Phenotypic identification and characterization of the PHA producers

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Možnosti získávání a využití PHA
Charakteristika mikroorganizmů vyskytujících se při tvorbě PHA
Vliv substrátů na tvorbu PHA
II. Praktická část
Cíl práce
Metody pro kultivační stanovení produkce PHA
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ABSTRAKT

Předložená diplomová práce je zaměřená na fenotypovou identifikaci a charakterizaci producentů PHA. Teoretická část diplomové práce se zabývá charakterizací polyhydroxyalkanoatu s jejich mozností získávání z producentů a jejich využitím. Dále v teoretické části najdeme charakterizaci samotných mikroorganismů produkujících PHA a zkoumání vlivu substrátu na produkci PHA.

Praktická část zkoumá vlivy různých druhů půd na produkci PHA, a to se třemi vzrorovými mikroorganismy *Cupriavidus necator, Escherichia coli* a *Pseudomonas mendocina*, kdy se PHA detekuje fenotypovými metodami - barvení fluorescenčním barvivem Nile Blue A a barvivem Sudan Black s následným ověřením pomocí metody FTIR. Po vytipování půd pro nejlepší produkci PHA jsme dále produkci PHA zkoumali na celkem 77 mikroorganismech se stejným postupem pro jejich detekci, kdy bylo cílem zjistit, které kmeny PHA produkují a porovnat všechny 3 metody detekce.

Klíčová slova: polyhydroxyalkanoáty, biopolymery, Nile Blue A, Sudan Black, FTIR, detekce

ABSTRACT

This master thesis is focused on the phenotypic identification and characterization of microbial polyhydroxyalkanoates (PHA) producers. The theoretical part of the master thesis deals with the characterization of PHA with their possibility of obtaining from bacterial producers and their use. Furthermore, in the theoretical part we find the characterization of the microorganisms producing PHA and the study of the influence of the substrate on the production of PHA.

The practical part examines the effects of different medium types on PHA production with three growth microorganisms *Cupriavidus necator, Escherichia coli* and *Pseudomonas mendocina*, where PHA is detected by phenotypic methods - staining with fluorescent dye Nile Blue A and Sudan Black dye followed by verification using FTIR. After selecting the soils for the best PHA production, we further examined the production of PHA on microorganisms with the same procedure for their detection, the aim was to find out which strains of PHA produce and compare all 3 detection methods.

Keywords: polyhydroxyalkanoates, biopolymers, Nile Blue A, Sudan Black, FTIR, detection

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

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INTRODUCTION

Microorganisms (MO) are all around us, so it is no wonder that their application is also in almost all possible industries. Thanks to microorganisms, a number of drugs for various incurable diseases have been invented in the pharmaceutical industry, and we include their contribution to the development of antibiotics. In the food industry, we can find microorganisms in a wide range of products, from dairy products, where microorganisms help fermentation and pasteurization, to alcoholic ones, such as beer, where microorganisms are found during fermentation. In the agricultural industry, microorganisms play an important role in increasing herbicide and pesticide resistance and finally, they can even be found in the production of biofuels.

They have also found their way into the plastics industry in the form of polyhydroxyalkanoates (PHA), which have the potential to replace synthetic plastics. Their properties are very similar to polyolefins, which we know primarily as packaging materials, which are difficult to recycle and in nature resist decomposition for several decades, unlike PHA. World consumption of these synthetic polymers is more than 200 million tonnes and is growing by 5% each year, with more than 215 million tonnes of fossil fuels being required to process, and this may be another major disadvantage in the future as the price increases. [1,2]

Thus, the general trend today is to find a material that has similar properties to these synthetic plastics but is also biodegradable and its production is not dependent on fossil fuels. The ideal plastic should therefore be durable, biodegradable, recyclable and produced from renewable sources. Most such criteria are met by PHA. [2]

One of the reasons why PHA have not yet replaced current plastics is their high production cost, where almost 50% of the money is paid on substrates for the cultivation of microorganisms and their isolation itself. A crucial role, however, is also played by its producers themselves, who do not create the PHA themselves evenly, but each producer specifically. The aim of this master thesis is the phenotypic identification of these producers and the influence of substrates on the amount of PHA produced. [2]

I. THEORY

1 POLYHYDROXYALCANOATES

PHA are a group of polymers produced by various types of microorganisms. Bacteria synthesize PHA as an energy storage and as a carbon storage in the form of lipophilic granules (Fig. 1). PHA granules with dimensions of 0.2–0.5 µm occur in the cytoplasm of bacteria. [3,4]

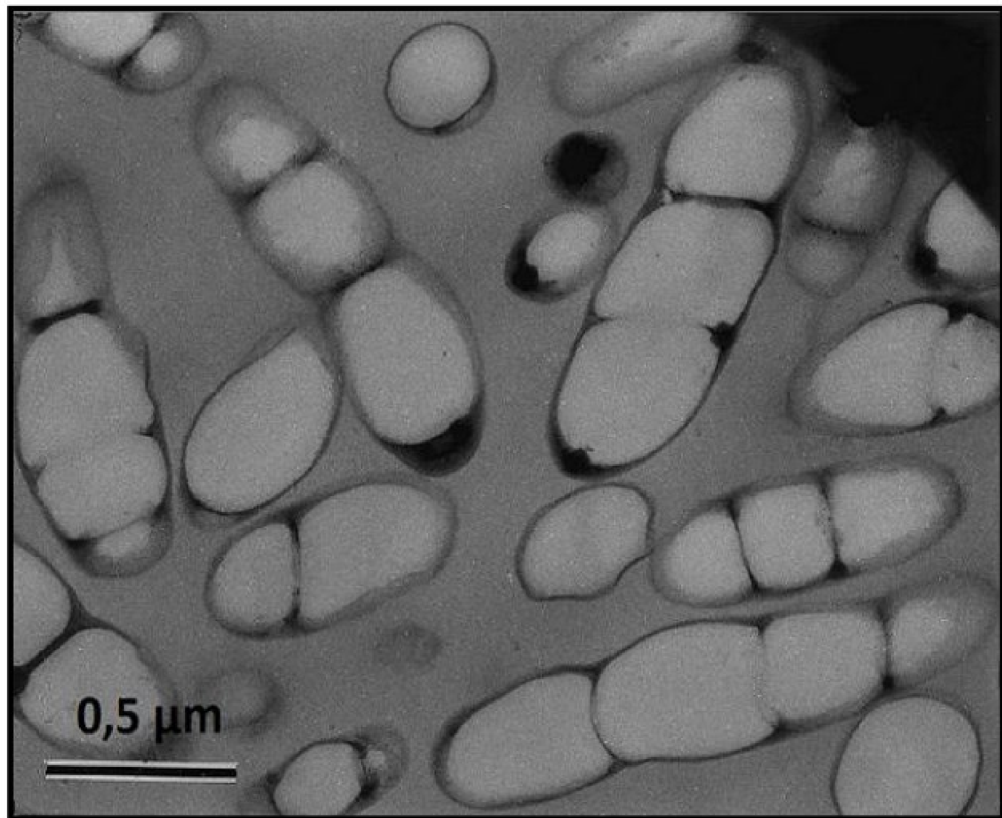

Fig. 1 Transmission electron micrograph of recombinant *Cupriavidus necator*. Containing PHA cells. Bar represents 0.5 µm. [3]

These granules are formed as a result of the synthesis of PHA by the enzyme PHA synthase, which is found freely in the cytoplasm. PHA synthase converts suitable precursors into PHA polymer fibers. These fibers later clump together due to hydrophobic interactions and a lipophilic PHA granule is formed. PHA synthases remain on the surface of the granules and further contribute to the elongation of PHA chains, thus enlarging the granules (Fig. 2). [3,4]

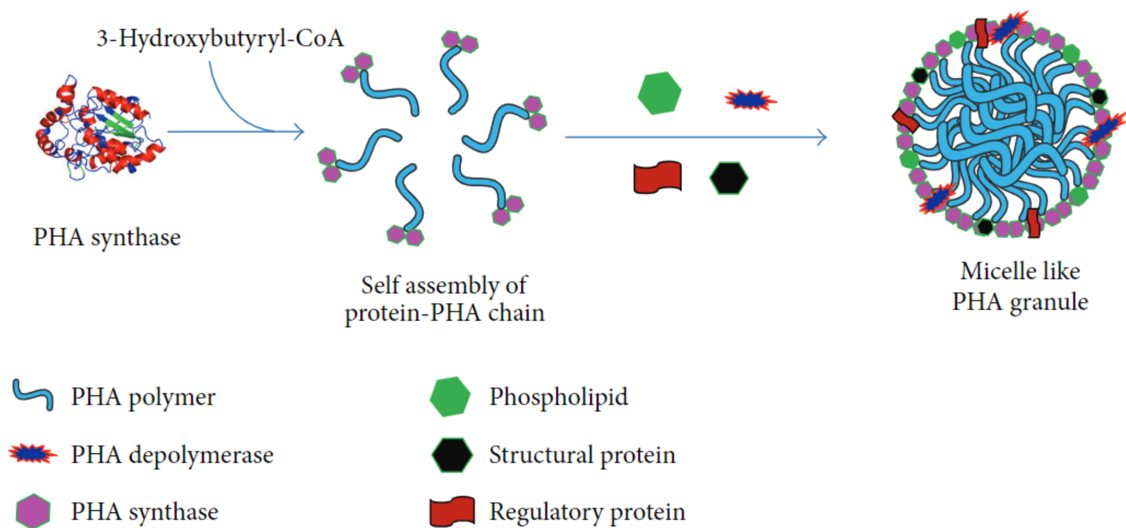

Fig. 2 Formation and structure of PHA granules [5]

PHA can be isolated from biomass and processed into a pure plastic-like form. Thanks to their biodegradability, they accumulate plastic waste in the environment as a promising solution. Because of their physical properties, they are in many respects comparable to conventional plastics produced from petroleum, but unlike them, they are biodegradable, they can be degraded by organisms present in the environment. Another advantage of PHAs is the fact that they can be produced exclusively from renewable sources. Finally, the biocompatibility of PHA should be mentioned, thanks to which their potential presence in the environment does not cause any negative effects on this environment. In addition, due to this property, they are also applicable in the field of medicine. [3–5]

1.1 History

The first mention of PHA dates back to 1926 and was named and described by the French microbiologist Maurice Lemoigne. He was able to isolate and later describe natural polyester granules from Bacillus megaterium cells, which he later named poly-3-hydroxybutyrate (P3HB). The significance of Lemoign's discovery was long overlooked, in large part because oil was cheap at the time and there was a surplus everywhere. The oil crisis in the mid-1970s brought renewed interest in finding alternatives to oil products. Until then, it has been found that P3HB granules act as a carbon and energy store in the cell for periods of adverse conditions. In 1974, other monomer units besides 3HB were identified. P3HB was designed in 1976 by Imperial Chemical Industries as one of the alternatives to replace some petrochemical plastics. The company launched a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer (P(3HBco-3HV) otherwise known as BiopolTM. However, this

product had its disadvantage in the order of magnitude higher price compared to traditional plastics, however, it started a more environmentally friendly production of plastics, as other companies did not have to wait long. When, for example, in 1996, the German company Wella used BiopolTM PHA as a packaging material for its shampoos. [6–8]

Today, PHA are already manufactured by a number of companies under various commercial names, such as Tepha, Ecogen, Mirel and Biocycle. From Czech companies, it is Nafigate corporate, which came to the market with the Hydal concept. Hydal uses waste frying oil as a suitable substrate for microorganisms to produce P(3HB). On the one hand, Nafigate sells P(3HB) production know-how and also produces P(3HB) exfoliating pieces for shower milk, thus preventing microplastics from entering the environment. Her other product is sunscreen, which uses P(3HB) as protection against ultraviolet (UV) radiation. [9,10]

1.2 Structure and composition

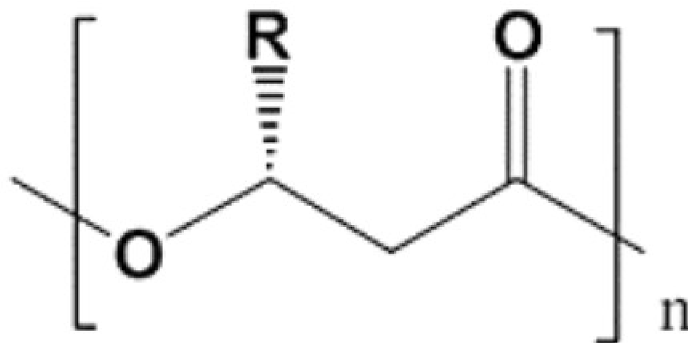

Fig. 3 General structure of polyhydroxyalkanoates [13]

As already mentioned, PHA are polyesters containing various hydroxylated acids as monomeric units. The acids used to build PHA by biological systems are mostly hydroxylated on the third carbon. The chiral carbon in the R-configuration occurs in the monomer structure due to the stereospecificity of the enzymes responsible for polymer synthesis. The general structure of the polymer is shown in Figure 3. The average number of monomer units in a polymer is 1000-30,000 units, but larger polymers can be found. The molecular weight of PHA produced by microorganisms ranges from 2.10^5 to 3.10^6 Da. [3,11]

PHA are classified according to the number of carbons in the monomer molecule:

SCL PHA (short-chain-length) - whose monomer units consist of 3–5 carbon compounds. It is one of the most studied groups of biopolymers of this type. The best known is poly(3-

hydroxybutyrate) (PHB) and its copolymers. Oligomers of 3-hydroxybutyrate play important roles in natural systems – an example is the ion channels formed by a calcium-polyphosphate complex in biological membranes. Chains containing up to 200 monomer units can be found in almost all types of organisms – from microbes to human cells. Polyhydroxybutyrate is produced by various bacterial cells using a wide variety of substrates. [3,11,12]

MCL PHA (medium-chain-lenght) – The second group contain 6–14 carbon atoms. It differs from SCL PHA in its chemical and mechanical properties. The biggest difference against SCL PHA is their low melting point and crystallinity, which limit their usefulness as thermoplastics. [12]

LCL PHA (long chain length PHA) – are formed by hydroxy acids with a carbon chain of more 15 carbon atoms. So far, LCL PHA have only been prepared in vitro and their presence in nature has not yet been observed. [12]

1.3 Physical and chemical properties of carboxylic acid esters

Polyesters are formed by a polycondensation mechanism in which small molecules (usually water) are cleaved. An important requirement is the so-called bifunctionality of the monomers, which means that the monomers must contain at least two functional groups. Hydroxylated carboxylic acids meet these requirements. Another example of polycondensation is the formation of amides from amino acids, in living systems this occurs during the formation of a peptide bond. [14]

Esterification is the reaction of a hydroxyl group with a carboxyl group, whereby one molecule of water is cleaved. It is a nucleophilic reaction taking place by an addition-elimination mechanism. The nucleophilic particle is added to the carbon of the carboxyl group, which occurs due to the detection of the carbon nucleus by more electronegative oxygens. This shifts the π-electrons of the carbon-oxygen bond. By further electron transfer, the carbonyl bond is renewed and the most easily leaving group, i.e. hydroxyl, is released. The most common is Fischer esterification, which is catalyzed by mineral or Lewis acid. [15, 16]

Carboxylic acid esters are susceptible to both basic and acid hydrolysis. Basic catalysis is generally known as saponification. By adding a lower alcohol to the ester in an acidic environment, the original alcohol can be exchanged for a lower one, this reaction is called transesterification and is used in the analysis of PHA obtained from biomass. [15]

1.3.1 Physical properties

From almost all available studies, it follows that the properties of polyhydroxyalkanoates depend mainly on their composition, ie monomers, and also on the molecular weight of the polymer. [15]

A substantial proportion of polyhydroxyalkanoates obtained from bacterial cells can achieve a high degree of crystallinity, which forms a number of its mechanical properties. In general, SCL PHA are crystalline, tensile but brittle. In contrast, MCL PHA are more flexible and elastic but have lower melting points. [17–19]

1.3.1.1 Short-chain-length (SCL) PHA properties

PHA with smaller monomers are generally highly crystalline after extraction from cells. The relative crystallites of SCL PHA range from 55–80%. The most common homopolymer, 12-poly(3-hydroxybutyrate), is similar in mechanical properties to polypropylene, but the relative elongation of polypropylene is significantly greater. The P3HB glass transition temperature is approximately 5 °C. [20]

The advantage of P3HB is its resistance to moisture, optical purity and its piezoelectric properties. On the contrary, the huge disadvantages of this polymer are mainly its low thermal stability – the decomposition temperature is approximately 200 °C, which is very close to their melting point. Another major disadvantage is embrittlement during storage under normal ambient conditions. [20]

Based on these properties, P3HB is subject to high requirements for modifications that would favor its material use. The modification consists in incorporating comonomers so as to preserve the biodegradability and biocompatibility of the polymer. Selected mechanical properties of P3HB and several different heteropolymers are given in table 1. For comparison, the properties of polypropylene are also given in the table 1. [20]

Table 1: Physical and mechanical properties of selected PHA and polypropylene (PP)

Polymer	Tm [°C]	E [GPa]	Ts [MPa]	ε [%]
P(3HB)	178	3	41	6
P(3HB-co-3 mol% 3HV)	169	3	38	-
P(3HB-co-9 mol% 3HV)	161	2	37	-
P(3HB-co-3 mol% 4HB)	167	-	28	44
P(3HB-co-10 mol% 4HB)	160	-	24	241
P(3HB-co-90 mol% 4HB)	51	100	64	1081
P(4HB)	52	150	104	1000
Polypropylene	169	2	35	405

Modifications of P3HB using 3-hydroxyvalerate can be observed in the table 1. With increasing 3HV content, notched toughness increases, which can generally be understood as a reduction in the brittleness of the polymer. As the 4HB content increases, the relative elongation increases, which is expressed as a percentage of the stretching of the polymer until it breaks. [20]

By comparing these modifications with the given properties of polypropylene, an order of magnitude of overcoming this commercial polymer can be observed. However, the P3HB modification also has a negative impact on other properties, such as melting point, in some cases elasticity (Young's modulus) or tensile strength. This significantly predetermines modifications for special applications. [20]

1.3.1.2 Meidum-chain-lenght (MCL) PHA properties

The general properties of MCL PHA result from their low crystallinity. Compared to SCL PHA, these polyesters are characterized by high flexibility and elasticity (large elongation values), and thus low tensile strength, as well as a low glass transition point. The best known monomer composing MCL PHA is 3-hydroxyhexanoate (3HH). Polyesters of these acids usually form blends with SCL PHA of various compositions, which are the subject of research. The higher the MCL content in the blended polymer, the higher the crystallization

temperature of the polymer, but the glass transition temperature decreases. These and other thermal properties and some mechanical properties are listed in table 2. [21]

Table 2: Selected properties of heteropolymers MCL PHA with PHB

Polymer	**Tg [°C]**	**Tc [°C]**	**Tt [°C]**	**E [GPa]**	**TS [MPa]**	**ε [%]**
P(3HB-co-6,4 mol% 3HO)	-1.41	65.2	159	0.18	10.4	54
P(3HB-co-14,7 mol% 3HO)	-2.22	77.7	157	0.03	3.1	111
P(3HB-co-31,3 mol% 3HO)	-6.24	-	-	0.02	1.8	230

Compared to SCL PHA, these polymers are relevantly less flexible and tensile strength, but with increasing amounts of MCL monomers, the relative elongation increases significantly. [22]

1.3.2 Thermal stability of PHA

Another general rule is relatively low thermal stability. Under the action of temperatures close to their melting temperatures, they decompose and this leads to the spontaneous decomposition of the ester bond to form alkenoic acids – carboxylic acids with one double bond. This relatively disadvantageous feature significantly limits the commercial use of natural polyesters. The thermal degradation scheme is shown in Fig 4. [17]

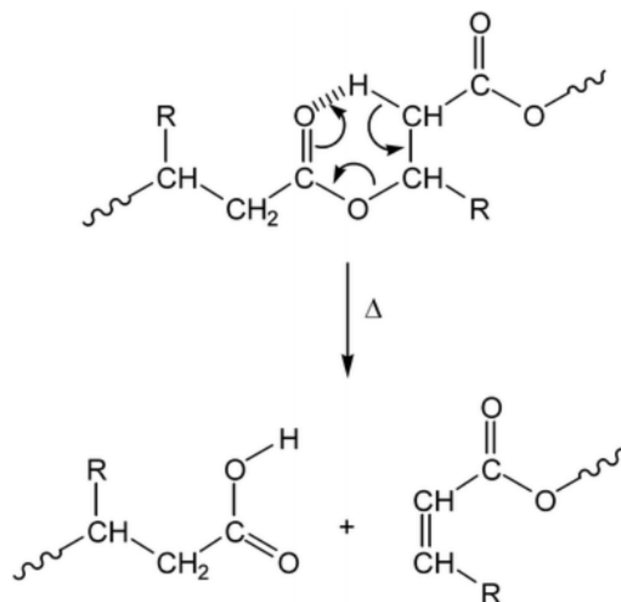

Fig. 4 Thermal degradation of PHA [17]

Modification of the PHB monomers of other hydroxy acids does not significantly alter the thermal stability of the polymer. This can be achieved by modification with polymeric additives, for example carboxylated butadiene-acrylonitrile or biocompatible polyvinylpyrrolidone. The addition of small amounts of said components can achieve a significant improvement in the thermal stability of the polymer, acceleration of crystallization and, in general, a change in the degradation kinetics. However, this can have an adverse effect on biodegradability. [23]

2 POSSIBILITIES OF ACQUISITION AND USE OF PHA

Downstream is an integral part of the biotechnological process and is often more demanding than the biotechnological production of the desired compound alone. It is a process of separating and purifying the compounds produced. The biotechnological product can occur both outside the producer cells and directly inside the cells. This is the second case with PHA. Thus, the phospholipid bilayer that forms the cytoplasmic membrane of all organisms needs to be disrupted in order to be able to separate water insoluble PHA from cells. The following methods are used to downstream PHA.

2.1 Pretreatment of samples

This isolation step is included immediately after the culturing step, where the grown culture is centrifuged and washed. Pretreatment is performed as a single or multi-stage using various methods to facilitate later disruption of the cell membrane. [24]

The basic method of pre-treatment is heating the biomass to a certain temperature. This heating results in denaturation and dissolution of the genetic information and proteins that form the cell wall, which leads to a violation of the membrane stability and prevents an increase in solution viscosity. Denaturation is preferably used to inactivate DNA depolymerase, which is subsequently no longer able to denature the obtained polymer. [25,26]

For example, for the bacterium *Cupriavidus necator* DSM545, it has been reported in the literature that a temperature of 85 °C can be used for pre-treatment for 15 minutes. [25]

Another possible purification is the application of salts - sodium chloride or potassium chloride. However, these salts show lower cell wall disruption efficacy when applied at higher temperatures. In such a culture, the cells are disrupted, but rather by the effect of temperature. [27]

Other possible methods of chemical pre-treatment include the addition of alkaline sodium hydroxide solution, which was used in the pre-treatment of *Allcaligenes latus* cells before subsequent mechanical disruption at room temperature. An effective pretreatment of biomass before mechanical disruption is the addition of 0.01% sodium dodecyl sulfate (SDS), which, like other surfactants, removes lipids. As the concentration of SDS increases, so does the degree of cell membrane disruption. [27,28]

Other physical pre-treatments of the samples include freezing. Lowering the temperature below freezing leads to the formation of crystals in the cell, which gain in volume and are thus able to disrupt the cell. Freezing is also one of the techniques of long-term storage of culture. [24]

2.2 Solvent extraction

The solvent-only method of separating PHA from biomass is one of the longest used. The course of solvent isolation is divided into two phases. In the first step, the permeability of the cytoplasmic membrane of the cells in which PHA occurs is impaired by the solvent, in the second step the polymer is dissolved in the solvent. This principle was first described in 1951 and subsequently in 1967 by two different authors. The first experiments were then performed with Bacillus megaterium and Rhodospirillum rubrum. The solvents used in this case were chloroform, 1,2-dichloroethane, chloromethane and some cyclic compounds. One of these cyclic compounds was propylene carbonate (4-methyl-1,3-dioxolan-2-one). In addition to the use of pure solvents, mixtures such as chloroform/methanol or dichloromethane/ethanol in various ratios have also been used. Isolation, specifically P(3HB), was then carried out by evaporation of the solvent. [24,29,30]

Later, the issue of solvent extraction was investigated using *C. necator* and liquid chlorinated solvents such as chloroethane and chloropropane. The best results have been obtained using solvents which contain at least one chlorine atom and one hydrogen atom in their structure. Another finding was that if 100% polymer yield was not achieved, the purity of the polymer was very high. In addition to chlorinated solvents, the extraction capabilities of various solvents were studied. The results of the study are shown in table 3 [31,32].

Table 3: Extraction results with non-halogenated solvents [32]

	Yield [%]	**Purity [%]**	**Extraction temperature [°C]**
1,2-propanediol	78	99.3	145
Glycerol	86	99.8	125
Diethyl succinate	89	99.9	120
Butyrolactone	89	99.4	120

Another method of isolating PHA was described for the first time in the Procter & Gamble patent. The method consists in mixing biomass with a mixture of solvents. In one of them

the PHA is soluble, in the other the polymer cannot be dissolved. PHA is separated from the biomass by means of a mixed solvent, solid residues of biomass depleted of polymer are removed and in the last step the extracted component precipitates after separation (distillation) of a good solvent from the mixture. Thus, PHA occurs in a suspension of a compound in which it is insoluble. [33]

This method of PHA isolation by precipitation was then used for further experiments. The Austrian company Chemie Linz applied the method to the cells of *Alcaligenes latus* and chose dichloromethane as the solvent. A similar principle was also chosen by Imperial Chemical Industries, located in the United Kingdom. However, the process was declared too expensive due to the finding that the solvent, which already contained 5 wt. % of polymer, was very viscous and the removal of the insoluble portion of depleted biomass was complicated. This would mean that in practice, a 20-fold amount of solvent would be required to separate a certain amount of PHA. The high costs associated with the amount of solvent used can be addressed by recycling. After adding water, the organic solvent can be distilled off from the mixture. [24]

The negative impact of the use of organic solvents can be a violation of the natural morphology of the PHA particles. The particles can be imperfectly crystallized after isolation from the solvent and this can fundamentally affect their properties. However, the advantage is that the solvents do not degrade the polymer (the molecular weight does not decrease) provided that the solvent is used in a short period of time and does not have too high a temperature. Another advantage of using solvents is the elimination of endotoxins that produce gram-negative bacteria. It has been shown that after extraction of P(3HB) with chloroform from the biomass of *Escherichia coli*, the polymer contained a permissible amount of endotoxins, which means that it is easily suitable for medical purposes. [24,34]

Isolation of PHA from biomass using halogenated solvents has significant environmental impacts. Due to the fact, that generally halogenated solvents are most often used on a laboratory scale, they receive a lot of attention. Many halogenated solvents are strong carcinogens and are also volatile substances, which is a very undesirable combination. For this and many other reasons, the initiative to use halogen-free solvents has emerged. For this purpose, aliphatic and cyclic alcohols, esters, amides and ketones whose carbon chain consisted of 4-10 carbon atoms were studied. Some of the non-halogenated solvents have been tested in the past for PHA extraction efficiency, see Table 3. [24,35,36]

2.3 Chemical disintegration methods

Disintegration methods generally work on the principle of decomposition, disruption, or chemical interaction with microbial biomass cells in order to release accumulated PHA from the cells. One method of cell disintegration is chemical. The principle is to add various chemical compounds that are able to react with parts of the biomass cells. Depending on the chemicals used, chemical disintegration involves the following specific methods. [24]

2.3.1 Surfactants

Surfactants (PAL), such as SDS, are able to disrupt cell structure through their incorporation into the phospholipid bilayer of the cytoplasmic membrane. From a structural point of view, PALs are a group of organic substances that already accumulate them at low concentrations at the interfacial interface and, by their action, reduce the energy that is at the junction of two different phases. SDS is then ionic in nature and has an ambivalent character. Sodium dodecyl sulfate contains a long non-polar carbon chain and at the same time a polar sulfate group to which sodium is ionically bonded. These properties can then be used to separate PHA from bacterial cells. Surfactant molecules accumulate at the interfacial interface until the entire interfacial area is completely filled. Once the interphase surface of the cell is fully occupied, the cytoplasmic membrane is disrupted and the cytosol of the cell is mixed with the environment in one phase. After disruption of the membrane, PALs can no longer accumulate at the interface and form aggregates called micelles. The result is a solution in which free PHA is present and the surfactants, in addition to disrupting the membrane, also ensure the dissolution of proteins and other cellular components in addition to PHA itself. [37,38]

Different PALs were used for this method, as well as different methods for their addition to biomass. An experiment was performed with the carnitine ester of palmitic acid, which is a representative of natural surfactants. It is palmitic acid, which is a source of a long non-polar carbon chain to which carnitine is attached by an ester bond, a compound that ensures the transport of acyl-CoA into the mitochondrial matrix during fatty acid metabolism. The disadvantage of using this surfactant is the need to treat the biomass for extraction, the advantage being that these particular PALs do not cause polymer degradation. [39]

Another method tested was the separation of PHA from biomass without treatment of the medium in which the organisms were cultured. In this case, only SDS was added and the mixture was stirred at elevated temperature. The main advantage of this method is the

possibility of performing isolation with large concentrations of biomass in solution. However, the use of PAL does not ensure high PHA purity (below 97 %) and another problem occurs when using a larger dose of PAL. As surfactants increase, the cost of wastewater treatment increases. [24]

2.3.2 Sodium hypochlorite

One of the most widely used chemicals in chemical digestion is hypochlorite, most often sodium, but potassium or calcium can also be used. This PHA extraction method is based on the direct treatment of cells with hypochlorite. It is an oxidizing agent which, at suitable concentrations, is more or less selectively able to degrade all polymer components of cells, with the exception of PHA, which mostly resists the chemical action of hypochlorite and remains in its original form. PHA, which does not dissolve in sodium hypochlorite and remains solid, can then be easily separated by filtration or centrifugation. One of the advantages of the hypochlorite method is that it is not strictly necessary for the cells to be dried before processing, this saves time and energy and thus reduces the overall cost of processing the product. In general, this process is easy to carry out and can be used on a large scale with certain measures. However, care must be taken to produce heat during application, as treatment of biomass with hypochlorite is a strongly exothermic process. The temperature in the reaction vessel must be controlled and a suitable cooling device must also be used. [40]

2.3.3 Chelates

Chelates are compounds used in working with metal cations such as Ca^{2+} and Mg^{2+}. These compounds are able to react with these ions and incorporate them into their structure, which is used, for example, in the production of food supplements with high absorption in the human body. Another use can be chelatometric titration in analytical chemistry, for example for the determination of magnesium ions in water. One of the most commonly used chelating agents is ethylenediaminetetraacetic acid (EDTA). In the context of PHA isolation from biomass, chelates are used, for example, in surfactant extraction. The addition of these compounds increases the speed of the process. The effect is then attributed to the occurrence of Ca^{2+} and Mg^{2+} cations on the outer membrane of some gram-negative bacteria. The chelates react with these cations, which leads to destabilization of the cytoplasmic membrane, which is then more susceptible to, for example, PAL. Although the method is very effective and provides a high-quality product, its main problem is the high production

of wastewater. For this reason, a continuous recycling production process was designed, consisting in the reuse of water with the addition of the required amount of PAL and chelates. Water that can no longer be used is then treated with hydrochloric acid with the simultaneous addition of activated carbon in order to adsorb pollutants. [24,41,42]

2.4 Isolation by enzymes

Another method of digestion is the use of enzymes. Some types of enzymes, such as proteases, nucleases, lysozyme, alkalase, and lipases, have high hydrolytic effects on proteins and other polymers of the bacterial cell mass and initiate cell lysis. Their advantage is high selectivity, when enzymes show high hydrolytic activity against proteins and other polymeric components of cells, but have no or little effect on PHA. The process of PHA regeneration by enzymatic digestion is a relatively complex procedure. It usually consists of steps such as solubilization of cellular components other than PHA, in heat treatment, enzymatic hydrolysis, and then washing with surfactants. The advantages of the method are, in addition to the selectivity of the enzymes, their low consumption and the fact that the necessary operating conditions are generally mild. However, the high cost of enzymes, combined with the fact that regenerated PHA is not so pure and the process itself is very complex, outweighs the benefits. [39,40,43]

2.5 Utilization of cell fragility

For some microorganisms such as *Azotobacter vinelandii*, the possibility of an increase in osmotic pressure during PHA accumulation has been reported. At high osmotic pressure, the cell suspension dilutes and undergoes an osmotic shock that the cell cannot withstand, ruptures and releases PHA granules. [39,44]

2.5.1 Flotation

In general, this is a technological process that uses different wettability (good, bad, selective wetting) of materials to separate them. It finds practical use, for example, in water purification. The hydrophobic surface particles adhere to the air bubbles which entrain them to the water surface. Here they are collected in the form of foam, which is mechanically removed and transported for further processing. Air bubbles do not adhere to particles with a hydrophilic surface, well wetted with water, so they sediment and form flotation waste at the bottom. [45]

There are records of the use of flotation methods in PHA isolation, mainly flotation in dissolved air. It was found that the isoelectric points of PHA granules and cell debris are approximately the same at a certain pH. Thus, after the granules were released, selective aggregation was performed first, followed by selective flotation controlled by interactions between particles, bubbles and hydromechanics. Using such successive steps, a relatively pure PHA was obtained. [39,40,46]

2.6 Mechanical disturbance

2.6.1 Pearl Mill

The bead mill insulates the PHA on the principle of solid phase shear stress. This means that the bead mill is in direct contact with the cell suspension containing PHA and releases the polymer from them due to mechanical damage. The mill itself consists of a vertical cylindrical grinding chamber with small steel or glass balls, in the center of which there is a cylindrical rotor with adjustable speed. The cell suspension is fed to the mill, the biomass cells undergo mechanical disruption and exit through an opening on the other side of the cylinder. During this mechanical intervention, the mill is heated due to friction, which is solved by water cooling by means of a cooling jacket around the chamber through which the cooling water can flow. The concentration of the input cell suspension must be chosen in the range of 8-66 kg of dry matter per 1 m3 of suspension. It was also found experimentally that the diameter of the balls in the grinding chamber does not speed up the process, but significantly affects the capacity of the chamber. According to the results of the experiment, eight passes of one particular suspension under specific conditions were needed to completely disrupt all cells: [47,48]

• 51 800 rpm

• 84% filling of the grinding chamber

• 514 μm balls

• 90 ml per minute of suspension flow

2.6.2 High pressure homogenization

The principle of high-pressure homogenization is based on the transition of the culture medium from a high-pressure space to a low-pressure space. As a result of cavitation, the cells break down. In order for cell disintegration to be effective, it is necessary to control the

operating pressure, the temperature of the suspension and the number of passes through the homogenizer. One pass is usually not enough and the process needs to be repeated. Another problem may be the release of DNA from the nuclei and the associated increase in viscosity. Other parameters that affect cell disintegration are microbial physiological parameters, more precisely the type and growth phase of microorganisms, as well as cell concentration. High pressure homogenization is better at lower biomass concentrations. Cell lysis is usually easier for gram-negative bacteria than for gram-positive ones. Disadvantages include the possibility of thermal degradation of products and the formation of fine cell debris, which make further processing of PHA more difficult. [39,43]

2.6.3 Ultrasound disruption – sonication

This method works on the principle of cell wall disruption using ultrasound. The method is based on a theoretical model that combines cell survival, acoustic power, disruption magnitude index, and sonication operation time. Purification by P3HB was achieved purely by sonication and subsequent centrifugation in *Haloferax mediterranei* in greater than 98% purity and in 80% yield. [24,49]

2.7 Supercritical fluid extraction

Supercritical fluids have unique physicochemical properties that are very suitable for extraction purposes. By supercritical fluid we mean a compound that can no longer be liquefied by a change in pressure or temperature. Such a phase is then called supercritical fluid, which has the following properties:

• Compressibility - density can be changed

• Order of magnitude lower viscosity than liquids

• Absence of surface tension [50,51]

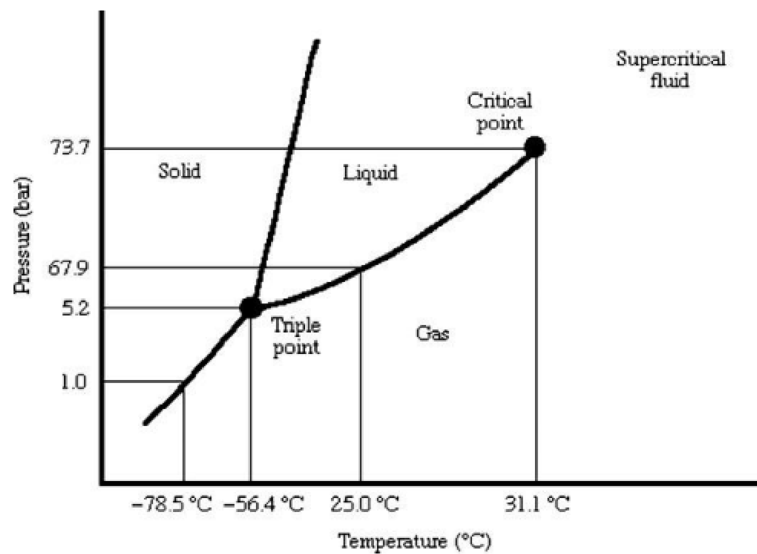

Fig. 5 Phase diagram of carbon dioxide [51]

Carbon dioxide (CO_2) is most often used for SFE. The main advantages are low toxicity and reactivity, easy conditions for supercritical fluid transition (31 °C and 7.5 MPa), wide availability, low cost and non-flammability. The SFE process takes place in the following steps:

1) CO_2 in the liquid compressed state is removed by means of a pump from the storage vessel.

2) It is then compressed to supercritical pressure (still in a liquid state).

3) Proceeds to the extraction chamber, which is equipped with a thermostat - the temperature rises to supercritical.

4) The injection of the substance that is extracted is usually carried out in countercurrent extraction agent.

5) The extract continues from the extraction chamber to the capture solvent via a capillary (restrictor). This leads to a pressure gradient of the extract and it remains in the supercritical fluid phase.

6) The collection chamber with solvent is already exposed to atmospheric pressure - CO_2 is evaporated and then sucked in by the pump and liquefied again, the extract is collected in a collection tank [50,51].

Supercritical fluid extraction, which uses carbon dioxide as an extractant, then has the following specifics:

• Extraction conditions must be precisely optimized.

• Use of co-solvents - modifier. (CO_2 is a completely non-polar extractant, a modifier such as methanol, acetonitrile, water is used to extract polar compounds)

• The water content of the extracted matrix affects the extraction efficiency.

• Aggregates (lumps) may form during extraction.

• Sample preparation for extraction is essential - particle crushing to 10 –50 μm is ideal.

• Extraction efficiency is higher at temperatures up to 200 °C. [50,51]

In addition to isolating intracellular proteins, SFE can also be used in PHA extraction. A study of this method of separation of specifically P(3HB) from *Cupriavidus necator* was performed. The optimal process conditions were then designed as follows: process lasting 100 minutes, pressure 20 MPa, temperature 40 °C, methanol modifier (due to higher polarity of PHA), CO_2 as extractant. The yield of this process was 89%. The experiment was further the subject of further studies that focused on the use of NaOH and NaCl as compounds for the SFE preparatory step to disrupt cell walls. [52,53]

2.8 Air sorting

The method was developed by Procter & Gamble for the isolation of PHA from *Cupriavidus necator*. The process begins on cells by exposure to an ultrasonic sonicator to form a suspension of polymer granules. The suspension is then lyophilized and pulverized using a fluid bed mill. The ground sample is then air sorted to produce a 38% fine fraction and a 62% coarse fraction. The fine fraction was then subjected to extraction with chloroform and subsequent precipitation with methanol. The isolated particles reach a purity of approximately 95% with a yield higher than 85%. [54]

2.9 Use of PHA

Until a few years ago, investors saw the potential of PHA in the field of packaging technology. However, today the use of PHA focuses mainly on medicine, or application in agriculture. The goal is to look for sales with the greatest possible added value. High molecular weight polymers can then find application in fisheries for high-strength net fibers. [9]

PHAs are considered a suitable alternative to petrochemical polymers especially for the following properties:

• Thermoplasticity

• Biocompatibility

• Biodegradability

• Compostability

Taking into account the properties, PHA has the greatest potential in biomedicine. In particular, copolymer P(3HB-co-3HV) is a research target for use in this field. A study of hybrid blends of PHA copolymers with collagen was performed to create a potential product for tissue engineering. In order to improve properties that would meet the biological requirements of human tissue, PHA are also enriched with components other than the collagen already mentioned. Other ingredients may be hydroxyapatite (HA), gelatin or silk. The use of ceramic nanoparticles together with PHA then has the potential for applications in bone tissue. Said hydroxyapatite mixtures have been studied in connection with cell proliferation and differentiation in vitro. A mixture of P(3HB-co-3HV) and hydroxyapatite served as a substrate for mesenchymal stem cells (MSCs). The results of the experiment showed that MSC proliferation performed better on the random copolymer P(3HB co-3HV), which did not contain HA admixture. At the same time, it was shown that after one to two weeks of MSC development, osteoprogenitor markers such as alkaline phosphatase (ALP) and osteocalcin (OCN) began to appear in the mixture, which demonstrated the initiation of MSC differentiation into bone cells – osteoblasts. Based on these results, P(3HB-co-3HV)/HA nanofibers were implanted into a rabbit suffering from defective bones. There was a significant improvement in the critical bone disorders of the rabbit studied. [55–59]

PHA applications can be as follows:

• Materials industry – Packaging materials, everyday consumables, smart materials (memory gel), hygiene products

• Medical purposes – Bio-implants, orthopedic supplies, drug carriers, tissue engineering materials, nutritional supplements

• Fuel production – biofuel additives

• Industrial microbiology – PHA synthesis in microorganisms as a metabolic regulator [9]

3 CHARACTERISTICS OF MICROORGANISMS OCCURRING IN PHA CREATION

For the biological production of PHA, the choice of microorganism, as the producer of PHA, is the most important factor, which depends not only on the synthesis of PHA but also on the chemical structure and properties, which are essential for its subsequent use across the entire taxonomy. [60]

Today, more than 75 genera of microbial organisms are associated with production, including *Acinetobacte, Azobacter, Bacillus, Bulkholderia, Klebsiella, Pseudomonas, Marinobacter, Rhizobium* and many others. Of course, a potential producer must meet many preconditions, which undoubtedly include simple gene manipulation. Related to this assumption is that recent advances in systems and synthetic biology allow, with the help of gene modifications, to construct an extremely efficient PHA-producing strain. A very desirable feature is the wider pH and temperature range at which producers can grow faster. An essential criterion is that the selected microorganism does not show pathogenicity and production of toxic substances. [60]

Bacteria are generally distinguished into gram-positive and gram-negative, with both groups able to accumulate PHA, with some studies stating that gram-positive bacteria are more resistant to extreme conditions than gram-negative bacteria and are thus considered better candidates for PHA production. Another advantage of gram-positive bacteria is the ability to synthesize PHA copolymers from inexpensive carbon sources such as molasses, whey and activated sludge, while gram-negative bacteria predominantly require structurally related carbon substrates such as fatty acids, which are, however, expensive. Gram-positive bacteria are also suitable candidates to produce PHA applicable in medical applications in terms of the absence of lipopolysaccharides (endotoxins) in the PHA produced, which in contrast are found in the outer membrane of gram-negative bacteria and release an immunogenic response. The composition of the outer membrane of gram-positive bacteria thus facilitates, among other things, the extraction of PHA from cells. Gram-positive bacterial producers PHA is mainly represented by a diverse genus of *Bacillus* bacteria. However, their current disadvantage is sporulation under adverse conditions. However, gram-negative bacteria such as *Cupriavidus necator* and recombinant *Escherichia coli* are currently used for PHA production because they show faster and better growth and higher accumulation of PHA compared to gram-positive bacteria. Although some gram-positive bacteria from the genus *Bacillus* have accumulated PHA approaching 90% of the dry weight of cells. [61–64]

3.1 Mesophilic microorganisms

Mesophilic microorganisms are the most common type because they have optimal conditions for growth at normal temperatures (15–45 °C). This group has the most PHA producers. The use of these microorganisms is very widespread at present. However, due to their susceptibility to contamination, the cost of producing PHA is increasing because sterilization of bioreactors is necessary to prevent contamination by other mesophilic microorganisms. [65]

One of the most commonly used bacteria is *Cupriavidus necator*. It is a gram-negative soil bacterium. It grows most often on a fructose substrate. The PHA content can reach up to 80% cell dry matter. Another widely used bacterium is the gram-negative *Pseudomonas oleovorans*, which has the shape of a rod. It can use alkanes and alcohols as a carbon source. Produced by MCL PHA. It uses a pathway associated with β-oxidation and is able to produce PHA from oils or fatty acids. *Alcaligenes latus*, a gram-negative, rod-shaped aerobic bacterium, is also used. It is a suitable bacterium for the production of SCL PHA, especially for the PHB homopolymer. It can accumulate PHB under favorable conditions up to 60% in dry matter. It collects the polymer during the growth phase, which significantly shortens the fermentation process. The mutant strain of *Azotobacter vinelandii* is a gram-negative aerobic bacterium in the shape of a short rod to a *cocobacillus*. *A. vinelandii* is a suitable candidate for PHA production because it can reach a PHA content of up to 75% dry matter. Produces PHB polymer. [20,66,67]

3.2 Thermophilic microorganisms

These are microorganism that require temperatures higher than 45 °C for their optimal growth. Microorganisms that have a growth maximum and temperature optimum at 80 °C and higher are called hyperthermophils. These microorganisms use thermostable enzymes whose optimal temperature for functioning is 70 °C and more, in some cases even 110 °C. Isolation and purification of these enzymes is less demanding compared to the isolation and purification of enzymes of mesophilic microorganisms, due to their thermostability and resistance to denaturing agents. Thermostable enzymes are then used in molecular biology, biochemical and biotechnological processes. Molecular biology uses thermostable DNA polymerase from the bacterium Thermus aquaticus, which enabled the development of the PCR (polymer chain reaction) method. In biochemistry, a thermostable protease from the bacterial strain *Thermus* is used for DNA and RNA purification processes. Biotechnology

uses thermostable α-amylase for starch liquefaction, obtained in most cases *from Bacillus licheniformis* and *B. stearothermophilus*. B-amylase from *Thermus thermosulfurigenes or T. maritima* is also used for starch saccharification, depending on the process temperature. Two types of thermophiles can be found in nature - acidophilic and neutrophilic, or slightly alkalophilic. Acidophilic thermophiles require a pH in the range of 1-3, depending on the type. Neutrophilic thermophiles require a pH of 7–9. [68,69]

Cellular metabolism generates thermal energy along with agitation. If thermophiles were used for cultivation, intensive cooling would not be necessary as with other microorganisms, which would reduce water and energy consumption. This fact would lead to a reduction in the production price. At the same time, elevated temperature would prevent contamination with mesophilic bacteria. [70]

The *Tepidimonas taiwanensis* strain was isolated from a hot spring in southern Taiwan. These are gram-negative sticks. This bacterium has optimal conditions for growth at 55 °C and neutral pH. [71]

3.3 Halophilic microorganisms

Halophiles are microorganisms that need a high concentration of salt in the medium for their growth. Their representatives can be found in the domains of Eukarya, Archaea and Bacteria. Halophilic microorganisms can be found in places with very high salt concentrations, such as salt lakes, salt pans or swamps. Depending on how high a salt concentration these microorganisms need, we divide them into halotolerant, mild, borderline extreme and extreme halophiles. *Halotolerants* do not necessarily require salt for growth, but they also grow in high salt concentrations. Slightly halophilic microorganisms grow at a salt concentration of 0.5−2 M. For borderline extreme halophiles, the best salt concentration is from 1.5 to 4.0 M. Extreme halophilic microorganisms have an optimal concentration between 2.5−5.2 M. [72]

The use of these organisms would reduce the overall cost of PHA production, because only halophilic organisms grow in high-salt culture media, so the sterility of the process would not have to place so much emphasis. The pretreatment of the substrate is usually carried out by acid hydrolysis with HCl. The subsequent acidic pH is most often adjusted with NaOH, which produces a certain amount of NaCl, which contributes to the salinity of the culture medium. [70]

Haloferax mediterranei is currently the best producer of PHA in the *Halobacteriaceae* strain. This is a representative of the Archaea domain, which was isolated from ponds near the Mediterranean Sea on the Spanish coast. Research has shown that *H. mediterranei* can accumulate up to 65% of the cell's PHA weight when grown on a substrate containing starch or glucose. Using continuous culture, the PHA content was about 46% of the cell weight, but this production is only possible if the PHA is synthesized during cell growth. Most microorganisms synthesizing PHA synthesize it in the stationary phase. *H. mediterranei* was found to be able to produce a poly(3-hydroxybutyrate-co-hydroxyvalerate) copolymer. The salt concentration in the culture medium must be higher than 22% to achieve optimal growth and production conditions. [73]

3.4 Psychrotrophic and psychrophilic bacteria

Psychrophilic microorganisms are those that like a cold environment. Ideal conditions for growth are from 15 °C below. The maximum growth temperature is around 20 °C. Psychrotrophic are cold tolerant, so they have the ability to grow at temperatures between 15 °C and 20 °C. There are large areas on earth where temperatures do not reach more than 5 °C (polar region, permafrost regions, deep oceans). In these areas, they have to cope not only with low temperatures, but also with other unnatural conditions. Microorganisms living in the deep oceans must adapt not only to low temperatures but also to high pressures. In the polar regions, microorganisms are exposed to nutrient deficiencies and increased UV radiation. These microorganisms are adapted to these conditions and are therefore able to survive longer in cryobiosis. Those bacterias are able to produce extracellular hydrolytic enzymes. These enzymes are interesting in terms of reducing energy consumption. They produce lipase enzymes, aspartate trans-carbamylase, malate dehydrogenase, which are effective at low temperatures. These enzymes could be used, for example, in the food industry. [74,75]

4 INFLUENCE OF SUBSTRATES ON PHA CREATION

There is considerable interest in the world to find suitable and at the same time cheap carbon substrates for PHA production, as the price of the substrate currently represents about 50% of the total cost of PHA production. Therefore, waste materials from agriculture and the food industry are becoming the subject of research as carbon and nitrogen sources for microbes producing PHA. The use of agricultural waste for PHA production is proving crucial for reducing the production costs of biopolymers and at the same time plays an important role in waste management. [76,78]

It is assumed that finding a suitable and renewable carbon source for PHA production will reduce production costs by up to the mentioned 40–50%. Various inexpensive substrates are currently used, such as whey, melon, corn steep liquor, starch wastewater and many others. Nevertheless, there is a continuing search for new substrates that could prove more suitable for PHA production. Table 4 shows the world market prices of substrates in 2010 and the possible theoretical profit P(3HB) from these substrates. [76,78]

Table 4: World market prices of substrates in 2010 and theoretical profit of PHB from individual substrates [76]

Substrate	Price per kg [CZK]	Gain of PHB [%]	Substrate price per kg PHA [CZK]
Sucrose	9.10	39	22.62
Glucose	10.66	39	27.82
Ethanol	8.06	51	16.38
Cane molasses	2.60	42	6.24
Soy oil	20.54	70	34.06
Palm oil	23.92	65	31.72

The choice of a suitable medium is important not only in terms of ensuring optimal conditions for the production of different types of PHA using different types of bacteria, but especially in terms of volume production of a given polymer that will be economically competitive for traditional plastics. The choice of media depends on several factors. Whether the microorganism is recombinant or not and whether it needs nutritional and nutrient limiting conditions. Another factor in choosing a suitable medium is whether we want to produce homopolymers or copolymers. This is because there are a large number of

homopolymers and copolymers that contain more than 100 monomers and have molecular weights in the range of 50,000–1,000,000 Da. [77]

Table 5 indicates the basic carbon source that is usable for microbial PHA production. In addition to the basic carbon source, the waste substrates also contain secondary carbon substrates, which can serve as precursors of monomer units. Another advantage is the frequent presence of nitrogenous substances and other compounds that can promote cell growth and PHA formation. Their presence often saves money. Cultivation of bacterial biomass on waste substrates leads to the production of the desired polymer, but the obtained PHA concentration is lower (around 65%) than when culturing on pure substrates, where the PHA concentration reaches up to 80% of biomass in fermenter cultures. However, this problem could be solved by working with recombinant strains of bacteria, where genes for the enzyme equipment necessary for PHA production are inserted into the genome of the bacterium, most often *Escherichia coli*. [79-81]

Table 5: Overview of waste substrates broken down by the basic component of the carbon source [80]

Substrate type	**Cheap waste substrates**
Carbohydrates	Meolasses Starch and its hydrolysates Lactose in whey Cellulose hydrolysates from the paper industry
Fats and oils (fatty acids)	Wastes from biodiesel production (methanol)
Alcohols	Vegetable and animal waste
Organic acids	Lactic acid from diary

4.1 Utilization of waste substrates containing carbohydrates

An important waste saccharide substrate is whey arising as a waste product in the production of dairy cheese. Whey makes up 80–90% of the milk used for production. The disadvantage of this substrate is the low solubility of lactose (210 g/L) compared to glucose (700 g/L), therefore for fermentation it is necessary to perform on a relatively large volume of wastewater. [82,83]

A whey PHB production study showed that this waste product could be utilized by recombinant *Escherichia coli* CGSC 4401, which is able to utilize lactose, but the genes for enzymes involved in PHA synthesis were inserted from *C. necator*. Biomass containing up to 81% PHB (5.2 g/L) in biomass can be obtained by this cultivation. PHA production on lactose substrates can also be achieved by preparing recombinant *C. necator*, which has the LacZ, Lac1 and Lac0 genes for *E. coli* bacterial lactose utilization in place of the PHA depolymerase gene. This genetic modification has a higher yield due to the removal of depolymerizing enzymes from the cell. [84]

Another sugar substrate that can be used for PHA production is xylose contained in lignocellulosic waste substrates from agriculture. Xylose is a suitable substrate for *Pseudomonas cepacia*, but PHA production is relatively low. A recombinant *E. coli* strain with a gene isolated from *C. necator* is currently used. Biomass containing up to 35.8% PHA was obtained by this fermentation. Significantly higher results were achieved by enriching the medium with a nitrogen source (soybean hydrolyzate), where the obtained polymer contained up to 74% biomass. [85]

Molasses is an important food waste product. For example, soy molasses produced during the processing of soybeans. At present, it has no significant industrial use because the oligosaccharides present in it are only partially digestible for animals and indigestible for humans. However, soy molasses contains 30% fermentable carbohydrates, of which raffinose, sucrose and stachyose are the most represented. [86,87]

It is already used today in fermentation processes involving, for example, the synthesis of lactic acid by the bacteria *Lactobacillus salivarius* or the production of butanol by *Clostridium beijerinckii*. For PHA production, MCL PHA-producing *Pseudomonas corrugata* was inoculated on soy molasses. *P. corrugata* utilizes only sucrose and high biomass concentrations (3.2–3.6 g CDW/L) can be achieved using 5% CDW soy molasses, but the PHA concentration is very low (5–17% CDW). [86]

In a later study, bacteria were isolated from soybean soil and sediments in the United States and were included in the genus *Bacillus sp.* CL1. This strain is able to utilize raffinose and form up to 90% of PHA in biomass. Equally high PHA production is observed for other carbohydrate substrates (glucose, fructose, sucrose, galactose, stachyose). [87]

Another waste product is waste containing a high starch content. A two-stage utilization was proposed for this waste product, where in the first step the starch was converted by fermentation into organic acids - acetic acid (60–80%), propionic acid (10–30%) and butanoic acid (5–40%), which resulted in conversion of up to 43 % starch to organic acids. In the second step, *C. necator* was cultured on the obtained organic acid solution, which resulted in the production of copolymer P(3HB-co-3HV). The total PHA concentration was 55 g relative to 100 g total carbon concentration. [88]

The production of maple syrup in Canada also brings with it a large amount of wastewater containing mainly sucrose. This can be advantageously used as an important substrate for the bacterium *C. necator*, which is able to produce biomass with 77.6% of the desired polymer, in this case P(3HB). [89]

4.2 Utilization of oil-based waste substrates

Fats and oils are an excellent and inexpensive source of carbon for the growth of *C. necator* bacteria, in the removal of which the bacteria may contain 80% of the polymer content in the bacterial cells. For example, in culturing *C. nector* in olive oil, polymer P(3HB) with a molecular weight in the range of 200–400 kDa was used. [90,91]

The disadvantage of culturing on *C. necator* oils is the synthesis of a homopolymer, but if the genome is recombinantly modified, this bacterium is able to produce copolymers that have better physical and chemical properties. Enzymes for the production of copolymer P(3HB-co-HH) can be obtained by gene expression of PHA synthase of the bacterium *Aeromonas caviae* vector inserted into *C. necator,* where the 3-hydroxyhexanoate monomer represents 4–5 mol % of polymer and the total polymer content in biomass reaches up to 80%, especially when cultivated in soybean oil. [91-93]

Pseudomanas aeruginosa is a motile, gram-negative rod-shaped bacterium that is able to utilize a variety of substrates, especially fatty acids. High production of PHA copolymer (54.5% CDW) was achieved in its cultivation on waste technical oleic acid (oleic acid content up to 80.7%), even greater gain of PHA of 66.1% was obtained in cultivation on waste free fatty acids, while cultivation on glucose reached only 16.8% of the biomass content. Waste frying oil from food plants was also used to advantage. [94]

By-products and waste substrates for palm oil production have very promising properties for PHA production and *C. necator* - H16 was chosen as the best bacterial strain for production. The same bacterium is able to utilize the carbon source from soybean oil, while the obtained biomass of the culture grown in the fermenter at a concentration of 118–126 g/L contained 72–76% PHB. [90,76]

Cultivation of *Pseudomonas sp*. DR2 in corn oil led to the production of PHA making up 37.34% of the biomass. This polymer is composed of 3-hydroxyoctanoate, 3-hydroxydecane and 3-hydroxydodecane monomer. [76]

4.3 Utilization of waste substrates containing alcohols

Not only waste substrates from food production can be used for microbial production of PHA, but also waste products from biodiesel production. Biomass with a concentration of 68.8 g/L was obtained by culturing the bacterial strain *Cupriavidus necator* DSM 545 using waste glycerol from biodiesel production, of which 38% of the biomass was formed by a polymer with a molecular weight ranging from $7.9 \cdot 10^5$ to $9{,}6 \cdot 10^5$. However, the disadvantage of this substrate is the high content of sodium and other impurities, which significantly shorten the production phase of PHB. [76, 95]

Compared to culturing on pure glycerol, only half of the possible PHA was obtained. However, if the nitrogen concentration and cultivation time are adjusted, an increase in productivity of up to 30% can be observed and thus a biomass with a polymer content of up to 50% can be obtained. [96]

4.4 Utilization of waste substrates based on organic acids

POME (colloidal suspension) was used for the production of PHA, which is an aqueous solution that is formed in large volumes during the grinding of palm fruits. Although it is an oily substrate, it is first converted by anaerobic fermentation to volatile organic acids – acetic, butyric and propionic in a ratio of 3:1:1, which are then used to produce a copolymer of PHA. POME is a colloidal suspension containing 95–96.5% water, 0.6–0.7% oil and 4–5% solids. [97]

Other authors proposed the production of PHA from starchy wastewater, which was subjected to two steps. First, the waste was decomposed into organic acids, which were then filtered into a bioreactor, where *C. necator* was cultured. The obtained polymer represented 34.1 % of biomass, while the concentration of biomass reached 1.2 g/L [96].

II. ANALYSIS

5 AIM OF THE STUDY

The aim of this study was to phenotypically identify (Nile Blue A and Sudan Black stainig) the PHA producer and to investigate the effect of various PHA detection mediums on PHA production in individual bacterial strains with subsequent detection using the FTIR method.

6 USED MATERIALS

6.1 Tested bacterial strains

Pseudomonas Mendocina, *Cupriavidus necator* and *Escherichia coli* strains were selected for testing the most suitable soils for PHA production. Then the best soil for phenotype identification of PHA detection was found. The following microorganisms were cultured on these soils to monitor PHA production. Temperature of cultivations, soil, relation to oxygen and the origin is shown on the table below in table 6.

Cupriavidus neactor CCM 3726, *Pseudomonas mendocina*, *Pseudomonas aeruginosa* CCM 3955 were obtained from the Czech Collection of Microorganisms (CCM). In addition, garlic-derived isolate D117, a chilli isolate D68 identified as *Pseudomonas putida*, was used.

The remaining 52 microbial strains were obtained from various fruit and vegetable varieties, which were identified by laser desorption and ionization mass spectrometry using a flow-through analyzer (MALDI-TOF). Furthermore, gene screening was performed on 20 strains of *Escherichia coli* provided by Mgr. Magda Janalíková, Ph.D. All strains used with culture conditions are listed in table 6.

Table 6: Used microbial strains with their culture conditions

Cultural designation	**Microbial strain**	**Origin**	**Temperature of cultivation [°C]**	**Soil**	**Relation to oxygen**
CCM 3590	*Pseudomonas mendocina*	-	37	BHI	aerobic
CCM 3726	*Cupriavidus neactor*	-	37	BHI	aerobic
CCM 3955	*Pseudomonas aeruginosa*	-	37	BHI	aerobic
GK CIP 5/1	*Stenotrophomonas maltophilia*	Dairy products	30	BHI	aerobic
DH5α	*Escherichia coli*	-	37	BHI	aerobic

Cultural designation	**Microbial strain**	**Origin**	**Temperature of cultivation [°C]**	**Soil**	**Relation to oxygen**
D10	*Pantoea agglomerans*	Cucumber	37	BHI	aerobic
D11	*Bacillus amyloliquefaciens* ssp. *amyloliquefaciens*	Onion	37	BHI	aerobic
D13	*Pantoea agglomerans*	Carrot	37	BHI	aerobic
D16	*Pseudomonas koreensis*	Pepper	37	BHI	aerobic
D17	*Pantoea agglomerans*	Cucumber	37	BHI	aerobic
D23	*Lysinibacillus xylanilyticus*	Kohlrabi	37	BHI	aerobic
D27	*Pantoea agglomerans*	Cucumber	30	M17	aerobic
D28	*Pseudomonas koreensis*	Kohlrabi	30	M17	aerobic
D28b	*Pantoea agglomerans*	Kohlrabi	30	M17	aerobic
D31	*Bacillus simplex*	Lettuce	30	BHI	anaerobic
D32	*Bacillus megaterium*	Lettuce	30	BHI	anaerobic
D36	*Acinetobacter calcoaceticus*	Kale	37	BHI	aerobic

Cultural designation	**Microbial strain**	**Origin**	**Temperature of cultivation [°C]**	**Soil**	**Relation to oxygen**
D37	*Pseudomonas oryzihabitans*	Lettuce	37	BHI	aerobic
D38	*Pseudomonas brassicacearum*	Letuce	37	BHI	aerobic
D47	*Acinetobacter calcoaceticus*	Cabbage	30	BHI	aerobic
D52	*Lelliottia amnigena*	Cabbage	30	BHI	aerobic
D55	*Acinetobacter calcoaceticus*	Cabbage	30	M17	aerobic
D61	*Oceanobacillus caeni*	Lettuce	37	BHI	aerobic
D68	*Pseudomonas putida*	Chilli pepper	37	BHI	aerobic
D69	*Citrobacter braakii*	Eggplant	37	BHI	aerobic
D78	*Bacillus amyloliquefaciens* ssp. *plantarum*	Pepper	30	M17	aerobic
D80	*Bacillus cereus*	Tomato	30	M17	aerobic
D93	*Pseudomonas fragi*	Carrot	6.5	BHI	aerobic
D100	*Pseudomonas corrugata*	Cucumber	30	BHI	aerobic
D102	*Pseudomonas antarctica*	Garlic	30	BHI	aerobic
D105	*Oceanobacillus kimchii*	Red onion	37	BHI	aerobic

Cultural designation	**Microbial strain**	**Origin**	**Temperature of cultivation [°C]**	**Soil**	**Relation to oxygen**
D109	*Pantoea agglomerans*	Garlic	37	BHI	aerobic
D111	*Staphylococcus*sp.	Cucumber	37	BHI	aerobic
D116	*Bacillus*	Red onion	30	BHI	anaerobic
D117	*Bacillus megaterium*	Garlic	30	BHI	anaerobic
D124	*Pseudomonas marginalis*	Garlic	30	M17	aerobic
D140	*Oceanobacillus*	Celery	37	BHI	aerobic
D146	*Pseudomonas putida*	Beet	30	BHI	aerobic
D165	*Acinetobacter calcoaceticus*	lettuce	37	BHI	aerobic
D171	*Acinetobacter calcoaceticus*	Lettuce	37	BHI	aerobic
D173	*Acinetobacter calcoaceticus*	Lettuce	30	BHI	aerobic
D180	*Pseudomonas gessardii*	Peach	30	BHI	aerobic
D183	*Pseudomonas frederiksbergensis*	Peach	30	BHI	aerobic
D186	*Pseudomonas veronii*	Apple	30	M17	aerobic
D199	*Bacillus megaterium*	Raddish	30	BHI	anaerobic

Cultural designation	Microbial strain	Origin	Temperature of cultivation [°C]	Soil	Relation to oxygen
D201	*Bacillus megaterium*	Raddish	30	BHI	anaerobic
D212	*Klebsiella oxytoca*	Raddish	37	BHI	aerobic
D214	*Pseudomonas extremorientalis*	Raddish	30	BHI	aerobic
D218	*Rahnella aquatilis*	Leek	30	BHI	aerobic
D220	*Pseudomonas chlororaphis*	Dill	30	M17	aerobic
D221	*Stenotrophomonas* sp.	Raddish	30	M17	aerobic
D223	*Kosakonia cowanii*	Tomato	30	M17	aerobic
D228	*Pseudomonas tolaasii*	Leek	30	BHI	aerobic
D233	*Pseudomonas rhodesiae*	Raddish	6.5	BHI	aerobic
D234	*Guehomyces pullulans*	Peach	6.5	BHI	aerobic
D237	*Pseudomonas frederiksbergensis*	Raddish	6.5	BHI	aerobic
D238	*Pseudomonas libanensis*	Raddish	6.5	BHI	aerobic
D239	*Pseudomonas fluorescens*	Dill	6.5	BHI	aerobic
114	*Escherichia coli*	Chicken	37	BHI	aerobic
119	*Escherichia coli*	Chicken	37	BHI	aerobic

Cultural designation	**Microbial strain**	**Origin**	**Temperature of cultivation [°C]**	**Soil**	**Relation to oxygen**
123	*Escherichia coli*	Chicken	37	BHI	aerobic
126	*Escherichia coli*	Chicken	37	BHI	aerobic
F10	*Escherichia coli*	Mungo seeds	37	BHI	aerobic
F53	*Escherichia coli*	Mungo seeds	37	BHI	aerobic
F77	*Escherichia coli*	Spring onion	37	BHI	aerobic
F108	*Escherichia coli*	Zucchini	37	BHI	aerobic
I/2	*Escherichia coli*	Pheasant	37	BHI	aerobic
I/3a	*Escherichia coli*	Pheasant	37	BHI	aerobic
I/6a	*Escherichia coli*	Pheasant	37	BHI	aerobic
II/2	*Escherichia coli*	Pheasant	37	BHI	aerobic
S7	*Escherichia coli*	Duck	37	BHI	aerobic
S10	*Escherichia coli*	Duck	37	BHI	aerobic
S17	*Escherichia coli*	Pheasant	37	BHI	aerobic
S23	*Escherichia coli*	Pheasant	37	BHI	aerobic
S32	*Escherichia coli*	Pheasant	37	BHI	aerobic
S33	*Escherichia coli*	Pheasant	37	BHI	aerobic
S34	*Escherichia coli*	Pheasant	37	BHI	aerobic
S35	*Escherichia coli*	Pheasant	37	BHI	aerobic

6.2 Used culture and detection media

Brain-heart infusion (BHI, Himedia, Bombaj, India) and M17 solid medium (Himedia, Bombaj, India) were used for cultivations of bacterial strains mentioned in table 6.

BHI solid soil at 25 °C has a final pH of 7.4 ± 0.2 and contains:

Table 7: Composition of BHI medium

Substance	Amount [g/L] in 1 L destilled H_2O
Calf brain, infusion form	200.0
Bovine heart, infusion form	250.0
Protease peptide	10.0
Dextrose	2.0
Sodium chloride	5.0
Sodium phosphate	2.5
Agar	15.0

The final pH of solid soil M17 at 25 °C is 7.1 ± 0.1 and contains:

Table 8: Composition of M17 medium

Substance	Amount [g/L] in 1L destilled H_2O
Tryptone	2.50
Peptone	2.50
Soya peptone	5.00
Yeast extract	2.50
Beef extract	5.00
Ascorbid acid	0.50
Magnesium sulphate	0.25
Lactose	5.00
Disodium β-glycerophosphate	19.00
Agar	15.00

PHA detection medium, E2 medium, mineral medium (MM) and minimum salt medium (MS), were used as a detection agar medium for the *Pseudomonas mendocina*, *Cupriavidus necator* and *Escherichia coli* strains. Their composition is described below (table 9–13)

The final pH of PHA detection medium at 25 °C is 7.3 ± 0.1 and contains:

Table 9: Composition of PHA detection medium

Substance	**Amount [g/L] in 1 L destilled H_2O**
Glucose	20.0
KH_2PO_4	13.0
M_gSO_4	1.3
$(NH_4)SO_4$	0.2
Citric acid	1.7
Trace elements MES	10.0
Agar	15.0

Table 10: Composition of trace elements for PHA detection medium

Substance	**Amount [g/L] in 1 L 1M HCL**
$FeSO_4.7H_2O$	10.00
$ZnSO_4.7H_2O$	2.25
$CuSO_4.5H_2O$	1.00
$MnSO_4.5H_2O$	0.50
$CaCl_2.2H_2O$	2.00
$Na_2B_4O_7.10H_2O$	0.23
$(NH_4)6M_{O7}O_2$	0.10
35% HCl	10.00

The final pH of E2 detection medium at 25 ° C is 7.3 ± 0.1 and contains:

Table 11: Composition of E2 detection medium

Substance	Amount [g/L] in 1 L destilled water
Glucose	20.0
KH_2PO_4	3.7
$Na(NH)_4NPO_4.4H_2O$	3.5
$K_2HPO_4.3H_2O$	7.5
$MgSO_4.7H_2O$	1.2
Trace elements MES	1.0
agar	15.0

Table 12: Composition of trace elements for E2 detection medium

Substance	Amount [g/L] in 1 L 1M HCL
$FeSO_4.7H_2O$	2.78
$ZnSO_4.7H_2O$	0.29
$CoSO_4.7H_2O$	2.81
$CuCl_2.2H_2O$	0.17
$CaCl_2.2H_2O$	1.47
$MnCl_2.4H_2O$	1.98

The final pH of MM detection medium at 25 ° C is 7.3 ± 0.1 and contains:

Table 13: Composition of MM detection medium

Substance	**Amount [g/L] in 1L destilled H_2O**
Glycerol	20.00
KH_2PO_4	2.80
Na_2HPO_4	3.32
NH_4Cl	0.50
$MgSO_4.7H_2O$	0.25
Trace elements MES	1.00
Agar	15.00

Table 14: Composition of trace elements for MM detection medium

Substance	**Amount [g/L] in 1L 1M HCL**
$CrCl_3.6H_2O$	0.11
$CaCl_2$	7.80
$CuSO_4.5H_2O$	0.16
$CoCl_2.6H_2O$	0.22
$FeCl_3$	9.70
$NiCl_2.6H_2O$	0.12

The final pH of MS detection medium at 25 ° C is 7.3 ± 0.1 and contains:

Table 15: Composition of MS detection medium

Substance	**Amount [g/L] in 1 L destilled H_2O**
Glucose	1.00
Oil	20.00
KH_2PO_4	1.00

$Na_2HPO_4.12H_2O$	5.58
NaCl	0.20
$MgSO_4.7H_2O$	0.20
NH_4NO_3	3.00
$CaCl.2H_2O$	0.05
Trace elements MES	1.00

Table 16: Composition of trace elements for MS detection medium

Substance	**Amount [g/L] in 1 L 0.5 M HCL**
$FeSO_4.7H_2O$	5.56
$ZnSO_4.7H_2O$	0.58
$CoSO_4.7H_2O$	5.62
$CuCl_2.2H_2O$	0.34
$MnCl_2.4H_2O$	3.96
$NiCl_2.6H_2O$	0.04
H_3BO_3	0.60
$Na_2MoO_4.2H_2O$	0.06

The final pH of CN detection medium at 25 ° C is 7.3 ± 0.1 and contains:

Table 17: Composition of CN detection medium

Substance	**Amount [g/l] in 1 l destilled H_2O**
$(NH_4)_2SO_4$	3.00
$Na_2HPO_4.12H_2O$	11.10
KH_2PO_4	1.05
$MgSO_4.7H_2O$	0,2
After sterilization:	
Carbon source	20
MES	1

Table 18: Carbon source for CN detection medium

Carbon source:	Medium name
Oil	CNO
Propionic acid	CNP
Glucose	CNG
Fructose	CNP

Table 19: Composition of trace elements for CN detection medium

Substance	Amount [g/l] in 1l 0.1 M HCL
$FeCl_3.6H_2O$	9.700
$CoCl_2.6H_2O$	0.119
$CuSO_4.5H_2O$	0.156
$CaCl_2.2H_2O$	7.800
$NiCl_2.6H_2O$	0.118
$ZnSO_4.7H_2O$	0.100

Upon detection on these soils, it was found that PHA was ideally grown on CN culture agar medium. Prof. Ing. Obruča Ph.D., provided us with this detection medium, which was enriched in the first case with propionic acid on the recommendation, then with oil, glucose and fructose. All 77 bacterial strains mentioned in table 6 were cultured on these detection media with subsequent phenotypic PHA detection. The composition of this media is shown in table 17.

6.3 Chemical used

- Tryptone (Penta, CZE)
- Peptone (Penta, CZE)
- Soya peptone (Penta,CZE)
- Protease peptide (LACHEMA, CZE)
- Bovine heart, infusion form (Penta, CZE)
- Calf brain, infusion form (Penta, CZE)
- Dextrose (Penta, CZE)
- Glucose (Penta, CZE)
- Lactose (Penta, CZE)
- Fructose (Penta, CZE)
- Yeast extract (Penta, CZE)
- Beef extract (Penta, CZE)
- Ascorbic acid (LACHEMA, CZE)
- Agar powder (Penta, CZE)
- disodium β-glycerophosphate (Penta, CZE)
- $MnSO_4.5H_2O$ (LACHEMA, CZE)
- $NiCl_2.6H_2O$ (Penta, CZE)
- $CaCl_2.2H_2O$ (LACHEMA, CZE)
- $CuSO_4.5H_2O$ (LACHEMA, CZE)

- $FeCl_3.6H_2O$ (Penta, CZE)
- $Na_2HPO_4.12H_2O$ (Lukeš, CZE)
- $MgSO_4.7H_2O$ (LACHEMA, CZE)
- $CrCl_3.6H_2O$ (LACHEMA, CZE)
- $FeSO_4.7H_2O$ (Lukeš, CZE)
- $ZnSO_4.7H_2O$ (Lukeš, CZE)
- $CaCl_2.2H_2O$ (LACHEMA, CZE)
- Na2B4O7.10H2O (Penta, CZE)

6.4 Used devices

- FTIR spectrophotometer Nicolet iS10
- Hermle Z300K centrifuge
- Centrifuge minispin plus Eppendorf
- Vortex Mixer Labnet
- Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA)

7 CULTIVATION OF THE MICROORGANISM

The microorganisms were stored in cryotubes using 10% glycerol as a cryoprotectant. Subsequently, the cultures were inoculated using appropriate sterile bacterial loops onto the appropriate culture prepared media according to table 6 (BHI, M17).

It is necessary to pay attention to the conditions for the growth of microorganisms, because 7 of the bacteria strains, we monitor are anaerobes. Such bacteria are cultured in a desiccator without access to oxygen.

The microorganisms were cultured for 24 hours at different temperatures depending on their culture conditions at 7, 30 and 37 ° C. After 24 hours, the control was performed and those cultures that did not grow were inoculated and cultured again for 48 hours. Subsequently, we inoculated all grown cultures onto CNO, CNP, CNF and CNG culture media.

Every bacterium was inoculated on each of the culture media 3 times for 3 PHA phenotypic detections, namely Nile blue A staining, Sudan Black staining and FTIR detection method.

8 METHODS FOR CULTIVATION DETERMINATION OF PHA PRODUCTION

8.1 Sudan Black staining

To prepare the solution for subsequent colony staining, we added 0.04 g of Sudan Black dye to 200 mL of ethylene glycol.

10 mL of this solution on each of the detection agar in the petri dishes was pipped and. The petri dishes was placed on a shaker, allowing the Sudan Black solution to act for 30 minutes. Subsequently, the remaining solution was discarded and the medium was washed with 96% ethanol. After this step we performed visual PHA detection. The colonies that turned black were PHA producers.

8.2 Nile Blue A staining

To prepare this solution for subsequent colony staining, we added 0.1 g of Nile Blue dye to 200 mL of ethanol.

We then pipetted 10 mL of this solution on each of the detection agar in the petri dishes and placed the petri dishes on a shaker in the dark, allowing the Nile Blue solution to act for 20 minutes. Subsequently, we poured the remaining solution and washed the medium with distilled water. Colonies stained in this way were observed under UV light. Colonies that contained PHA shone.

8.3 FTIR

The last phenotypic method for PHA detection is Infrared spectroscopy, which is a method that analyzes the rotational and vibrational states of molecules. Vibrational spectra of biomass samples obtained from the prepared production media were recorded by a NicoletiS10 FTIR spectrometer in the spectral range 400-4000 cm^{-1} in steps of 2 cm^{-1}. The resulting spectra were generated from an average of 64 scans. The measurement was performed by the ATR method on a single-reflecting diamond crystal with specially treated samples.

Sample preparation for this method was performed by sampling the grown organisms from the media using an eppendorf loop, which are microtubes, to which 10 mL of saline of the exact composition was added, see table 20. The next step was to shake the eppendorf cultures

on a Vortex Mixer (Labnet,Labnet international, Inc.) and centrifuged to ensure the cleanest possible PHA. Next, we added 5 mL of saline to the centrifuged samples and transferred the sample to a microscope slide using a loop.

To measure the sample on an FTIR instrument, we placed the samples on a microscope slide in an oven preheated to 100 °C for 15 minutes to dry the samples.

The dried samples were prepared for the determination of FTIR method.

9 EVALUATION AND PROCESSING OF RESULTS

First, a medium on which bacteria would produce as much PHA as possible was investigated. *Cupriavidus necator, Pseudomonas mendocina* and *Escherichia coli* bacteria were used for this step, which were cultured on E2, MM, MS,PHA and CN detection media under detection by Nile Blue A and Sudan Black staining method.

The cultures that were lit were marked as positive for PHA and according to the intensity of the lighting, the results were recorded in table 20, where - means that the detection was negative and + means a positive detection.

Table 20: Results for PHA detection for *C. necator, E. coli* and *P. mendocina*

	MS		MM		E2		PHA		CN	
	S	N	S	N	S	N	S	N	S	N
C. necator	+	+	+	+	-	-	+	+	+	+
P. mendocina	+	-	-	-	+	+	+	+	+	+
E. coli	-	-	+	-	-	+	-	-	-	-

*the symbol S is means Sudan Black staining, N means Nile Blue A staining

According to table 20, we can see that *C. necator* was detected by PHA on all media examined except E2. PHA produced by *P. mendocina* was positive in MS, CN and E2 media, and in *Escherichia coli* we recorded positive detection only in MM medium in Nile Blue A staining and in E2 also in Nile Blue A staining. Examples of Nile Blue A and Sudan Black can be seen in Fig. 7 and 8.

Fig. 6 MM medium under UV – *C. necator, E. coli, P. mendocina*

Fig. 6 shows the phenotypic detection of PHA producer after Nile Blue A staining, with *Cupriavidus necator* and *Escherichia coli* shining under UV, which is attributed to the production of PHA on this MM medium, and this sample is therefore evaluated as positive for these bacteria.

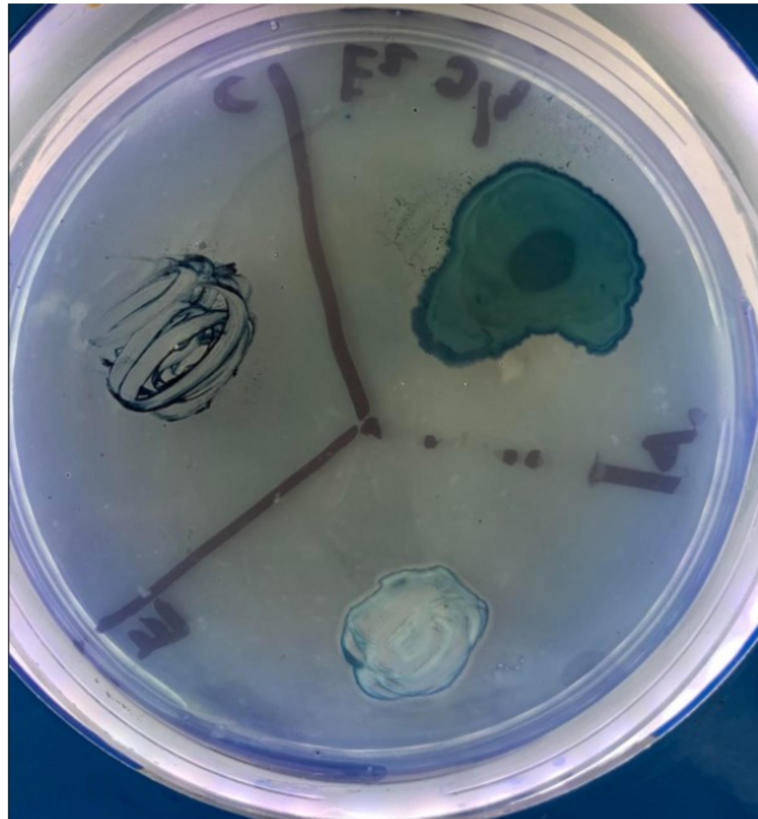

Fig. 7 E2 medium stained with Sudan Black - *C. necator, E. coli, P. mendocina*

Fig. 7 shows a sample of Sudan Black staining on E2 medium. In this method, PHA-producing bacterial colonies stain black, which can be seen in the bacterium *Pseudomonas mendocina* and is therefore evaluated as a positive producer on this medium.

After evaluating the best soil for cultivation, we performed the detection on the bacteria listed in table 6. As mentioned earlier, each bacterium was cultured 3 times on each agar (CNO, CNP, CNF, CNG) for 3 phenotypic detections - Nile Blue A staining, Sudan Black staining and FTIR detection method. The first detection method was Nile Blue A staining, in which petri dishes were transferred to darkness and UV shone on them. The cultures that were lit were marked as positive for PHA and according to the intensity of the lighting, the results were recorded in table 22, where n means that the culture did not grow so that it

couldn't be phenotypically identified. The - means that the detection was negative, and the bacterial strain was not lit and the + means that the detection was positive, and the bacterial strain was lit and was producing the PHA. Bacterial strains that are marked as red did not grow on the culture media even after the 2nd spread.

Table 21: Bacterial strains with results of phenotypic identifications and FTIR detection

Bacteria		CNO			CNP			CNG			CNF	
	F	N	S	F	N	S	F	N	S	F	N	S
CCM 3590	n	-	n	n	-	n	n	-	n	+	+	n
CCM 3726	-	n	-	-	n	-	+	++	+	++	++	+
GK CIP 5/1	-	-	n	-	n	n	-	n	n	-	-	-
DH5α	-	-	n	-	-	-	-	-	-	-	-	+
D10	-	n	n	-	-	-	+	-	+	+	+	+
D11												
D13	n	n	n	-	-	-	-	+	-	+	+	+
D16	n	n	n	-	-	n	n	n	n	n	n	n
D17	n	n	n	-	-	-	-	+	+	+	+	+
D23	n	n	n	-	-	-	-	-	-	-	-	-
D27	n	-	-	-	-	-	-	+	+	+	+	+
D28	-	-	-	-	+	-	-	+	+	+	++	+
D28b	n	-	-	-	-	-	-	+	+	-	+	+
D31	-	-	-	-	-	-	-	+	-	-	+++	-
D32	-	n	n	-	-	+	-	+	+	+	+++	+
D36	-	+	-	-	-	+	-	-	-	-	+	+
D37	+	+	-	-	++	-	+	++	+	n	+	+
D38	-	n	n	n	-	n	n	-	n	n	n	n
D47	-	+	-	n	+	-	-	-	-	-	-	-
D52	n	-	-	n	-	-	-	+	-	-	-	-
D55	-	-	-	-	+	-	-	-	+	-	-	+
D61	-	-	n	+	++	-	-	-	-	-	-	-
D68	-	n	n	n	-	-	n	+	+	-	+	+
D69	n	n	n	n	-	-	-	-	-	-	-	+
D78	-	-	-	-	-	-	n	+	+	-	-	+
D80	-	-	-	-	-	-	-	-	-	-	-	-
D93												
D100	-	-	-	-	-	-	n	+	+	-	++	+
D102												
D105	-	n	n	-	-	-	n	-	-	-	-	n
D109	-	n	n	-	-	-	-	-	-	-	-	-
D111	n	n	n	-	-	+	n	+	+	-	+	+
D116	-	-	-	-	-	-	-	+	-	-	+++	-
D117	n	n	n	-	+	-	-	+	+	+	+++	+
D124	-	-	-	n	-	-	-	+	+	-	++	+
D140	n	n	n	-	-	n	-	+	n	-	n	n
D146	-	-	-	-	-	-	-	-	-	-	-	-

		CNO			CNP			CNG			CNF	
	F	**N**	**S**	**F**	**N**	**S**	**F**	**N**	**S**	**F**	**N**	**S**
D165	+	++	-	n	+	+	-	+	+	-	+	+
D171	+	++	-	n	-	+	n	+	+	-	++	+
D173	-	+	-	n	-	+	-	-	+	-	-	-
D180	-	+	-	-	+	+	-	+	+	-	+	+
D183	-	-	-	-	-	+	+	+	+	-	+	+
D186	-	+	-	-	+	+	-	-	+	-	+	+
D199	-	-	-	+	+	-	+	+	+	+	+++	+
D201	-	-	-	-	+	-	+	+	+	+	+++	+
D212	n	n	n	-	-	-	+	++	+	-	++	+
D214	n	-	-	+	+	+	+	+	+	-	+	+
D218	+	+	-	-	-	-	-	-	-	-	-	-
D220	-	-	-	-	-	+	-	-	+	-	-	+
D221	-	-	-	+	+	-	-	-	-	n	-	-
D223	-	-	-	-	-	-	-	-	-	n	-	-
D228	-	-	-	+	-	+	n	-	+	-	+	+
D233												
D234												
D237												
D238												
D239												
I12	-	-	n	-	-	-	-	+	n	-	-	n
I13a	n	n	n	-	-	-	-	-	n	-	-	n
I16a	n	n	n	-	n	+	-	-	n	n	-	n
II12	-	n	n	n	n	+	-	-	n	n	+	n
114	n	-	n	-	-	-	-	+	+	n	+	+
119	-	n	n	-	-	+	+	+	+	-	+	+
123	-	n	n	-	-	+	+	+	+	-	-	+
126	+	n	n	-	n	+	-	+	+	-	+	+
F10	-	-	n	-	+	+	-	+	+	-	-	+
F53	n	-	n	n	+	-	-	-	+	-	-	+
F77	n	-	n	n	-	+	-	+	+	-	+	+
F108	n	-	n	n	+	-	-	-	-	+	+	+
S7	-	n	n	n	-	+	+	+	+	-	+	+
S10	-	n	n	-	+	-	-	-	-	-	+	-
S17	n	-	n	-	-	-	-	+	+	-	+	-
S23	n	-	n	-	-	-	-	-	-	n	+	+
S32	-	n	n	-	-	-	-	-	-	-	+	-
S33	-	n	n	n	-	-	-	-	-	n	+	-
S34	n	-	n	n	-	-	-	-	-	n	+	+
S35	-	-	n	-	-	-	-	-	-	n	-	+

In table 21 we can see that when measuring FTIR, a lot of bacterial strains are negative for PHA formation. This may be due to the fact that the PHA were used as a source of energy

by bacterial strains before the measurement took place. We can notice that the bacterial strains that grew on the CNO medium with the addition of oil, did not grow at all in many cases. On the contrary, PHA growth thrived in a medium enriched with fructose.

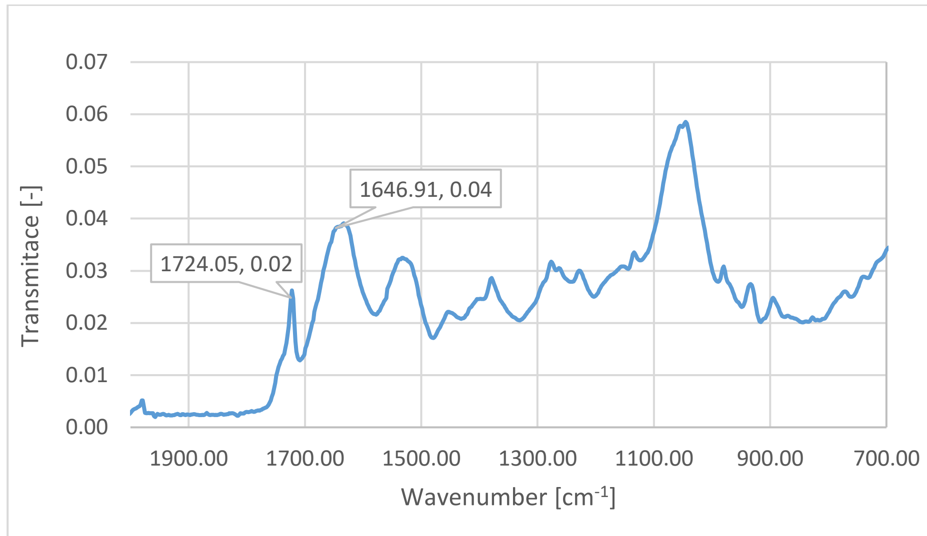

Fig. 8 FTIR evaluation for bacteria D201 on CNG medium

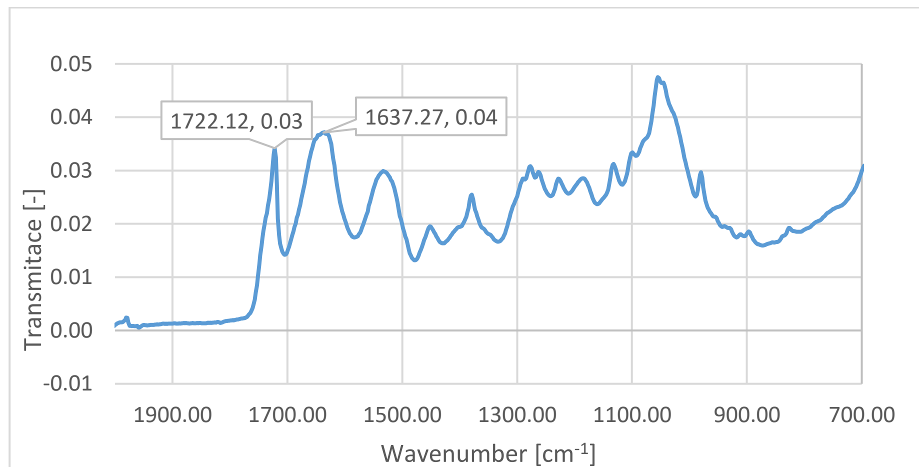

Fig. 9 FTIR evaluation for bacteria D201 on CNF medium

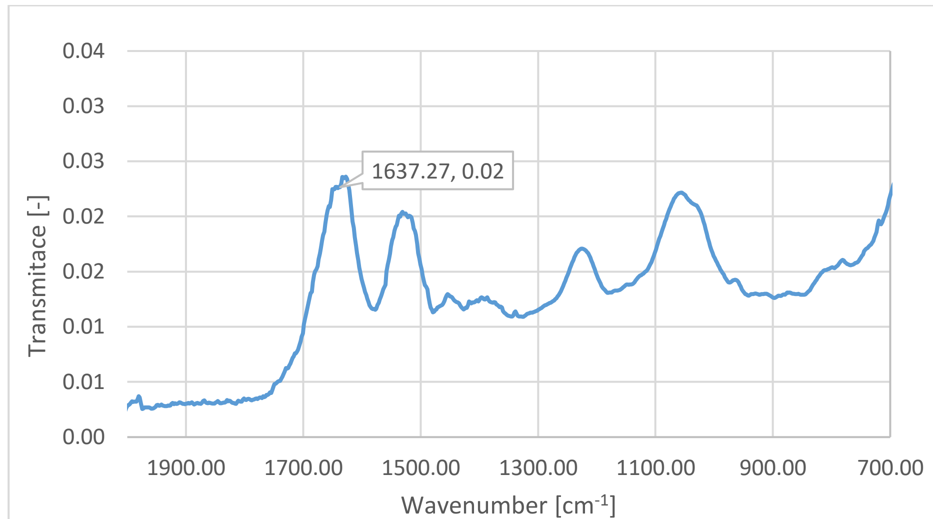

Fig. 10 FTIR evaluation for bacteria D201 on CNO medium

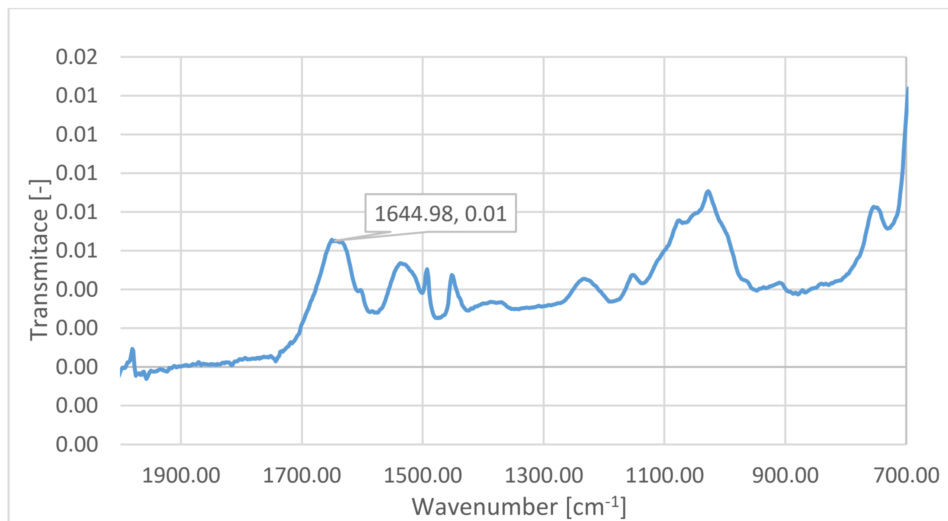

Fig. 11 FTIR evaluation for bacteria D201 on CNP medium

In Fig. 9–11 we see an example of FTIR measurements for D201 bacteria on all 4 media. In the Fig. 8 and 9 we see a characteristic peak at 1722 cm^{-1} and 1724 cm^{-1}, which characterizes PHA, which is visibly not in the Fig. 10 and 11. Thus, the bacteria produces PHA on CNG and CNF media.

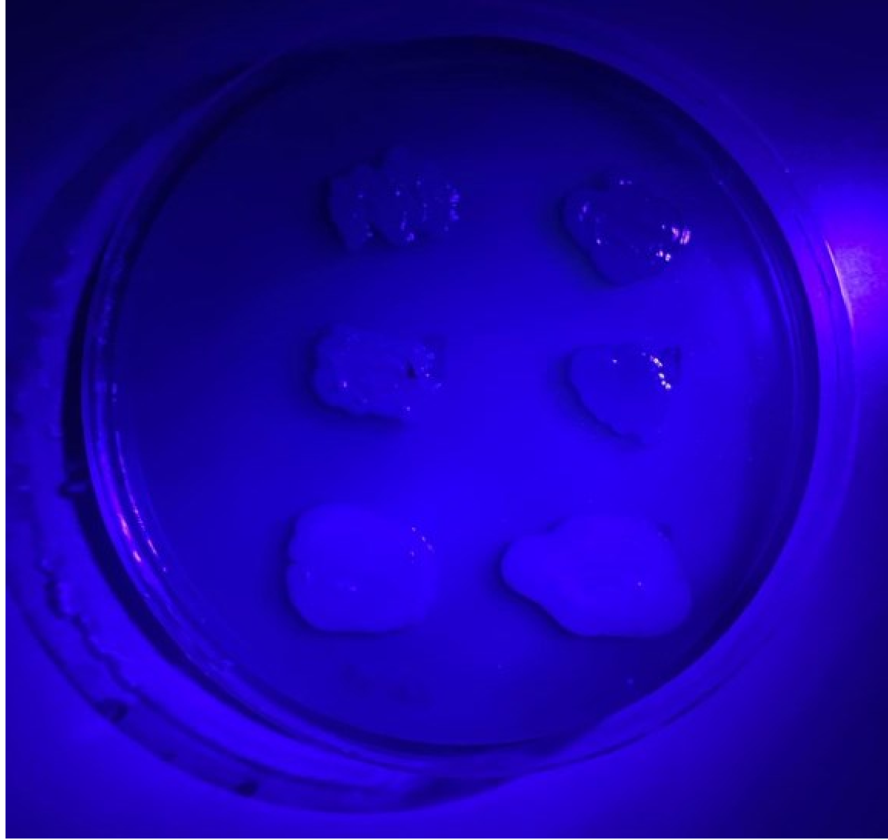

Fig. 12 Bacterias D180, D183, D186, D199, D201, D212 on CNF medium under UV

In the Fig. 12 we see positively grown bacteria that shine under UV-stained Nile Blue A and thus producing PHA.

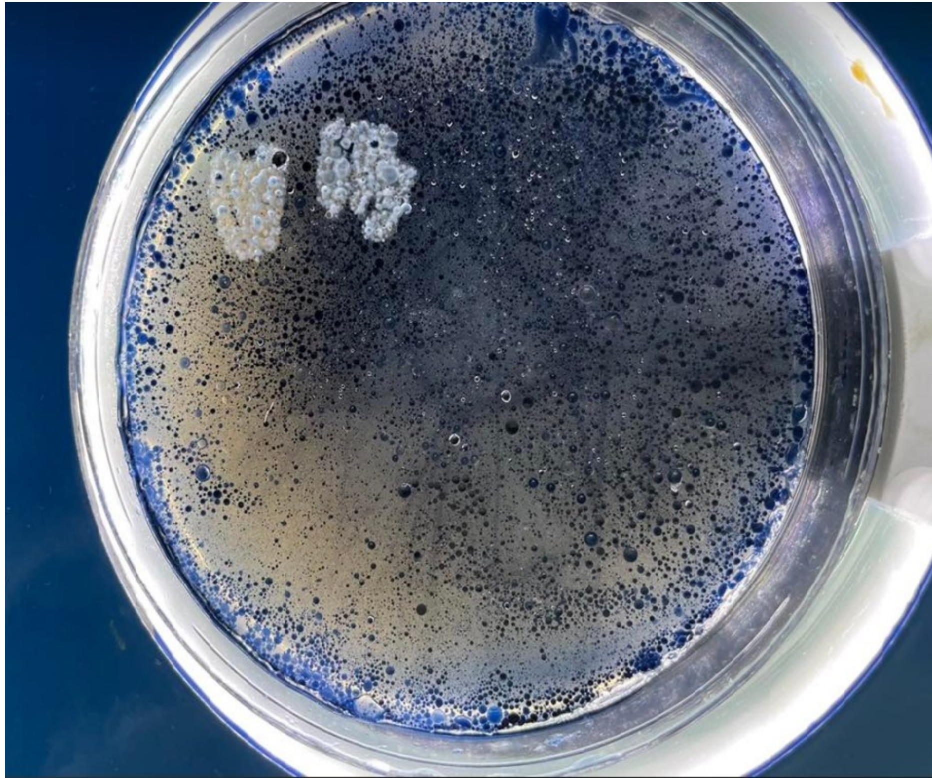

Fig. 13 Bacterias D199 and D201 on CNO medium stained with Sudan Black

Fig. 13 shows an example of Sudan black staining on CNO medium. We see that only 2 bacterias (D199 and D201) have grown, but they do not produce PHA because the colonies did not turn black.

10 DISCUSSION AND FORMULATION OF CONCLUSIONS

The aim of this diploma thesis was the phenotypic identification of PHA producers. Many microorganisms can synthesize PHA, which could replace synthetic, non-degradable plastics in the future, such as olefins, which are used in packaging materials. Unfortunately, the production of PHA is still too expensive, so they are still used mainly for special applications such as in the medicine.

The culture medium plays an important role, which can reduce the cost of production of this bioplastic by up to 50%, so in this diploma thesis the phenotypic production of PHA (Nile Blue A and Sudan Black staining) and subsequently detection by FTIR method was investigated.

First, 5 different media were phenotypically examined - E2, MM, MS, PHA and CN detection medias on 3 bacterial strains *Cupriavidus necator, Pseudomonas mendocina* and *Escherichia coli*, staining with Nile Blue A and Sudan Black. It was found that *C. necator* grew on all mentioned media except E2. According to the results, *P. mendocina* produced PHA on MS, CN and E2 media and *E. coli*, on the other hand, did not produce PHA on any of these media. It was positively marked only when staining Nile Blue on MM and E2 media.

Subsequently, this method was used for detection with the same measurements of PHA in a total of 77 microbial strains comprising 16 different microbial genera. (*Acinetobacter, Bacillus, Citrobacter, Cupriavidus, Escherichia, Guehomyces, Klebsiella, Kosakonia, Lelliottia, Lysinibacillus, Oceanobacillus, Pantotea, Pseudomonas, Rahnella, Staphylococcus* and *Stenotrophomonas*). Detection was performed on the best production medium we choosed from the first detection, which was recommended as a suitable detection by prof. Ing. Obruča Ph.D., where the carbon source was changed, when it was either oil, propionic acid or the addition of the sugar component fructose or glucose. The results can be seen in table 21.

It was found that bacterial strains did not grow on oil-added medium even after successful cultivation, although, for example, D165 and D171 bacteria thrived in oil-medium and produced PHA. In contrast, most colonies that produced PHA were recorded in fructose-enriched media.

For bacterial strains that tested positive for at least 2 trials, we concluded to be producers of the PHAs we detected. For the medium with oil there were 4 bacterial strains, for the medium with propionic acid it was 8 bacterial strains, for the medium with glucose 22 bacterial strains

and most producers recorded the medium with fructose as a carbon source, namely 28 bacterial strains.

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LIST OF ABBREVIATIONS

3HH	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
ε	elongation
ALP	Alkaline phosphatase
CDW	Cell dry weight
E	Young 's modulus
EDTA	Ethylenediaminetetraacetic
FTIR	Fourier transform infrared spectroscopy
LCL PHA	Long chain length
MCL PHA	Medium-chain-lenght polyhydroxyalkanoates
MO	Microorganism
MSC	Mesenchymal stem cells
OCN	osteocalcin
PAL	Surfactants
PCR	Polymer chain reaction
PHA	Polyhydroxyalkanoates
P3HB	Poly-3-hydroxybutyrate
P4HB	Poly-4-hydroxybutyrate
(P(3HBco-3HV)	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-90 mol% 4HB)	poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 5 mol% 3HB and 95 mol% 4HB
SFE	Supercritical fluid extraction
SCL PHA	short-chain-length polyhydroxyalkanoates
SDS	Sodium dodecyl sulfate

Tm	Melting temperature
Ts	Tensile strength
UV	ultraviolet

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