. In this bacterium, the accumulation of PHA was described for the first time, so for this reason its metabolism was well researched and used to serve as a model system. The biosynthetic pathway is similar to that of *C. necator*, but the amount of polymer produced is less than about 50% dry matter. However, the bacterium is still receiving attention mainly due to its wide range of enzyme equipment, high resistance and low nutritional requirements, which makes it more attractive for the utilization of waste materials of various origins. [55,12]

3.1.3 Halophilic microorganisms

This microorganism was discovered in 1972 in the Dead Sea and named Hallo-bacterium after it. Later, however, it turned out to be *Haloarcula marismortui*. Its importance is in the ability to accumulate P(3HB) or copolymers P(3HB-co-3HV), such as the genera *Haloferax, Halobiforma* or *Haloquadratum*. [43]

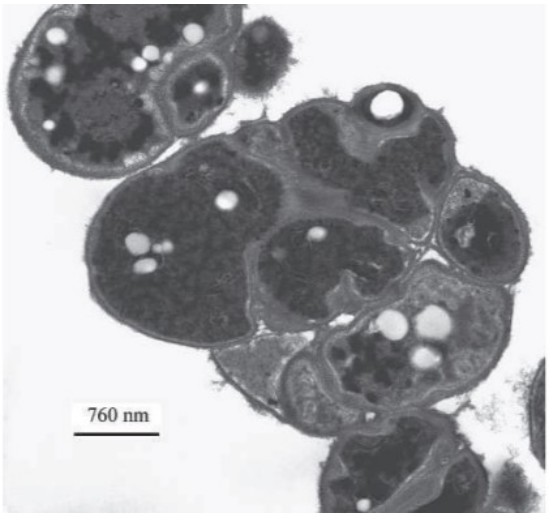

Figure 11: PHA accumulating microbial strain *Haloferax* [45]

3.1.4 Methanotrophic and methylotrophic microorganisms

These are microorganisms that use monocarbon organic compounds such as methane as a carbon source. The recovery of P3HB in the nitrogen limit state, where methane of *Methylobacterium organophilum* was used as a carbon source, was 57% in dry matter. In addition to its higher content, however, it also has potential as a connection with the technology of anaerobic disposal of biological waste. For the time being, however, its use in such operations is quite problematic. [57.58]

3.1.5 Photosynthetic microorganisms

The ability to accumulate PHA is also assigned to some photosynthetic microorganisms. An example of such a microorganism is *Spirulina subsalsa*, which has been able to increase PHA production by increasing the salinity of the culture medium. Despite this, PHA production is very low. [59]

3.1.6 Production by yeast

Yeasts are characterized by a larger cell size than the vast majority of bacteria, and therefore it is easier to isolate PHA, which is carried out, for example, by filtration. The possibility of streamlining production, which is being researched and paid attention to, is therefore very promising. An example is the transgenic yeast *Saccharomyces pombe*, which has been genetically modified with plastic containing enzymes from the bacterium *Cupriavidus necator* and was able to accumulate 9% P3HB in dry matter. [59] [60]

3.2 Mcl-PHA producers

3.2.1 Pseudomonas mendocina

Bacteria of the genus *Pseudomonas* are gram-negative rod-like, widespread microorganisms. A variety of substances can be used as the carbon source. *Pseudomonas oleorans* produces a mixture of mcl-PHA and P(3HB) when grown on glucose-octanoic medium. Upon growth on nonanoic acid, P(3HB-co-3HV) was produced. Good results have been obtained in the production of PHA with *Pseudomonas stutzeri* using a medium with glucose and fatty acids. However, the monomer composition again varies depending on the strain used, the nutrient medium and the culture conditions. Most often, the genus *Pseudomonas* produces up to several different monomers in different proportions. However, a wild strain was also discovered which was able to produce a pure homopolymer. [48][12]

3.2.2 Plants

Another option with great potential is to obtain PHA, where the producers are plants. Unlike gaining PHA with bacteria, however, production costs could be much lower. The main aspect that could reduce production costs is the use of CO_2 as a carbon source and sunlight as energy. Another aspect is that complex sterile conditions do not have to be created in an expensive fermentation process as in the production of PHA by bacteria. The first attempts to obtain PHA from plants date back to 1992. It has been discovered that wild plants can produce 0.1% in dry matter, which was a prerequisite for the combination of a gene from PHA-producing bacteria. In the first experiments, it was possible to produce up to 14% of PHA in dry matter, thanks to a modification of the genus *E. eutropha*. The studied goal is to obtain genetically stable recombinant plants. [72]

3.3 Characteristics of some methods detecting PHA producing bacteria

3.4 Nile Blue A and Nile red staining

Nile Blue A and Nile Red are lipophilic dyes that are used to identify substances of a lipophilic nature. The nature of the PHA granule is lipophilic, so these dyes are well suited as a suitable method for rapid PHA analysis. Both dyes are almost insoluble in water and show no fluorescence in the polar environment. Dyes dissolve in lipophilic granules and do not

chemically interact with PHA. When the microorganism is then exposed to UV radiation, the granules will emit it in the form of bright yellow to red in the case of Nile Red and bright blue in the case of Nile Blue A. This method is relatively fast, but does not serve as a quantitative analysis or PHA type analysis. In addition, these dyes are relatively expensive. We will pay 3.500CZK for 5g. This method is used only for quick identification, if the bacterial strain is producing PHA. It is not a kvantitative analysis. [41,42]

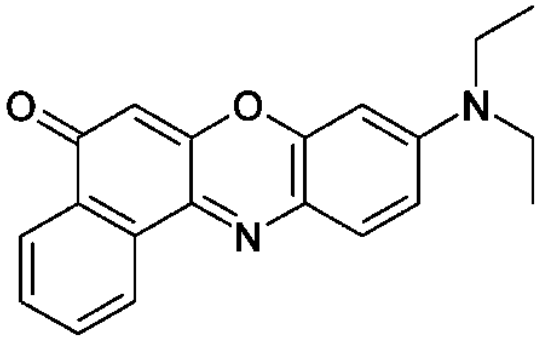

Figure 12: Nile Red stain structure [41]

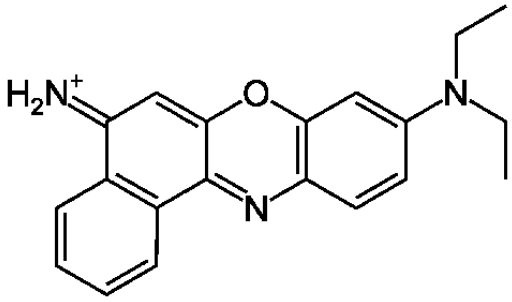

Figure 13: Nile Blue A stain structure [41]

3.5 Sudan Black staining

Like Nile Blue A and Nile Red, Sudan Black is a lipophilic dye that stains various lipids, such as phospholipids in cell membranes, neutral fats, or PHA granules, which, as mentioned earlier, are lipid characteur. This dye can be stained directly on a petri dish during cultivation or a small sample of the bacterial strain on a slide, as a sample for microscopy. If the sample contains a lipid component - PHA, it will form black pigments. This method as a Nile Blue A and Nile Red is used only for quick identification, if the bacterial strain is producing PHA. It is not a kvantitative analysis. [2.43]

3.6 PCR

Polymerase chain reaction (PCR) is a method by which a selected gene can be amplified from a DNA sample. The method was developed by Kary B. Mullis in 1985. To amplify a particular gene, so-called primers are needed, which are short oligonucleotide strands with an 18-specific base order that is compatible with the region-defining regions of the selected gene. After thermal denaturation of the DNA, the primers anneal to opposite polynucleotide strands. From each primer, a new strand is synthesized by DNA polymerase. The resulting formations are denatured again and the whole process is repeated. Several consecutive cycles are performed, and with each cycle, the copy number of the gene is doubled. If there is no region complementary to the primers used in the sample, the primers do not bind to the template DNA and the reaction is negative. Using specific primers, the phaC gene conditioning the production of the enzyme PHA synthase can be amplified. Bacterial producers of PHA can be detected by amplification of this gene. To verify the bacterial origin, it is possible to perform the so-called multiplex PCR, which amplifies several genes in one reaction, in this case the phaC gene and at the same time the 16S rRNA gene specific for bacteria. However, this method may discriminate against longer DNA fragments, in this case the 16S rRNA fragment. PCR products can be detected by gel electrophoresis. [46,47]

4 STRATEGIES OF ISOLATION PHA

PHA isolation plays an important role in the final price of the total PHA production. The aim is to achieve low-cost insulation that would reduce the overall cost of the polymer obtained and thus be in competition with petrochemical plastics. Another goal is to achieve the cleanest possible product, which can then be used in all sectors. [61] [67]

However, this process is not so simple. As mentioned earlier, there are 3 types of PHA according to the monomer chain length and there are also copolymers. Thus, each type is suitable for a different extraction method, which greatly complicates the isolation process. [61]

In general, we can divide the process of obtaining PHA from biomass into 3 parts.

4.1 Concentration of the culture medium

This step is performed immediately after culturing, where the grown culture is either lyophilized, filtered or centrifuged to concentrate the fermentation broth and then washed. This will greatly facilitate the later disruption of the cell membrane in the next step. Furthermore, the PHA needs to be inactivated by thermal heating around 85 ° C, otherwise the PHA may become graded there. Heating results in denaturation and dissolution of the genetic information and proteins that form the cell wall, which leads to a violation of membrane stability and the prevention of increased solution viscosity. Denaturation is preferably used to inactivate DNA depolymerase, which is subsequently no longer able to denature the polymer obtained. [61-63,67]

4.2 Isolation of PHA

A number of techniques have been developed over the years. The best investigated methods are those where P(3HB) isolation plays a role, as the most common type of PHA. Isolation occurs after concentration and can be divided into several groups, see below. [67]

4.2.1 Methods of isolation PHA

4.2.1.1 Method using PHA solubility

This is one of the oldest methods for isolating PHA, and this method is based on the solubility of PHA in an organic solvent, while the cellular components remain undissolved and are often filtered off from the obtained PHA solution. [61]

In the first step, the permeability of the cell membrane is modified and in the next, the PHA itself is dissolved. This is subsequently obtained from the solution either by precipitation in solutions which do not dissolve the PHA or by evaporation of the solvent. The best solvents appear to be chlorinated hydrocarbons (chloroform), their azeotropic mixtures, cyclic hydrocarbons or mixtures of chloroform with other hydrocarbons. In later years, for safety and ecological reasons, non-chlorinated solvents (ethylene carbonate) began to appear. However, the disadvantage is that, due to the nature of the solvents used, they take place at higher temperatures, which at the same time cause thermal degradation of the isolated PHAs. From this point of view, the use of chlorinated hydrocarbon extractants is most advantageous, as it allows the separation of PHA from them at lower temperatures (generally up to about 100 to 120 °C), at which thermal degradation of PHA does not yet occur. However, when testing these methods, it has been found that chlorinated hydrocarbon extractants extract biomass components from the biomass in addition to PHA, which precipitate together with the PHA during subsequent separation by precipitation into water and substantially reduce their final purity. Thanks to this, it reaches a maximum of about 90%. These components can be removed by digestion processes, which are, however, expensive and, in addition, cause the viscosity of the culture medium thus treated to increase, which makes processing difficult. This includes the method of evaporating the solvent from the solution, which can be carried out in several ways, the most common of which is spraying into a spray dryer or below the level of boiling water in the tank. In the first case we get an already dry product and in the second we need to dry the polymer. [61,67]

The Czech Republic is proud of the invention of the method of injecting a PHA solution into a loop with circulating hot water, where the solvent is evaporated, and the precipitated polymer is removed by overflow for drying. [61,67]

4.2.1.2 Methods based on dissolving other cellular components

These methods are, in principle, opposite to the extraction methods. In these processes, the PHA is not converted to a liquid state, but on the contrary, there is an interest in keeping it in a solid state and dissolving other parts of the cell. It usually consists of 3 parts, where in the first part the biomass is heated to a higher temperature after cultivation in order to denature proteins and DNA. In the second part, the proteolytic distribution of cellular components occurs. In the last phase, the obtained product is purified, which is carried out with a surfactant. [65]

The disadvantage is the production of a large amount of difficult-to-treat wastewater and the relatively high cost of the input chemicals. The advantage over extractions is that they make it possible to process fermentation liquids in large volumes and with a high concentration of cells. Therefore, a process has been proposed in which once used wastewater is re-enriched with a small amount of surfactant and chelating agent. If the purity of the polymer obtained falls below 96%, which usually occurs after 7 cycles, the resulting wastewater is purified by the addition of hydrochloric acid and activated carbon. [69]

The protein isolation method was first published by Zeneca as an alternative to extraction isolation. Proteolytic enzymes are used in this isolation, which have a high ability to cleave bonds between individual amino acids of the cell membrane and thus disrupt the PHA envelope. [61]

4.2.1.3 Mechanical insulation and disintegration

This includes, for example, high-pressure homogenization, where under very high pressure, the culture medium is injected into the lower pressure space, where the cavitation effect disintegrates the cells. In most cases, however, one pass through the homogenizer is not enough and the procedure must be repeated. Another complication is that DNA is also released from the cell nuclei, which greatly increases the viscosity of the fluid and makes subsequent operations difficult. Homogenization is followed by isolation of the released PHA granules without the use of chemicals. The process takes place mechanically, whereby the PHA suspension can be fed, for example, to a plate separator, which needs to be repeated several times. However, this method produces a substantially impure polymer that needs to be purified. [67]

4.3 Purification of the obtained product

The most commonly used methods for purification are mainly the application of hydrogen peroxide combined with other methods such as enzyme activity or chelating ions or ozone, which causes bleaching, solubility of any impurities and deodorization. Ozone is thought to limit the use of hydrogen peroxide, due to its instability and the high temperature at which it is used. [70]

CONCLUSION

Due to the high accumulation of plastics and their difficult recycling, new environmentally friendly polymers are being studied to avoid this problem. Such a solution can be polyhydroxyalkanoates, which meet the requirements for biodegradability and, in addition, are very similar to fossil fuel-based polymers. Their diversity offers many practical applications, but there are still problems which prevent the greater production of these polymers. Most notably, their high production cost, which is up to six times higher with polymers produced from fossil fuels. Thus, new investment and various techniques are being investigated to reduce the cost of producing these polymers as well as the amount produced, which is claimed for their purity as a starting material. Further research is also needed on PHA producers, using new or genetically modified microbial strains and plants, along with finding suitable raw materials. There is also room for many improvements in the insulation process itself. The right solvents are still being sought which can be regenerated and thus the chemical footprint is reduced and, last but not least, the waste from these production processes must be optimized. There are a lot to improve in this sector, the question remains whether the investment in this material will pay off. The best-known P(3HB), although biodegradable, still has limited uses, although due to its biocompatibility it is very interesting in the field of biomedical applications. In this respect, its copolymers are promising, which considerably increase its properties.

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LIST OF ABBREVIATIONS AND SYMBOLS

Alphabetically ordered

3HV	hydroxyvalerate
3HB	hydroxybutyrate
ASTM	American Society for Testing and Materials
CEN	European Committee for Standardization
CZK	Czech Koruna
CO_2	carbon dioxide
DIN	German Institute for Standardization
DNA	deoxyribonucleic acid
ISO	International Organization for Standardization
Lcl-PHA	long chain lenght PHA
M	Molar weight
Mcl-PHA	medium chain lenght PHA
OECD	Organisation for Economic Co-operation and Development
PhA	β-ketothiolase
P(3HB-co-3HV)	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-3MP)	poly (3-hydroxybutyrate-co-3-mercaptopropionate)
P(3HB)	poly-(3-hydroxybutyrate)
P(3HC)	poly(3-hydroxycaproate)
P(3HD)	poly(3-hydroxydecanoate)
P(3HDD)	poly(3-hydroxydodecanote)
P(3HH)	poly(3-hydroxyheptanoate)
P(3HN)	poly(3-hydroxynonanoate)
P(3HO)	poly(3-hydroxyoctanoate)

P(3HP)	poly(3-hydroxypropionate)
P(3HV)	poly(3-hydroxyvalerate)
P(3HUD)	poly(3-hydroxyundecanote)
P(4HB)	poly(4-hydroxybutyrate)
P(5HV)	poly(5-hydroxyvalerate)
PCR	polymeric chain reaction
PE	polyethylen
PHA	polyhydroxyalcanoates
PHB	polyhydroxybutyrate
PhaB	acetoacetyl-CoA
PhaC	PHApolymerase
PhaJ(R)	enoyl-CoA hydratase
PhaP	phasin
PhaZ	PHAdepolymerase
rRNA	ribosomal ribonucleic acid
Scl-PHA	short chain lenght PHA
UV	ultraviolet

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