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METHODS OF POLY-3-HYDROXYBUTYRATE EXTRACTION AND THEIR OPTIMIZATION

METODY EXTRAKCE BIOPOLYMERU POLY-3-HYDROXYBUTYRÁTU A JEJICH OPTIMALIZACE

DOCTORAL THESIS

DIZERTAČNÍ PRÁCE

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Assignment Doctoral Thesis

Department:	Institute of Materials Science	Academic year: 2022/23
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Title of Doctoral Thesis:

METHODS OF POLY–3–HYDROXYBUTYRATE EXTRACTION AND THEIR OPTIMIZATION

Doctoral Thesis:

1. Conduct a literature review on the topic of PHB isolation from bacterial biomass.
2. Next, review the processing and material use of the polymer, with an emphasis on specialty chemicals, alternatives to microplastics in cosmetics, and materials for 3D printing.
3. Design and experimentally verify new or modified isolation methods, describe their advantages and disadvantages.
4. After agreement with the industrial partner, verify the selected methods on a pilot–plant scale

Deadline for Doctoral Thesis delivery: 28.10.2022:

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In Brno dated 1.10.2019

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ABSTRACT

Poly-3-hydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic polymer with a wide range of potential applications. The production of PHB typically involves the fermentation of renewable feedstocks by bacteria. However, the recovery of PHB from bacteria is often challenging and inefficient. In this study, different methods for PHB recovery, including digestion, mechanical disruption, and extraction, are reviewed and compared. Various optimization strategies that can be used to improve the efficiency of PHB recovery are discussed. The experimental and results section describe efforts to improve recovery methods, especially acid and soap-based digestion, which have been selected as potentially economical, safe and environmentally friendly. Together with our industrial partners, we have developed these technologies to a pilot-plant scale and further development is planned. Possible applications and commercializations of PHB were also addressed. It has been found that PHB can replace some microplastics in cosmetics and that this effort has potential, as the presence of microplastics in common products is still high and their phase out is planned at European Union level. Furthermore, the thermal stability of PHB depending on the recovery method was studied, and two stabilization methods were developed, which made it possible to use PHB obtained by the soap-based digestion in material applications.

KEYWORDS

Poly hydroxy alkanoate, PHA, polyhydroxy butyrate, PHB, recovery, isolation, extraction, digestion, thermal stability, microplastics, biopolymer, polyester

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DECLARATION

I declare that I have written the Doctoral Thesis titled "Methods of Poly-3-hydroxybutyrate Extraction and Their Optimization" independently, under the guidance of the advisor and using exclusively the technical references and other sources of information cited in the thesis and listed in the comprehensive bibliography at the end of the thesis.

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Contents

Introduction

Poly[(R)-3-hydroxybutyrate] (PHB) is a polyester naturally occurring in a wide range of organisms. It attracts attention as a possible bio sourced and biodegradable alternative to currently used plastics [1]. Research of PHB has a long tradition at Brno University of Technology. Especially biotechnological production of PHB (and other polyhydroxyalkanoates) has been extensively studied [2, 3, 4, 5, 6, 7, 8]. It was found out that different waste materials from agricultural and food industry can be used as a carbon source for bacteria, including waste cooking oil [9, 10]. Main goal of this work is to improve downstream process. Isolation of PHB has been studied for almost a century, but the conclusions of previous researchers are not automatically transferable to our practice. The main reasons are different starting material and a scale. In collaboration with Nafigate Corporation, we aim to produce PHB on a large scale, while available literature often ends up on lab scale. In this work, previous knowledge is summarized and evaluated. On the basis, new or improved methods of PHB isolation are suggested and tested on our biomass. Another goal is to explore chemical derivatives of PHB, such as organic reagents, chiral building blocks, green solvents or polymeric specialty chemicals.

This text recapitulates a number of results that we published in peer-reviewed journals ([11, 12]). Here, they are supplemented by unpublished experiments and an extended discussion so that it would serve not only as a thesis, but also as a comprehensive summary of literature and laboratory test results for our industrial partners.

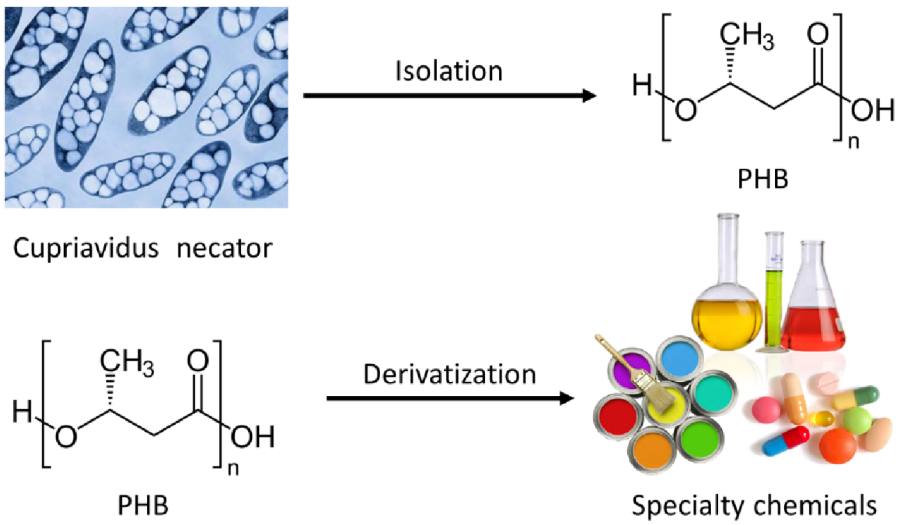

Fig. 1: Topics of this work

1 Theory

1.1 PHB isolation from biomass

During literature search, it was found out that there are three basic approaches for separation of PHB from other constituents of biomass:

- extraction of polymer with organic solvent
- dissolution (digestion) of non-polymer cell material
- mechanical disruption of cells and physical separation

In following sections, these methods will be discussed. Their advantages and disadvantages will be summarized and a feasibility of transfer to industrial scale will be evaluated. Some literature presents a combination of the above methods. In that case, it will be classified according to prevailing method.

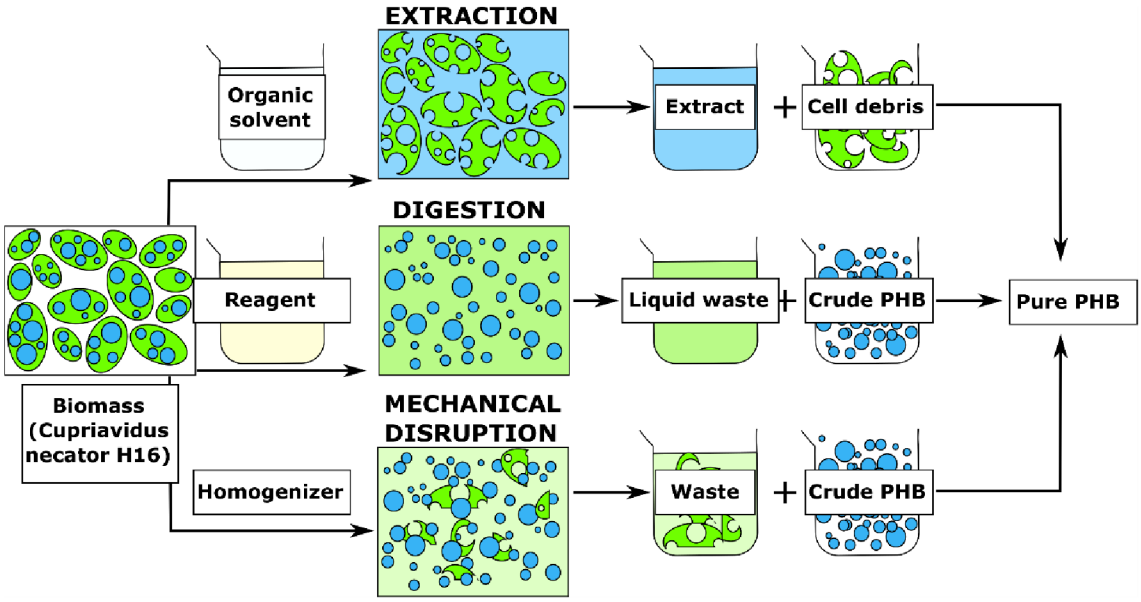

Fig. 1.1: Three basic approaches for PHB isolation

1.1.1 Extraction methods

Extraction methods take advantage of the fact that PHB is soluble in some organic solvents due to its lipophilicity, while other cell components, like proteins, polysaccharides or nucleic acids, are mostly hydrophilic. Therefore, a suitable organic solvent can be added to wet or dried biomass and selectively extract polymer, remaining majority of other cell components solid or dissolved in water (fig. 1.2).

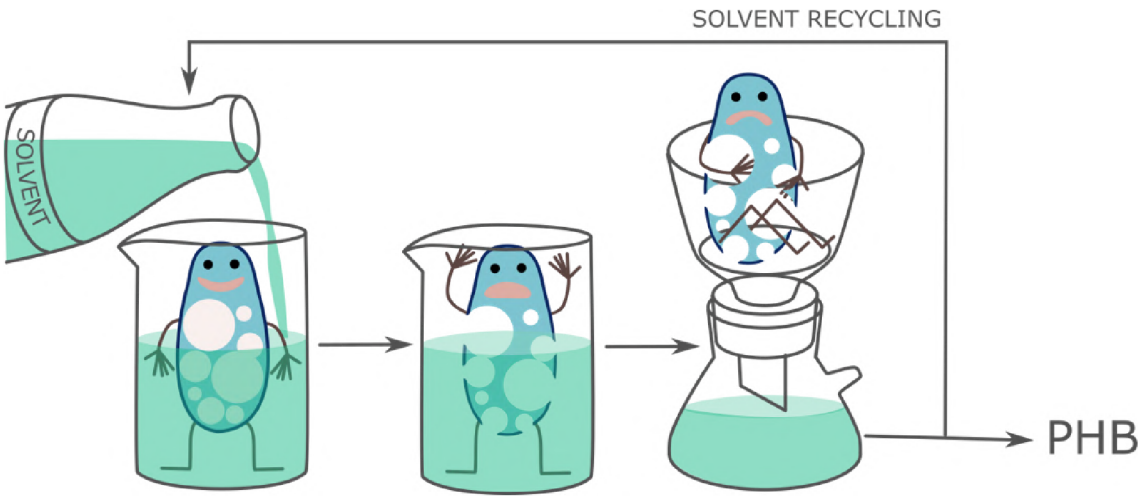

Fig. 1.2: Extraction of PHB from bacteria using solvent

Polymer in the extract can be recovered by evaporation or precipitation. Precipitation is usually preferred, because it enables removal of lipophilic non polymer cell components, like oils or phospholipids.

In extraction methods, proper choice of the solvent is crucial. The solvent should selectively dissolve PHB, resulting solution should be concentrated, but of low viscosity, recycling of the solvent should be easy and energy-saving. Conditions of extraction should not cause severe polymer degradation. Safety and environmental consideration also play important role. Among common solvents, only a few dissolve PHB. In general, moderately polar organic solvents are capable of dissolving PHB. Examples are chlorinated solvents, pyridine, dioxane, cyclic esters of carbonic acids, dimethyl formamide, dimethyl sulfoxide or carboxylic acid esters. Of course, temperature matters. Only solvents that dissolve PHB below their boiling point at atmospheric pressure are considered "solvents for PHB" in this section. High pressure methods of extraction, that would enable use of many more solvents, are out of the scope of this work.

1.1.1.1 Halogenated solvents

Historically, the first solvents known to dissolve PHB were halogenated solvents (fig. 1.3), especially chloroform [13]. Therefore, first detailed descriptions of PHB isolation focus on chlorinated solvents [14, 15, 16]. In Hayward's study [14], bacterial biomass is first centrifuged and dried, and then extracted with chloroform. Organic extract is then precipitated with ether and PHB precipitate is dried to obtain pure PHB. Simply said, nothing has changed since then. All procedures published afterwards basically follow the same scheme. The four elemental steps (biomass

pretreatment, extraction, precipitation and drying) are always there, with slightly different settings.

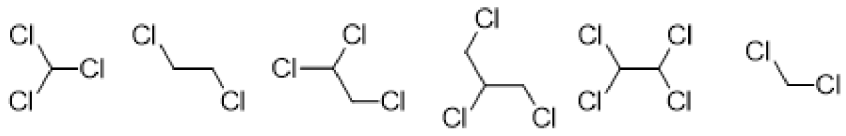

Fig. 1.3: Example chlorinated solvents for PHB extraction

Biomass pretreatment Biomass, naturally, is obtained in a form of diluted aqueous suspension of cells, minerals, organic metabolites and residues of feed material. Overall content of polymer in this mixture is low and direct extraction is not feasible. All procedures, therefore, first concentrate biomass using centrifugation. Cells concentrate in a pellet, while non-cell material remains in supernatant. Resulting supernatant then can be reused for next fermentation or discarded. Pellet, which is a starting material for next steps, usually contains 20 – 60 % dry mass, out of which 50 – 80 % is PHB that is located inside the cells. Cell walls and envelope on a surface of PHB granules may be a barrier for a solvent that should extract PHB. Some authors use drastic pretreatment methods to disrupt the barriers, while others don't. This apparent disagreement on necessity of pretreatment may be explained by differences in biomass. Some microorganisms are more fragile than others and mere presence of solvent can disrupt the barriers. In case of other microorganisms, more severe chemical or physical disruption is needed.

In any case, logical question must be answered – to dry or not to dry? Some experts use drying of biomass in their processes [14, 17, 18, 19, 20, 21]. It has many advantages – it is possible to store dried biomass before extraction for very long time without deterioration. Grinding and sieving can be used before extraction to obtain optimally extractable and filtrable particles. Water – solvent contact doesn't happen so troubles with contaminated wastewater don't exist. On the other hand, complexity of the process is higher. If drying is preferred, necessity of another pretreatment must be considered. There are many authors that don't use any and directly dry the concentrated biomass before extraction [18, 22, 23, 13, 14]. But many others specifically focus on biomass pretreatment before drying. The reason probably is that drying without any pretreatment isn't easy. Concentrated biomass is a sticky, pasty matter and during drying, compact impermeable crust is being formed on its surface, making drying slow and inefficient. Due to high temperature and long time, colored byproducts may form and later contaminate final polymer. Many methods of pretreatment exist. For example, one study [15]

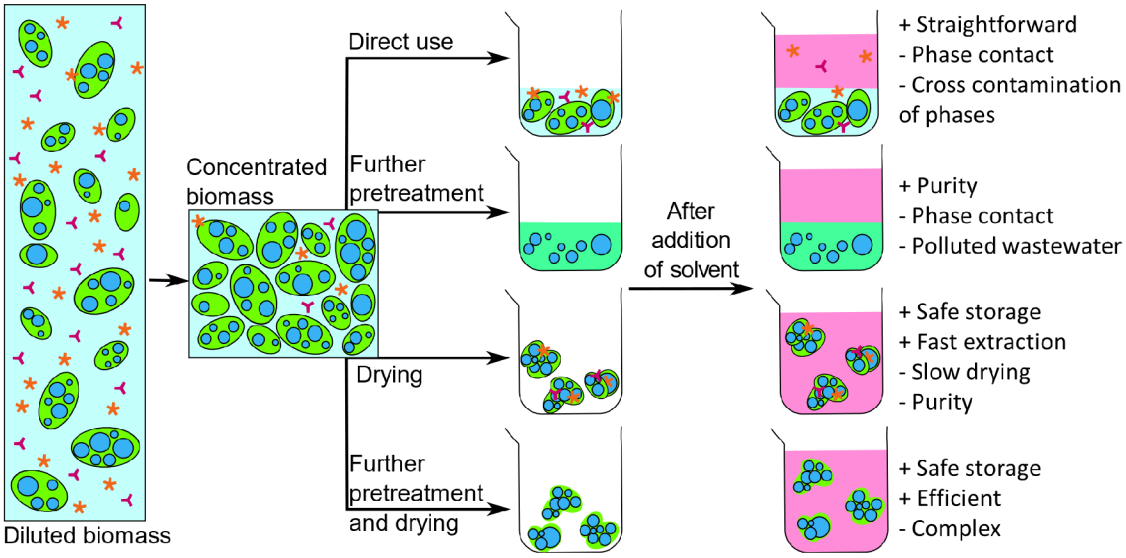

Fig. 1.4: Possible methods of biomass pretreatment, advantages and disadvantages

reports that acetone wash disrupts cell walls, removes lipids and enables easy drying. Similarly, acetone wash is reported to increase extractability of freeze-dried biomass [17]. Other source [16] uses ultrasonication or high-pressure homogenization to disintegrate biomass prior to centrifugation and drying. It is also believed that after such pretreatment, proteins from biomass remain mostly in supernatant and don't contaminate final product. Other studies [20, 21] suggest drying the cells using water vapor. The high temperature of vapor disintegrates biomass enough to enable extraction. Lipid removal is performed prior to extraction and may also contribute to cell wall weakening. Flocculation by changes of pH is also reported [19]. pH of biomass is adjusted to alkaline and then acidic or vice versa. Flocks of biomass form during pH manipulation. Flocks are collected, dried and extracted. Degreasing of flocks can also be done before extraction. Work with wet biomass is another option [24, 25, 26]. It overcomes all drawbacks of drying, but brings others – most importantly, how to ensure phase contact during extraction and then how to quickly separate water from organic phase or how to deal with wastewater that contains chlorinated hydrocarbon. Wet biomass doesn't have to be pretreated at all [24, 26] or can be prepared in different ways. One patent [25] uses alcohol solution to remove lipids. Biomass is then heated with water to remove residual alcohol and wet residue is used in extraction. Flocculation by pH changes can also be used and the flocks can be extracted without drying [19].

Extraction The most popular halogenated solvent is chloroform. Most academic papers, together with patents that are focused on other aspects of technology, use it as model solvent. Temperatures range from room temperature to boiling point,

time is chosen accordingly from several minutes to several days. After extraction, 1 – 10% solution of PHB should be obtained and the amount of chloroform is designed accordingly [17, 14, 13, 16, 26, 25]. Other works use different solvents to overcome limits of chloroform. For example, extraction with dichloromethane/ethanol mixture (5:1) is described [15]. Other work proposes use of chloroethanes and chloropropanes instead of chloroform. Due to their higher boiling point, higher extraction temperature can be used, which leads to higher efficiency, shorter time of extraction and higher molecular weight of product [22]. Another study systematically compares several halogenated solvents and investigates role of extraction time and temperature. It confirms that in case of all tested solvent, increased temperature is necessary. At room temperature, yields are very low even at long extraction times (>1 day). Kinetics of PHB degradation is also described. For example, in refluxing chloroform, maximum recovery of polymer is reached in 15 minutes. Longer stirring of the mixture at high temperature slowly decreases molecular weight [17].

After extraction, polymer solution must be separated from cell debris. Other methods than filtration are used only sporadically. It can be centrifugation [18, 25] or sedimentation. If aqueous biomass suspension is extracted, phases can be left to separate as usual in liquid – liquid extraction. Cell debris concentrates in water phase and so can be easily removed [26]. However, there is a risk that in a presence of phospholipids, fatty acids and other surface-active biomolecules, hard-to-process emulsions may be formed.

Extract processing The polymer can be isolated from solvent by evaporation or precipitation. Evaporation only is unusual, because purity of the product thus obtained is low. Evaporation and subsequent wash of the residue with other organic solvent is an option [16]. Most common way of polymer recovery is precipitation. For example, petroleum ether or other hydrocarbon, water or alcohol can be used, extract to precipitant ratio can be 3:1 [15]. Injecting polymer solution to hot water is cheap and elegant way how to obtain precipitate [26, 25]. If the polymer is produced on a large scale, the solvent - antisolvent mixture resulting from precipitation must be somehow recycled. Common method is distillation. Azeotrope formation is an important issue that limits possible solvent – antisolvent combinations. Combination of solvent and antisolvents that can or cannot be separated by distillation are described in one work [18].

Halogenated solvent methods - summary In summary, all procedures with halogenated solvents deal with similar issues. The first is, how to prepare the biomass for extraction. Working with wet biomass is an option, but it may lead to hydrolytic decomposition of polymer during extraction. Further, at the end there will be some

wastewater contaminated with halogenated hydrocarbon. Common halogenated hydrocarbons are at least slightly soluble in water and can't be completely removed by distillation due to existence of azeotropes [27]. Even though the concentrations are very low, in industrial scale it may still mean unacceptably large quantity of pollutant in water. Choice of minimally soluble solvent is an option, but rather expensive. Purification of wastewater by means of membrane techniques, salting out or other common methods is possible, but makes the whole process more complex.

If one decides that wet biomass should be avoided, drying must be used. But, no matter how easy it seems, drying of PHB-containing biomass is not a straightforward task. Direct drying seems feasible only in small-scale and any pretreatment that would enable large-scale production requires complex apparatus or additional reagents. One of the most promising ways seems to be flocculation using small amounts of alkalis and acids [19]. We decided to further explore possibilities of the procedure as described in Experimental section.

Contrary, rest of the process is quite straightforward compared to methods with other organic solvents. Dissolution of polymer is fast, low to medium temperature usually suffice and degradation of polymer isn't severe. Halogenated solvents usually don't decompose significantly during extraction and can be easily recycled using distillation. Separation of cell debris from polymer extract can be achieved by simple filtration. Extraction efficiency can be close to quantitative and yield-limiting factor is usually remainder of some extract on filtration cake. Washing the filtration cake with additional solvent can increase the yield. Filtrate is easily processable by evaporation or precipitation. During precipitation, PHB is obtained as easy-to-separate precipitate. Purity and molecular weight of thus obtained PHB can be very high.

The main issue is toxicity and environmental impact of halogenated solvents. Any leakage in technology can have severe consequences to health of workers and citizens in surrounding areas, environment and manufacturer's reputation. Overall feasibility of this route in industrial scale is therefore questionable.

1.1.1.2 Alkylene carbonates

After chlorinated solvents, cyclic esters of carbonic acid are the most common solvents for PHB extraction (fig. 1.5). Propylene carbonate and ethylene carbonate are of special interest. Lower toxicity and good availability make them practical extraction agents. However, higher price and corrosivity at high temperature may limit their use [28].

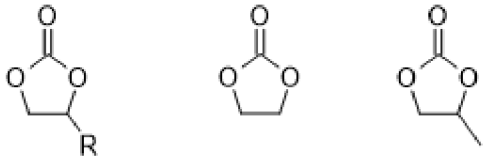

Fig. 1.5: Alkylene carbonates

Biomass pretreatment Biomass is prepared for extraction in the same way as for the extraction with halogenated solvents (see section 1.1.1.1). It was observed that heat or pH shock pretreatment doesn't significantly improve extraction yield but has positive effect on purity [29].

Extraction Extraction with alkylene carbonates requires much higher temperature than extraction with halogenated solvents. For example, a kinetic study [30] describes dissolution of PHB in propylene carbonate. The study shows that PHB dissolves within 6 min at 125 °C and only 2 minutes at 145 °C. Strong temperature dependency of solubility is also shown. Below 115 °C, the material is practically insoluble. At 115 °C, solubility is around 5 g/l. At 140 °C, solubility is >350 g/l. Unfortunately, high temperature may have negative effect on molecular weight because PHB undergoes thermally induced chain scission [31]. Time and temperature must be carefully chosen to obtain good yield and high molecular weight. Both extraction of wet and dried biomass can be performed. Preferred extraction temperature is 120 – 150 °C. Resulting solution is reported to be of relatively low viscosity (compared to chloroform, pyridine, DMF or DMSO extract) and easily filtrable even at high concentration (>10% w/w). During extraction, however, significant depolymerization occur, especially with ethylene carbonate [32].

Extract processing After extraction, solution can be filtered to remove cell debris. Unlike in halogenated solvent extraction, PHB in alkylene carbonate extracts readily precipitates upon cooling. Therefore, precipitation doesn't require antisolvent. After cooling to ambient temperature, precipitate can be separated and dried. Filtrate don't contain the antisolvent and its regeneration is therefore easier or, in some cases, can be used repeatedly without purification [32].

Alkylene carbonate methods – conclusion Alkylene carbonates dissolve exceptionally high amounts of PHB which makes them very appealing. The less solvent must be used, the more economical process in theory. Another advantage is that strong temperature dependency of solubility enables quantitative precipitation of polymer by cooling. No antisolvent is then needed and recycling of solvent is easier.

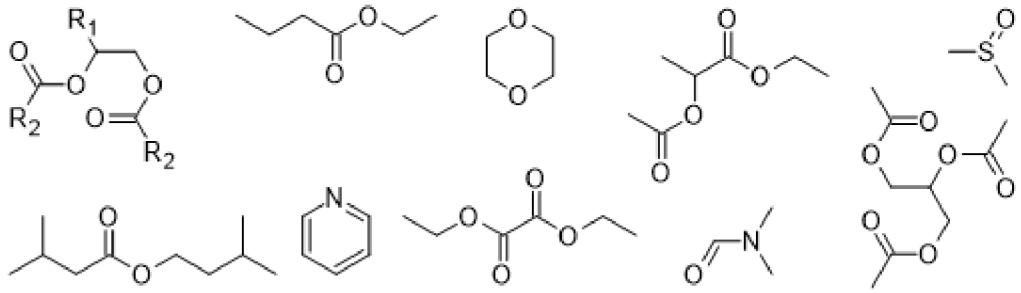

Fig. 1.6: Examples of other solvents for PHB

The main drawback is necessity of high temperature and consequent degradation of polymer. Conditions must be carefully controlled to avoid it. Alkylene carbonates are susceptible to hydrolytic degradation which may bring additional costs to the process. But favorable toxicological profile of alkylene carbonates, together with wide usage and good availability makes them promising.

1.1.1.3 Other solvents

Attempts to use other polar organics were also found in early literature [33, 16, 34, 35]. The early literature (except for [33]) only briefly proposes use of other solvents and demonstrates their use in a lab scale. More detailed studies came later (starting from 1990s), with increasing pressure on environmentally safe technologies. Therefore, mainly so-called "green" solvents have been studied. The solvents differ significantly in their properties and therefore there is different process suitable for each of them.

Example procedures with other solvents Pyridine method starts with biomass centrifugation that reduces water content. Pellet is added to acetone in ratio 1:1 to 1:10 and stirred, the acetone is then filtered off. The purpose of acetone step is to remove lipids and residual water. Dried biomass is then mixed with pyridine (10x – 100x excess) and refluxed for 5 – 30 min. Pyridine is filtered off and polymer is precipitated from filtrate using petroleum ether, benzene or alcohol [33]. The main advantage of this method over chloroform- based methods should be higher yield, but due to many disadvantages, like toxicity, basicity and probable solvent recycling difficulties, the method didn't get much attention. Author of the patent himself, only two months later, focuses again to use of halogenated solvents [15].

A recent study [36] compares different solvents for extraction of PHB. Tested solvents are DMSO, DMF, ethylene carbonate, hexane, propanol, methanol and acetic acid, standard solvent is chloroform. Compared to chloroform, only ethylene carbonate at 150 °C was found to reach similar efficiency in terms of yield. The

study quantifies the long-known fact that only organic solvents of medium polarity are suitable.

A rapid method to isolate PHA for screening studies uses DMSO as a solvent. DMSO at 70 °C supposedly dissolves everything, including non-polymer cell material, and forms a clear solution with biomass. After addition of ethanol, non-polymer material precipitates and can be removed. PHB remains in DMSO/ethanol solution and can be precipitated using water. The method is intended for fast gravimetric measurement of PHB content in biomass sample, not for large scale production [37].

Diols and acetalized triols, tricarboxylic acid esters, mixtures of dicaboxylic acid esters or butyrolactone can be used. Biomass is centrifuged and dried or, preferably, used watermoist. It is added to extracting agent, heated to 100 – 150 °C and stirred for 5 – 20 min at this temperature. Extract is then separated from debris, usually by filtration. Polymer-containing filtrate is precipitated or cooled to obtain solid polymer or polymer gel, respectively. Polymer can be separated from solvent by means of filtration, vacuum filtration, or, if obtained as gel, squeezing. Crude solid is washed using alcohol, acetone, water or mixtures thereof and dried. Reported yields, purities and molecular weights of obtained polymer are very high, but certain issues of technology are not fully discussed. Although reported that solvent/washing agent mixtures can be recycled by distillation and reused indefinitely, there is no example of successful recycling procedure [28].

Extraction with glycerol acetates is preferably accompanied with mechanical operation (pulverizing, milling, high-pressure homogenization etc.) to increase extraction efficiency. Mechanical operation can be used as a pretreatment method, too. After extraction, solid cell debris is separated (typically filtration) and polymer is obtained by drying the extract. No details regarding yield, molecular weight and purity are given [38].

Other aliphatic esters can be also used, for examples isoamyl propionate, propyl butyrate, isoamyl valerate and isoamyl isoamylate. Extraction temperatures are then around 100 – 150 °C. Exceptionally high concentrations of extracts (up to 27.8 %) are reported. PHB extract processing (specifically precipitation with water or vapor) can be conducted in such manner that powdered PHB is obtained. Moderate loss of molecular weight is reported [39].

Detailed study of extraction with non-traditional ester solvents diethyl oxalate or acetoxy ethyl lactate comes from FCH BUT [23]. Dried biomass is extracted at high temperature (120 – 180 °C) for 5 – 60 minutes and filtered. PHB precipitates from solvent upon cooling and forms gel. The gel is squeezed to remove solvent, residue is repeatedly washed with ethyl acetate and pure polymer is dried. Changes in molecular weight and extraction yield in time are studied at different conditions. The study revealed that both solvents are suitable for extraction at temperature 140

– 160 °C. Reasonable time of extraction is between 5 to 30 min, when maximum yield is reached, and molecular weight decline is not yet significant. Yield, molecular weight and purity are excellent. Size of dry biomass particles also matter, 1-2 mm diameter being optimal for efficient extraction and filtration. Both solvents undergo hydrolytic degradation, which can lead to significant loss of solvent after several cycles of distillation, that is necessary step in industrial process. Acetoxy ethyl lactate is more stable then diethyl oxalate.

Other solvent methods – conclusion Aggressive solvents, such as acids (acetic acid) and bases (pyridine) don't have any future. DMSO is suitable only for lab scale procedure. Dioxane should be avoided due to its toxicity. Most promising are polar carboxylic acid esters, but the drawbacks are alike alkylene carbonates – corrosivity and instability of solvent and polymer at the conditions of extraction. Ester solvents differ in price, availability and performance in extraction. So far, none of the solvents was found to be superior in all measures. Compared to halogenated solvents, esters are usually more expensive. Yield, purity and molecular weight can be the same. Relatively low toxicity of ester solvents is the main benefit.

1.1.2 Digestion methods

The goal of digestion methods is to solubilize non-PHB cell material and remain PHB granules intact (fig. 1.7). There is one obvious challenge - cells are complicated. Including cells of PHB-producing microorganisms. There are not only PHB granules, even though their content in cell can be enormous. There is still some non-PHB cell material and it consists of large number of different compounds. There are low molecular weight compounds, such as amino acids, lipids or inorganic salts, but also large biomolecules, namely proteins, polysaccharides, and nucleic acids. Moreover, molecules in living creatures are assembled to different supramolecular structures – biomembranes, organelles etc. that can have properties that differ significantly from a chemistry of isolated compounds. Therefore, the task of solubilizing everything but PHB may seem unattainable. Fortunately, the "everything" doesn't have to be taken literally. Main target of digestion methods is to disrupt cell walls and release cell content, including PHB granules. There are proteins and phospholipids attached to PHB granules [40], so the next step can be solubilization of these. Other cell constituents can remain intact, if separable in their native form.

Then, solid PHB can be separated by any practical method for solid – liquid separation. PHB granules usually have a diameter around 1 micron and a density of 1.24 g/cm^3. Because of small particle size and high viscosity of liquor, filtration may

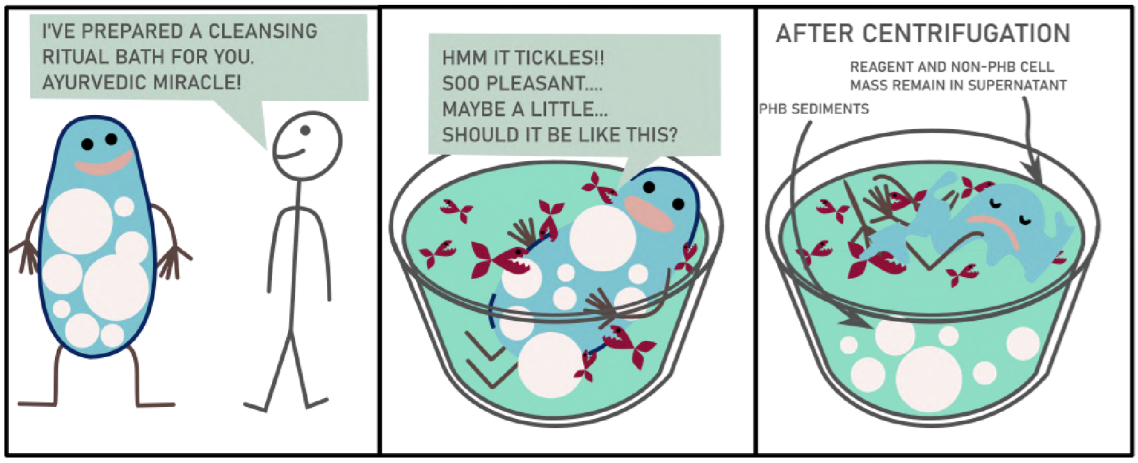

Fig. 1.7: Isolation of PHB from biomass by digestion

be challenging. Centrifugation is usually preferred. After separation, crude product is usually washed several times to remove residual impurities and dried.

Common reagents for biomass digestion are oxidants (mainly hypochlorite), acids and bases, surfactants, enzymes and other microorganisms. Procedures described in a literature usually combine several agents. Mechanical homogenization is often combined with chemical digestion, too.

1.1.2.1 Digestion with oxidants

Sodium hypochlorite is among the first agents used for PHB separation. Williamson used sodium hypochlorite and observed the digestion under microscope for the first time [41]. Cells were treated with sodium hypochlorite solution at 37 °C. Cells started to fade, but PHB granules remained apparently intact. After a short while, all that could be seen from the cells were PHB particles, that also started to agitate violently due to Brownian motion. Still, their motion was somehow restricted by barely visible cell walls. Later, cell walls were completely dissolved and PHB inclusions started to freely move in the solution and seemed to remain stable in the medium indefinitely. This pilot work nicely shows how stable the PHB is. Despite being polyester, due to its hydrophobicity and compact form, it is much less susceptible to digestion by aqueous reagent than all other cell components. However, only a direct observation is described in the study. No information on molecular weight, purity and yield is given. Later, PHB samples from different bacteria obtained using different methods were tested in more detail [42]. The study shows that hypochlorite treatment significantly decreases molecular weight. PHB obtained by extraction instead of Williamson's digestion is much less degraded. It refines Williamson's observation – PHB granules withstand hypochlorite treatment without any change in

their physical appearance, but molecular weight is affected.

Later, conditions of hypochlorite digestion were further explored. Temperature, time, concentration of biomass and hypochlorite, pretreatment and other factors were investigated. The outcome is a method that uses much less hypochlorite then previous [41] and consequently, resulting polymer is far less degraded. Best results are obtained when the biomass is pretreated with 0.125% SDS (pH = 10) and then reacted with 5.25% NaClO at pH = 10 for very short time (1 min + 15 min centrifugation), molecular weight can be up to 96 % of original value and purity can be up to 98 %. However, reported recovery is only 74 %. Very diluted biomass is used – only 1% concentration. Higher concentrations reportedly led to lower purity of product [43, 44]. The procedure significantly improves feasibility of hypochlorite method, but due to huge excess of water, necessity of careful optimization and low recovery, still seems impractical.

Another promising oxidant is hydrogen peroxide, preferably in combination with chelating agent. The chelating agent binds polyvalent metal ions and reportedly increases efficiency of oxidant. Biomass is pretreated by shock heating at 150 °C for 80 s. Then chelating agent and oxidant are added. The concentration of biomass is quite high (100 – 200 g/l) and a ratio of non-PHB cell material to hydrogen peroxide is roughly 1:1 (w/w) [45]. Information about yield and molecular weight are not given. Purity is reported, but the analytical method is quite pelicular – nitrogen is measured in a product, protein content is estimated from the % of nitrogen and it is assumed that everything else is PHB. Calculated purities are very high (98 – 100 %), but the method obviously stands on too many flawed assumptions. Otherwise, the process seems appealing because it uses reasonably high concentration of biomass, acceptable amount of relatively nontoxic oxidant and requires quite mild conditions.

1.1.2.2 Digestion with surfactants

Surfactants are known to disrupt biomembranes, solubilize lipids and denature proteins, which makes them ideal agents for biomass digestion. Most popular are anionic surfactants like sodium alkyl sulfates, sodium alkyl ether sulfates and linear alkyl benzene sulfonates due to their low cost and high efficiency. Sodium dodecyl sulfate (SDS) is of special interest.

Procedure with SDS as the only reagent can be used for analytical purposes. Biomass is mixed with SDS solution in a test tube and sonicated. After that, sample is centrifuged and washed with water and ethanol. After drying, pure PHB is obtained. The procedure requires only one test tube and enables simple, gravimetric determination of PHB. It correlates well with standard method, which is whole-cell methanolysis and subsequent determination of methyl 3-hydroxybutyrate by GC.

Molecular weight is not significantly affected by SDS [46].

If faster method suitable for large-scale operation is needed, action of anionic surfactant can be combined with alkaline pH. Mild temperature (10 – 50 °C) and short time (less than hour) then suffice to digest the biomass. Concentration of the biomass can be high. Polymer can be separated by filtration, preferably after addition of coagulant. Yield is around 85 %, purity exceeds 98 % and molecular weight is above 400 000 Da [47]. Another study further improves the SDS method. Concentrated biomass suspensions (50 to 300 g of dry cells/l) is treated with SDS at 30 °C for 1 h. After the reaction, mixture is heated to 121 °C for 15 minutes and is centrifuged. Solid PHB is washed with water and dried. Different doses of SDS were used to evaluate optimal amount. It was, finally, found out that 1 g of SDS digests 0.72 g non-PHB cell material, regardless other factors [48]. Excellent purity yield and molecular weight can be reached by this method. High concentration of biomass is another benefit. But it clearly demonstrates that SDS must be used in excess over non-PHB cell material. Use of SDS is problematic due to its cost (compared to inorganics) and troubles in wastewater disposal.

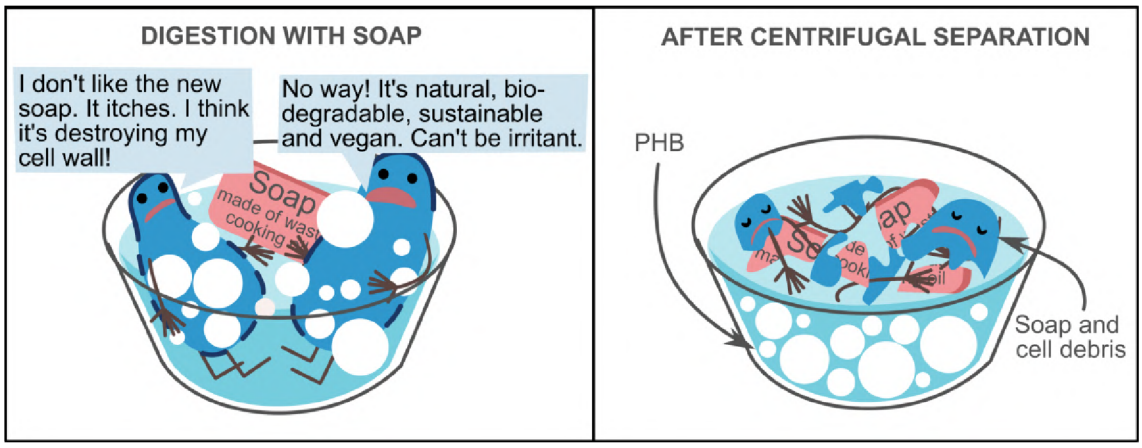

Fig. 1.8: Isolation of PHB by soap-based digestion [11]

These issues, apparently, can be solved if other anionic surfactant – ordinary soap. Genius of the method is that soap in wastewater can be easily precipitated by addition of acid and separated, together with other organic compounds, in a form of large flocks. Wastewater with much lower organic carbon content results from the process, which makes the process more economical and environmentally friendly than similar procedures with SDS [49]. Other authors [50] presented slightly different concept, with soap as "switchable anionic surfactant" and claim that after digestion and separation from the PHB, soap can be precipitated from wastewater by acidification and used again (they do not presuppose the precipitation of other substances). As can be seen, there are only few mentions of this interesting and

potentially economic method, they lack information about the essential steps of the process (or they are lost in the confusing translation from Japanese), and in some respects they contradict each other. For this reason, soap digestion was given attention in my thesis.

1.1.2.3 Digestion with acids and bases

Acids and bases act as catalysts for hydrolysis of different biomolecules and therefore are capable of solubilizing biomass. Common acids and bases seem to be ideal reagents for digestion, because they are cheap, readily available and their reaction with organic compounds don't produce hazardous byproducts. After digestion, they can be simply neutralized. On the other hand, PHB is known to hydrolyze in extreme pH, too. Some decrease of molecular weight always appears if biomass is treated with acid or alkali for a long time.

Acids and bases differ in performance and there is no consensus on the best reagent among this class. For example, one study tries to compare different digestion reagents with chloroform extraction. 5% biomass was treated with different chemicals for 1 hour at 30 or 37 °C. The chemicals tested for the digestion were 0.1 N acids (HCl and H_2SO_4), 0.1 N alkalis (NaOH, KOH, and NH_4OH), and 5% surfactants (dioctyl sulfosuccinate sodium salt [AOT], hexadecyltrimethylammonium bromide [CTAB], sodium dodecyl sulfate [SDS], polyoxyethylene-p-tert-octylphenol [Triton X-100], and polyoxyethylene(20)sorbitan monolaurate [Tween 20]). With this reaction conditions, purity above 95 % can be reached only with SDS. Similar results have hydroxides (KOH, NaOH: 92 %), other chemicals at the given conditions are insufficient (purity <90 %). Hydroxides are explored further, the effect of concentration on yield, purity and molecular weight is described. Digestion of 5% biomass with 0.2 N NaOH at 30 °C for 1 h, which will result in the purity of 98.5 % and recovery yield of 91.3 % is concluded to be ideal for large – scale operation. Molecular weight was found out to be only 16 % lower than the original molecular weight. The study also shows that overall cost of large - scale process with sodium hydroxide would be lower than sodium hypochlorite digestion [51]. It looks like a clear win for hydroxide, but another study gives contradictory results. It compares sulfuric acid method with alkaline digestion, hypochlorite digestion and combined $NaClO/CHCl_3$ technique. Here, reagents are tested at different conditions, not only one fixed concentration, temperature and time. Sulfuric acid at slightly higher concentration and temperature not only gives pure polymer but is also superior in both economic and environmental aspects. Calculations for industrial scale production are also given. It is shown that suggested route would produce polymer for 1.11 Eur/kg and carbon footprint would be 6 kg CO_2/kg of polymer [52]. Method with

sulfuric acid is in detail described in another work [53]. It reveals that concentration of the acid should be between 0.005 – 0.25 mol/l and biomass slurry should contain around 7 % of solids. Biomass is heated with the acid for up to 4 hours at 80 – 150 °C. The mixture is then alkalized to pH 7 – 11 and polymer is separated and washed. Molecular weight, yield and purity depend on concentration of the acid, temperature and time. The authors introduce "severe factor" (SF) that impacts quality of the product (Eq. 1.1).

$$SF = \log(te^{\frac{T-100}{14.75}}) - pH \tag{1.1}$$

Where t is time in minutes and T is temperature in °C. n is approximated by normality of the acid (Eq. 1.2).

$$SF = \log(te^{\frac{T-100}{14.75}}) + log(n_{acid}) \tag{1.2}$$

Where t is time in minutes, T is temperature in °C and n is normality of the acid in mol/l of H^+ equivalents. According to the authors, the severe factor should be between 0.8 and 1.2 to obtain good compromise between purity and molecular weight. Then, results can be as good as >95% yield, >97% purity and molecular weight >500 000 Da (if the original biomass has Mw > 1 000 000 Da). Further, it is reported that thus obtained PHB is highly crystalline and withstands bleaching with peroxide or hypochlorite without significant loss of molecular weight. The sulfuric acid method seems to be well explored and gives excellent results. We concluded that certain disadvantage is a presence of sulfates in wastewater – limits for sulfate disposal are quite low. Advantageous would be use of hydrochloric acid that produces chlorides instead. Results are shown in Experimental section.

1.1.2.4 Biotechnological methods of digestion

Use of more sophisticated biotechnological tools like enzymes or microorganisms is another way how to separate PHB. The advantage is extreme specificity and mild conditions. Therefore, molecular weight of resulting PHB is usually excellent. On the other hand, price or reaction time can be limiting. Purity may be lower because the highly specific agents are not capable of removing all constituents of the cell debris. Often, combination with other methods is used to increase quality of the product.

Enzymes – lysozyme and deoxyribonuclease, combined with sonic oscillation can be used to separate PHB granules. Mild conditions (30 °C, pH 7 – 8, small amounts of enzymes) proved to be enough, but no information on yield and purity of the material is given [54]. Described purification is unsuitable for large scale operation– it uses centrifugation in glycerol and dialysis as a last step. Other work tries to develop enzymatic process transferable to industrial scale. It combines use of enzymes,

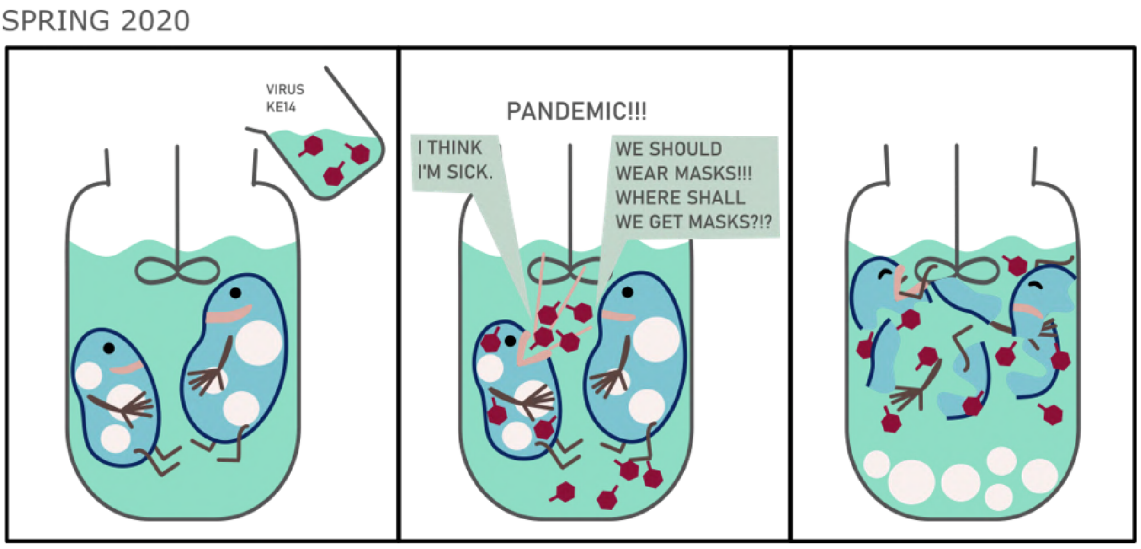

Fig. 1.9: Use of phages for biomass digestion

surfactants (namely SDS) and hydrogen peroxide as bleaching agent [55]. Quality of resulting PHB is good, but the process is quite complicated. It uses a large amount of SDS (10 % amount of dry cell mass) which would dramatically increase cost of wastewater treatment.

Different approach is use of phage culture as a digestion agent (fig. 1.9). Phages are introduced to biomass after cultivation and left to infect the cells. Cells start to disintegrate, produce more virus and liberate PHB. At the end, PHB is separated by centrifugation [56]. Predatory bacteria can also act as a digestion agent. *Bdellovibrio bacteriovorus* was used for this purpose, and was found effective in killing and disintegrating PHA-producing bacterie. However, its wild is able to degrade the polymer by hydrolysis with extracellular-like mcl-PHA depolymerase. But this obstacle can be easily overcome by disrupting phaZ_{Bd} gene by common genetical engineering methods (fig. 1.10). [57]

The idea of biological war in fermenter is indeed very attractive. As the "reagent" replicates itself, only small amount is needed at the beginning. Most of the "reagent", in fact, is produced in situ from non-PHB cell material by cells themselves. The liquid waste from the process, if sterilized, is safe and can be disposed to ordinary wastewater treatment plant. On the other hand, research on this topic is at the beginning and current results are not optimal. Purity of the obtained PHB is low, the process is time consuming and yield is quite low, too. Authors explain that some PHB is consumed by cells during their fight with the disease and another portion remains entangled in not-fully disintegrated cell debris.

Even higher organisms can be used to isolate PHA from biomass. This fact was discovered by serendipity when a team of researchers found rats in storage room

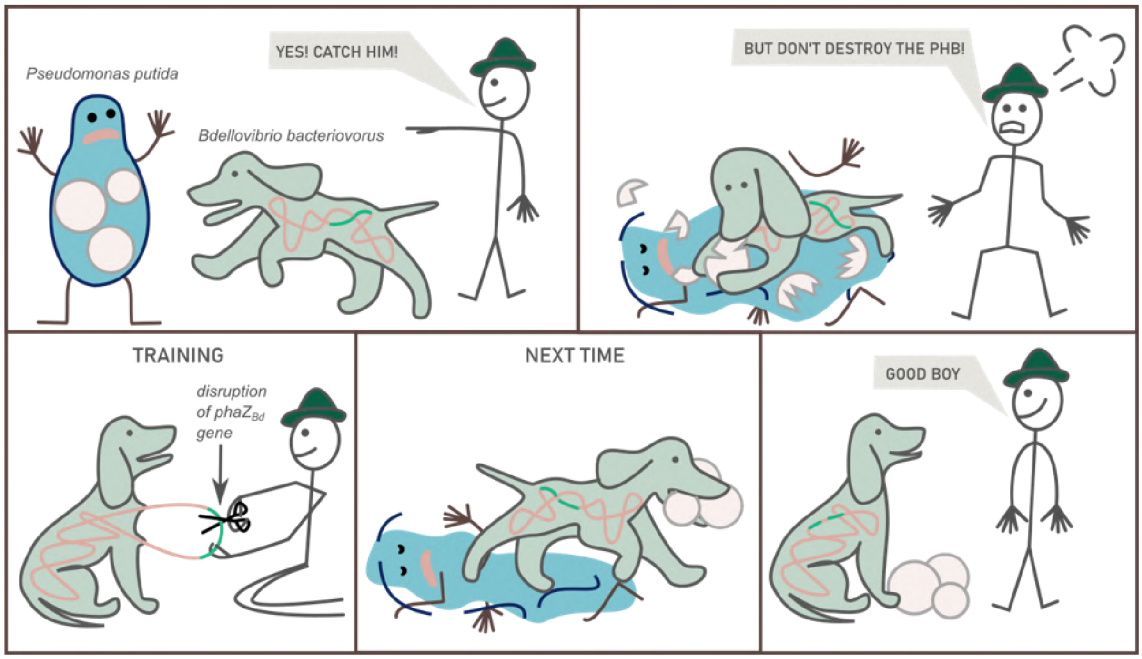

Fig. 1.10: Use of predatory bacterium for biomass digestion

with dried biomass. The researchers probably started thorough sanitation work and noticed that the rat excrements were almost white and remarkably resembling a powdered PHB (fig. 1.11). This led them to further experiments under more controlled conditions, which showed a surprisingly high purity of the obtained PHB. [58] Similar experiments were also performed with mealworms. [59]

Another strategy is a genetical engineering approach. Genome of bacteria can be manipulated to contain an autolysis system that turns on when certain outer condition change. In other words, cells can be programmed to commit suicide after physical or chemical command. The environmental change can be exhaustion of

Fig. 1.11: Use of rats for biomass digestion

nutrient (glucose) [60] or presence of lysis inducer (3-methylbenzoate) [61]. All these approaches promise very economical PHB separation but are still in a proof of concept stage.

1.1.2.5 Digestion methods – conclusion

Digestion methods, overall, are much less hazardous than extraction methods. Especially processes with alkalis, acids, microorganisms, enzymes and surfactants are not highly toxic or explosive in any stage. Solubilization with these agents don't produce hazardous byproducts. Methods with oxidants require slightly more precaution, but still are far safer than any of the solvent-based methods.

The physical form of polymer is very different from the extracted PHB – instead of rough precipitate, it is a fine powder. It may be a big benefit, especially if the target use requires powdery form (mainly cosmetics). But may be disadvantage in other cases, where dusts are unpopular (compounding etc.). Yield is usually good to excellent, typically higher than in extraction. After solvent extraction, filtration is usually used to separate solid cell debris and some extract always remains on the filtration cake. Due to high viscosity, the amount of retained extract may be significant. Researcher must always do the Sophie's choice – to wash the extract with additional solvent or to leave some product behind in the cake. Contrary, after digestion, it is easy to separate PHB quantitatively by filtration or centrifugation.

On the other hand, compromise must be typically made between purity and molecular weight. Harsh methods of digestion give very pure product, but molecular weight decreases a vice versa. This is valid mainly for methods based on acids, alkalis and oxidants. Surfactants and enzymes are much milder and possibly very efficient at the same time, but their price is discouraging. We can conclude that digestion methods are suitable, if resulting polymer doesn't have to be of extreme purity and/or molecular weight can be lower. Technical plastics or cosmetics are possible examples, while medicine seems not to be.

Instead of solid waste that results from extraction, aqueous solution is obtained after digestion. The solution contains variety of organic and inorganic compounds in quite diluted form. Direct disposal can be costly and due to complex composition, other uses do not seem feasible. Evaporation of water and disposal or incineration of solid waste may be advantageous but is energy-consuming. Appealing, but still hypothetical option is to use the liquid waste as a source of nutrients for next fermentation. The idea will be experimentally tested in the future.

1.1.3 Mechanical disruption methods

These methods have similar philosophy as digestion methods but deny (or at least minimize) use of chemical agents for cell disruption and employ mechanical energy instead. Milling, sonication, high pressure homogenization, high shear mixing and other common methods for cell disruption can be used. Mechanical disruption is often used as a part of chemical process, as described in the chapters above. However, some examples where mechanical disruption plays a key role also exist.

1.1.3.1 Example mechanical disruption methods

One work deals with PHA production from different plants, but in some examples, use of bacterial biomass is mentioned, too. Biomass is ultrasonicated to obtain a mass containing PHB granules of size <1 micrometer. Suspension is freeze-dried and pulverized using fluid energy mill. Air classification yields fine fraction containing PHB. To reach better purity, fine fraction is then extracted with chloroform and precipitated to methanol. Precipitate has purity higher than 95 % and yield is more than 85 % [62]. Efficiency of only the mechanical process, unfortunately, is not given but probably is insufficient for ordinary use of the polymer. Instead of drying and air classification, suspension can be injected to hydrocyclone. The effluent coming out of top section contains PHB. The effluent is spray-dried to obtain PHB that has purity higher than 95 % and yield is about 90 % [63].

In another patent [64], continuous jet separator is used. According to the authors, centrifuges failed for the separation, but jet separator gives good results. After high pressure homogenization, suspension is purified by the jet separator and diluted with distilled water. Diluted suspension is separated again, and PHB-containing stream is spray dried. 98% purity can be reached by this method. Process can be further improved by alkali and acid wash and possibly bleaching with hypochlorite or hydrogen peroxide in last stage. Purity increases from 95.5 % to 99.9 %. Another advantage is that purified polymer reportedly withstands high temperatures better [65].

Mechanical disintegration can be combined with two phase aqueous separation. After cultivation, cells can be ultrasonicated and mixed with thermoresponsive polymer solution. Example of the polymer is PEG-block-PPG. The solution is then heated to 65 °C and phases separate. Impurities concentrate in a top aqueous phase, while PHB and thermoresponsive polymer are in bottom phase. Bottom phase is centrifuged, and pellet is washed to obtain PHB. Supernatant containing thermoresponsive polymer can be used repeatedly [66]. Purity and yield are not any impressive and comparative example is missing; therefore, it is not clear if the procedure is more efficient than mere disintegration and centrifugation.

1.1.3.2 Mechanical disruption – conclusion

Purely mechanical processes have two main disadvantages – necessity of large investments to homogenizers and separators and low purity of the product. The main advantage is no need of other chemicals and that biomass debris is the only waste. Apparently, pros don't overcome cons because the authors of purely mechanical methods usually improve them by extraction or digestion step in their following publications. Purity then increases, but the price is increased complexity of overall process. Considering all the aspects, mechanical methods don't seem to be practically useful for our purposes.

1.2 PHB derivatives

Main goal of PHB technology is to obtain pure PHB that would be used as a plastic. But PHB can be also further treated (chemically or physically) to obtain its derivatives. For purposes of this work, the derivatives will be grouped to three categories:

- monomeric derivatives (crotonic acid, 3-hydroxybutyric acid, esters of the acids etc.)
- oligomers
- polymeric specialties (PHB micro- and nanoparticles, biofillers etc.)

Some derivatives are of high value, for example microparticles for cosmetics or nanoparticles for medical applications. Therefore, their technology may start with high quality virgin PHB. Other derivatives are of lower value than virgin PHB or can be easily manufactured from different starting material. For example, crotonic acid can be produced from cheap petrochemicals [67] and (R)-3-hydroxybutyric acid can be prepared by catalytic reduction of acetoacetate or fermentation [68]. In that case, it is meaningless to use virgin PHB as a resource. But they can be desired product of so-called chemical recycling of PHB.

1.2.1 Monomeric derivatives

Monomeric derivatives are obtained by depolymerization of PHB. Commonly used ways of depolymerization are hydrolysis, alcoholysis, elimination or reduction, yelding (R)-3-hydroxybutyric acid, its esters, crotonic acid or (R)-1,3-butanediol as products. In following section, these chemicals will be briefly discussed.

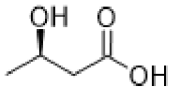

Fig. 1.12: (R)-3-hydroxybutyric acid

1.2.1.1 (R)-3-hydroxybutyric acid

Properties and uses (R)-3-hydroxybutyric acid (C4H8O3, M=104.106, CAS 625-72-9) has a melting point 44-46 °C [69], boiling point is 85-87 °C at 0.08 Torr [70]. It's a chiral compound with rotary optical power -24.7°(c=5g/100ml, water). It's naturally occurring metabolite and can be found in animal and human bodies [71]. It is a sex pheromone of spider Linyphia triangularis [72] (fig. 1.13). It is a useful building block in organic synthesis [70] and can be used as plasticizer precursor [73], but most uses are related to its bioactivity. One study shows that it improves locomotion and relieves hypersensitivity in mice after spinal cord injury [74]. It can induce ketosis and related effects on health, for example faster catabolism of fats, lower cholesterol levels, improved brain function [71, 75] or better control over seizures in patients with pharmaco-resistant epilepsy [76]. It was suggested as a treatment for non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, alcoholic steatohepatitis or non-alcoholic fatty liver [77]. It can be used as nutritional supplements to increase physical performance and as therapeutics to ameliorate symptoms of medical conditions, particularly neurological conditions, such as Alzheimer's and similar [78].

Fig. 1.13: Linyphia triangularis

Preparation Direct hydrolysis of the polymer is unsuitable because harsh conditions are required. Long times and high temperatures result in formation of crotonic acid (fig. 1.14) that is hard to remove [79, 70].

Enzymatical methods are possible [80], but availability and price of the enzymes limit their use. Most common method is alcoholysis of PHB and subsequent hydroly-

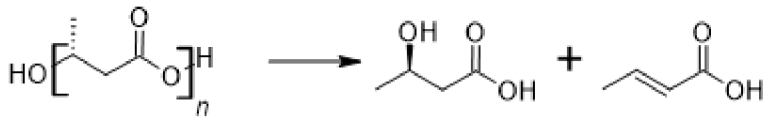

Fig. 1.14: Hydrolysis of PHB

sis of the ester (fig. 1.15). Yields are typically high, and racemization doesn't occur [81, 82, 83, 84]. Due to availability of reagents and easy separation, the method seems ideal.

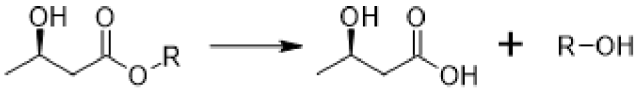

Fig. 1.15: Hydrolysis of (R)-3-hydroxybutyric acid ester

1.2.1.2 Esters of (R)-3-hydroxybutyric acid

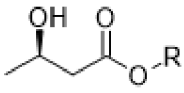

Fig. 1.16: (R)-3-hydroxybutyric acid ester

Properties and uses Methylester (CAS 3976-69-0) and ethylester (CAS 24915-95-5) are the most common. Ethylester finds its use as a solvent and food flavoring. Methylester and (-)-menthylester are also flavorings [85]. Methyl ester is considered promising biofuel with expected price around 1.2 USD/kg [86]. Butylester is marketed as a high-performance solvent for household cleaners [87]. Fatty alcohol esters can be intermediates for specialty surfactants [88]. Esters of (R)-3-hydroxybutyric acid are readily metabolized to free acid and therefore can be used as its precursors in dietary supplements. Common esters and free acid are reported to have unpleasant taste that can discourage users from treatment. Some unusual esters (for example butandiol monoester) were found to be more sensorially acceptable [89].

Preparation As mentioned previously, the esters can be prepared by alcoholysis of PHB [81, 82, 83, 84, 70, 69]. Moreover, waste PHB can be used as starting material [90] and alcoholysis is therefore promising way of chemical recycling of PHB (fig. 1.17).

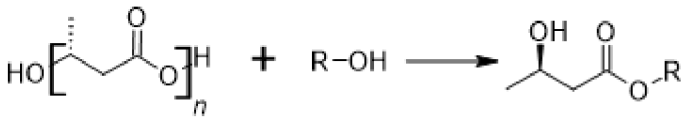

Fig. 1.17: Alcoholysis of PHB

Other, PHB-unrelated methods include for example reduction of acetoacetate esters [91] or alcoholysis of lacton [73] (fig. 1.18). Racemic or optically pure esters can be obtained, depending on catalyst and precursor, respectively.

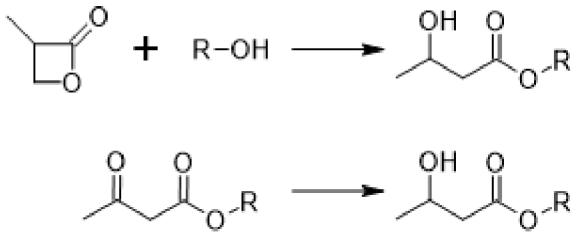

Fig. 1.18: Example methods for alkyl 3-hydroxybutyrate preparation

1.2.1.3 Crotonic acid

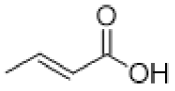

Fig. 1.19: Crotonic acid

Properties and uses Crotonic acid (CAS 107-93-7) is white crystalline solid with typical odor. Melting point is 72 °C, boiling point is 189 °C. It is slightly soluble in water and soluble in polar organic solvents (alcohols, ketones, ethyl acetate). It has widespread use in chemical technology. Free acid is used in motor fuels, for metal etching, electrochemical metal deposition or PVC heat stabilization. But its main use is in the synthesis of copolymers with a variety of comonomers. Especially important are copolymers with vinyl acetate. Copolymers of crotonic acid have a broad range of applications, for example in paints and coatings, adhesives, paper and textile coatings, flocculants, binders, agrochemicals and drilling additives. Esters of crotonic acid serve as antimicrobial agents in deodorants. Fatty alcohol esters of crotonic acid are used in leather industry. It is a precursor for several agrochemicals and pharmaceuticals, for example crotamiton is anti-itching drug [67].

Preparation Industrially, crotonic acid is produced by isomerization of vinyl acetate or, preferably, by oxidation of but-2-enal [67] (fig. 1.20).

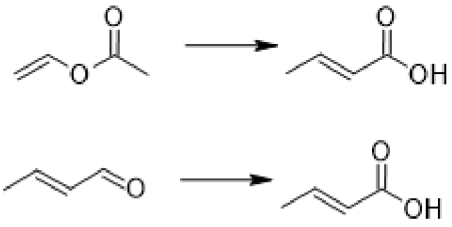

Fig. 1.20: Methods for industrial production of crotonic acid

On a laboratory scale it can be prepared from 2-hydroxybutanoic acid, by photochemical oxidation of crotonaldehyde, condensation of acetaldehyde and malonic acid, thermal degradation of PHB and many other methods [67] (fig. 1.21).

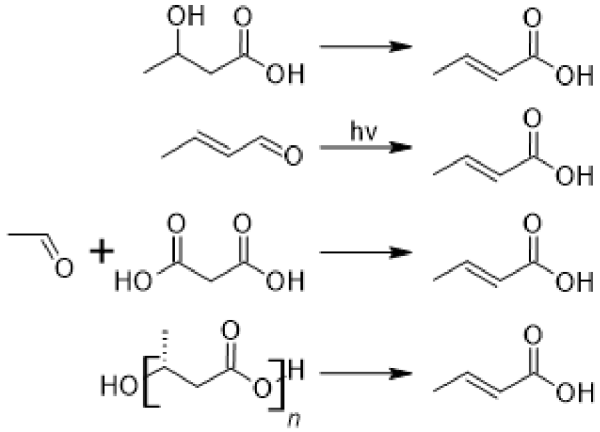

Fig. 1.21: Example methods for preparation of crotonic acid

1.2.1.4 (R)-1,3-butanediol

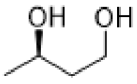

Fig. 1.22: (R)-1,3-butanediol

Properties and uses It is a colorless viscous liquid with boiling point 98-102 °C (at 11 Torr) [70]. It has a specific rotation (ethanol) of -18.8° [92]. It is a ketogenic compound and has similar physiological effects as 3-hydroxybutyric acid and its esters. Therefore, it can be used as pharmaceutical or dietary supplement for

similar diagnoses [93, 94]. For other than medical applications, cheaper racemic 1,3-butanediol is usually used. Example uses are synthesis of polyester resins [92] or as humectant in cosmetics. It retards loss of aromas and preserves cosmetics against spoilage by microorganisms. It is often used in hair sprays and setting lotions. [95].

Preparation It is usually prepared by reduction of naturally derived chiral precursor (fig. 1.23). Starting material can be (R)-3-hydroxybutyric acid [96] or its esters [97].

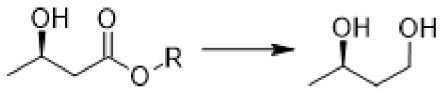

Fig. 1.23: Reduction of chiral ester

Procedures starting with PHB were also described, usually the reduction agent is LAH [98, 70] (fig. 1.24).

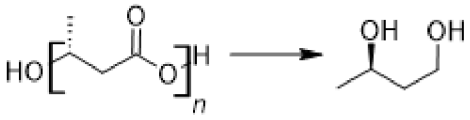

Fig. 1.24: PHB reduction

1.2.1.5 Other monomeric derivatives

3-(R)-hydroxybutyrate skeleton is a versatile building block for organic synthesis, due to enantiomeric purity for reasonable price [99, 100]. Other derivatives can be prepared by different reactions of the above. Derivatization on C1 (carboxylic group) can lead to amides, thiols, halogenides or unusual esters. Hydroxyl on C3 can be substituted or hydroxy group can be alkylated or acylated. Multistep reactions can lead to beta-lactam antibiotics precursors [101, 102].

1.2.2 Oligomers

Properties and uses Very short oligomers (dimer, trimer) have antioxidant activity and are suggested as treatment or prevention of oxidative stress related diseases [103]. In biomedical research, oligo-(R)-3-hydroxybutyrates are used for preparation of biocompatible polyurethanes [104] or block copolymer for drug delivery [105, 106]. Different compounds, like antioxidants, pesticides or pharmaceuticals can be bound to oligomer. Resulting conjugate is then able to controllably release the active compound [107, 108, 109, 110, 111]. Oligomers also find their place in material research.

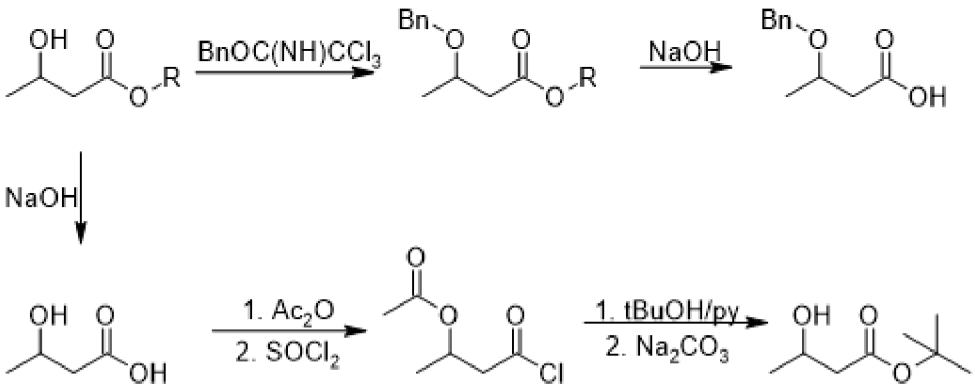

Fig. 1.25: Synthesis of protected 3-hydroxybutyric building blocks

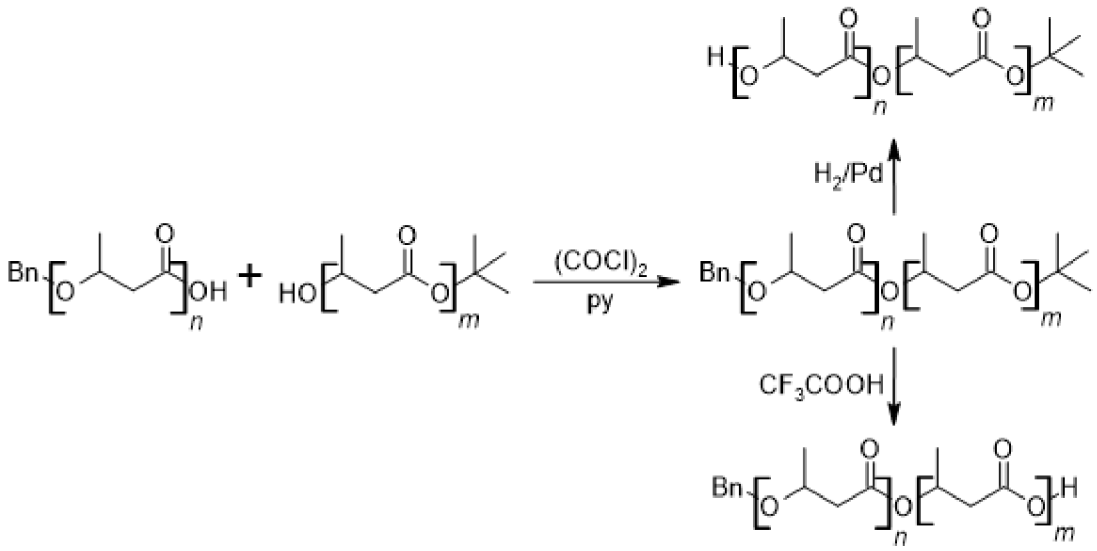

Fig. 1.26: Coupling and deprotection of the building blocs

They can be copolymerized with PEG or polyurethane. Copolymers can be useful in many material applications for which PHB on its own is too brittle or stiff [112, 113].

Preparation Most common way of oligomer preparation is "bottom-up" method, for example polymerization of beta-butyrolacton [114, 115, 116, 105, 117] or condensation of protected building blocks (fig. 1.25) developed by Seebach's team[118, 119]. Seebach's technique is multistep and laborious but enables preparation of monodisperse oligomers with total control over chain length (fig. 1.26). If needed, (S)-3-hydroxybutyric units can be incorporated, again with perfect control over their position in oligomeric chain. Seebach's oligomers were used for fundamental research of PHB crystallization and its metabolism. These methods, however, are out of our scope because they don't use PHB as starting material.

Preparation "top-down", from PHB, is possible by partial depolymerization.

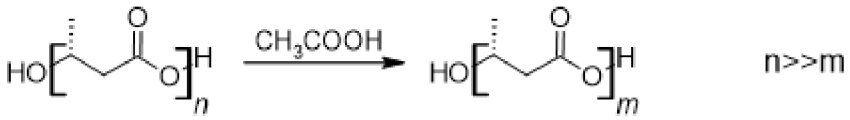

Fig. 1.27: Top-down synthesis of oligomers

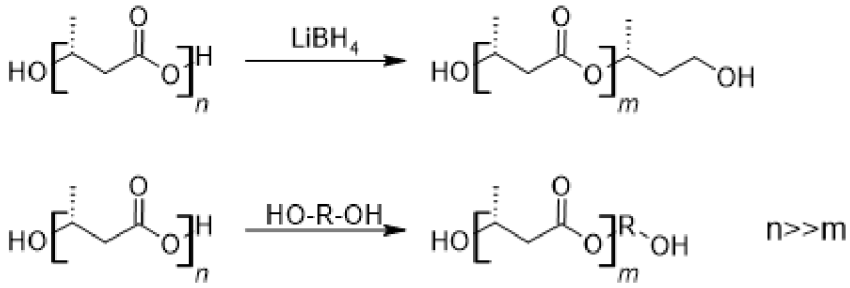

Fig. 1.28: Syntheses of telechelic -OH terminated oligomers

Conditions of depolymerization depend on the required end groups of the oligomer. Nonfunctionalized oligomers, with "natural" end groups -OH and -COOH can be prepared by partial hydrolysis in glacial acetic acid [120] (fig. 1.27).

If the termination should be double bond and carboxylic group, simple thermal degradation is an option [31]. If two or more -OH groups are required, the polymer can be partially alcoholyzed using polyol [121, 103, 122, 113]. The product can be used for example for preparation of Kolomaznik's copolymer [113]. Another strategy for getting -OH terminated telechelic polymer is partial reduction of PHB with, for example, LiBH4 [111] (fig. 1.28).

Other functional groups can be introduced by reaction of thus obtained hydroxyls, double bonds and carboxyls. For example, to obtain epoxy- terminated oligomer, double bond can be oxidized with peroxy acid or carboxy group can be reacted with epibromhydrin [123].

1.2.3 Polymeric specialties

1.2.3.1 Functional fillers

PHB in concentration 1 – 10 % can be used as a filler for polyurethane to improve its mechanical properties [124]. Powdered PHB may serve as nucleation agent in different plastics.

1.2.3.2 Cosmetic additives

Polymer additives are present in many cosmetic products. Currently used polymer additives are usually made of nondegradable polymers and are criticized for their

negative effect on the environment (“microplastic problem”) [125, 126, 127]. The additives can be visible particles, but more often, are present in a form of fine powders, synthetic waxes or heavy oils. Some authors consider soluble synthetic polymers microplastics, too. Polymers in cosmetics serve, for example, as abrasives in scrubs, thickeners in gel formulations, matting ingredients in decorative care, fillers in solid cosmetics (lipsticks, eyeshadows...) and stabilizers in emulsions. Porous particles can be used as carriers for active compounds [128]. Apparently, microplastics are widely used and the range of products with microplastic ingredients is bigger than one would expect.

Determining which of the synthetic polymers meets the definition of microplastics and which does not is not an easy task. The definition is relatively clear, but it is difficult to clearly determine whether the given ingredient is a microplastic. Only INCI names are publicly available, but they do not say anything about the physical form. E.g. under INCI "polyethylene" there may be a low molecular weight wax serving as a thickener (i.e. not a microplastic) but also abrasive microparticles (typical microplastics). Despite these ambiguities, ECHA has published two lists of synthetic polymers which could be considered microplastics [129]. The first, shorter list contains the polymers which are considered to be the most problematic. If a milder version of the envisaged restriction were passed, only these polymers would probably be restricted ("low scenario"). The larger list contains 520 polymers, that would be banned in the event of a tough restriction ("high scenario"). Given that a ban on these 520 synthetic polymers would lead to the end of the European cosmetics industry, only the shorter list might be considered authoritative.

PHB-based alternatives are under development. For example, colored PHB microparticles for use in decorative care are patented [130]. Scrub particles based on PHB were developed on FCH BUT [131] and are currently marketed. Our next research will be focused on developing fine PHB powders as another grade for different uses.

2 Aims

The main aim of this work was to improve methods for PHB separation from bacterial biomass. Work was focused on methods of extraction and digestion that, based on literature review and consulting with our industrial partners, were concluded to be the most promising strategies.

Regarding extraction, mainly biomass pretreatment methods were experimentally studied. As described in Theoretical section, dried biomass was preferable starting material for PHB extraction, but suitable pretreatment should be done to enable easy drying. Alkali/acid-induced flocculation was studied as a promising, economical and easy method.

Digestion methods based on nontoxic, readily available reagents were explored. It is known that purity and molecular weight of PHB from acid - catalyzed digestion correlate with so-called severe factor and that it can be calculated according to equation 1.2. Validity of the severe factor – based model was evaluated. Soap - based digestion was studied as a method that uses very economical surfactant, which is soap derived from waste cooking oil, and gives PHB with high molecular weight. Both methods were optimized for large-scale experiments and future industrial use.

Another goal was to investigate the potential uses of PHB. Based on a literature search and negotiations with the industrial partner, the use of PHB in cosmetics and 3D printing materials was evaluated as promising. *In silico* analysis of the composition of cosmetic products available on the Czech market was performed, focusing on the presence of polymers that could be replacd with PHB. For the purpose of assessing PHB as a 3D printing material, the thermal stability and its dependence on the isolation method used were studied.

3 Materials and Methods

3.1 Materials

Bacterial biomass of *Cupriavidus necator* H16 was donated by Nafigate Corp. a.s. (Czech Republic). According to the manufacturer, the biomass was obtained by fermentation of waste cooking oil and then concentrated by centrifugation to about 50 % cell dry weight (CDW). The biomass was then frozen and stored and transported at -20 ° C. The PHB content in the CDW was about 70 %, depending on the batch. Specific data on dry matter and PHB content in the batches used were also provided by the manufacturer. Waste cooking oil was also obtained from Nafigate. According to the manufacturer, saponification value of the oil was 186 mg KOH/g and acid value was 5 mg KOH/g and the oil contained mainly oleic, linoleic, palmitic and stearic acid triacylglyceroles, diacylglyceroles, free fatty acids and oxidised forms of the above. Iodometric analysis according to AOAC methods [132, 133] showed iodine value 96 g/100 g and peroxide value 20 meq/kg. Commercial PHB sample was provided by Biomer (Germany). EtOH, and MeOH were obtained from Lach-ner LLC (Czech Republic). Protease from *Bacillus licheniformis* (2.4 U/g), citric acid, succinic acid, stearic acid, maleic anhydride, and succinic anhydride were obtained from Sigma-Aldrich (Germany). NaClO solution (4.7%, technical) was provided by Bochemie a.s. (Czech Republic). All other chemicals were supplied by Penta Chemicals Unlimited (Czech Republic).

3.2 Extraction methods

3.2.1 Biomass pretreatment before extraction

3.2.1.1 Attempted flocculation with acid

Frozen biomass containing 50 % dry matter out of which 75 % is PHB was used. After reaching room temperature, the biomass was diluted to 8 % CDW with distilled water. Biomass suspension was acidified to pH 6 – 2 with different acids (HCl, H_2SO_4, H_3PO_4, CH_3COOH). No flocculation was observed nor at room temperature neither upon heating. Subsequent neutralization of the acidic suspension with alkali (NaOH) didn't lead to the flocculation either.

3.2.1.2 Attempted flocculation with alkali

Frozen biomass was left to reach room temperature and diluted to 8 % CDW. The suspension was treated with different bases (NH_3, NaOH, KOH, triethylamine).

Rapid increase of viscosity was observed, but without flocculation. Mixtures were heated up to 95 °C, which didn't lead to flocculation either.

3.2.1.3 Flocculation with alkali/acid, general procedure

Biomass suspension was alkalized (tested bases were NaOH, NH_3, triethylamin, $Ca(OH)_2$) to the required pH (7 - 12) as described above. The viscous gel-like mixture was heated to the required temperature (25–90 °C, 0–30 min). Then the mixture was acidified (tested acids were sulfuric acid, phosphoric acid, lactic acid, acetic acid and acetanhydride) to required pH (1-6). At certain pH, coagulation and formation of stiff flocs was observed. The flocs were left to stand in acidic solution for 0.5 h during which further stiffening and shrinking could be seen. Flocs were then separated from the liquid and dried at 100 °C until the weight was constant.

Different biomass batches were used: E_180620 Nafigate, E_180620, E_170919 and washed biomass (centrifuged after fermentation, mixed with distilled water and centrifuged again before freezing). Testing with fresh (unfrozen) biomass was also included. Influence of biomass suspension salinity was also evaluated, by experimenting with 8 % washed biomass suspensions containing 1 – 3 % NaCl. Further, concentration of biomass was explored, with samples containing 4 – 24 % dry mass. Separation of flocs was by vacuum filtration over standard filter paper, filtration over sieve (0.5 mm), with or without pressing of the flocs.

3.2.1.4 Flocculation with $NaOH/H_2SO_4$, large-scale procedure

The experiment was conducted at the Institute of Microbiology. 375 l of biomass E_181107 #107 (contained 8,94 % dry mass) was processed directly after fermentation. Biomass was stirred (30 Hz) in 400 l batch reactor with single external jacket. pH was increased to 9 by addition of 30% NaOH and the mixture was heated to 60 °C. Heating took 2 h and meanwhile, pH dropped to 7.2 and viscosity decreased. Afterwards, a sample was withdrawn and flocculated in laboratory. Direct flocculation lead to soft flocs and turbid liquor, but if pH of the sample was further increased, hard flocs and clear liquor were obtained. Therefore, another NaOH was added to reactor to increase viscosity of the mixture. Then the acid was added while monitoring pH and cooling the reactor. Sample of thus obtained flocs was filtered, dried and extracted using diethyl oxalate as described below.

3.2.1.5 Flocculation with $NaOH/H_3PO_4$, large-scale procedure

The experiment was conducted at the Institute of Microbiology. 1000 l of biomass E_181204 (contained 7.6 % dry mass, out of which 53 % is PHB) was stirred in 1500 l fed-batch fermenter (duplicated pressure vessel with 4 baffles, agitated with

3 Rushton turbines and 1 Hydrofoil on a shaft, with automatic pH, temperature and pressure control. Otherwise, procedure was as the $NaOH/H_2SO_4$ experiment described above. After flocculation, mixture of flocs and liquor was left to sediment. Liquor (containing 4.813g/l P (photometrically) and 0.379 % Na (ICP)) was discarded, flocs-rich phases (360 l) were collected, concentrated using drum centrifuge and dried using vacuum drier yilding 51.5 kg of dried flocs. Moisture content was 2 %. Sample of the material was used for extraction with diethyl oxalate as described below, yielding 9.25 g of white product.

3.2.1.6 Extraction with diethyl oxalate

To evaluate if thus obtained flocs are suitable for extraction, polymer was extracted by Jašek's method with diethyl oxalate [23]. Briefly, 25 g of the dried biomass was heated with diethyl oxalate (250 g) at 130 - 140 °C for 15 min. Extract was vacuum filtered while hot and then left to cool to room temperature upon precipitation of the polymer. The precipitate was filtered and gently squeezed to remove most of the mother liquor. Then it was washed twice with ethyl acetate and dried to constant weight.

3.2.1.7 Extraction with chloroform

For comparative experiments, standard method - chloroform extraction described earlier in the literature [12] was used. Briefly, biomass was dried to constant weight at 105 °C and coarsely grinded. 5 g of dried biomass was stirred with 95 g of chloroform under reflux. The mixture was filtered and the filtrate was precipitated by slow addition to 400 ml of cold methanol. Then the product was dried at 70 °C for 0.5 h.

3.3 Digestion methods

3.3.1 Alkaline digestion

3.3.1.1 Attempted alkaline digestion of untreated biomass

Frozen biomass was left to reach room temperature and incubated with NaOH (temperatures 25 – 90 °C, pH 8 – 12, time 5 min – 5 h). After addition of alkali, viscous gel was obtained. Homogenization by high shear mixer was used to decrease viscosity. Attempts were made to separate the product by centrifugation, however, due to the high viscosity of the mixture were not successful.

3.3.1.2 Alkaline digestion of dried biomass

Previously published procedure [52] was adapted. Biomass was dried at 105 °C to constant weight and grinded. 9.36 g NaOH was dissolved in 459 ml water and mixed with 12 g of the dry biomass. The mixture was stirred at 37 °C for 4 h and then centrifuged (10 000g/10min). White pellet was separated from brown supernatant and the pellet was washed by diluting with water to 460 ml and centrifugation. The washing was repeated once more with water and then with ethanol. Then the pellet was dried at 60 °C for 5 h.

3.3.1.3 Heat pretreatment and gelling

Frozen biomass was left to melt at room temperature and diluted with water to 25 % CDW. the mixture was stirred and heated in a pressure vessel at given temperature and time. Then it was left to cool to room temperature and tested for gelling upon alkalization by adding 20% NaOH to pH 9.

3.3.1.4 Alkaline digestion of heat-pretreated biomass

Biomass (5 g) was left to reach room temperature and diluted with water (5 ml) to 25 % CDW. Then it was heated for 1 h at 100 °C. Biomass was mixed with NaOH solution (10 ml) and stirred. After given time at required temperature, sample was centrifuged (10 000g/10 min). Pellet was washed by diluting to 20 ml with water and centrifugation. Washing with water was repeated twice. Then the pellet was dried to constant weight at 105 °C.

3.3.2 Acid digestion

3.3.2.1 Acid digestion - general procedure

Frozen biomass was left to reach room temperature and diluted with water. Acid (H_2SO_4, HCl, HNO_3, HCOOH or H_3PO_4) was added and the mixture was stirred at given temperature for given time. Then 20% NaOH was added until the pH was 9, the mixture was diluted and centrifuged at 7000g for 8 min. Supernatant was discarded and pellet was diluted with water to the original volume and centrifuged. The washing with water was repeated twice more. Then the product was dried at 105 °C to constant weight.

3.3.2.2 Digestion with HCl, optimized procedure

760 g of frozen biomass (with 50 % dry mass, out of which 60-70 % is PHB) was left to reach room temperature and mixed with 240 ml of water. 36% HCl was added to

(40 ml, 47g, 16.9 g HCl, 450 mmol) and mixture was heated and vigorously stirred. After reaching 90 °C, the mixture was stirred for 3 h at 90 – 95 °C. Pale brown suspension turned darker, viscosity first slightly increased and then decreased. After 3 h, mixture was diluted with 2500 g of water and alkalized to pH 9 by adding 20% NaOH. Consumption of NaOH depended on a batch of biomass and ranged from 152 to 162 g (760 to 810 mmol). Mixture was centrifuged (7500 g/6 min) and dark brown supernatant discarded. Pale pellet (containing mostly PHB) weighted around 430 g (typical value; depends on biomass batch) and contained 57 % dry matter. 3000 g of water was mixed with pellet until homogeneous. Pale yellow mixture was centrifuged (7500 g/6min), yellow supernatant discarded and white pellet (around 380 g, dry matter 63 %) was mixed with another 3000 g of water and centrifuged in the same manner. Supernatant was roughly neutral (pH around 7.5) and little yellowish. Pellet (370 g, 65 % of dry matter) was mixed with acetone (2500 g) if bright white color of product was required. Centrifugation (4000 g/4 min) led to pale yellow supernatant and while pellet (400 g, 60 % dry matter). Pellet was dried for 12 h at 105 °C.

3.3.2.3 Digestion with HCl, large-scale procedure

The test was conducted at Research Institute of Chemical Technology. 65 kg of water was heated to 95 °C in 250 l batch enameled reactor. 82.5 kg of frozen biomass (batch 181003, 181010, 181018; representative sample had 52 % dry matter out of which 59.8 % was PHB) was added and mixture was stirred for 25 min to homogenize. Then hydrochloric acid (5.3 kg) was added and charger was washed with 24 kg of water. Temperature dropped to 53.4 °C. Mixture was heated via external jacket (t= 130 °C) and stirred (80 rpm) until the temperature reached 90 °C (2 h 25 min). Then the temperature was maintained at 90 – 95 °C for 2.5 hours. A sample (140 ml) was withdrawn and worked up as described above. White product was obtained, indicating that digestion was successful. Mixture in the reactor was diluted with water (183 l) and alkalized to pH 3 – 4, which required 4 kg of 20% NaOH. The slightly acidic solution was stored at 4 °C and used for centrifugation tests. Sample of the mixture (100 g, 1/3650 of the total volume) was worked up in a laboratory. pH was set to 9 and sample was centrifuged and washed with water as described in section 3.3.2.1. Dry product weighted 6.8131 g.

3.3.3 Soap-based digestion

3.3.3.1 Soap preparation

Small-scale soap preparation was described elsewhere [11]. For making larger amounts, modified procedure was used: 318 g (7.95 mol, 1eq.) of NaOH was dissolved in 912 g of water. 2400 g of waste cooking oil (7.95 mol of saponifiable groups, based on saponification value) was thoroughly mixed with the hydroxide solution in a 5 l beaker using a hand mixer. The beaker was covered with a petri dish and placed to an oven heated to 80 °C. After 0.5 h, the mixture was homogenized again. To quantify residues of free hydroxide, sample (ca 5 g) was withdrawn, dissolved in 50 ml of hot water and titrated with 0.5 M HCl on phenolphthalein. turning from dark pink to pale pink was considered endpoint. Conversion was calculated as the difference between the concentration of free hydroxide at the beginning (ie 2.19 mmol/g) and the actual concentration, divided by the total concentration of alkalis (ie soap and free hydroxide, 2.19 mmol/g). The reaction in 80 °C oven with homogenization each 0.5 h continued until the conversion was above 95 %. Typically, this took 1 - 1.5 h. Then the mixture was poured on a tray with a baking paper and left to cool overnight. Resulting solid was cut into ca 50 g pieces and stored in sealed plastic bags.

3.3.3.2 Soap-based digestion, general procedure

Frozen biomass was mixed with water 1:1 to obtain the suspension containing 25 % CDW. The mixture was heated to 95 °C for 0.5 h and cooled to 80 °C. Soap was added and if needed, the pH was set to 9-9.5 by addition of small amount of 20% NaOH solution. The mixture was stirred for 1 h at 80 °C and then cooled to 55 °C. If required, protease was added (0.01 ml/g CDW). The mixture was stirred at 55 °C for 1 h and the pH was adjusted to 9 – 9.5 if needed. The mixture was heated to 95 °C and centrifuged (10 000g/10 min/60 °C). The supernatant was separated, same volume of boiling water was added to pellet and resulting mixture was centrifuged (10 000g/10 min/60 °C). Washing with hot water was repeated twice more. The supernatants were stored for further workup (see Section 3.3.3.4). The product was dried at 105 °C to constant weight. [11]

3.3.3.3 Soap-based digestion, optimized procedure

Frozen biomass (1000 g) was mixed with 1000 g of water in 2.5 l jacketed glass reactor. The mixture was heated to 95 – 100 °C for 0.5 h. Heating was stopped and soap (50 g, 0.1 g/g CDW) was added. The pH was adjusted to 8 by the addition of 10 ml of 20% NaOH. After 1 h, the temperature dropped to 90 °C. The mixture was

centrifuged (10 000g/10 min/60 °C) and the pellets were returned to the reactor. The volume was made up to 2 l with water and protease (5 ml, 0.01 ml/g CDW) and another portion of soap (50 g, 0.1 g/g CDW) were added. The mixture was stirred 1 h at 55 – 60 °C and the pH was maintained above 8 by additions of 20% NaOH (another 26 ml). The mixture was heated to 90 °C and centrifuged (10 000g/10 min/60 °C). The pellet was washed with 1 l of boiling water three times. Aqueous supernatants (5 l total) were stored at 4 °C for further workup (see Section 3.3.3.4). Pellet was washed with acetone (1 l) and vacuum dried at 40 °C to constant weight (ca 4 h).

3.3.3.4 Precipitation

The supernatants from PHB digestion were acidified to pH 3 using 2.5 M HCl. The mixture was centrifuged and the precipitate (stable, semisolid oily emulsion) was separated from transparent yellow liquid. Dry weight of precipitate was measured gravimetrically. The precipitate was stored at 4 °C. [11]

3.3.3.5 Cultivation

Cultivation experiments were performed by Ing. Iva Novackova. *C. necator* H16 (CCM 3726) was inoculated in NB medium (25 g/l) at 30 °C and 180 rpm for 24 hours. Subsequent cultivations were performed in mineral salts medium consisting of 1 g/l $(NH_4)_2SO_4$, 1.02 g/l KH_2PO_4, 11.1 g/l $Na_2HPO_4 \cdot 12\ H_2O$, 0.2 g/l $MgSO_4 \cdot 7\ H_2O$ and 1 mL/l mineral elements solution MES (9.7 g/l $FeCl_3$, 7.8 g/l $CaCl_2 \cdot 2H_2O$, 0.156 g/l $CuSO_4 \cdot 5\ H_2O$, 0.119 g/l $CoCl_2 \cdot 6H_2O$, 0.118 g/l $NiCl_2$ in 0.1 M HCl). Waste cooking oil (20 g/l), the precipitate (20 g/l) or their mixture (10 + 10 g/l) was used as carbon source. The inoculation ratio was 10 %. Each cultivation was performed in two parallel flasks at 30 °C and 180 rpm for 72 hours. The content of harvested biomass was analyzed gravimetrically. The data were obtained from two parallel cultivations, when two values for each Erlenmeyer flask were determined, i.e. averages were taken from four individual values. [11]

3.4 Thermal stability

3.4.1 Acid wash

Preparation of acid-washed samples followed the digestion methods described above (sections 3.3.1.2,3.3.2.2,3.3.3.3), but the last wash with water was modified - the aqueous suspension was acidified to pH = 3 – 3.5 with 3% H_2SO_4 before centrifugation. [11]

3.4.2 Incorporation of additives

1 g of dry PHB, prepared by the above methods (sections 3.3.1.2,3.3.2.2,3.3.3.3), was grinded with mortar and pestle with a given additive, until a fine homogeneous powder was formed. [11]

3.4.3 Thermal degradation under isothermal conditions

Each sample (ca 1 g) was heated 5 min at 190 °C in a laboratory hot press Qnubu Rosin Press. The degree of thermal degradation was then evaluated by GPC. [11]

3.4.4 Compounding and 3D printing

A prototype plastic object was prepared according to the procedure described previously. [134] PHB was compounded with PLA (25 %), tributyl citrate (15 %) and succinic anhydride (2 %). The object was printed from thus prepared filament on PRUSA i3 MK2 3D printer.

3.5 Analysis

Analytical methods are described in detail in the relevant articles. [11, 12, 135] Here, methods are briefly repeated for the sake of clarity.

3.5.1 GC-FID

The analyses were performed by Ing. Iva Novackova. Approximately 10 mg of dried sample was mixed with 1 ml of chloroform and 0.8 ml of internal standard consisiting of 5 mg/ml of benzoic acid in 15% sulfuric acid in methanol in crimping vial. During transesterification the vials were heated in a thermoblock for 3 h at 94 °C. Then the samples were cooled down, poured into vials with 0.5 ml of 0.05 M NaOH and shaked. After the phase separation, 50 µl of lower organic phase containing volatile methylesters of alkanoic acids were diluted with 900 µl of chloroform and analyzed via GC-FID (Thermo Scientific, Trace 1300; column: DB-WAX 30 m by 0.25 mm). Commercially available PHB standard was used for calibration. Every analysis was performed in triplicate.

3.5.2 GPC

5 mg of sample was dissolved in 1 ml of chloroform and measured using Agilent HPLC series 1100 chromatograph with PLgel mixed-c 5 µm, 7.5x300 mm column,

temperature 30 °C. Chloroform was used as mobile phase. 12 polystyrene standards (0.2 – 2000 kDa) were used for calibration.

3.5.3 FTIR

Infrared spectra were obtained using Bruker Tensor 27 spectrometer in ATR mode.

3.5.4 Laser diffraction

The analysis was performed by Zulfiya Cernochova of the Institute of Macromolecular Chemistry of the Czech Academy of Science. The particle size distribution (weighted by volume fraction) was determined by Mie scattering (Mastersizer 3000 instrument, Malvern Instruments Ltd., United Kingdom).

3.5.5 DSC

Experiments were performed by Ing. Veronika Melcova. Around 10 mg of sample was hermetically sealed into aluminum pans. Four heating cycles were measured using DSC 2500 from TA Instruments. In each cycle, samples were heated to 190 °C (10 °C/min) annealed for 5 min at 190 °C and cooled to 0 °C (10 °C/min).

4 Results and discussion

4.1 Extraction methods

4.1.1 Biomass pretreatment

As defined by our industrial partner, first obstacle in any industrial solvent extraction of PHB was concentration and drying of biomass. Concentration of fermentation medium by centrifugation was possible, however, the step was rather time-consuming and energy-demanding. Feasibility of drying of the concentrated biomass on industrial scale was questionable. There were problems with sticking drying biomass to parts of the dryers. In addition, drying was slow due to the formation of an impermeable layer on the surface. This created a requirement to convert the biomass into a better separable and dryable form. Based on the literature search and the requirements of the industrial partner, experiments with cell flocculation using pH adjustments were done.

First, experiments were performed by simply adjusting the pH to an acidic and basic region using various reagents. It was found that acidification to pH 2-6 at room temperature with any of the tested acids did not lead to any appreciable change in mixture consistency. On the contrary, alkalization led to a dramatic change in viscosity, when the liquid suspension became a semisolid gel (fig. 4.1. It was relatively stable - neither the increase in temperature nor the longer reaction time led to the desired conversion of the gel into flakes. But if the alkaline mass was then neutralized with acid upon stirring, formation of flocks surrounded with clear solution was observed. The reversed procedure (acid first, base second) didn't lead to any flocculation. Therefore, only the alkali/acid procedure was further developed.

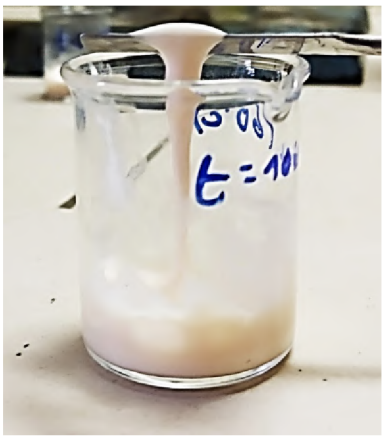

Fig. 4.1: Gelling of bacterial biomass upon alkalization

4.1.1.1 Flocculation with alkali/acid combination

Conditions necessary for successful flocculation were further explored. Series of experiments were conducted to evaluate influence of different factors. In all experiments, basic procedure consisted of alkalization, incubation of the alkaline mixture, acidification, standing in acidic solution in order to stiffen and concentrate the flocks, separation and drying. Procedures were optimized for getting stiff flocks containing minimal amount of water. Surrounding solution should be clear (shouldn't contain unreacted biomass). It was found out that all variables - biomass, base, acid, times and temperatures - influence optimality of the flocculation.

There are slight differences between batches of biomass. Clarity and color of liquor and also density and volume of flocks differ between batches (fig. 4.2).

Fig. 4.2: Flocculation of different biomass batches

Salinity is probable explanation. The starting frozen biomass contains not only cells, but also residues of culture medium that contains minerals. Best results were reached with washed biomass that, due to wash with distilled water, contains less salts than other samples. To confirm the hypothesis, different amounts of NaCl were added to washed biomass suspension. These samples were then processed in the same manner. As can be seen in figure 4.3 , without NaCl, dense, stiff flocks were obtained, and liquor was only slightly opalescent, while sample with 3 % of NaCl flocculated to a form of soft, moist gel.

Concentration of the biomass doesn't influence the result too significantly. The higher the concentration of biomass, the higher viscosity of alkaline mixture. To ensure efficient agitation, concentration therefore should be up to 24 % . In all proceeding steps, behavior of 4% sample is the same as 24% sample (fig. 4.4). There is only little difference in a moisture content in the flocks. flocks prepared from 4%, 8%, 16% and 24% biomass suspension contained 75 %, 73 %, 71 % and 66 % moisture, respectively.

Base and conditions of alkalization were very important. After alkalization and heating, viscous gel was formed. It was found out that viscosity of the gel influences

Fig. 4.3: Flocculated biomass after filtration (Petri dish) and its liquor (beaker above). Leftmost sample contains no added salt, others had 1%, 2% and 3% of added NaCl, respectively.

Fig. 4.4: Differently concentrated biomass suspensions after flocculation

Fig. 4.5: Spherical flocks obtained by dropping alkaline biomass gel to acid solution

clarity of the resulting liquor, regardless other conditions. In other words, if the viscous gel is formed, liquor will be clear after flocculation. Viscosity is affected by identity of base, temperature and pH. NaOH was found to be the most practical reagent. NH_3 and TEA are also efficient, but due to their volatility and irritancy of vapors are harder to handle. $Ca(OH)_2$ doesn't cause the desired reaction. The higher the pH and temperature, the higher viscosity. But too severe conditions should be avoided to maintain high molecular weight of polymer.

Acidification can be done with any acid, all tested acids proved to react in the same manner. Temperature was also found insignificant. Only the pH matters. The lower the pH after acidification, the harder and drier flocks are. But identity of the base used for previous step also plays a role – in experiments with NaOH, acidification to pH = 5 resulted in flocks with 60 % moisture. If NH_3 was used as a base, pH had to be decreased to 3 to get the same result.

Acidification can be done reversely, too. The gel can be dosed to solution of acid, which may be advantageous if uniform particles are needed. By dropping the gel to acidic solution, uniform spheres were obtained (fig. 4.5).

At the end, flocks had to be removed from the liquor. The most practical method was filtration with gentle pressing of flocks to remove maximal amount of water. Liquor is always rather viscous and little cloudy, filtration over too fine filter is therefore slow. Use of fine filter was unnecessary because flocks after successful experiment are quite big. 0.5 mm sieve was found out to be optimal filter in our experiments. Another option was centrifugation but had to be gentle. flocks don't withstand too high acceleration and very fast centrifugation leads to their breakage.

Drying was much easier than that of raw biomass. flocks were less adherent to

any of tested surfaces (glass, porcelain, steel) and were porous. If dried in one layer, they didn't stick together and remained as particles of suitable size for extraction (diameter 1 – 5 mm). If moisture of flocks was less than 60 %, constant weight was reached within one hour at 100 °C. For comparison, with untreated biomass (50 % CDW, otherwise same conditions) the constant weight was reached after 6 h.

4.1.1.2 Further laboratory experiments

Further experiments were conducted to find suitable conditions for large scale production. Efficient, robust, economical, nontoxic and energy saving procedure was the main goal. Therefore, further experiments were focused on common chemicals (NaOH, NH_3, H_2SO_4, H_3PO_4) in smaller amounts. Temperature changes were also reduced. Testing with fresh (unfrozen) biomass was also included. Two procedures were, afterwards, developed for large-scale testing. Economical and "zero waste". The economical procedure consists of alkalization with NaOH to pH 9, heating to 60 °C and acidification with H_2SO_4 or H_3PO_4 to pH 5. Zero waste procedure uses NH_3 and phosphoric acid. Their use results in liquor enriched with nitrogen and phosphorus, that can be theoretically used as medium for next biomass fermentation and doesn't have to be discarded. It was found out that slightly more severe conditions were needed. Series of experiments showed that higher temperature and later more acidic conditions of precipitation are essential for successful flocculation. If the temperature was less than 90 °C, gelling in alkaline stage wasn't sufficient and after acidification, soft flocks and cloudy liquor were obtained. At 90 °C, liquor was clear after acidification, but pH at least 3 was needed to obtain firm flocks (figure 4.6).

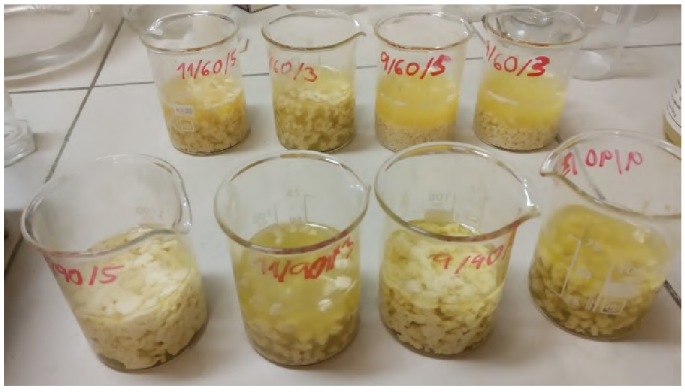

Fig. 4.6: Flocculation with NH_3 and H_3PO_4. Experiments at 90 °C in front, at 60 °C in behind. From left: alkalization to pH 11, acidification to pH 5; alkalization to pH 11, acidification to pH 3; alkalization to pH 9, acidification to pH 5; alkalization to pH 9, acidification to pH 3

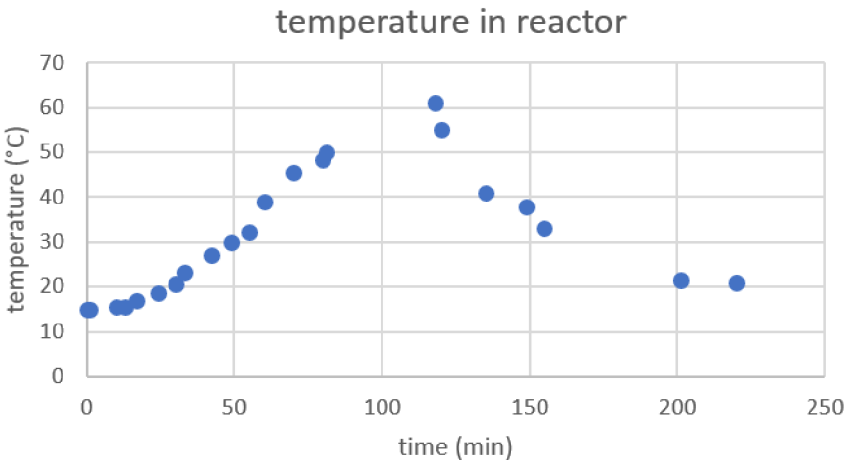

Fig. 4.7: Temperature during the $NaOH/H_2SO_4$ large-scale experiment. Reactor was heated after alkalization and cooled during acidification (from 120th minute).

To evaluate if thus obtained flocks are suitable for extraction, polymer was extracted by Jašek's method with diethyl oxalate 3.2.1.6. It was found out that yield and purity were same as if directly dried biomass of the same batch was used. Molecular weight was only 10 % lower.

4.1.1.3 Flocculation with $NaOH/H_2SO_4$, large-scale procedure

The experiment was performed in 400 l reactor as described in section 3.2.1.4. Observed values are shown in figures 4.7 and 4.8.

The experiment shows that flocculation can be done in simple batch reactor. Consumption of reagents was slightly higher than expected from lab-scale tests. Main reason is prolonged time of heating (caused by dimensions of the reactor) and subsequent drop of pH after alkalization. It had to be compensated by addition of more NaOH and therefore, consumption of the acid was also higher than laboratory test suggested. Due to prolonged stirring at rather high speed, the flocks were slightly smaller than required. Therefore, further workup of the whole volume was not performed. Sample of thus obtained flocks was filtered, dried and extracted using diethyl oxalate as described previously 3.2.1.6 to confirm good extractability of the polymer.

4.1.1.4 Flocculation with $NaOH/H_3PO_4$, large-scale procedure

The experiment was performed in 1500 l fermenter (fig. 4.9) as described in 3.2.1.5. Otherwise, procedure was as the $NaOH/H_2SO_4$ experiment. Less intense stirring

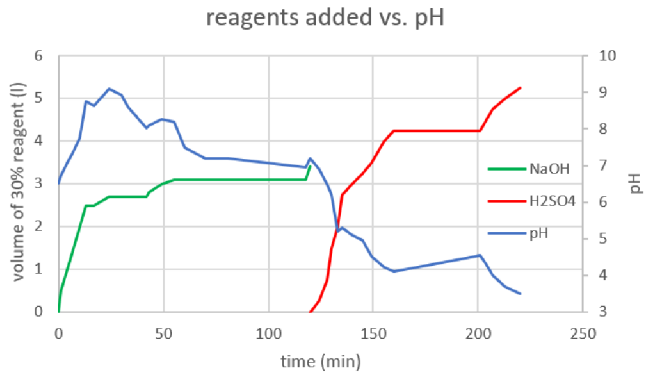

Fig. 4.8: pH in reactor during experiment. After addition of hydroxide, pH increases to 9, but later drops to almost neutral. Within 40 minutes, 4.25 l of acid was added and pH dropped to 4.1. During next 40 minutes, pH slightly increases, until another dose of acid was added.

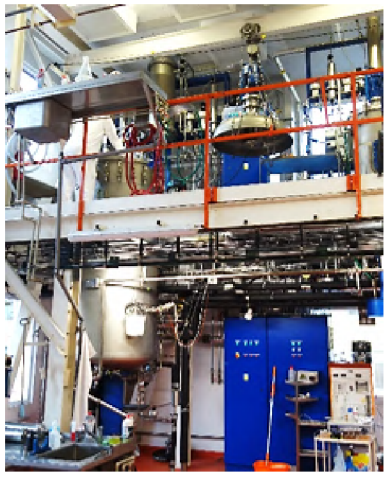

Fig. 4.9: Fermenter

was used in the precipitation phase, which resulted in larger, more suitable flocks. Data are shown in figures 4.10 and 4.11.

There was no dedicated device for separation of the flocks in the facility, and transportation of the whole volume was not feasible. Therefore, the flocks were pre-separated by sedimentation that resulted in floc-rich phase and floc-poor liquor, that was discarded. Floc-rich phase was transported, concentrated using drum centrifuge and flocks were dried using a vacuum drier (fig. 4.13). Overall yield of the dried flocks was 68 %, whereas the material loss may be attributed to the sub-optimal way of separation. Extraction with diethyl oxalate showed that a high quality polymer could be obtained from the flocks. Yield was 71 %, the extraction product was white, melted at 175 °C and formed white plastic upon melting (fig. 4.14).

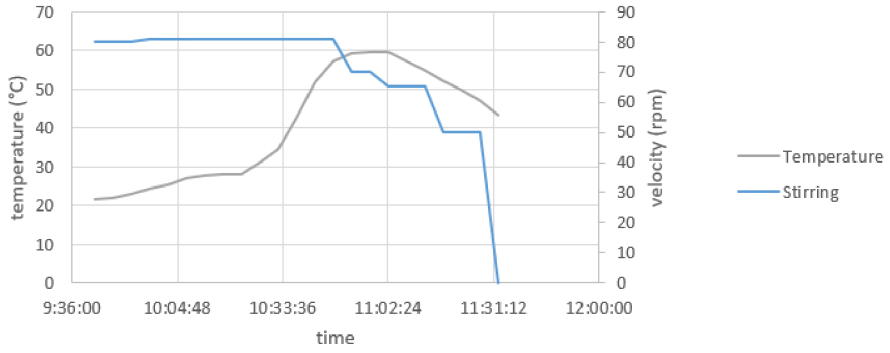

Fig. 4.10: Temperature and stirring during $NaOH/H_3PO_4$ experiment. Reactor was heated after alkalization and cooled after addition of acid. To avoid breakage of flocks, stirring speed was reduced after acidification.

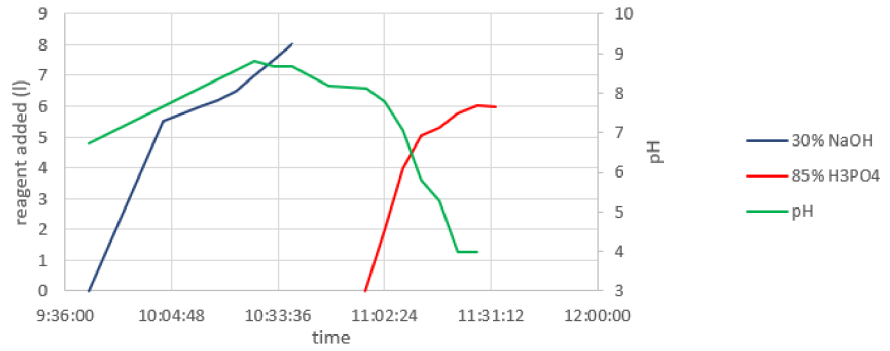

Fig. 4.11: Volume of reagents and pH in the $NaOH/H_3PO_4$ experiment

Fig. 4.12: Consistency of biomass during experiment. 9:52 – raw biomass, 10:37 – alkaline gel, 11:28 – flocculated

Fig. 4.13: Drum centrifuge and drum vacuum drier for flocks separation and drying

Fig. 4.14: PHB obtained by extraction of dried flocks from $NaOH/H_3PO_4$ experiment

4.2 Digestion methods

4.2.1 Alkaline digestion

4.2.1.1 Attempted alkaline digestion of untreated biomass

Although alkaline digestion of biomass is often mentioned in the literature, in our conditions it seemed very problematic at first. Mainly because the published procedures usually use dried or lyophilized biomass, while the priority for us was to work with fresh, untreated biomass, as it is obtained after fermentation and centrifugation. However, as mentioned above (section 4.1.1), the alkalization of this biomass leads to a thick, gel-like mixture, which is then difficult to stir and process. A number of experiments were performed with progressively more aggressive reaction conditions, but none led to the decomposition of the gel into a liquid mixture from which PHB could be obtained. Some thinning was observed when the mixture was homogenized, but even that was not sufficient.

4.2.1.2 Alkaline digestion of dried biomass

To test whether our biomass behaves differently if dried first, an experiment with dried biomass was performed (3.3.1.2). In this experiment, no gelling of the mixture was observed, workup was rather easy and good-quality product was obtained. The product was a white powder, with analytical purity of 98 %. Slight decrease in molar mass was observed - M_w was 263 kg/mol, compared to 423 kg/mol before isolation. The main drawback of the procedure was the necessity to dry and finely grind the biomass, high dilution of the reaction mixture, long reaction time and rather low yield, which was 76 %.

4.2.1.3 Heat pretreatment and gelling

This experiment showed that drying the biomass eliminated the problem of gelling in the mixture. Although there was no room for a thorough study of the thickening mechanism in this work, it can be assumed that it may be due to the release of bacterial macromolecules in an alkaline environment, and that these macromolecules degrade upon heating. Therefore, a number of experiments was performed with heating the biomass at different temperatures for different times. It was evaluated whether the biomass thus treated would be gel-like after alkalization or not. The results are summarized in fig. 4.15. It can be seen that temperature plays a key role. Heating to 90 °C had to be rather long to be effective, while heating to 100 °C or more worked almost immediately. 1 h heating at 95 °C seems to be a safe

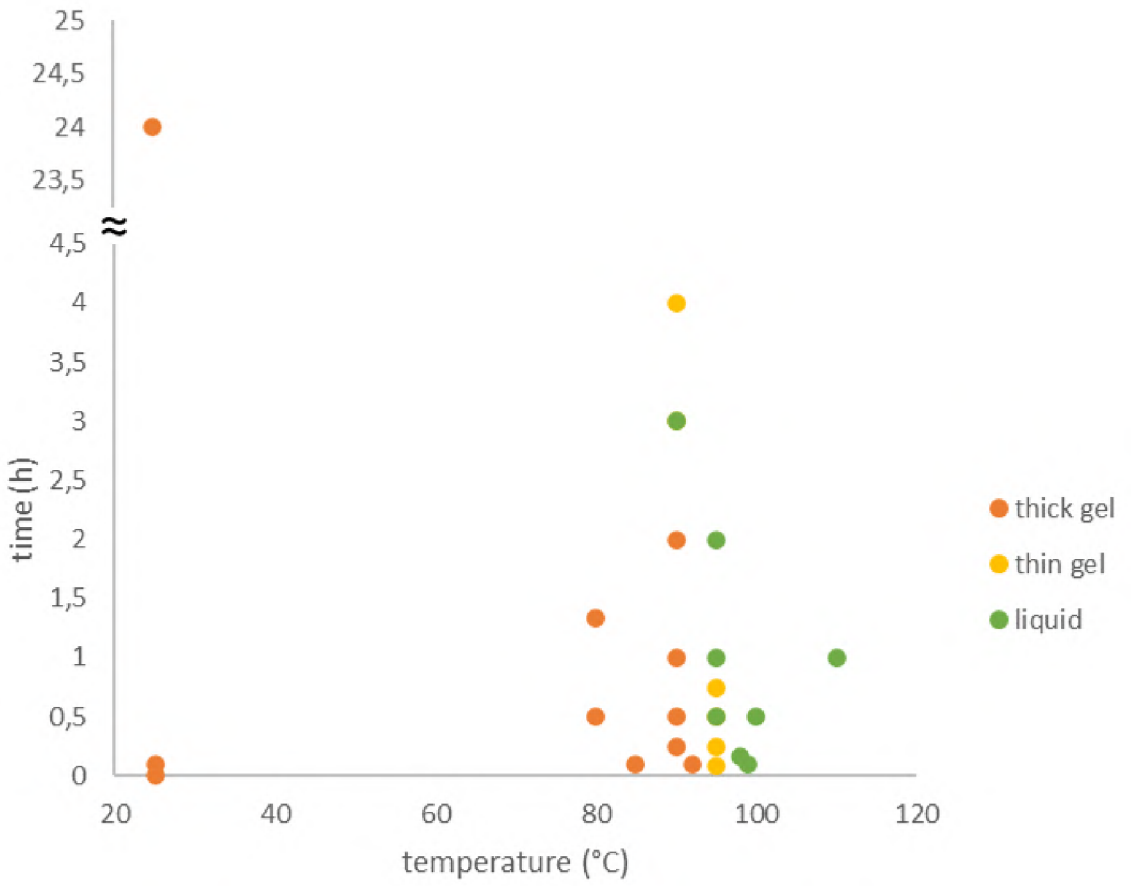

Fig. 4.15: Consistency of 25% biomass suspension after: 1. heating for given time at given temperature, 2. alkalization with NaOH to pH 9.

compromise in industrial settings, where boiling or pressure-heating of the mixture is not suitable.

4.2.1.4 Alkaline digestion of heat-pretreated biomass

Next experiments on alkaline digestion were done on heat-pretreated biomass. Getting white product with sufficient purity in nearly quantitative yield was the main goal. Here, workup by centrifugation and repeated washing with water was easy, due to low viscosity of mixtures. Therefore, more concentrated mixtures (up to 10 % CDW) could be successfully used. Relationship between temperature, time, NaOH concentration and purity was explored. We observed that if purity (analyzed by GC method) of PHB is above 97 %, white powder is obtained. The lower the purity, the more the sample resembles dry biomass (brown glassy solid). In pilot experiments, therefore, purity was evaluated visually. Results are summarized in fig. 4.16. As can be seen, yield is close to quantitative in only one experiment, with the mildest

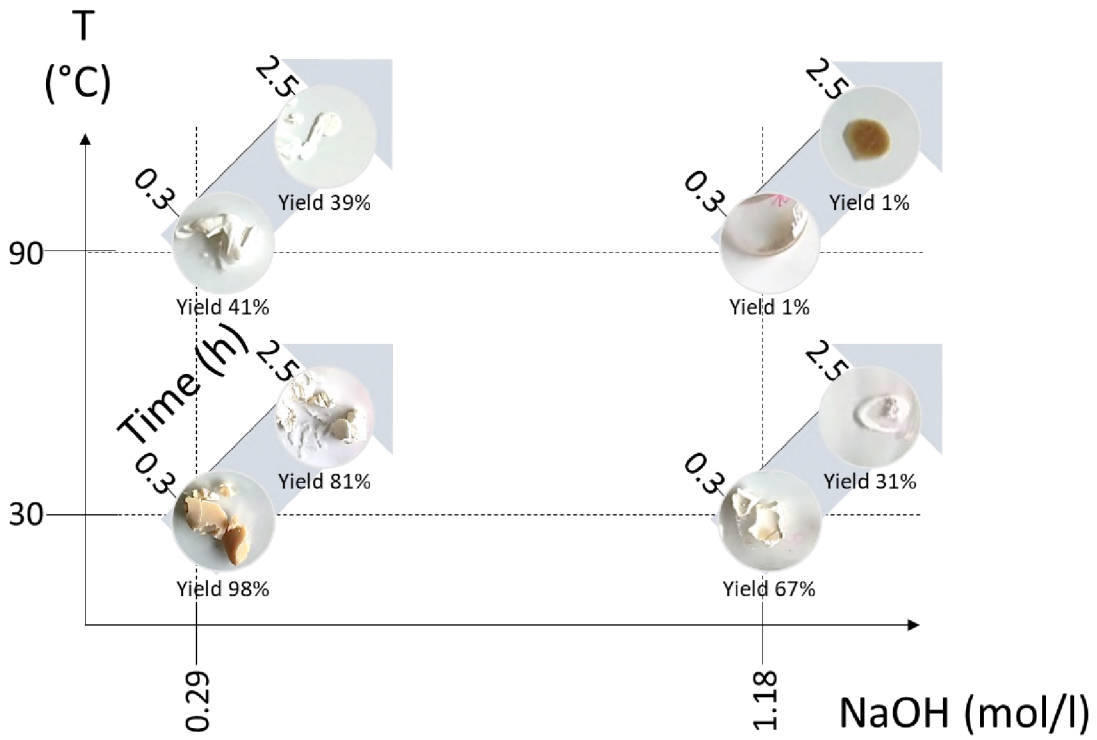

Fig. 4.16: Alkaline digestion of heat-pretreated biomass with NaOH. Influence of time, temperature and NaOH concentration on yield and product's color

reaction conditions. However, this experiment didn't give pure product, as can be seen in the photo. Low purity was further confirmed by FTIR (fig. 4.37), where rather intense protein signals at 1530 and 1650 cm^{-1} could be seen. At harsher reaction conditions, purity increased, but yields were significantly lower. At the highest temperature and NaOH concentration, yield was close to zero. Only a small amount of brown solid was obtained, and according to FTIR, it was mainly protein. The results show that NaOH separates PHB from other cell components, but at the same time, it reactively dissolves the polymer. Finding optimal conditions that would lead to sufficient purity and yield would be demanding. Therefore, this method was not explored further and other strategies were tested.

4.2.2 Acid digestion

4.2.2.1 Acid digestion - initial experiments

Our experiments were strongly inspired by work of Yu and Chen [53], who developed a method for isolation of PHB using sulfuric acid. The experimental design stems from their procedures, however, certain modifications were made to satisfy re-

Fig. 4.17: Acid digestion with different acids. Preparation followed general procedure 3.3.2.1, biomass concentration was 38 % CDW, concentration of acid in reaction mixture was 0.4 mol/l and the mixture reacted 3 h/95 °C.

quirements for scalable procedure. For the sake of simplicity and environmental and occupational safety, bleaching step was omitted, and more concentrated biomass was used. Initially, different acids were tested in order to assess their effectivity. Resulting products are shown in fig. 4.17. HCOOH a H_3PO_4 reacted too slowly and after the reaction time gave brown, biomass-like product. HNO_3 gave brightly yellow mixture due to xanthoprotein reaction. Even after repeated washing, the coloration couldn't be removed. Best results were obtained with H_2SO_4 and HCl, where the reaction led to white product. Furthermore, both acids are widely available for acceptable price.

Deciding which acids would be more suitable for potential industrial use was not easy. The advantage of H_2SO_4 is its non-volatility, and thus higher safety in the acid phase of the reaction. However, the treatment of the reaction mixture and the treatment of the wastewater also had to be taken into account. The consumption of NaOH for neutralization of HCl is lower than the consumption for neutralization of equally concentrated H_2SO_4. In addition, chlorides are considered less problematic in wastewater than sulphates. Therefore, the use of HCl is potentially more economical than H_2SO_4.

4.2.2.2 Digestion with HCl

In following experiments, different HCl concentrations were tested, together with different reaction times and temperatures. Unlike the alkaline digestion described above, no significant losses of product were observed. In all cases, yields were 80 - 100 % (data not shown for the sake of clarity) and losses were fully explainable by clumsy sample handling. Regarding purity, it was hypothesized that results should match Yu's model [53]. The model predicts that if SF (severe factor, expressed by the equation 4.1, where t is time in minutes, T is temperature in °C and c is concentration of the acid in mol/l) is higher than 0.8, pure product (>97 %) should

be obtained. As in previous section (4.2.1), our requirement was getting white product and we could assume that color roughly correlated with analytical purity. Severe factor for different reaction conditions was calculated and expected outcome was plotted together with experimentally obtained products (fig. 4.18 and 4.19).

$$SF = \log(te^{\frac{T-100}{14.75}}) + log(c_{acid}) \tag{4.1}$$

As can be seen in figures 4.18 and 4.19, the model doesn't match experimental results entirely. Slightly more severe conditions were needed in our experiments to obtain pure product. Probable reason is concentration of the biomass. Model was developed for digestion of 7% biomass, while we work with 36% biomass. The biomass has certain buffering capacity due to presence of amino acids, fatty acid, phosphates etc.. Therefore, the more concentrated biomass, the more acid is consumed for overcoming the buffering capacity and doesn't contribute to the reaction.

For further scale-up, minimal amount of reagents was the main requirement. It can be seen that 0.34 mol/l is the minimal HCl concentration for successful digestion. Temperature and time then have to be at least 90 °C and 3 h. There are differences between different batches of biomass. These minimal conditions are not suitable for all of them. Slightly higher concentration, 0.45 mol/l, and 3 h at 95 °C proved to be universally applicable for all available batches of biomass. Therefore, further experiments were conducted at these conditions. In following experiments, centrifugation procedure was modified. To conduct the series of spin tests, digestion in 1 – 2 l batch was performed and the above procedure proved to be applicable at the larger scale. Main goal of the spin tests was to reduce amount of wash water and reduce time and speed of the centrifuge, while maintaining high yield and purity of the product. Due to small particle size, 4000 – 10000 g is needed. Required time is inversely proportional to centrifugation force and ranges between 4 min and 30 min. Based on the knowledge, the optimized procedure, suitable for larger-scale testing was formulated. Specific procedure is described in section 3.3.2.2. Typically, product yield and quality were as follows:

- Yield: 94 %, white powder (fig. 4.20)
- Purity (GC): 99 %
- M_w (GPC): 200 000 g/mol
- Surface (BET): 5.7 m^2/g
- Particle size (Laser diffraction): 0.9 μm (fig. 4.21)
- Particle size (SEM): 0.8 μm (fig. 4.22)

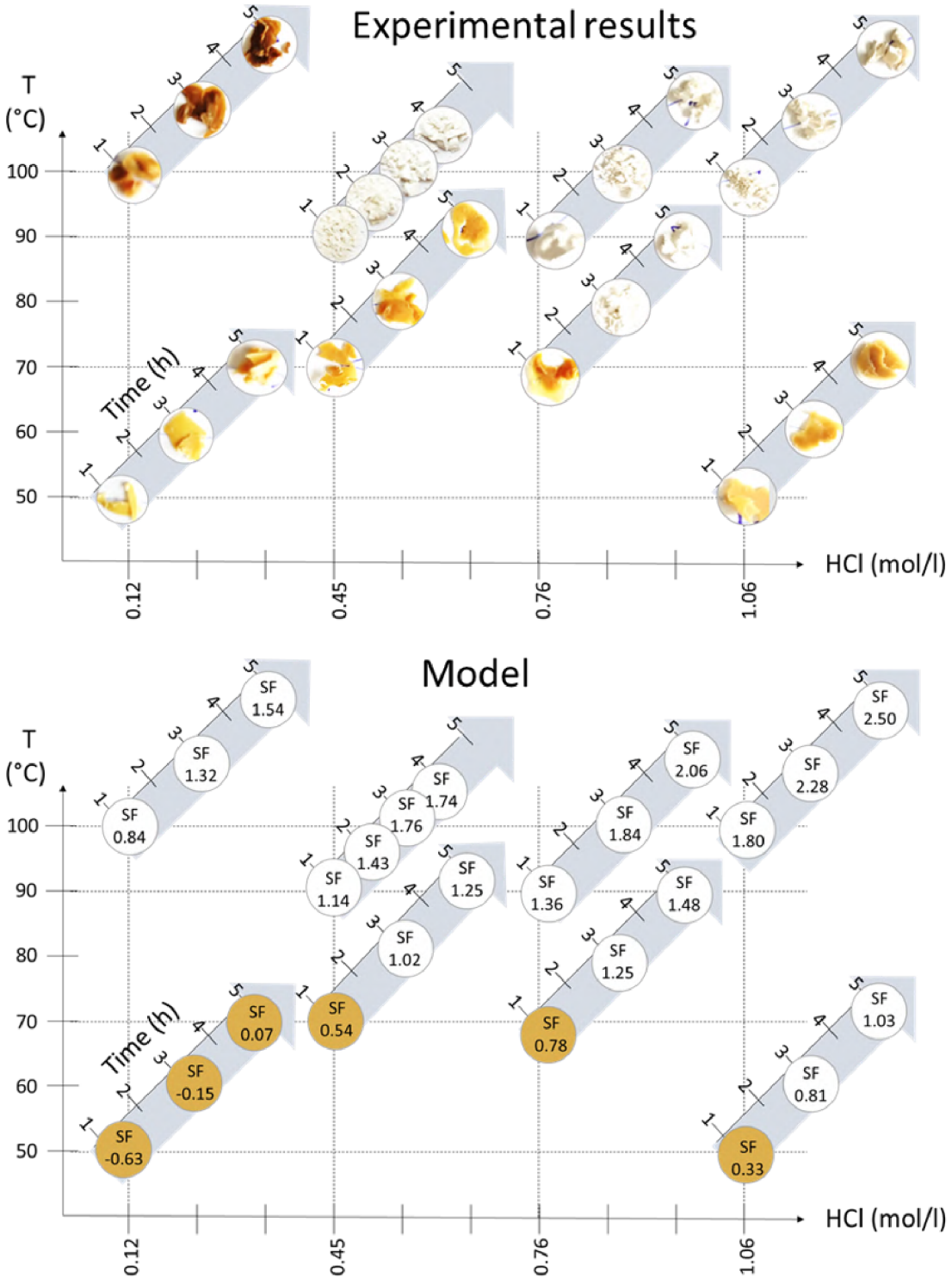

Fig. 4.18: Dependence of PHB purity on temperature, time and acid concentration. Experimental results are photos of products obtained by reaction at given conditions. Model shows severe factors for the reaction conditions. Pure product was expected for SF $>$ 0.8.

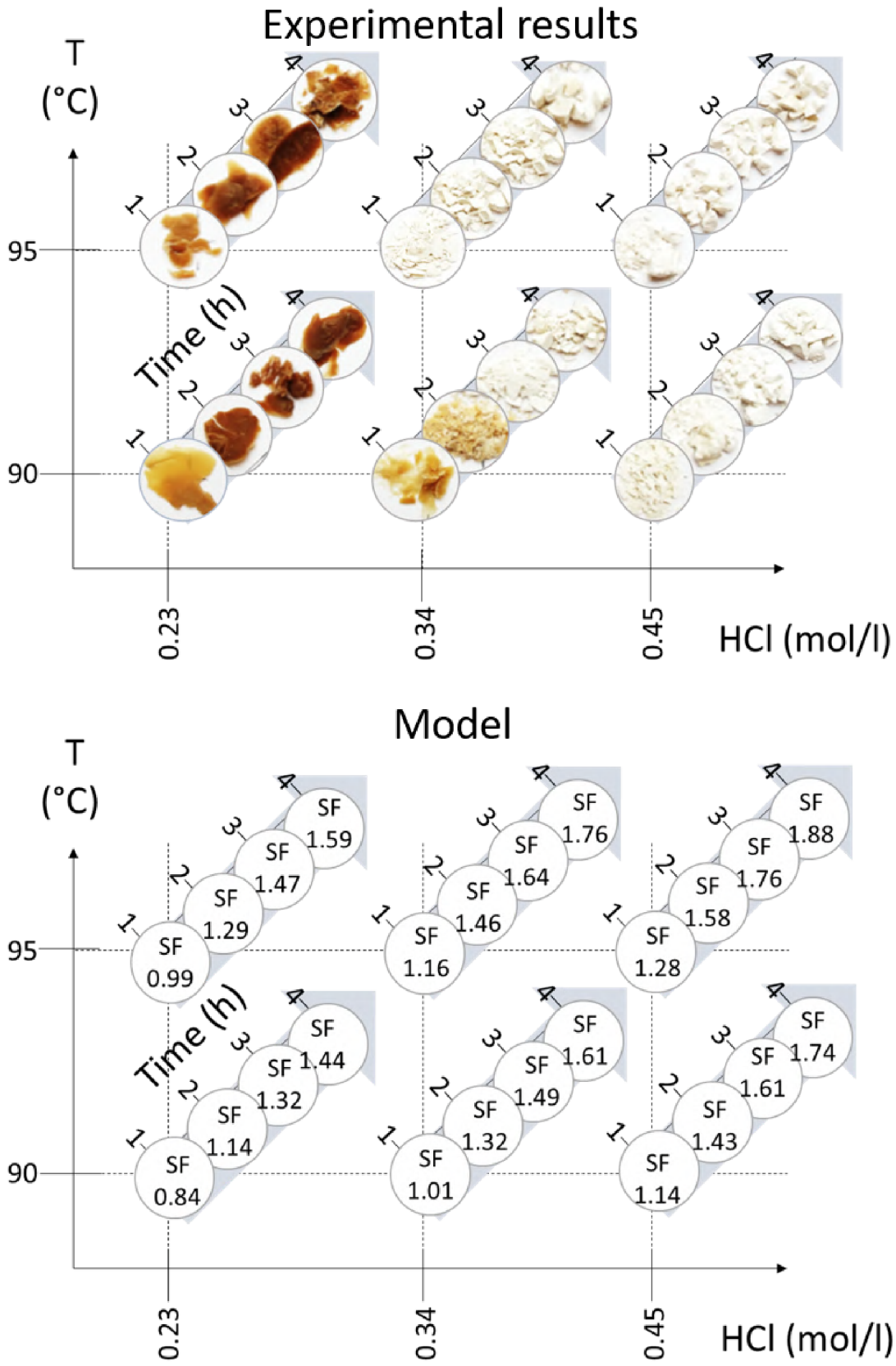

Fig. 4.19: Dependence of PHB purity on temperature, time and acid concentration. Experimental results are photos of products obtained by reaction at given conditions. Model shows severe factors for the reaction conditions. Pure product was expected for SF > 0.8.

Fig. 4.20: Isolated PHB

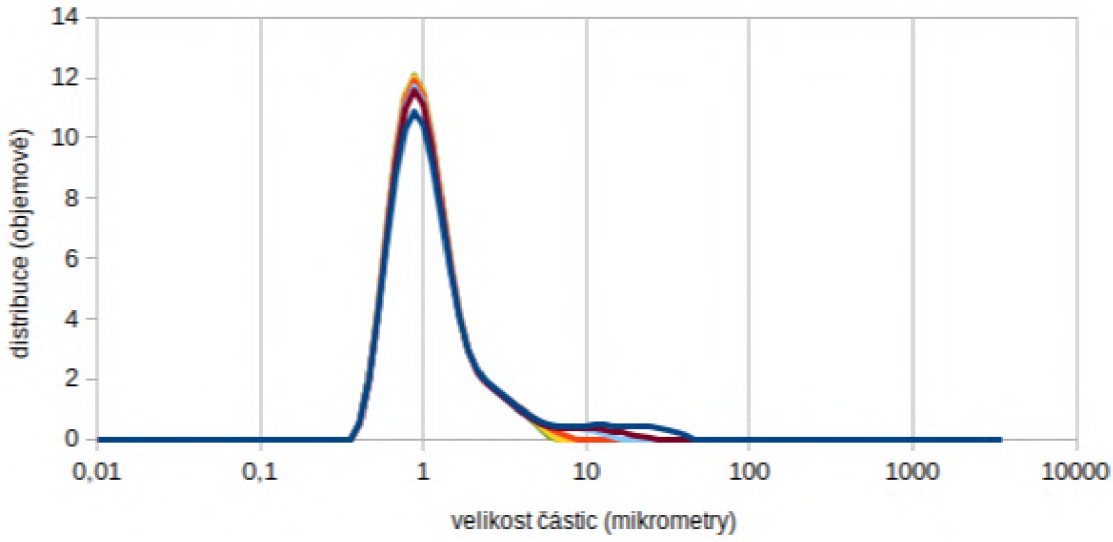

Fig. 4.21: Particle size distribution as measured by laser diffraction

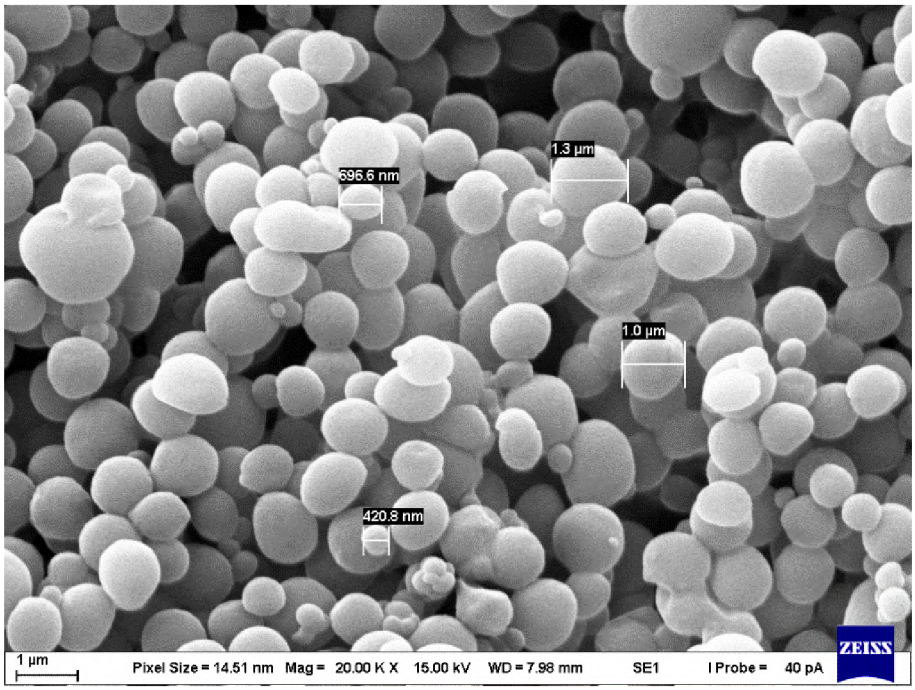

Fig. 4.22: SEM image of PHB isoalted by acid digestion

4.2.2.3 Digestion with HCl, large-scale procedure

The upscaled procedure, that was done in 250 l reactor (section 3.3.2.3) showed that the experiment proceeded similarly as in laboratory conditions. However, complete evaluation of the test was not possible due to the lack of equipment for workup. Sufficiently powerful centrifuge was not present at the site of the experiment, so most of the reaction mixture was only neutralized and further processed by our industrial partner elsewhere. Unfortunately, the details of further processing are not known. However, an aliquot of the reaction mixture was worked up in our lab to the final product, which was further analyzed. The data obtained are as follows:

- Yield 97 %, white powder
- Purity (GC): 99 %
- M_w (GPC): 70 000 g/mol
- FTIR: matches PHB standard (fig. 4.38)

As expected, the reached yield and purity were sufficiently high. Molar mass decreased substantially, much more than in comparable laboratory experiments. This may be attributed to longer heating times (reaching 90 °C took more than two hours, compared to several minutes in a laboratory) and longer storage of the acidic mixture before workup. This shows that if higher molar mass is needed, these steps must be carefully optimized.

4.2.3 Soap-based digestion

4.2.3.1 Soap preparation and initial experiments

As described in Theoretical section (1.1.2.2), main advantage of soap, compared to most industrial surfactants, is the possibility to precipitate it from wastewater. We have decided to validate and use this approach, and to improve its economy. Above all, instead of pure reagent, we used crude soap prepared from waste oil. In order to reduce water consumption and plant size, we used higher concentrations of biomass in the reaction mixture. We have also explored the possibilities of processing the generated waste.

The preparation of soap from waste oil was rather straightforward. On a small scale, it was feasible using ordinary laboratory glassware. The only difficulty in preparing a larger amount was the homogenization of the mixture, because in the initial phase of the reaction it is necessary to ensure good contact of the aqueous phase with the oil phase. There was a need for more powerful equipment than a small laboratory homogenizer. Quality kitchen mixer was found appropriate.

As in alkaline digestion (4.2.1), gelling of untreated biomass upon reaction with soap, which is slightly alkaline, was observed. Again, it was solved by heat pretreatment of the biomass. Unlike in acid and alkaline digestion, here, the reagent was not readily soluble in cold water. Therefore, it was advantageous to conduct the experiments at higher temperatures. In practice, 50-80 ° C has proven to be suitable, with the soap dissolving relatively quickly but without undesired boiling of the mixture or significant degradation of the polymer. Another important aspect was the use of less extreme pH. The soap is fully effective in the slightly alkaline conditions, which enabled experimenting with industrial enzyme (protease used in washing powders) as additional reagent. Based on these assumptions, the general procedure outlined in chapter 3.3.3.2 was proposed and a series of experiments were performed to examine the relationship between the amount of soap in the mixture, the addition of protease and analytical purity. Results are shown in fig. 4.23. Plotted values are similar to those for SDS-based method published previously, [48] shown in fig. 4.24. In all experiments, yields were close to quantitative and all losses could be explained by clumsiness in handling. For the sake of clarity, yields are not reported. Analytical purity was monitored because, unlike the acidic and alkaline methods, it was not possible to reliably evaluate visually. Even very analytically pure products, after isolation, sometimes took the form of a glassy, slightly yellowish solid, not a white powder.

In an effort to scale up, the viscosity of the mixture began to have observable negative effect. Although the mixture was heat-pretreated, centrifugation was difficult at a given biomass concentration. The viscosity also increased with increasing

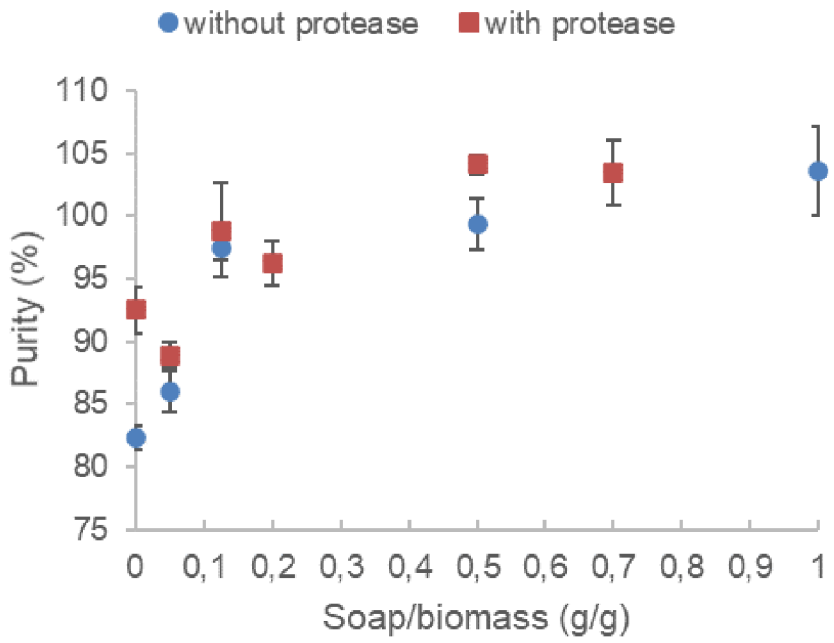

Fig. 4.23: Purity of PHB obtained by soap-based digestion [11]

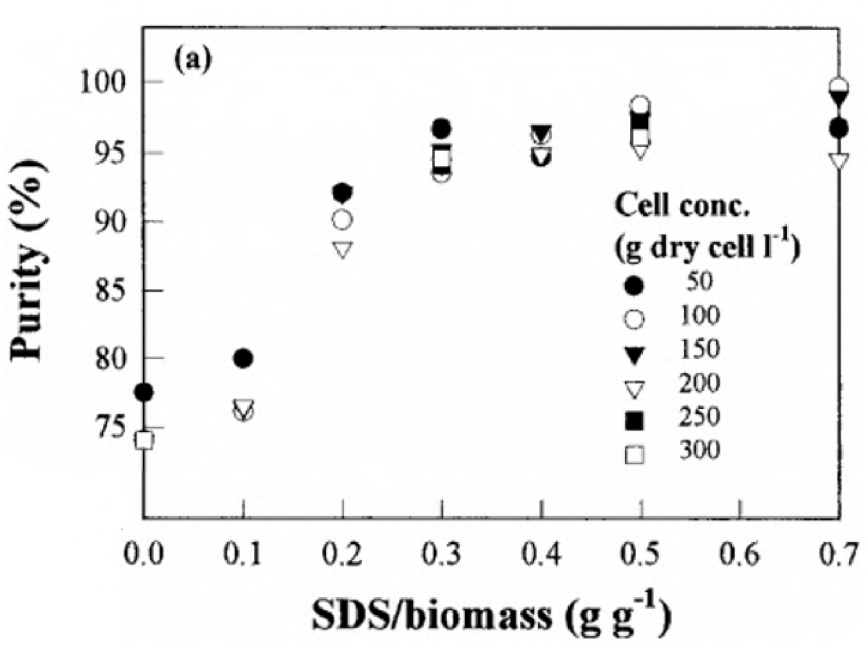

Fig. 4.24: Purity of PHB from SDS-based method. Obtained from previous publication.[48]

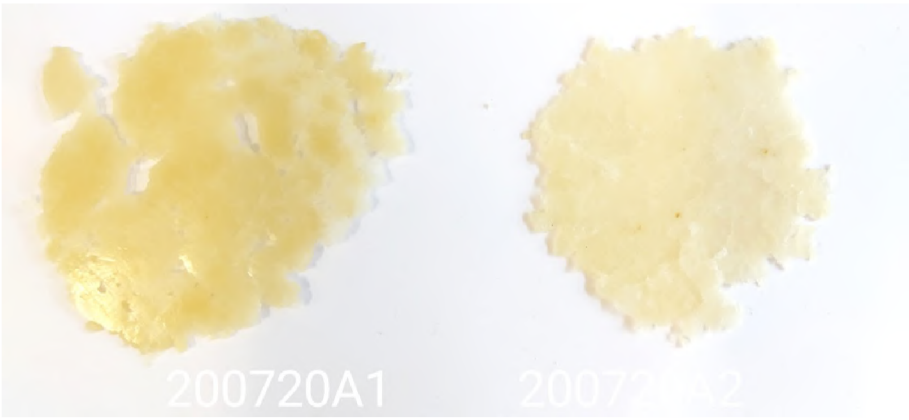

Fig. 4.25: PHB from soap-based digestion with 0.25 g soap/g CDW and protease. Left: soap was added in one dose, according to General procedure 3.3.3.2. Right: first half of soap was used for pre-purification, then the mixture was centrifuged, pallet was made up to the original volume and treated with enzyme and rest of soap.

soap dose. In order to achieve a reasonable centrifugal separation efficiency at lower speeds, which was required for large-scale tests, a slight reduction in the biomass concentration and the division of the soap into two doses were further experimented. The first batch of soap was used for the initial pre-purification, followed by centrifugation to remove most of the cell debris, dilution of PHB-rich pellet to the original volume, and reaction with a second batch of soap together with the enzyme at a lower temperature (so as to maintain enzymatic activity). Afterwards, workup was the same as in General procedure 3.3.3.2, but only two washing with water were done in order to maintain the same total number of centrifugations in both experiments. The procedure with two doses of soap proved to be advantageous because the viscosity of the mixtures was lower and the coloration of the product was less pronounced than when the same dose of soap was used at the same time (fig. 4.25).

4.2.3.2 Soap-based digestion - optimized

To maximize quality of PHB and minimize the amount of reagents and waste, the procedure with only 0.2 g of soap per 1 g of BDW was employed and the purity was improved by use of protease and final wash with easily recyclable solvent acetone (for details see Section 3.3.3.3). Outcome of the experiment was following:

- Yield 97 %, white powder(fig. 4.26)
- (GC-FID): 96 %
- M_w (GPC): 389 000 g/mol
- M_n (GPC): 167 000 g/mol
- FTIR: matches PHB standard (fig. 4.39)

Fig. 4.26: PHB isolated by soap-based digestion

Microstructure of the product (fig. 4.27) was different from the PHB obtained by acid digestion (fig. 4.22). Acid digestion gave spherical particles, that resembled free PHB granules in the bacterial cells, while the product from soap-based digestion resembled whole cells. This may be caused by trace residues of cellular material, that holds the shape together, or by the tendency of granules to coagulate during isolation. This phenomenon will be further investigated in a future. Nevertheless, color, yield, purity and molar mass of the product were satisfying. This PHB was later processed to 3D printing material 3.4.4. At the same time, aqueous supernatants collected from the experiment were used as model wastewaters for subsequent precipitation (3.3.3.4).

4.2.3.3 Processing of wastewater from soap-based digestion

Supernatants from soap-based PHB isolation contained mainly soap and non-PHB cell mass. Therefore, one would expect that primarily fatty acid would be formed upon the acidification and hopefully could be neutralized with lye and used as a soap again. Alike result is reported by Samori [50] who claims that after the acidification, fatty acid is obtained in 98% yield and can be easily recycled back to soap without further purification. The Samori's method is slightly different (ammonium laurate instead of sodium soap, acidification with CO_2 instead of HCl) and uses significantly higher amount of soap (up to 2 g/g BDW) but otherwise the precipitation step is close to ours. To our surprise, the titration curve showed that not only soap reacts with hydrochloric acid in our process. The titration curve had practically flat shape

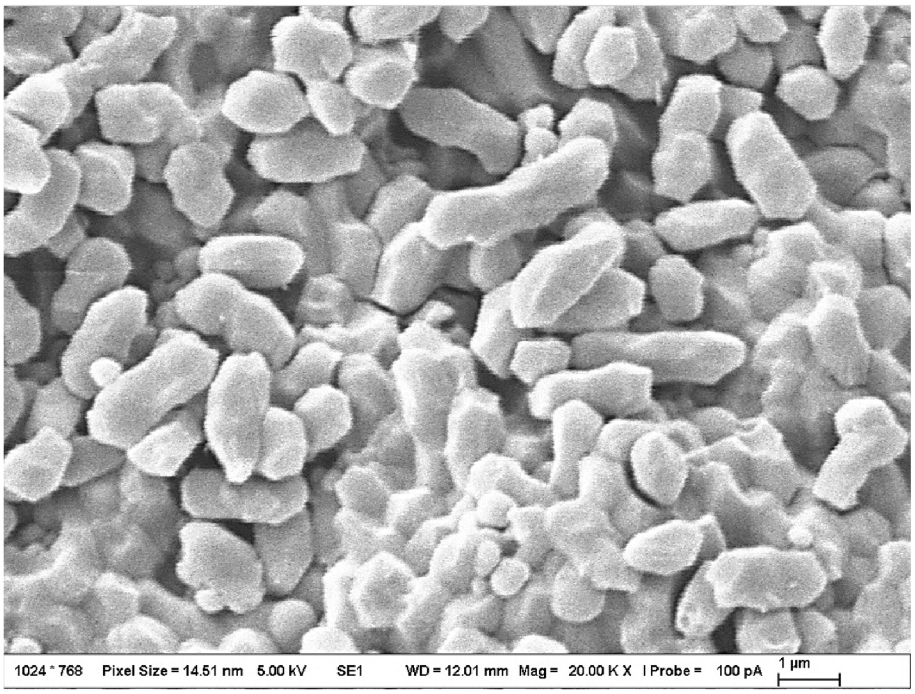

Fig. 4.27: SEM image of PHB isolated by soap-based digestion

(fig. 4.28), which indicated the acid-base reaction of more compounds with different pK_A values. The finding corresponds with the gravimetry – the yield of precipitate dry weight was higher than expected. In other words, the precipitate contained not only fatty acids, but also significant portion of non-PHB cell mass (NPCM). This fact was also confirmed by FTIR of dried precipitate (fig. 4.40), which showed strong fatty acid signals (aliphatics at 2972 and 2853 cm^{-1}, carboxyl at 1709 cm^{-1}) together with protein, which was manifested mainly by the amide signal at 1530 and 1650 cm^{-1}. Yield depended on the soap:BDW ratio – the more soap, the smaller percentage of NPCM in precipitate. This may explain sufficient purity of Samori's fatty acid – with higher amount of soap, the amount of NPCM in precipitate could be negligible. In our experiments, that typically work with minimal amount of soap, the yields of precipitate range from 136 % (experiment with 0.5 g/g BDW) to 193 % (experiment with 0.125 g/g BDW). Therefore, our precipitates were not suitable for soapmaking because they contained 26 – 48 % of NPCM as impurity. Due to complex and diverse character of NPCM, we didn't attempt to remove it. Instead, we decided to use the precipitate as substrate for fermentation.

Laboratory-scale fermentation tests show that the precipitate can be readily used as substrate for bacteria to produce bacterial biomass. For reference, standard substrate (waste cooking oil) and mixture of both was fermented in the same manner. There was a synergy between precipitate and waste cooking oil – more substrate was converted to PHB if a mixture of the above is used as a carbon source. The

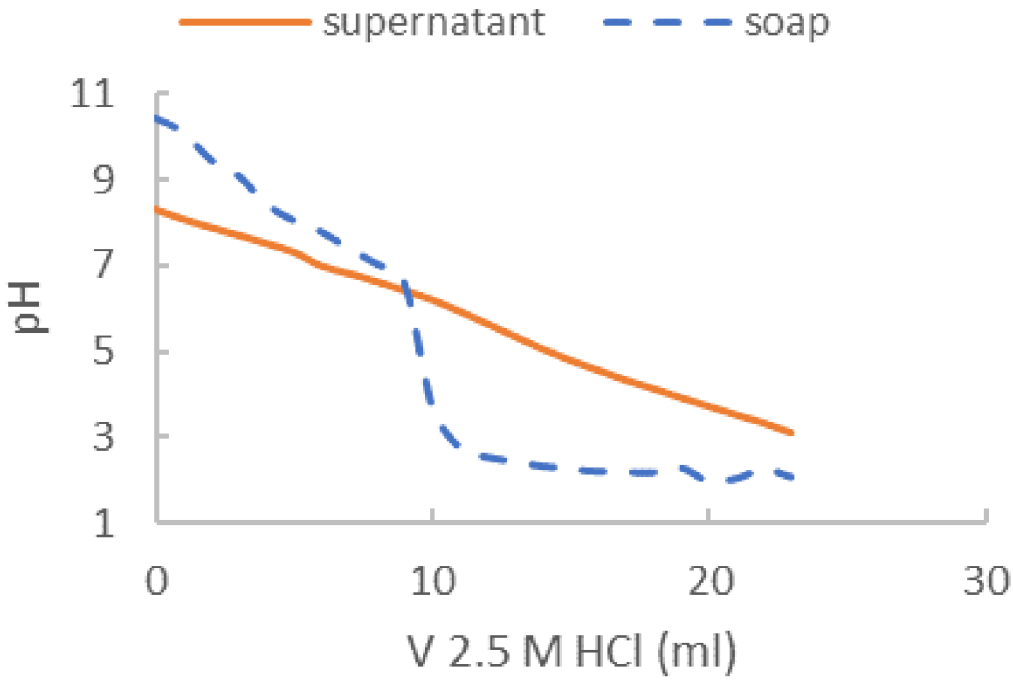

Fig. 4.28: Titration curves: 1. supernatant (0.2 g/g BDW of soap used for digestion; supernatant contains 10 g of soap in 550 ml), 2. soap (10 g in 550 ml of water) [11]

reason may be emulsifying properties of the precipitate which make the oil more bioavailable. Pure precipitate didn't perform so well as the mixture or oil alone. High amount of hydrolyzed protein in the precipitate may be the reason. It is known that *Cupriavidus necator* accumulates PHB faster in N-deficient environment [**?**] and thus nitrogen-rich precipitate may slow down the process. But we assume that oil + precipitate mixture will usually serve as a substrate for fermentation. Therefore, the synergy of oil and substrate brings positive result.[11]

4.3 Microplastics in cosmetics - market research

In order to assess how widespread microplastics are in cosmetics, and if any of them could be replaced by PHB, compositions of cosmetic products available on Czech market were analyzed with a help of dedicated python scripts. As a source of data, we used the composition of products (under INCI names) that manufacturers are obligated to publish on packaging, and which vendors usually publish on websites. Manufacturers have to list all ingredients in descending order (except for ingredients with concentration below 1 %, which can be listed in arbitrary order) but are not obligated to inform about specific concentrations. Therefore, this research is in-

evitably of a qualitative nature. Because it would be difficult to obtain data for the entire Czech market, it was approximated by assortment on e-shop of Teta drogerie. Although this is not an optimal approach, we consider it sufficient for a rough orientation. Large chains, such as Teta, serve majority of Czech consumers so we assume that Teta's assortment reflects the demand of typical consumers. Teta was chosen because at the time of solving this task (april 2020) it published composition of all cosmetic products, had a well-arranged website, suitable for web scrapping.

The analysis began by downloading information about all relevant products from the e-shop. Cosmetic products appeared in a total of ten categories or subcategories. The pages with the entire assortment in the given category were manually searched, and the link to it was saved in link_finder.py (appendix 4.6). The script written in link_finder contains the find_links function, which opened each link, found all links to the pages of individual products, and appended them to the list. The obtained lists were saved in links.py (for its lenght not shown in printed version; available online in Appendix II). For the purpose of archiving and possibility of offline work, the pages with product information were downloaded and saved in text files by downloader.py (appendix 4.7). It contains DownloadPage function, that opens the link to individual product, finds product's name and saves the html code to a file with product's name. It also includes a command to print the product name to the console and a short stop, and basic error handling for skipping unsaved or non-existent files. This allowed control of downloading entire categories, which were downloaded by simple for loops listed below in the downloader.py. E.g. When downloading the "hair care" category, some non-cosmetic items such as clippers or hair bands also appeared among cosmetic products. The download delay made it possible to monitor what was being downloaded while manually deleting non-cosmetic products. In this manner, information about total of 2478 products was downloaded.

Another goal was to find and sort key data for each product. This was done by the analyzer.py file (appendix 4.8), which includes the analyze_products function. It opens text files with stored html codes, parses them and searches for the required information. Based on this, it creates objects of type "Product". The attributes of these objects are the product name, its price, category, subcategory and sub-subcategory, and composition, with the composition being divided into six groups by the categorize_ingredients function. Groups are microplastics, other synthetic polymers, polyethers, degradable ingredients, minerals and others.

The division of ingredients into these groups was semi-automatic, as a sufficiently large and freely available database of cosmetic ingredients and their nature was not found. For microplastics, ECHA's list of polymers that are considered microplastics [129] was used. It contains 19 polymer groups, each including many different com-

pounds with different INCI names. Other ingredients were initially sorted only by short lists stored in ingredient_lists.py, and by simple logic - if the ingredient name is "extract" or "juice", it can be assumed that it will be a biobased and biodegradable ingredient. "ci" in turn means pigments that generally do not belong to microplastics. Nevertheless, automatic decision about most ingredients was not possible at first. Therefore, when categorize_ingredient function encountered an unknown ingredient, it asked the user (ie me) to which group the ingredient belonged. I decided it either on the basis of existing knowledge or according to information available online. The function stored the information for further use. Therefore, the lists of categorized ingredients grew and necessity for manual labor gradually decreased. At the end, all ingredients were categorized, and lists with individual ingredient groups were stored in the updated version of ingredient_lists.py (for its lenght not attached to printed version; available online in Appendix II).

After going through all the products in a given category, the analyze_products function generates a short report on the number of analyzed products in the given product category, and on the occurrence of synthetic polymers in them. This was done for all categories. The whole report is given in the 4.10 appendix. Furthermore, the analyze_products function returns a list of lists with product attributes, together with a table of productID - microplasticID relations. These data are useful for constructing .csv files as sources for a visualization software. Another .csv with microplasticID - microplastic_name assignment was constructed using microplastic_table_generator.py file (appendix 4.9).

The data were visualized in PowerBI Desktop. The model connecting the three tables is given in fig. 4.29. The whole interactive dashboard can be found on https://app.powerbi.com/links/xY92HUv_K2?ctid=c63ce729-ca17-4e52-aa2d-96b79489a542&pbi_source=linkShare. A brief overview is in fig. 4.30. As can be seen, microplastics are quite widely used, especially in decorative cosmetics. At the same time, products from this category most often contain more than one ingredient from the category of microplastics. Some mascaras and lipsticks contained up to five microplastic species. Other categories rich in microplastics were skin and hair care.

The most used microplastics were acrylate and methacrylate copolymers, which appeared in products from all categories. This class is very wide in terms of physical and chemical properties of the polymers, performing a wide range of functions, from thickening and film-forming to enhancing sensory properties. Silicone polymers were the second most popular group of microplastics, finding use mainly in decorative, skin and hair care. Their main role is enhancement of sensorial properties, such as skin feel or slipperiness and shine of hair. Polyamides were the third most popular ones, finding use in decorative and skin care. Polyamides are solids that do not dissolve in water or common cosmetic oils, alike PHB. Therefore, it seems that

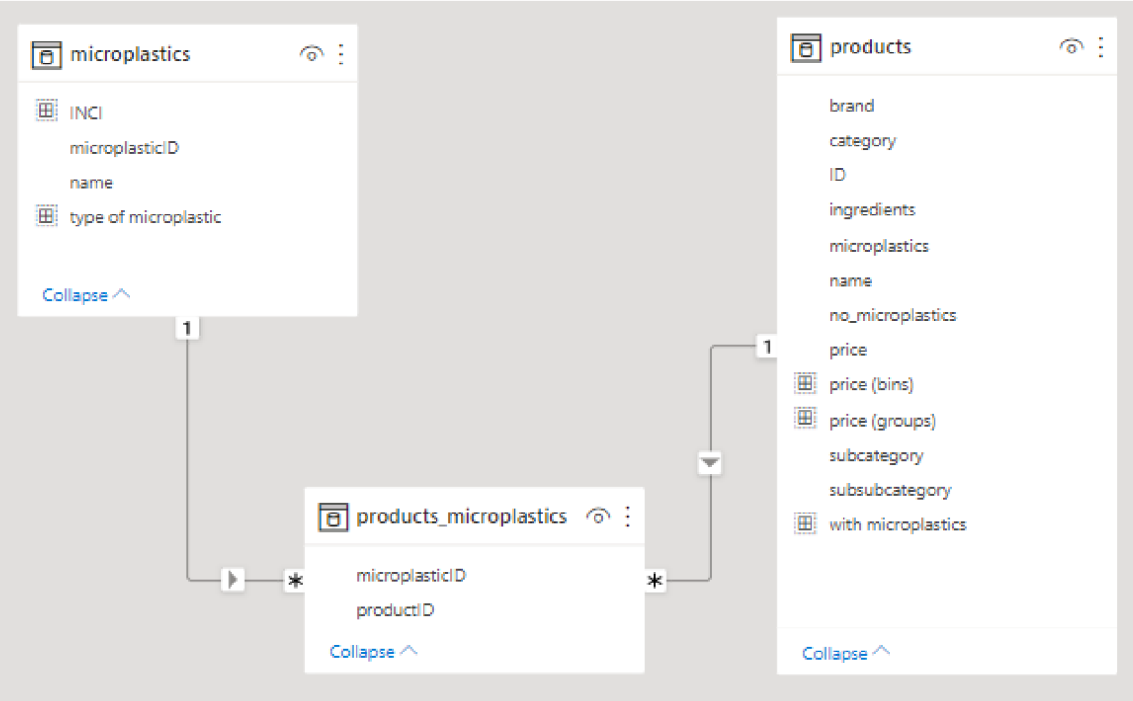

Fig. 4.29: Data model

PHB could find application in the same segments. In their microparticulate form, polyamides serve as sensory enhancers, rheology modifiers and "soft focus" agents in high-end products.

Contrary to popular belief, it turned out that the occurrence of microplastics is more common in more expensive products. In the group of about 500 cheapest, microplastics were in only 18 % of products. In a similarly large group of the most expensive it was already 42 %. It shows that microplastics were not cheap bulking agents, which is good news for the possible commercialization of PHB in this industry.

Differences also existed between cosmetic brands. While some rarely used microplastics, others put microplastics in most products. The influence here is mainly due to the segments on which the given brands focus. Those that focus on decorative and skin cosmetics use microplastics more often.

4.4 Thermal stability and melt-processing

One of the proposed applications of our PHB is the production of materials for 3D printing via FDM technology. Melt processability is a necessary condition for this application. The material undergoes melting both during filament production and during 3D printing itself. For this reason, the thermal stability of the prepared PHB

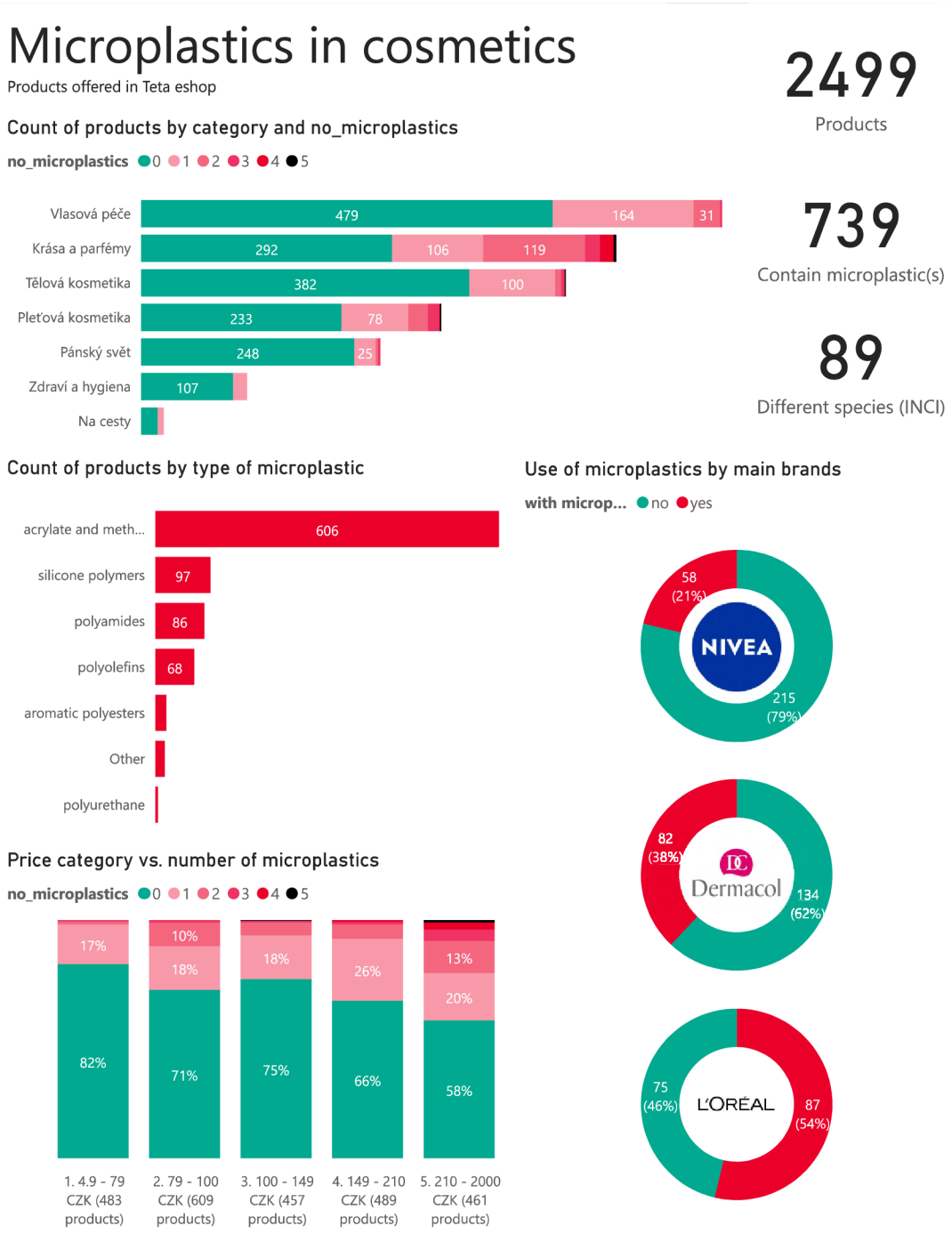

Fig. 4.30: Report on microplastics in cosmetic products

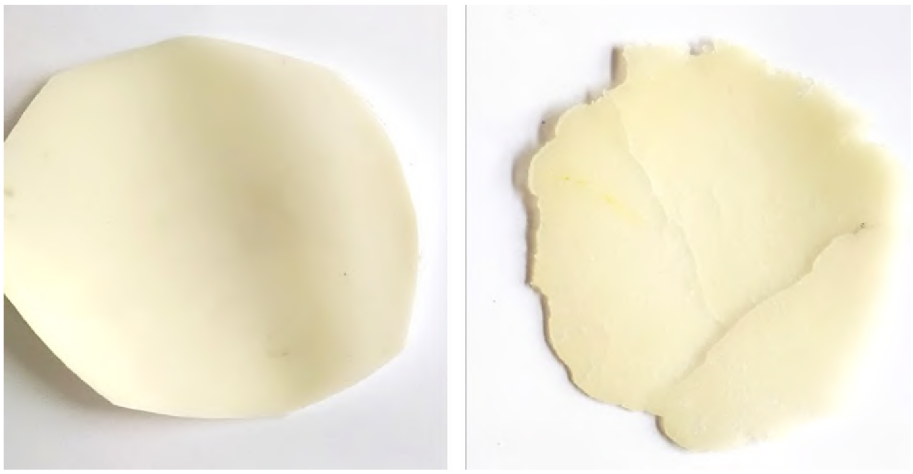

Fig. 4.31: PHB samples after melting at 190 °C/5 min and cooling. Left: commercial PHB, right: PHB obtained by soap-based digestion. The sample spontaneously cracked upon cooling.

samples was investigated. As a simple and straightforward test, a small sample of the material was melted at 190 ° C for 5 minutes. These conditions roughly simulate the thermal stress that the sample would undergo during melt processing. [**?**] In this way, samples prepared by diethyl oxalate extraction (described in section 3.2.1.6, chloroform extraction (3.2.1.7), and alkaline, acid and soap digestion (3.3.1.2,3.3.2.2 and 3.3.3.3) were tested and compared with standard commercial product. The commercial sample and samples isolated by extraction resulted in a relatively durable plastic, while those obtained by digestion methods gave an extremely brittle material (fig. 4.31).

For acid and alkaline digestion polymers, the problem may be due to the low molecular weight of the polymer, which was about half the molecular weight of chloroform isolated or commercial. However, this does not explain the fragility of the sample from soap-based digestion, which had a molecular weight comparable to that of a commercial one. Thus, the method of isolation seemed to play a major role. As a detailed study of the literature has shown, the lower thermal stability of the digestively obtained PHB has been observed by a number of authors (literature search was summarized in [12]). Low stability is probably associated with the fact that the polymer is isolated from an alkaline environment and contains basic impurities that dramatically accelerate thermal degradation via E1cB mechanism.

If alkaline impurities are the issue, the logical solution is to remove or neutralize them. Efforts to eliminate these impurities would lead to higher complexity of isolation procedures, and so were not considered feasible. We focused on neutralization. Acid washing (3.4.1) and stabilization with base neutralizing additives (3.4.2)

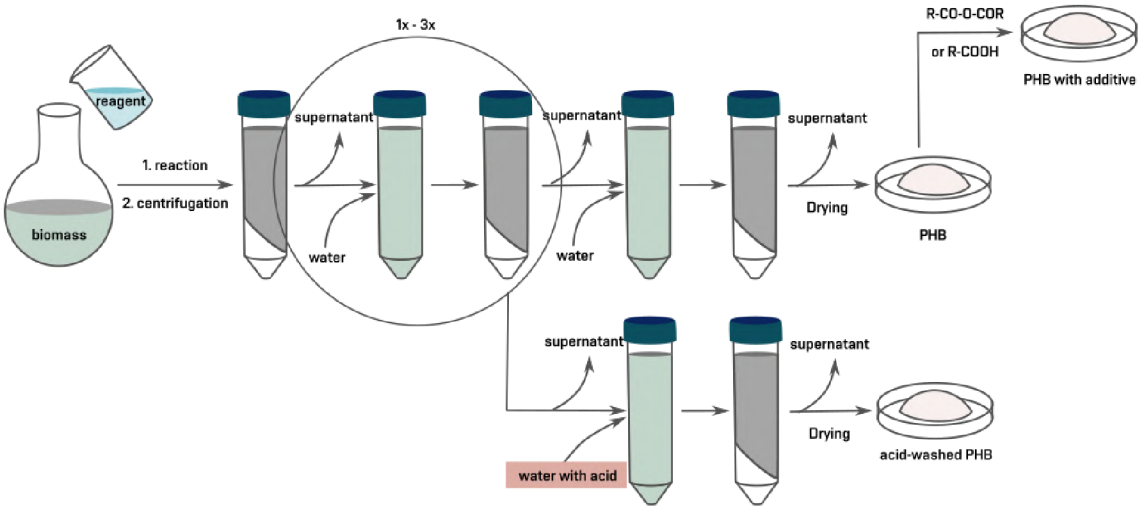

Fig. 4.32: Schematic representation of PHB isolation by a digestion method and incorporation of methods for thermal stabilization.

were tested. These steps are simple and do not complicate the potential production process (fig. 4.32). Nevertheless, they have proven to be highly effective. Sulfuric acid was chosen as the model washing agent and succinic anhydride as the model additive, but the phenomenon seems to be more general. Washing with other acids (HCl, CH_3COOH, citric acid) and addition with other reagents (maleic anhydride, succinic acid, citric acid) were also experimented with, and these interventions also visibly increased heat resistance (data not shown). The melting of the additive and acid-washed samples resulted in a significantly lower decrease in molecular weight than the melting of untreated sample (fig. 4.33). The same phenomenon could be seen in DSC (fig. 4.34). Melting point (which is known to decrease with decreasing molecular weight) decreased significantly for unstabilized materials. Stabilized samples were much less susceptible.

To test the stabilization protocol in a real-world application, PHB was prepared by soap-based digestion (3.3.3.3) and stabilized using succinic anhydride (3.4.2). For making the PHB-based plastic product, we followed the method that was earlier developed for the processing of commercially available PHB (3.4.4). The stabilized PHB was compounded with bio-based additives and resulting filament was used for 3D printing. Resulting object had pale beige color and good mechanical properties (fig. 4.35). The material successfully withstood significant heat stress during the process with only medium loss of molecular weight. The molecular weight dropped from M_w = 541 000 g/mol in biomass to M_w = 172 000 g/mol in 3D printed object.

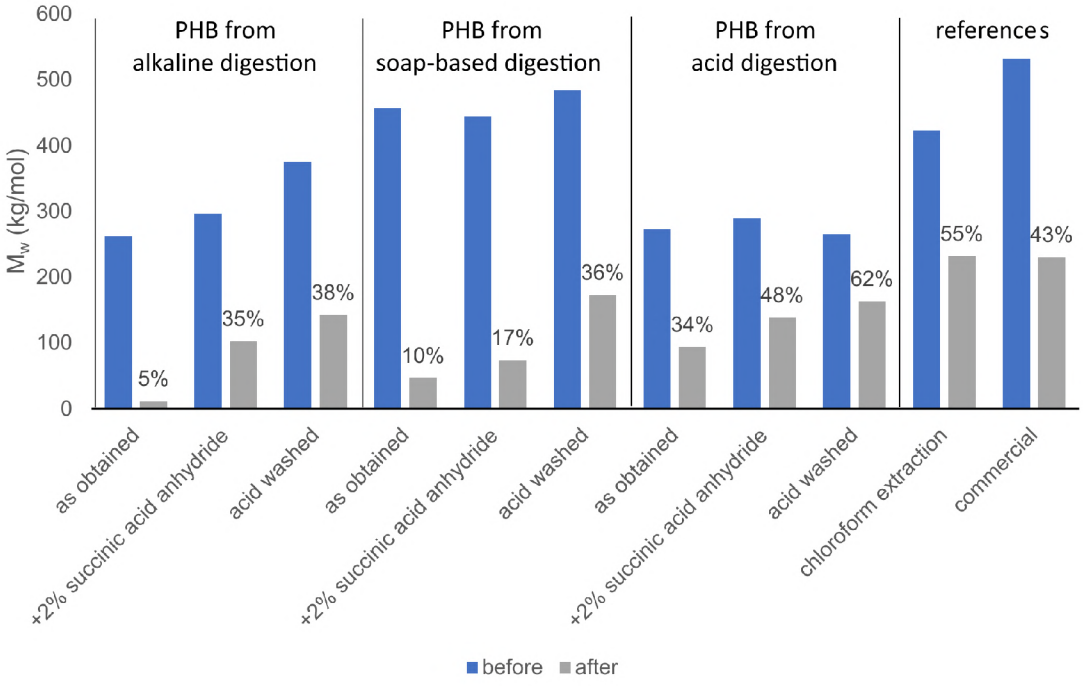

Fig. 4.33: Molecular weight of the polymer before and after thermal treatment Numbers above columns represent percentage of original molecular weight [12]

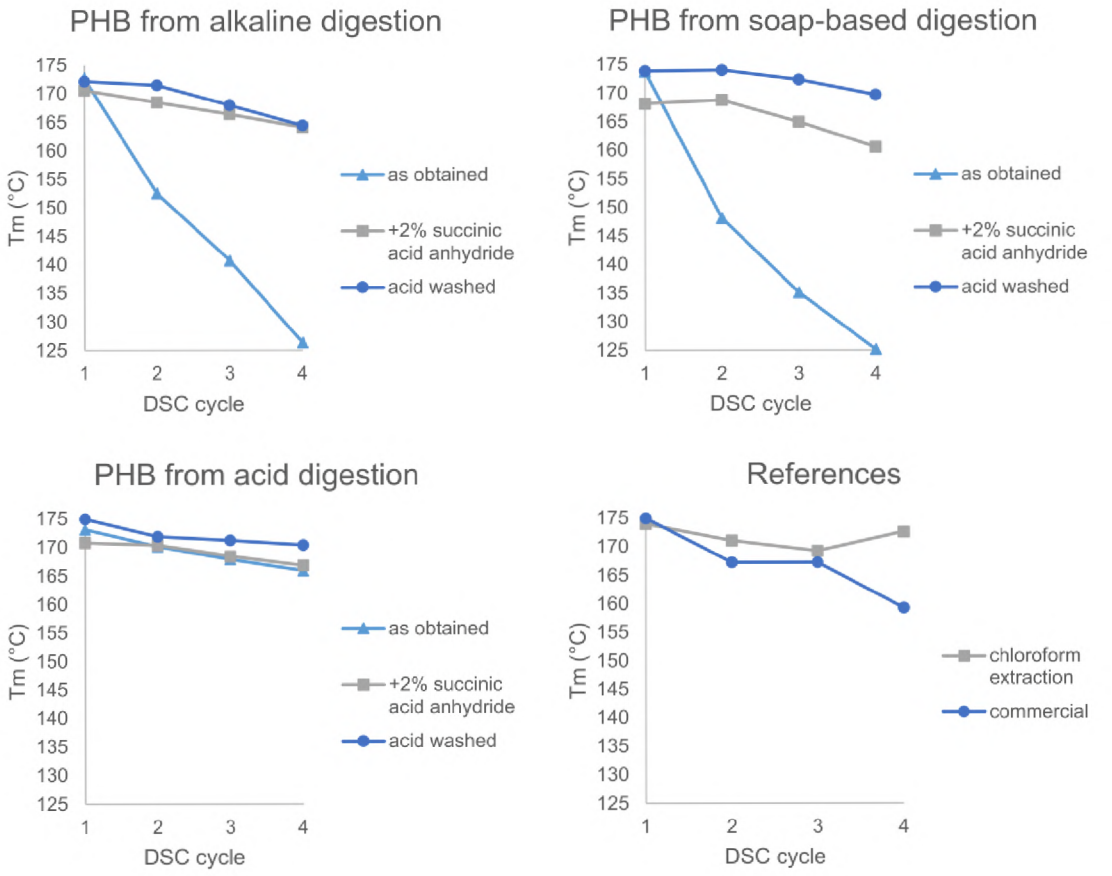

Fig. 4.34: Melting point of PHB after several cycles of differential scanning calorimetry. Each cycle consisted of heating to 190 °C, annealing at the temperature for 5 min and cooling. [12]

Fig. 4.35: 3D printed object made from PHB. The polymer came from soap-based digestion and was stabilized with 2 % succinic anhydride before melt processing

Conclusion

The main task in this work was to improve the recovery of PHB from bacterial biomass. In solvent extraction, the first of many demanding steps, i.e. the treatment of biomass before extraction, has been addressed. Alkali/acid biomass precipitation was used, which greatly facilitated concentrating and drying prior to extraction. However, due to the high complexity, cost and risk of solvent extractions, this direction has not been further developed yet. Instead, great attention was paid to the so-called digestion methods. Two selected methods - acid and soap digestion - have been developed for use in pilot operations and have proven to be feasible on a large scale. Furthermore, the applicability of the obtained PHB and possible commercialization were addressed. Acid digestion has been shown to give a polymer of relatively low molecular weight but in the form of a fine powder which has potential for use in cosmetics. We are currently working with several cosmetic companies to commercialize this product. Soap digestion, on the other hand, provides PHB, which had a relatively high molecular weight, and thus material applications were conceivable. Initially, its low temperature stability was an obstacle, but this was explained and solved as described in the chapter on thermal stabilization. The success of this process is demonstrated by the production of 3D printing material from this polymer.

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Appendix

4.5 FTIR spectra

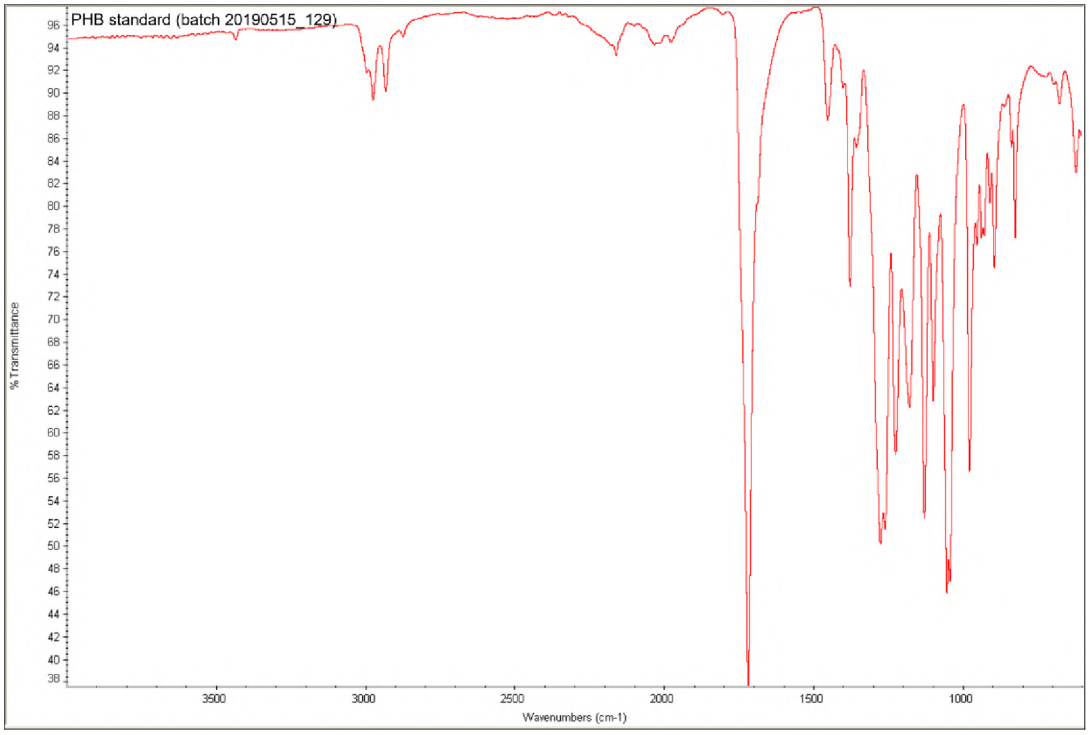

Fig. 4.36: FTIR spectrum of commercially available PHB

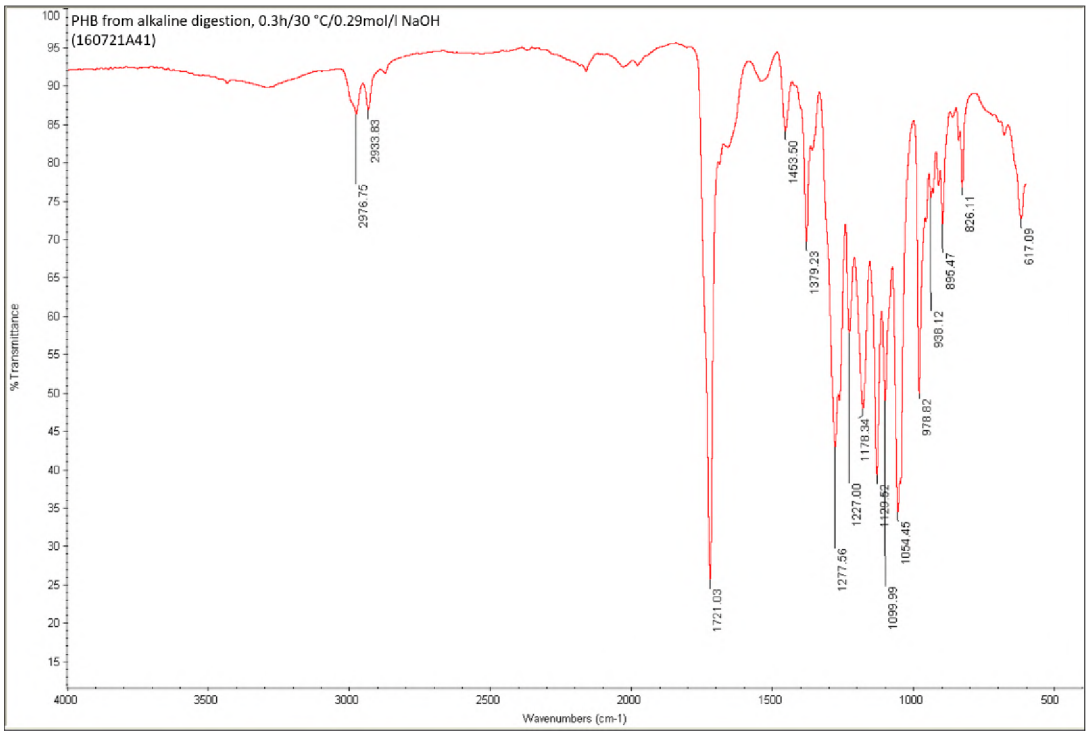

Fig. 4.37: FTIR spectrum of PHB obtained by alkaline digestion

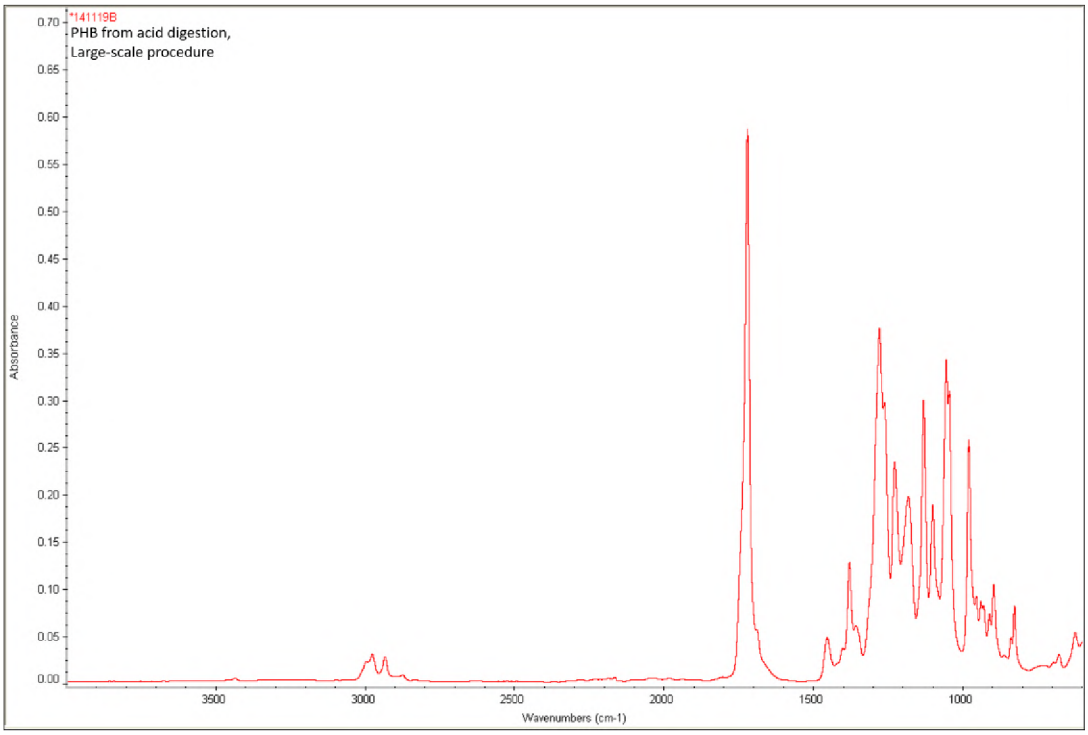

Fig. 4.38: FTIR spectrum of PHB obtained by acid digestion

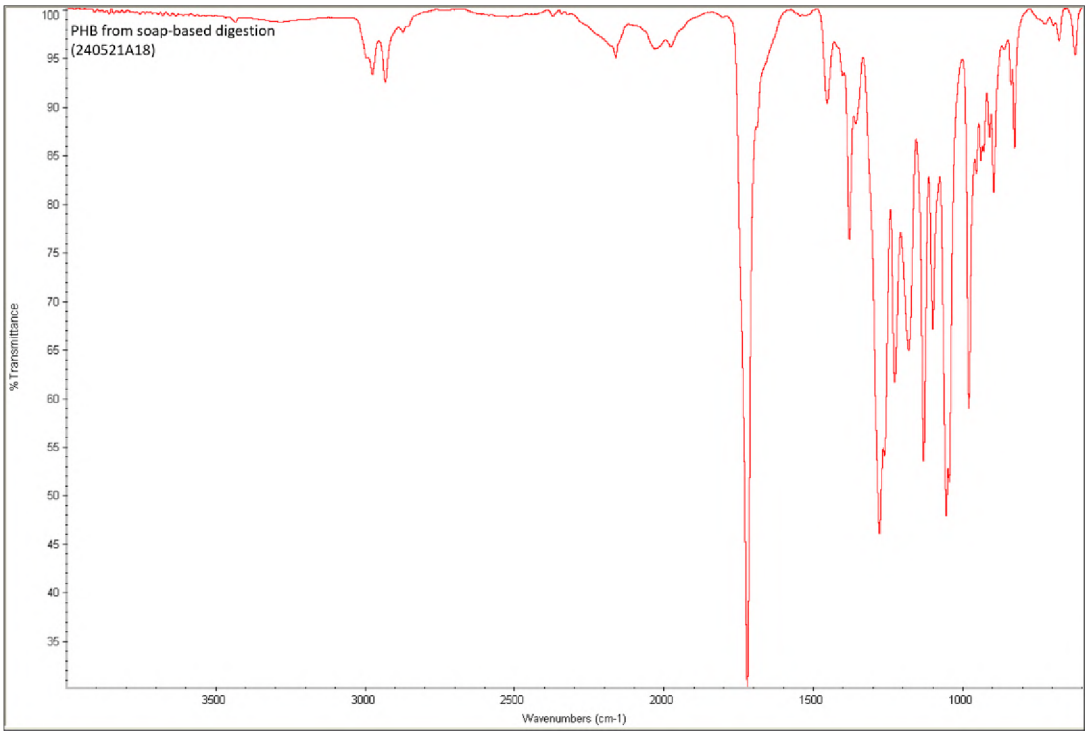

Fig. 4.39: FTIR spectrum of PHB obtained by soap digestion

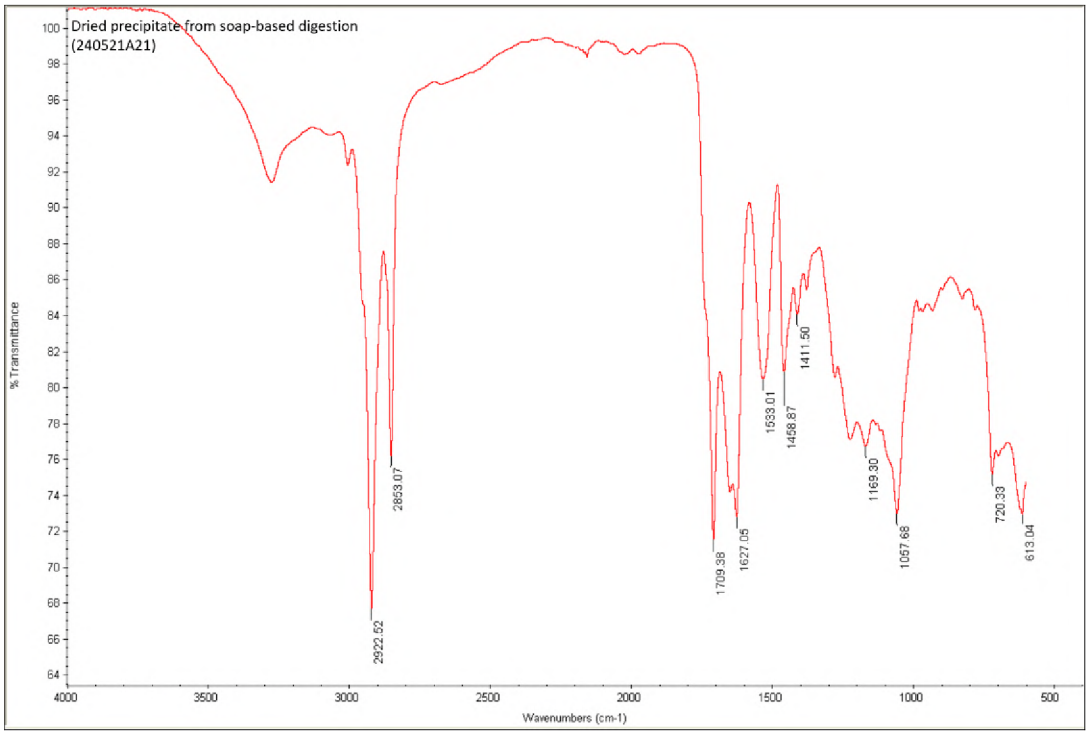

Fig. 4.40: FTIR spectrum of precipitate from soap-based digestion

```python
# -*- coding: utf-8 -*-
"""
Created on Mon Apr  6 18:44:25 2020

@author: Aneta
"""
import urllib.request
from bs4 import BeautifulSoup

#níže manuálně vyhledané linky na katalog produktů z dané kategorie
pletova="https://www.tetadrogerie.cz/eshop/produkty/pletova-kosmetika?stranka=9&pocet=60"
telova="https://www.tetadrogerie.cz/eshop/produkty/telova-kosmetika?stranka=18&pocet=60"
vlasova="https://www.tetadrogerie.cz/eshop/produkty/vlasova-pece?stranka=19&pocet=60"
krasa_a_parfemy="https://www.tetadrogerie.cz/eshop/produkty/krasa-a-parfemy?stranka=28&pocet=60"
parfemy="https://www.tetadrogerie.cz/eshop/produkty/krasa-a-parfemy/parfemy?stranka=4&pocet=60"
zuby_a_usta="https://www.tetadrogerie.cz/eshop/produkty/hygiena/pece-o-zuby?stranka=7&pocet=60"
lubrikanty="https://www.tetadrogerie.cz/eshop/vysledky-vyhledavani?searchtext=lubrika%C4%8Dn%C3%AD"
intimni="https://www.tetadrogerie.cz/eshop/produkty/hygiena/damska-hygiena/intimni-hygiena"
panska="https://www.tetadrogerie.cz/eshop/produkty/pansky-svet?stranka=9&pocet=60"
na_cesty="https://www.tetadrogerie.cz/eshop/produkty/hygiena/hygiena-na-cesty"


def find_links(url):
    """
    otevře stránku, v html kodu najde odkazy na všechny zobrazené produkty
    vrací list s odkazy
    """
    r=urllib.request.urlopen(url).read()
    b=BeautifulSoup(r,"html.parser")
    l=b.find_all("div",class_="sx-item j-ajax-content j-item")
    links=[]
    for item in l:
        link="https://www.tetadrogerie.cz"+str(item.a["href"])
        #    print(link)
        links.append(link)
    return links

for item in [pletova, telova, vlasova, krasa_a_parfemy, parfemy, zuby_a_usta, lubrikanty, intimni,panska,na_cesty]:
    print(find_links(item))
    print("")
```

```python
# -*- coding: utf-8 -*-
"""
Created on Mon Apr  6 19:08:11 2020

@author: Aneta
"""

import urllib.request
from bs4 import BeautifulSoup
import random
import time
import links

def DownloadPage(url):
    """
    pro stahování infa o kosmetice z eshopu Teta drogerie
    html ukládá do texťáku ve složce cosmetics
    """
    html=BeautifulSoup(urllib.request.urlopen(url).read(),"html.parser")
    name=(html.find("a",class_="j-scroll-to-target").string).strip()
    textfile="cosmetics/"+name+".txt"

    try:
        with open(textfile,"w") as notebook:
            notebook.write(str(html))
        print("finished: "+name)
    except UnicodeEncodeError:
        print("error")
    except FileNotFoundError:
        print("notfounderror")

    time.sleep(random.random())

# for link in links.pletova:
#     DownloadPage(link)

# for link in links.telova:
#     DownloadPage(link)

# for link in links.vlasova:
#     DownloadPage(link)

# for link in links.krasa_a_parfemy:
#     DownloadPage(link)

# for link in links.parfemy:
#     DownloadPage(link)

# for link in links.zuby_a_usta:
#     DownloadPage(link)

# for link in links.lubrikanty:
#     DownloadPage(link)

# for link in links.intimni:
#     DownloadPage(link)

# for link in links.panska:
#     DownloadPage(link)

for link in links.na_cesty:
    DownloadPage(link)

"""
stahování po kategoriích se dělalo postupně, aby se stíhalo manuální
dotřídění souborů. Např. jsem ze stažených odstranila věci, co nejsou
kosmetika - kartáče, pilníky, vlhčené ubrousky apod.
"""
```

```python
# -*- coding: utf-8 -*-
"""
Created on Mon Apr  6 19:35:44 2020

@author: Aneta
"""

from bs4 import BeautifulSoup
import os
import csv
from ingredient_lists import echa_microplastics, other_synthetic_polymer, polyether, mineral, readily_degradable, other
from brands import brand_list

class Product(object):
    def __init__(self,ID,name,price,category, subcategory, subsubcategory, ingredients):
        """
        předpokládá, že v textu se seznamem ingrediencí jsou jednotlivé složky oddělené čárkami
        rozseká ingredients na jednotlivé složky a hodí je do listu, a roztřídí
        """
        self.ID=ID
        self.name=name
        self.add_brand()
        self.price=price
        self.category=category
        self.subcategory=subcategory
        self.subsubcategory=subsubcategory
        self.ingredients=ingredients
        self.ingredient_list=[i.lower().strip() for i in ingredients.split(",")]
        self.categorize_ingredients()

    def add_brand(self):
      global brand_list
      possible_brands=[brand for brand in brand_list if brand in self.name]
      if len(possible_brands)==1:
        self.brand=possible_brands[0]
      elif len(possible_brands)==2:
        possible_brands.remove("Palette ") #neb nejčastější kiks je, že Palette v názvu není značka, ale slovo
        self.brand=possible_brands[0]
      else:
        self.brand = self.name.split()[0] #pokud není značka v seznamu, vzít jako značku první slovo názvu. Podle printu zkontrolovat

    def categorize_ingredients(self):
        """
        podívá se, jaké jsou v produktu ingredience, a zda jsou v některém seznamu známých složek
        pokud ano, zařadí ingredienci do příslušné kategorie
        pokud ne, zkusí to sám dovodit z názvu
        při nejhorším se zeptá uživatele, co to je. podle toho to zařadí a navíc zapíše do seznamu známých složek
        """
        global echa_microplastics
        global other_synthetic_polymer
        global polyether
        global readily_degradable
        global other
        global mineral
        self.microplastics=[]
        self.syntpolymers=[]
        self.degradable_ings=[]
        self.uncategorized=[]
        self.minerals=[]
        self.polyethers=[]
        for ingredient in self.ingredient_list:
            if ingredient in echa_microplastics:
                self.microplastics.append(ingredient)
            elif ingredient in other_synthetic_polymer:
                self.syntpolymers.append(ingredient)
            elif ingredient in polyether:
                self.polyethers.append(ingredient)
            elif ingredient in readily_degradable:
                self.degradable_ings.append(ingredient)
            elif ingredient in other:
                self.uncategorized.append(ingredient)
            elif ingredient in mineral:
                self.minerals.append(ingredient)
            elif "extract" in ingredient or "juice" in ingredient: #zavádím předpoklad, že rostlinné extrakty jsou rozložitelné
                self.degradable_ings.append(ingredient)
                readily_degradable.append(ingredient)
            elif "ci " in ingredient: #zde předpoklad, že barvy patří do 'other'
                self.uncategorized.append(ingredient)
                other.append(ingredient)
            else:
                self.ask(ingredient)

    def ask(self,ingredient):
        """
        zeptá se uživatle, kam zaředit neznámou surovinu. Podle toho ji zařadí produktu
        a připíše do příslušného listu známých surovin
        """
        global echa_microplastics
        global other_synthetic_polymer
        global polyether
        global readily_degradable
```

```
            global other
            global mineral
            i=str(input("kam patří "+ingredient+"? (echa mikroplast:e, synt. polymer:s, polyether: p, minerál:m, degradovatelné:d, jiné: o): "))
            if i=="e":
                echa_microplastics.append(ingredient)
                self.microplastics.append(ingredient)
            elif i=="s":
                other_synthetic_polymer.append(ingredient)
                self.syntpolymers.append(ingredient)
            elif i=="d":
                readily_degradable.append(ingredient)
                self.degradable_ings.append(ingredient)
            elif i=="m":
                mineral.append(ingredient)
                self.minerals.append(ingredient)
            elif i=="o":
                other.append(ingredient)
                self.uncategorized.append(ingredient)
            elif i=="p":
                polyether.append(ingredient)
                self.polyethers.append(ingredient)
            else:
                self.ask(ingredient)

    def __str__(self):
        print("-------")
        print("ID:"+str(self.ID))
        print(self.name)
        print("  "+self.category)
        print("  "+str(self.price)+" Kč")
        print("   ")
        print("složení: "+self.ingredients)
        print("   ")
        print("mikroplasty: "+str(self.microplastics))
        print("syntetické polymery: "+str(self.syntpolymers))
        print("polyethery: "+str(self.polyethers))
        print("degradovatelné složky: "+str(self.degradable_ings))
        print("minerální složky: "+str(self.minerals))
        return "jiné: "+str(self.uncategorized)

    def list_attributes(self):
        return [self.ID,
        self.name,
        self.brand,
        self.category,
        self.subcategory,
        self.subsubcategory,
        self.price,
        self.ingredients,
        len(self.microplastics),
        self.microplastics]

    def list_fact_table(self):
      global echa_microplastics
      if self.microplastics:
        return [[self.ID, echa_microplastics.index(microplastic)+1] for microplastic in self.microplastics]
      else:
        return [[self.ID, 0]]

def analyze_products(categ):
    """

    Parameters
    ----------
    categ : str, musí se shodovat s názvem složky v cosmetics

    vyhledá složku s některou kategorií produktů, pootvírá soubory
    z každého souboru s infem o produktu vycucne název, cenu, ingredience
    vytvoří objekt Product
    pokud obsahuje syntetické polymery, zapíšou se
    vypíše se krátký report

    Returns
    -------
    list listů se seznamy atributů pro jednotlivé produkty

    """
    global ID

    folderPath = 'cosmetics/'+categ
    products=[]
    with_microplastics=0
    with_other_synt_polymers=0
    analyzed_products=0
    all_microplastics=[]
    errors=[]
    facts=[]
    for root, dirs, files in os.walk(folderPath):
        for file in files:
            path="cosmetics/"+categ+"/"+str(file)
            with open(path) as item:
```

```
                b=BeautifulSoup(item.read(),"html.parser")
                # print(b.prettify())
                name=str(file).rstrip(".txt")
                # print(name)
                category_list =[text.strip() for text in b.find("div", class_ = "sx-breadcrumbs").strings if text.strip()]
                cat, subcat = category_list[2:4]
                if len(category_list) > 5:
                  subsubcat = category_list[4]
                else:
                  subsubcat = ""
                price_list=[text for text in b.find("div",class_='sx-item-price-group').strings]
                #dává [koruny,halíře,akční cena koruny, akční cena halíře, \n]
                # print(price_list)
                try:
                    price=float(price_list[0]+"."+price_list[1]) #sečte koruny s halířema a zapíše neakční cenu
                except ValueError: #úprava cen nad tisícovku (1 299,90)
                    #print(name)
                    #print(price_list)
                    if "'>" in price_list[0]:
                        price=float("".join([item.strip() for item in (price_list[1]).split()])+"."+price_list[2])
                        #print(price)
                    else:
                        errors.append(str(file))
                try:
                    ings=b.find("div",id="slozenialergeny").p.string
                    #print(ings)
                    p=Product(ID,name,price,cat, subcat, subsubcat,ings)
                    analyzed_products+=1
                    # print(p)
                    products.append(p.list_attributes())
                    facts+=p.list_fact_table()
                    if p.syntpolymers!=[]:
                        with_other_synt_polymers+=1
                    if p.microplastics!=[]:
                        with_microplastics+=1
                    ID+=1
                except AttributeError:
                    errors.append(path)


    print("kategorie: "+categ)
    print("analyzovaných produktů: "+str(analyzed_products))
    print("s mikroplasty: "+str(with_microplastics))
    print("s jinými syntetickými polymery: "+str(with_other_synt_polymers))
    print("mikroplasty v kategorii: ")
    for polymer in echa_microplastics:
        c=all_microplastics.count(polymer)
        if c>0:
            print("   "+polymer+":"+str(c)+"x")
    print("")

    return products, facts

ID = 1
with open('products.csv', 'w', newline="") as products, open('products_microplastics.csv', 'w', newline = "") as fact_table:
  product_writer = csv.writer(products)
  fact_writer = csv.writer(fact_table)
  product_writer.writerow(["ID","name","brand","category","subcategory","subsubcategory","price",
                           "ingredients","no_microplastics","microplastics"])
  fact_writer.writerow(["productID","microplasticID"])
  for category in ["cestovni","dekorativni","intimni","lubrikanty","panska","parfemy_a_deodoranty",
                   "pletova","telova","vlasova","zuby_a_usta"]:
    result=analyze_products(category)
    product_writer.writerows(result[0])
    fact_writer.writerows(result[1])
```

```
from ingredient_lists import echa_microplastics
import csv

with open('microplastics.csv','w', newline="") as microplastic_table:
  writer=csv.writer(microplastic_table)
  writer.writerow(["microplasticID","microplastic_name"])
  writer.writerows([[echa_microplastics.index(microplastic)+1, microplastic]
                    for microplastic in echa_microplastics])
```

mikroplasty v kosmetických produktech ze sortimentu Teta drogerie (duben 2020)

kategorie: cestovni
analyzovaných produktů: 26
s mikroplasty: 7
s jinými syntetickými polymery: 2
mikroplasty v kategorii:
   styrene/acrylates copolymer:4x
   styrene/acrylate copolymer:1x
   acrylates copolymer:1x
   octylacrylamide/acrylates/butylaminoethyl methacrylate copolymer:1x

kategorie: dekorativni
analyzovaných produktů: 415
s mikroplasty: 240
s jinými syntetickými polymery: 252
mikroplasty v kategorii:
   polyethylene:43x
   polyethylene [+/- ci 77891:3x
   oxidized polyethylene:4x
   polyethylene wax:2x
   polymethyl methacrylate:20x
   methyl methacrylate crosspolymer:12x
   ptfe:1x
   nylon-6ti/dimethicone copolymer:3x
   nylon-611/dimethicone copolymer:15x
   nylon-6:4x
   nylon-12:59x
   styrene/acrylates/ammonium methacrylate copolymer:13x
   styrene/acrylates/ ammonium methacrylate copolymer:1x
   styrene/acrylates copolymer:37x
   styrene / acrylates copolymer:1x
   styrene / acrylates / ammonium methacrylate copolymer:2x
   polyethylene terephthalate:13x
   polyethylene terephithalate:1x
   polybutylene terephthalate:5x
   terephthalate:1x
   acrylates copolymer:81x
   acrylates/c10-30 alkyl acrylate crosspolymer:1x
   sodium polyacrylate:3x
   glyceryl polymethacrylate:1x
   acrylonitrile/methyl methacrylate/vinylidene chloride copolymer:15x
   glyceryl polymethacrylate:1x
   sodium polymetnacrylate:1x
   rosin acrylate:3x
   sodium polymethacrylate:9x
   acrylates/dimethicone copolymer:10x
   lauryl methacrylate/glycol dimethacrylate crosspolymer:9x
   ethylene/acrylic acid copolymer:14x
   sodium polemethacrylate:1x
   ethylene/methacrylate copolymer:4x
   acrylic acid/isobutyl acrylate/isobornyl acrylate copolymer:5x
   acrylonitrile/methyl methacrylte/vinylidene chloride copolymer:1x
   acrylonitrile/methyl methacrylate/vinylidene chloride copolymer aluminum

hydroxide:3x
   acrylates/c12-22 alkyl methacrylate copolymer:2x
   c30-45 alxyldimethylsilyl polypropylsilsesquioxane:1x
   polymethylsilses-quioxane:1x
   polypropylsilsesquioxane:5x
   c30-45 alkyldimethylsilyl polypropylsilsesquioxane:19x
   vinyl dimethicone/methicone silsesquioxane crosspolymer:14x
   vinyl dimethicnone/methicone silsesquioxane crosspolymer:2x
   diphenyl dimethicone/vinyl diphenyl dimethicone/silsesquioxane
crosspolymer:4x
   vinyl dimethicone / methicone silsesquioxane crosspolymer:1x
   c30-45 alkyldimethylsilyl popypropylsilsesquioxane:3x

kategorie: intimni
analyzovaných produktů: 19
s mikroplasty: 1
s jinými syntetickými polymery: 0
mikroplasty v kategorii:
   acrylates/c10-30 alkyl acrylate crosspolymer:1x

kategorie: lubrikanty
analyzovaných produktů: 8
s mikroplasty: 0
s jinými syntetickými polymery: 1
mikroplasty v kategorii:

kategorie: panska
analyzovaných produktů: 278
s mikroplasty: 30
s jinými syntetickými polymery: 27
mikroplasty v kategorii:
   polyethylene:4x
   polymethyl methacrylate:1x
   ptfe:2x
   polyurethane crosspolymer-2:2x
   styrene/acrylates copolymer:2x
   styrene / acrylates copolymer:1x
   acrylates copolymer:13x
   acrylates/c10-30 alkyl acrylate crosspolymer:1x
   pvm/ma copolymer:2x
   glyceryl acrylate/acrylic acid copolymer:2x
   sodium polyacrylate:2x
   ammonium polyacryloyldimethyl taurate:1x
   acrylamidopropyltrimonium chloride/acrylamide copolymer:2x
   ammonium polyacryldimethyl tauramide/ammonium polyacryloyldimethyl taurate:1x
   sodium polynaphthalenesulfonate:1x

kategorie: parfemy_a_deodoranty
analyzovaných produktů: 139
s mikroplasty: 21
s jinými syntetickými polymery: 0
mikroplasty v kategorii:
   acrylates/octylacrylamide copolymer:21x

kategorie: pletova
analyzovaných produktů: 351
s mikroplasty: 117
s jinými syntetickými polymery: 145
mikroplasty v kategorii:
   polyethylene:6x
   methyl methacrylate crosspolymer:2x
   ptfe:2x
   nylon-12:7x
   styrene/acrylates copolymer:2x
   acrylates/vinyl neodecanoate crosspolymer:1x
   sodium acrylates copolymer:6x
   acrylates/vinyl isodecanoate crosspolymer:8x
   acrylates copolymer:12x
   sodium acrylate/sodium acryloyldimethyl taurate copolymer:7x
   acrylates/c10-30/alkyl acrylate crosspolymer:1x
   polyacrylate crosspolymer-6:4x
   acrylates/c10-30 alkyl acrylate crosspolymer:37x
   hydroxyethyl acrylate/sodium acryloyldimethyl taurate copolymer:4x
   pvm/ma copolymer:2x
   glyceryl acrylate/acrylic acid copolymer:2x
   polyacrylate-13:4x
   methyl methacrylate/glycol dimethacrylate crosspolymer:1x
   sodium polyacrylate:11x
   glyceryl polymethacrylate:1x
   acrylonitrile/methyl methacrylate/vinylidene chloride copolymer:1x
   ammonium polyacryloyldimethyl taurate:6x
   acrylamide/sodium acryloyldimethyltaurate copolymer:8x
   acrylonitrile/methyl methacrylate/vinylidene chloride:3x
   copolymer:3x
   ammonium acryloyldimethyl-taurate/steareth-25 methacrylate crosspolymer:1x
   ammonium acryloyldimethyl - taurate/steareth-25 methacrylate crosspolymer:1x
   acrylamide / sodium acryloyldimethyltaurate copolymer:2x
   ammonium polyacryldimethyltauramide/ammonium polyacryloyldimethyl taurate:1x
   dimethicone/polyglycerin-3 crosspolymer:3x
   sodim acrylates copolymer:1x
   ammonium polyacryldimethyltauramide / ammonium polyacryloyldimethyl
taurate:2x
   acrylonitrile / methylmethacrylate / vinylidene chloride copolymer:1x
   polyacrylate-21:1x
   acrylates/dimethylaminoethyl methacrylate copolymer:1x
   glyceryl polymethacrylate:1x
   sodium acryloyldimethyltaurate/vp crosspolymer:1x
   acrylates/beheneth-25 methacrylate copolymer:2x
   acrylates crosspolymer-4:2x
   potassium acrylates copolymer:1x
   acrylic acid/acrylamidomethyl propane sulfonic acid copolymer:1x
   acrylamide/ammonium acrylate copolymer:1x
   polymethylsilsesquioxane:11x

kategorie: telova
analyzovaných produktů: 496
s mikroplasty: 113
s jinými syntetickými polymery: 116

mikroplasty v kategorii:
   polyethylene:8x
   polymethyl methacrylate:1x
   ptfe:1x
   polyurethane crosspolymer-2:2x
   styrene/acrylates copolymer:33x
   styrene / acrylates copolymer:2x
   styrene/arcylates copolymer:1x
   sodium acrylates copolymer:3x
   acrylates/vinyl isodecanoate crosspolymer:6x
   acrylates copolymer:14x
   sodium acrylate/sodium acryloyldimethyl taurate copolymer:4x
   acrylates/c10-30 alkyl acrylate crosspolymer:26x
   hydroxyethyl acrylate/sodium acryloyldimethyl taurate copolymer:3x
   pvm/ma copolymer:3x
   glyceryl acrylate/acrylic acid copolymer:3x
   sodium polyacrylate:12x
   ammonium polyacryloyldimethyl taurate:1x
   acrylamidopropyltrimonium chloride/acrylamide copolymer:2x
   carbober:1x
   ammonium acryloydimethyltaurate/vp copolymer:1x
   ammonium polyacryldimethyltauramide/ammonium:1x
   sodium acrylates/c10-30 alkyl acrylate:1x
   sodium acrylate/acryloyldimethyltaurate/dimethylacrylamide crosspolymer:3x

kategorie: vlasova
analyzovaných produktů: 676
s mikroplasty: 197
s jinými syntetickými polymery: 177
mikroplasty v kategorii:
   polyethylene:1x
   styrene/acrylates copolymer:7x
   styrene/acrylates:1x
   acrylates copolymer:14x
   polyacrylate crosspolymer-6:1x
   acrylates/c10-30 alkyl acrylate crosspolymer:15x
   sodium polyacrylate:4x
   copolymer:1x
   acrylates/beheneth-25 methacrylate copolymer:2x
   octylacrylamide/acrylates/butylaminoethyl methacrylate copolymer:41x
   acrylates/octylacrylamide copolymer:5x
   acrylamidopropyltrimonium chloride/acrylamide copolymer:7x
   sodium polynaphthalenesulfonate:4x
   polyacrylate crosspolymer-9:3x
   acrylates/t-butylacrylamide copolymer:7x
   sodium acrylate/acryloyldimethyltaurate/dimethylacrylamide crosspolymer:2x
   dimethylacrylamide/ethyltrimonium chloride methacrylate copolymer:1x
   vinyl caprolactam/vp/dimethylaminoethyl methacrylate copolymer:12x
   vp/dmapa acrylates copolymer:4x
   acrylates/hydroxyesters acrylates copolymer:6x
   acrylates/steareth-20 methacrylate crosspolymer:5x
   acrylamidopropyltrimonium chloride/acrylates copolymer:27x
   ethylhexyl acrylate/methyl methacrylate copolymer:1x
   acrylates/steareth-20 itaconate copolymer:3x

```
   acrylamidopropyltrimoniumchloride/acrylates copolymer:1x
   amp-acrylates/allyl methacrylate copolymer:1x
   vinyl capropactam/vp/dimethylaminoethyl methacrylate copolymer:1x
   acrylates/stearyl acrylate/ethylamine oxide methacrylate copolymer:1x
   acrylates/steareth-20 methacrylate copolymer:1x
   pvp/va copolymer:5x
   vp/methacrylamide/vinyl imidazole copolymer:1x
   vp/dimethylaminoethylmethacrylate copolymer:2x
   vp/vinyl caprolactam/dmapa acrylates copolymer:1x
   butyl ester of pvm/ma copolymer:1x
   polymethylsilsesquioxane:1x
   polypropylsilsesquioxane:2x
   amodimethicone/morpholinomethyl silsesquioxane copolymer:37x
   amodimethicone/morpholinomethyl silsequioxane copolymer:2x
   dimethiconol/silsesquioxane copolymer:1x

kategorie: zuby_a_usta
analyzovaných produktů: 96
s mikroplasty: 15
s jinými syntetickými polymery: 11
mikroplasty v kategorii:
   pvm/ma copolymer:7x
   calcium/zinc pvm/ma copolymer (33%):3x
   calcium/zinc pvm/ma copolymer:1x
   calcium/zinc pvm/ma copolymer (35%):1x
   calcium/sodium pvm/ma co-polymer:1x
   calcium/sodium pvm/ma copolymer:2x
```

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Education and employment

9/2021-11/2021 **Erasmus+ internship – Centre of Polymer and Carbon materials, Polish Academy of Science**

- Research topic: Direct synthesis of PHA oligomers from biomass
- Found a simple, economical, and environmentally friendly way to prepare oligomers by thermal degradation. Presented the results at the conference, preparing an article.

From 10/2018 **Doctoral study – Brno University of Technology, Faculty of Chemistry**

- Main field of study: Chemistry, technology and properties of materials
- Topic of thesis: Methods of Poly-3-hydroxybutyrate Extraction and Their Optimization

From 10/2018 **Senior Scientist – Nafigate Corporation**

- Research and development of new methods for isolation of poly-3-hydroxybutyrate from bacterial biomass. Developed acid-based and soap-based methods for use in pilot-plant scale, assisted with large scale experiments.
- Found ways to increase the thermal stability of less stable PHB batches. Proposed the use of fine-powdered PHB as a substitute for synthetic polymers in cosmetics, manages the Nafigate's technical cooperation with several cosmetic companies interested in this solution.

8/2017–4/2018 **Visiting student researcher – University of South Australia**
Future Industries Institute

- Project: Commercialization of a Microfluidic Swimming Pool Water Quality Sensor.
- Optimized reagent composition for measurement on microfluidic sensor. Modified total chlorine method so that single reagent could be used instead of two component reagent. My modification of free chlorine method led to fourfold increase in sensitivity.
- In collaboration with partner company's developer, created a Raspberry Pi-based prototype. Tested standards and real samples, contributed to design of hardware modifications for increase of accuracy.
- Regularly presented results for the partner company, wrote experimental parts for publications and patents.

10/2016-7/2017 **Junior Scientist - Ratiochem s.r.o.**

- Developed original non-infringing route of synthesis for a non-sedating antihistamine.
- Found a new customer - TestLine Clinical Diagnostics. Prepared several commercially unavailable luminescence enhancers for TestLine.
- Explained source of irreproducibility of a key step in synthesis of a semi-synthetic bile acid. Contributed to development of alternative routes.

8/2016-10/2016 **Intern - TestLine Clinical Diagnostics s.r.o.**

- Prepared and analyzed key components of ELISA test kits.
- Looked for a cause of decreased reactivity of certain batches of substrate solution. Suggested and conducted kinetic experiments that partially revealed origin and nature of the inhibitor.

2/2012–7/2016 **Laboratory Assistant - Institute of Macromolecular Chemistry of the Academy of Sciences**

- Research related mostly to bachelor and master thesis topics: designed and synthesized new polymers with potential use in cancer treatment.
- Analyzed products and intermediates (NMR, MS, IR, UV/Vis, HPLC...), evaluated biological experiments.
- Presented results at conferences and via research papers.

9/2014–6/2016 **Master's study - University of Chemistry and Technology, Prague, Faculty of Chemical Technology**

- Main field of study: Organic Chemistry
- Thesis: Polymer conjugates of muscarinic acetylcholine receptor ligands as targeted moieties for oncological diagnostics and therapy

9/2011–7/2014 **Bachelor's study - University of Chemistry and Technology, Prague, Faculty of Chemical Engineering**

- Main field of study: Chemistry
- Thesis: Multimodal thermoresponsive polymer nanodiagnostics for cancer applications – analysis with fluorescent probes and light scattering

Conferences

03/2022 **The Silesian Meetings on Polymer Materials,** CMPW PAN
09/2021 **European Biotechnology Congress,** EBTNA
11/2020 **Chemistry is Life student's conference,** FCH BUT
09/2020 **2nd PHA platform World Congress – webinar,** Bioplastics Magazine
6/2019 **Plastics beyond petroleum**, Innoplast Solutions
11/2015 **Student's Scientific Conference,** UCT Prague
9/2015 **67. Zjazd Chemikov,** Slovak Chemical Society
2/2015 **24. Kolokvium,** IMC AS CR
11/2014 **Student's Scientific Conference**, UCT Prague
9/2014 **5th French-Czech "Vltava" Chemistry Symposium,** Institut de Chimie des Substances Naturelles
9/2013 **65. Zjazd Chemikov,** Slovak Chemical Society

Awards

12/2021 **Dean's Award**, FCH BUT
11/2020 **Special award** for the most intriguing presentation at the Chemistry is Life student's conference
07/2020 **3rd place** at Make Our Planet Great Again prize for environment & climate research
06/2016 **Dean's Award** for excellent study results at UCT Prague.
11/2015 **2nd place** at Student's Scientific Conference, UCT Prague.
02/2015 **Makroush Iuvenilis Optima** at 24. Kolokvium, IMC AS CR
Award for the best Ph.D. student and junior scientist presentation

Publications

- Pospisilova, Aneta, et al. "Effects of Differing Monomer Compositions on Properties of P(3HB-co-4HB) Synthesized by Aneurinibacillus Sp. H1 for Various Applications." *Polymers*, vol. 14, no. 10, May 2022, p. 2007.
- Vodicka, Juraj, et al. "Degradation of P(3HB-Co-4HB) Films in Simulated Body Fluids." *Polymers*, vol. 14, no. 10, May 2022, p. 1990.
- Pospisilova, Aneta, et al. "Techniques for increasing the thermal stability of poly [(R)-3-hydroxybutyrate] recovered by digestion methods." *Polymer Degradation and Stability* 193 (2021): 109727.
- Pospisilova, Aneta, Ivana Novackova, and Redek Prikryl. "Isolation of poly (3-hydroxybutyrate) from bacterial biomass using soap made of waste cooking oil." *Bioresource Technology (2021)*: 124683.
- Kontárová, Soňa, et al. "Printability, Mechanical and Thermal Properties of Poly (3-Hydroxybutyrate)-Poly (Lactic Acid)-Plasticizer Blends for Three-Dimensional (3D) Printing." *Materials* 13.21 (2020): 4736.

- Microfluidic Sensor For Continuous Or Semi-continuous Monitoring Of Quality Of An Aqueous Solution, Patent no. WO2021077153
- Elmas, Sait, et al. "Photometric Sensing of Active Chlorine, Total Chlorine, and pH on a Microfluidic Chip for Online Swimming Pool Monitoring." *Sensors* 20.11 (2020): 3099.
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