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METHODS OF POLY-3-HYDROXYBUTYRATE EXTRACTION AND THEIR OPTIMIZATION

METODY EXTRAKCE BIOPOLYMERU POLY-3-HYDROXYBUTYRÁTU A JEJICH OPTIMALIZACE

SUMMARY OF DOCTORAL THESIS

ZKRÁCENÁ VERZE DIZERTAČNÍ PRÁCE

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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. In collaboration with Nafigate Corporation, we aimed 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This text recapitulates a number of results that we published in peer-reviewed journals ([11, 12]), supplemented by unpublished experiments and an extended discussion so that it would serve also as a comprehensive summary of literature and laboratory test results for our industrial partners.

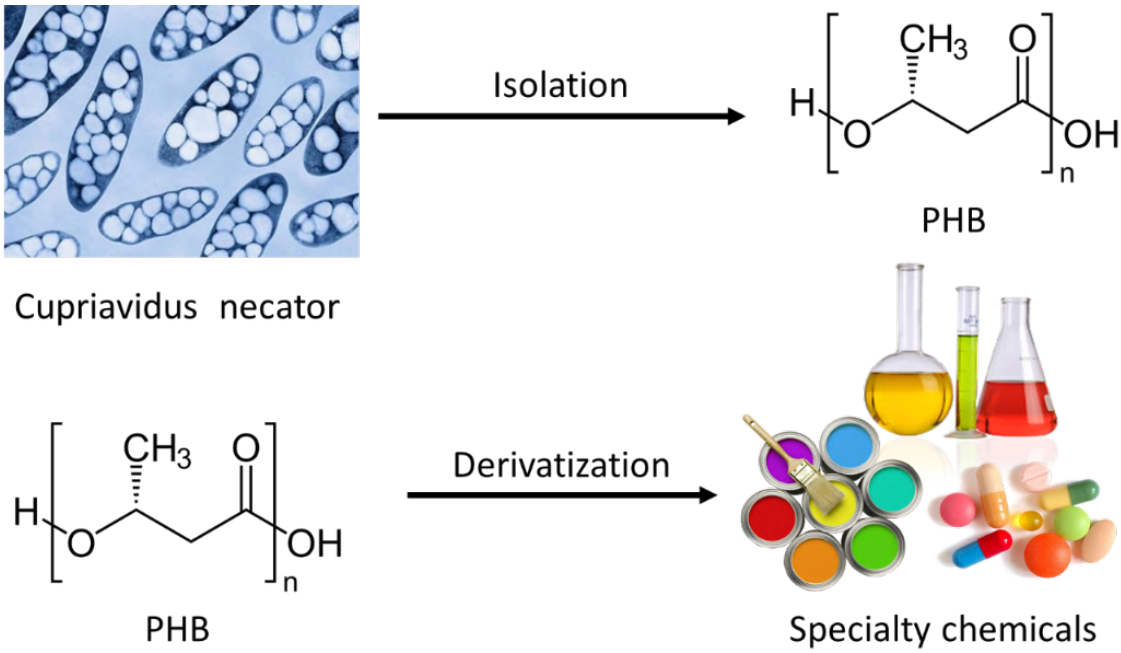

Fig. 1: Topics of this work

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.

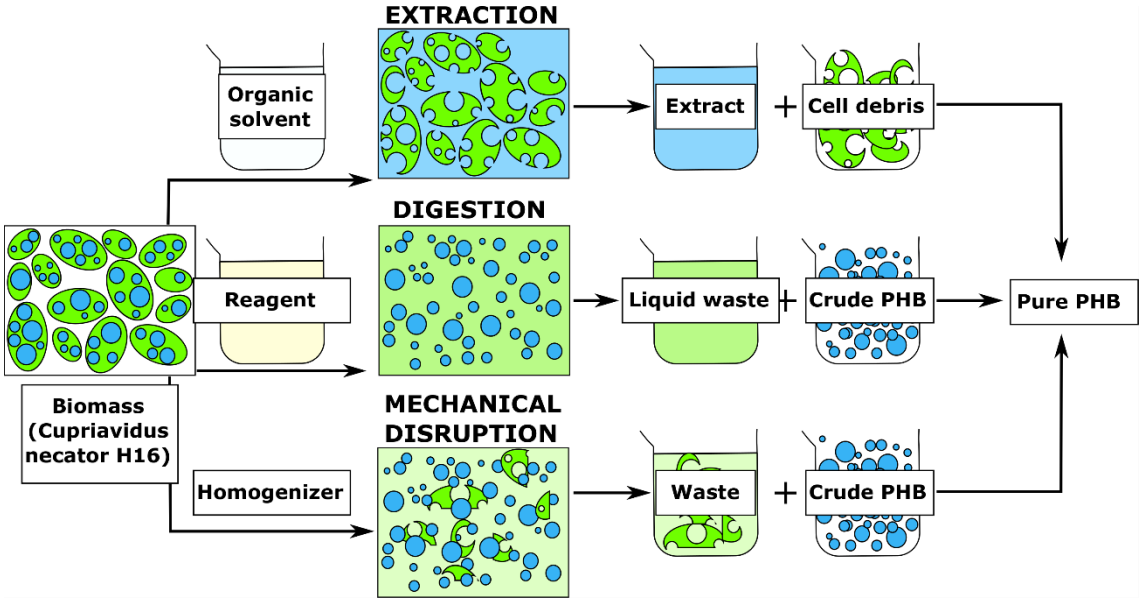

Fig. 1.1: Three basic approaches for PHB isolation

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).

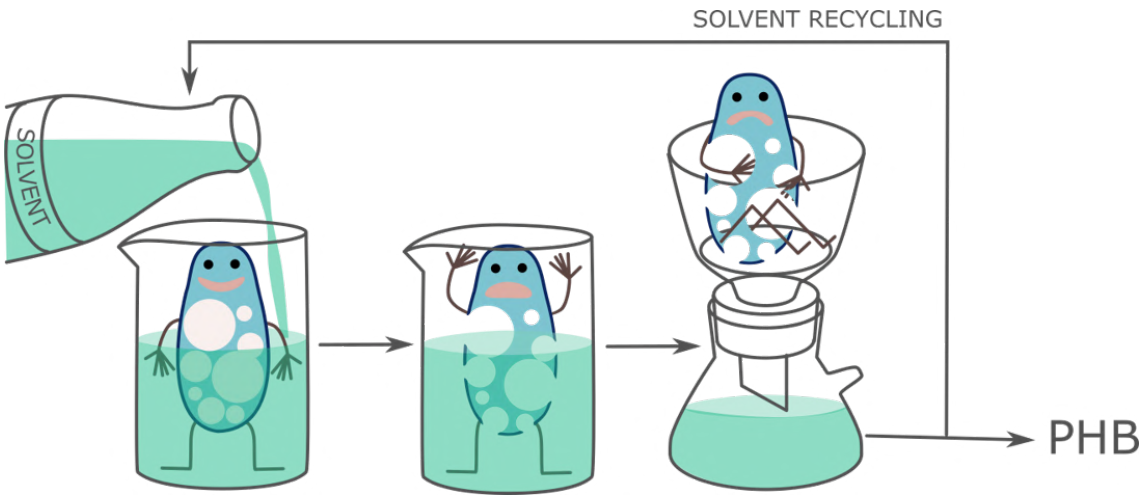

Fig. 1.2: Extraction of PHB from bacteria using solvent

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.

1.1.2 Digestion methods

The goal of digestion methods is to solubilize non-PHB cell material and remain PHB granules intact (fig. 1.3). 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,

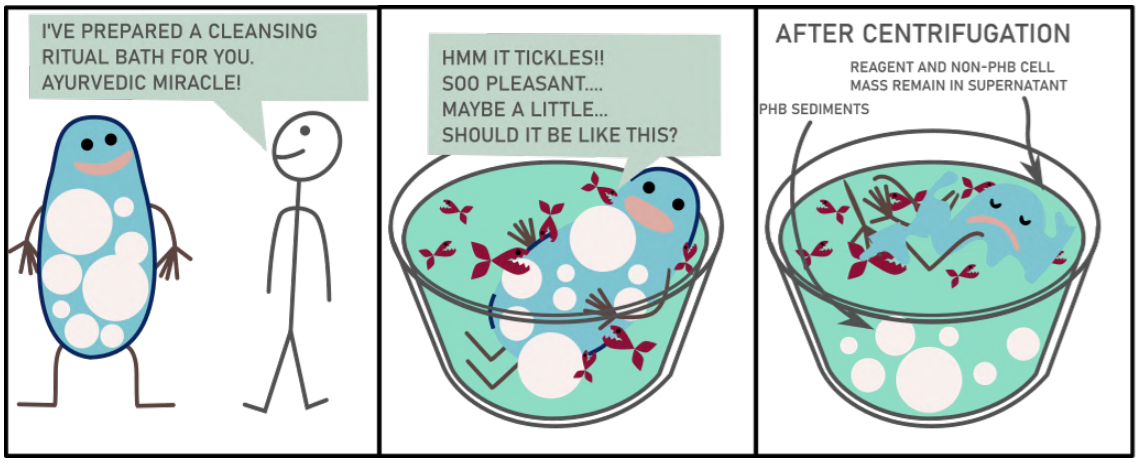

Fig. 1.3: Isolation of PHB from biomass by digestion

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 [13], 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 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 [14, 15, 16, 17]), acids and bases [18, 19], surfactants [20, 21, 22], enzymes [23], and other organisms [24, 25, 26, 27]. Procedures described in a literature usually combine several agents. Mechanical homogenization is often combined with chemical digestion, too.

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 [28, 29, 30, 31].

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 [32] and (R)-3-hydroxybutyric acid can be prepared by catalytic reduction of acetoacetate or fermentation [33]. 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.

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 4.1. 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 [34, 35] 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 Extraction with diethyl oxalate

To evaluate if thus obtained flocs are suitable for extraction, polymer was extracted by Jašek's method with diethyl oxalate [36]. 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.

3.2.1.5 Extraction with chloroform

For comparative experiments, standard method - chloroform extraction described earlier in the literature [12] was used.

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 [19] was adapted.

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]. Large-scale preparation follows a slightly modified method described in a full-length version of this thesis.

3.3.3.2 Soap-based digestion, general procedure

The procedure is described in detail in recent publication [11]. The supernatants from centrifugation were stored for further workup (see Section 3.3.3.4). The product was dried at 105 °C to constant weight.

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. The procedure is described in a paper [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. [37] 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, 38]

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. Centrifugation and drying of the concentrated biomass on industrial scale was time-consuming and energy-demanding. 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.

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 the flocculation. Findings are described in detail in the full-lenght version of this thesis.

After the flocculation, flocks had to be removed from the liquor. As the flocs are rather big, fine filter wasn't needed. 0.5 mm sieve was found out to be suitable. Another option was gentle centrifugation.

Drying was much easier than that of raw biomass. flocks were less adherent to tested surfaces (glass, porcelain, steel) and were porous. If dried in one layer,

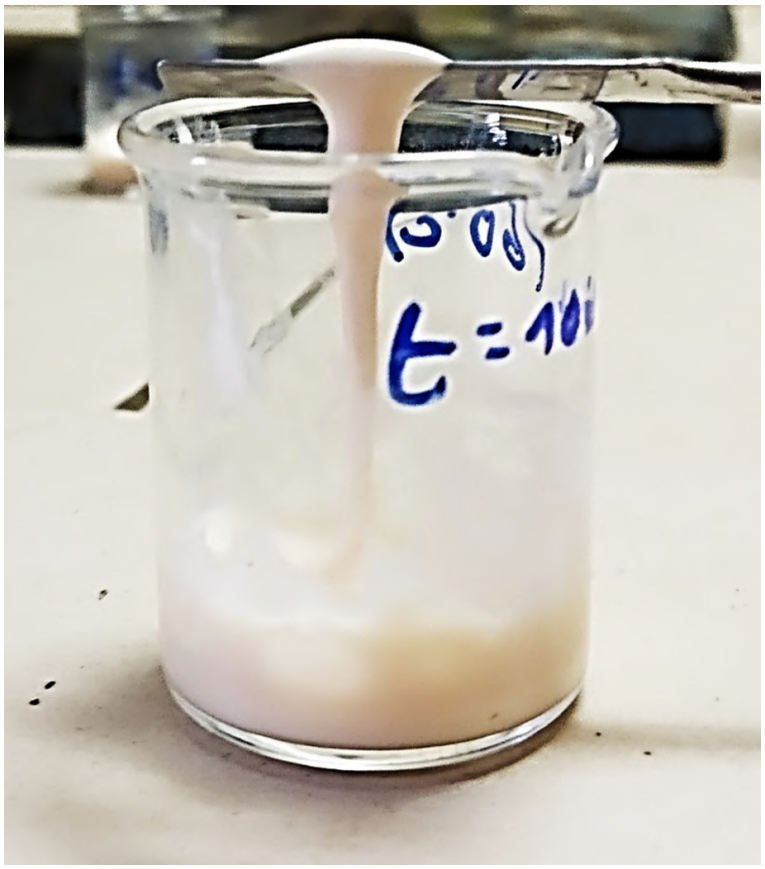

Fig. 4.1: Gelling of bacterial biomass upon alkalization

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 and 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. 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 4.2).

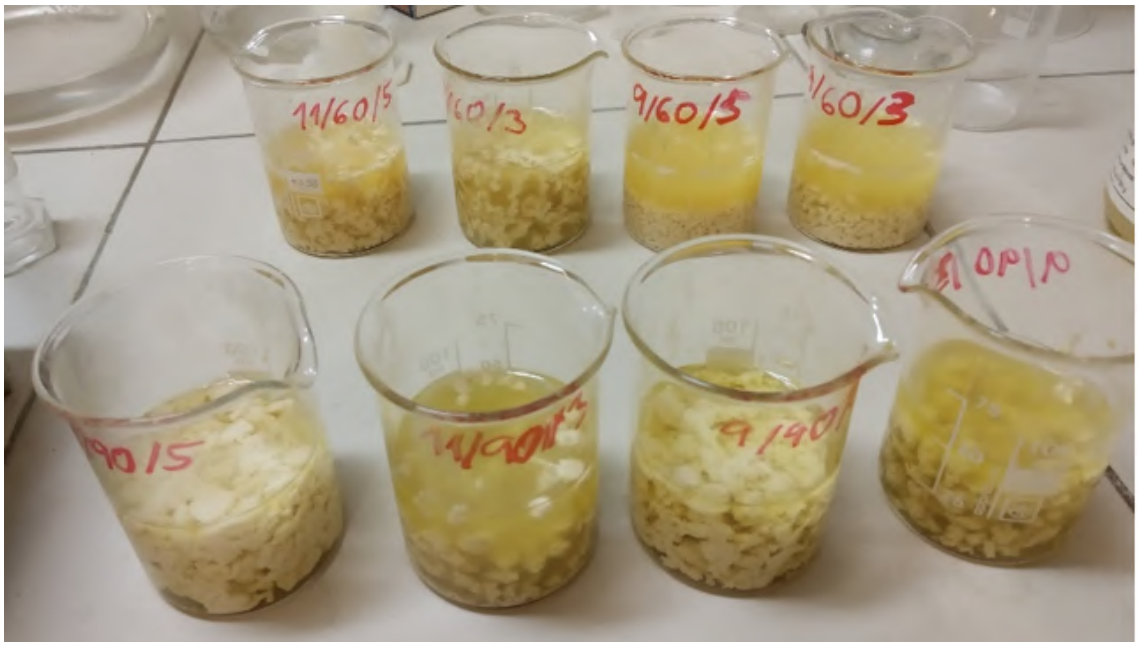

Fig. 4.2: Flocculation with NH_3 and H_3PO_4. Experiments at 90 °C in front, at 60 °C in behind. From left: alkalization to pH 11, acidification to pH 5; alkalization to pH 11, acidification to pH 3; alkalization to pH 9, acidification to pH 5; alkalization to pH 9, acidification to pH 3

4.1.1.3 Large-scale procedures

The experiment was performed in 400 l and 1500 l scale. Both experiments are described in detail in a full-lenght version of this thesis. The experiments show 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. Sample of thus obtained flocks was filtered, dried and extracted using diethyl oxalate as described previously 3.2.1.4 to confirm good extractability of the polymer. 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.

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. 4.3. 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

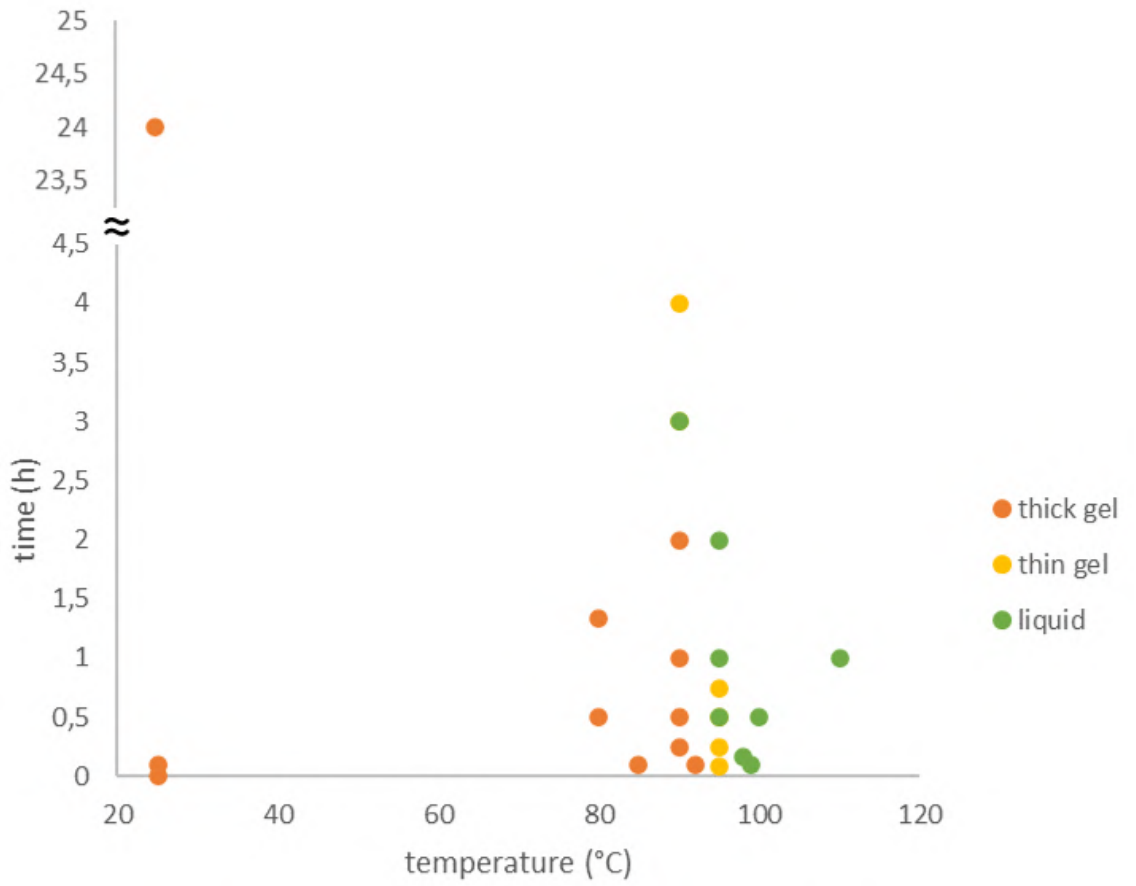

Fig. 4.3: Consistency of 25% biomass suspension after: 1. heating for given time at given temperature, 2. alkalization with NaOH to pH 9.

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. 4.4. As can be seen, yield is close to quantitative in only one experiment, with the mildest

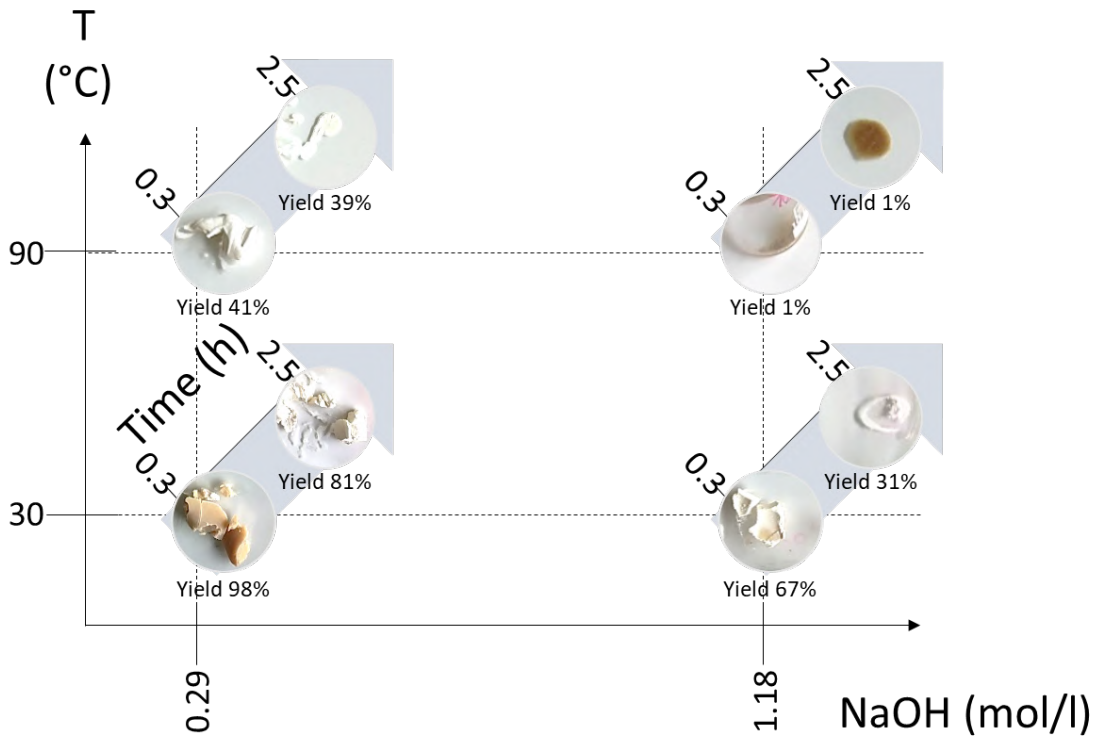

Fig. 4.4: Alkaline digestion of heat-pretreated biomass with NaOH. Influence of time, temperature and NaOH concentration on yield and product's color

reaction conditions. However, this experiment didn't give pure product, as can be seen in the photo. Low purity was further confirmed by FTIR, 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, 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

Fig. 4.5: 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.

requirements 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. 4.5. 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 sample handling. Regarding purity, it was hypothesized that results should match Yu's model [**?**]. 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. 4.6 and 4.7).

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

As can be seen in figures 4.6 and 4.7, 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. Details are given in the full-lenght version of this thesis. Final procedure, suitable for large-scale operation, is described in section 3.3.2.2. Typically, product yield and quality were as follows:

- Yield: 94 %, white powder (fig. 4.8)
- 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. 4.9)
- Particle size (SEM): 0.8 μm (fig. 4.10)

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:

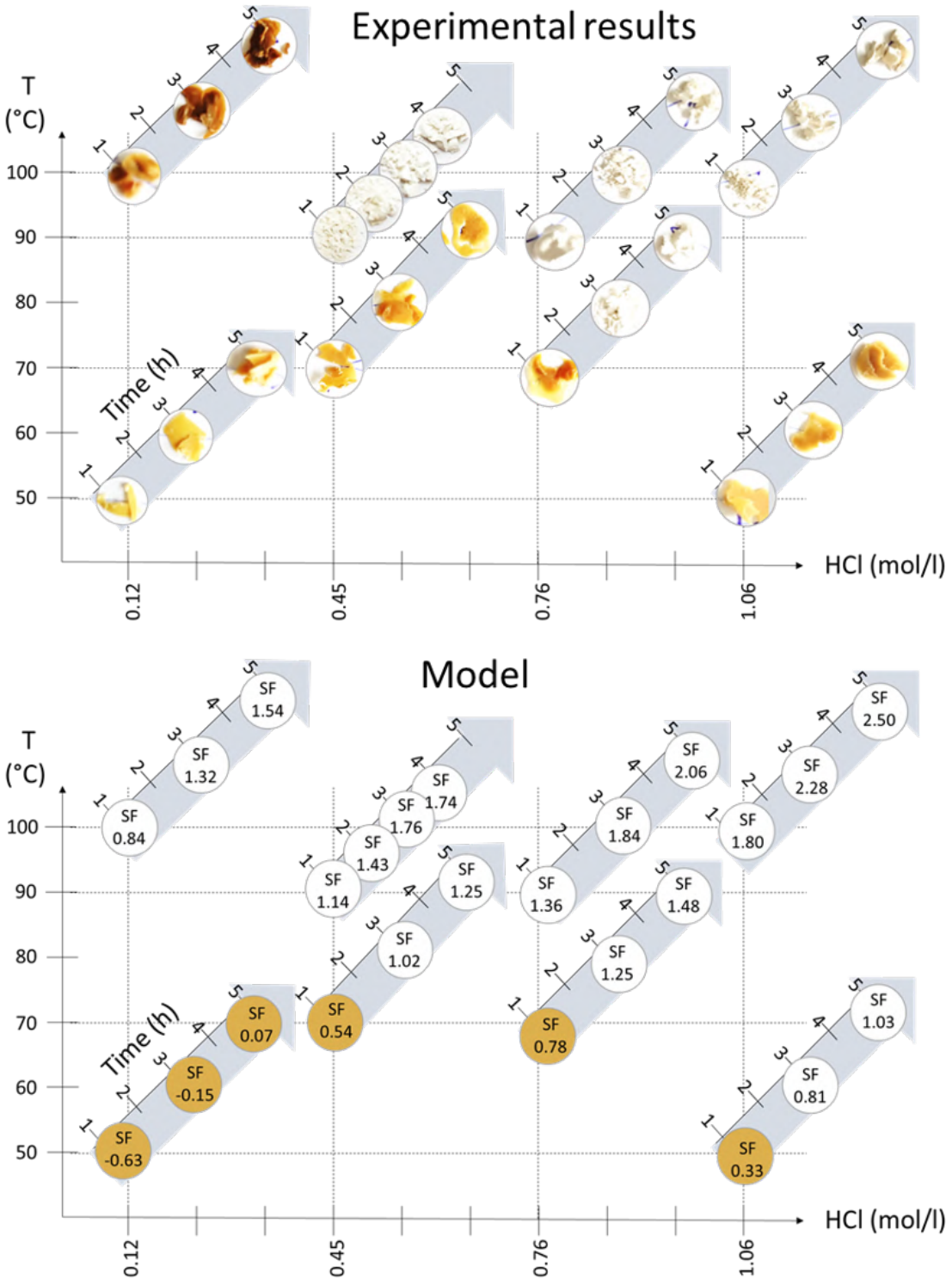

Fig. 4.6: Dependence of PHB purity on temperature, time and acid concentration. Experimental results are photos of products obtained by reaction at given conditions. Model shows severe factors for the reaction conditions. Pure product was expected for SF $>$ 0.8.

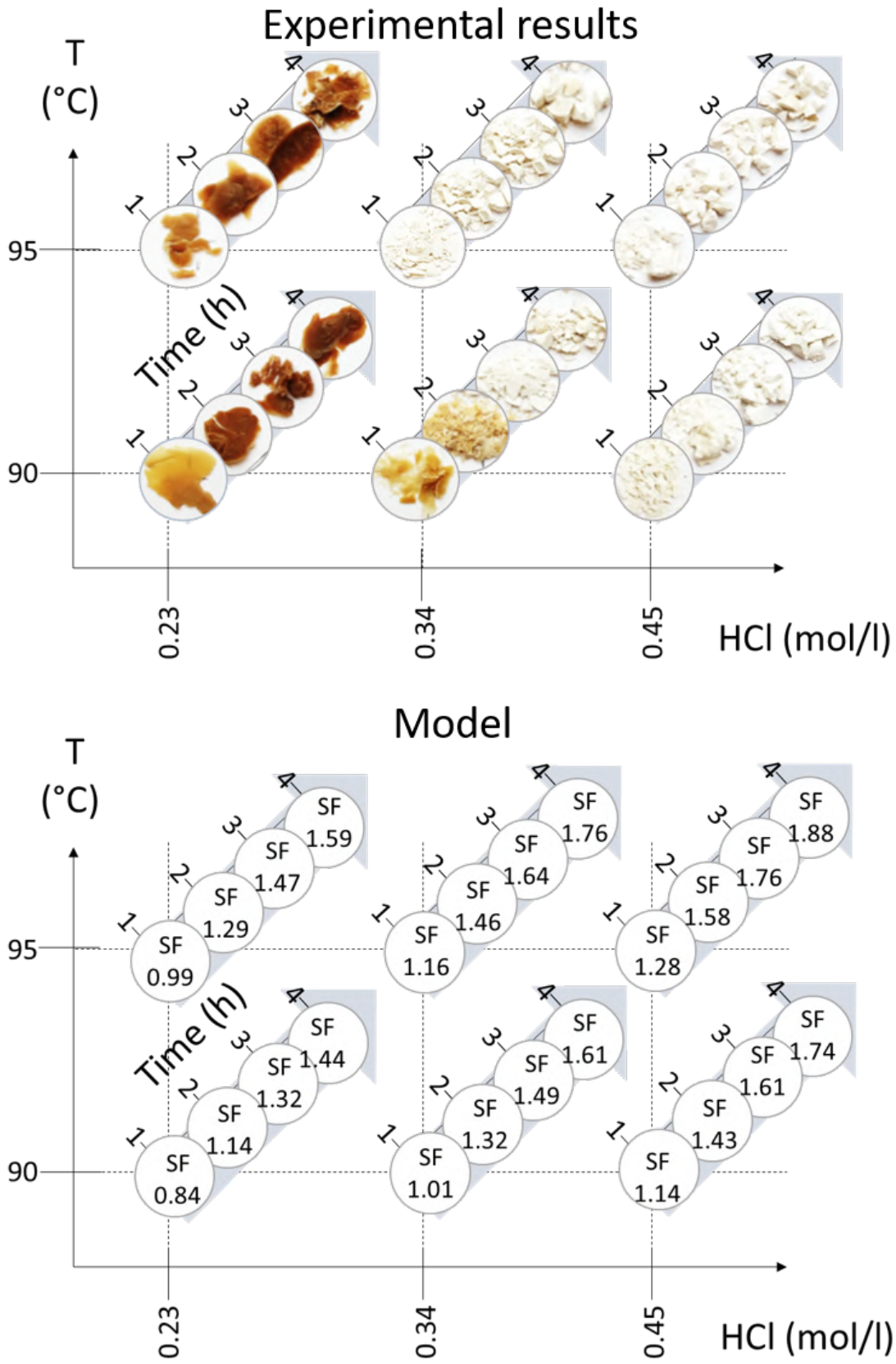

Fig. 4.7: Dependence of PHB purity on temperature, time and acid concentration. Experimental results are photos of products obtained by reaction at given conditions. Model shows severe factors for the reaction conditions. Pure product was expected for SF > 0.8.

Fig. 4.8: Isolated PHB

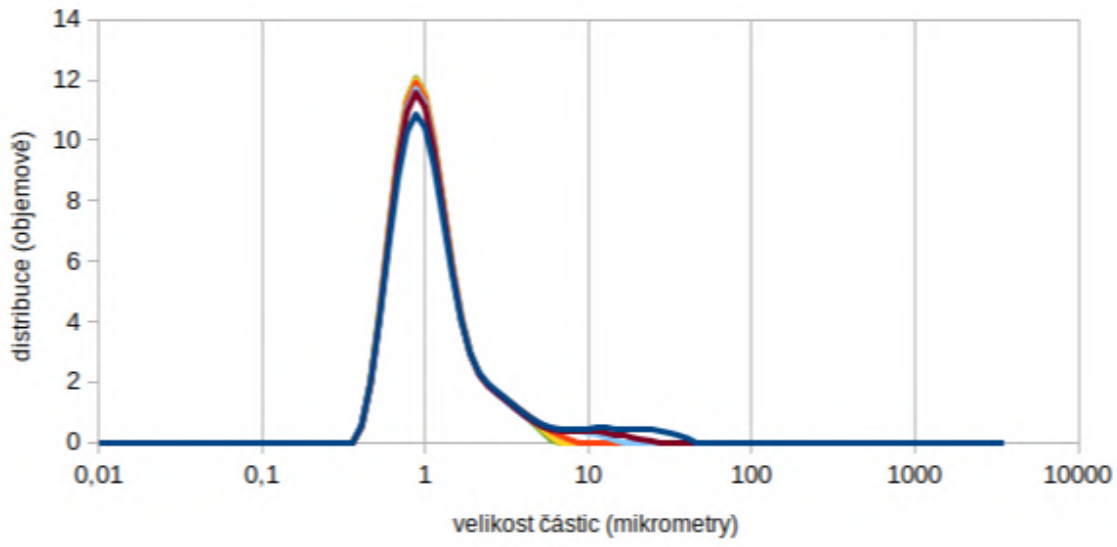

Fig. 4.9: Particle size distribution as measured by laser diffraction

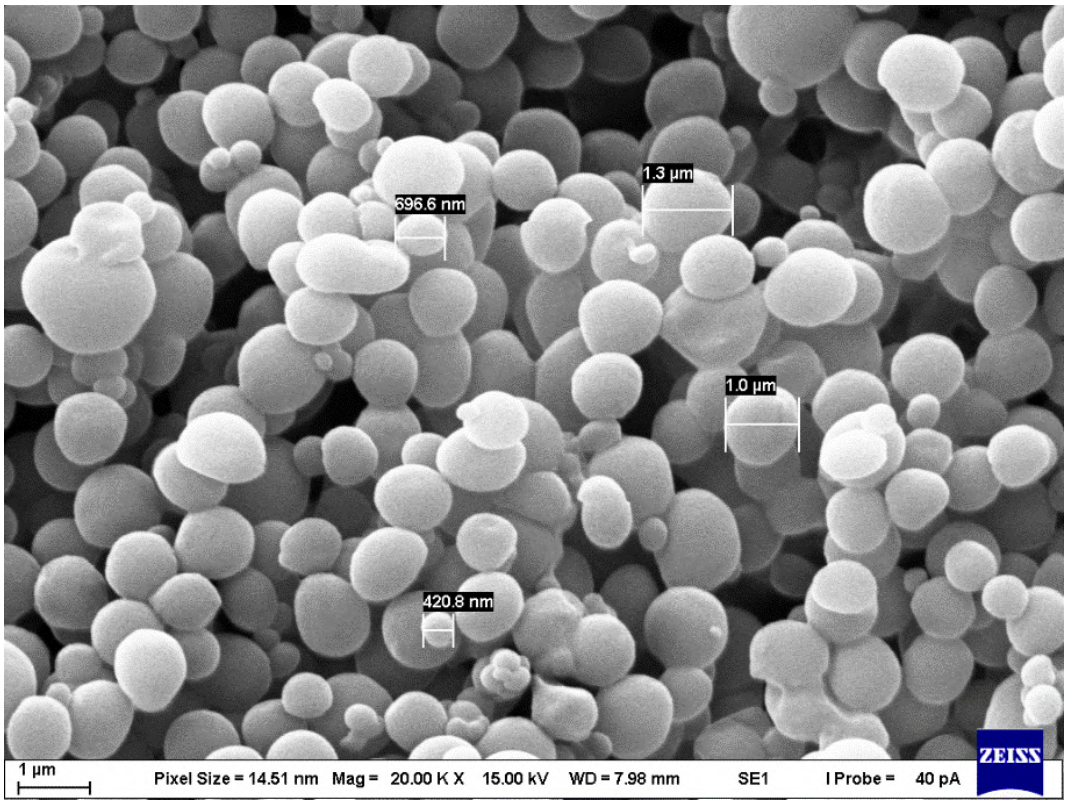

Fig. 4.10: SEM image of PHB isoalted by acid digestion

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

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

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. 4.11. Plotted values are similar to those for SDS-based method published previously, [22] shown in fig. 4.12. 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 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

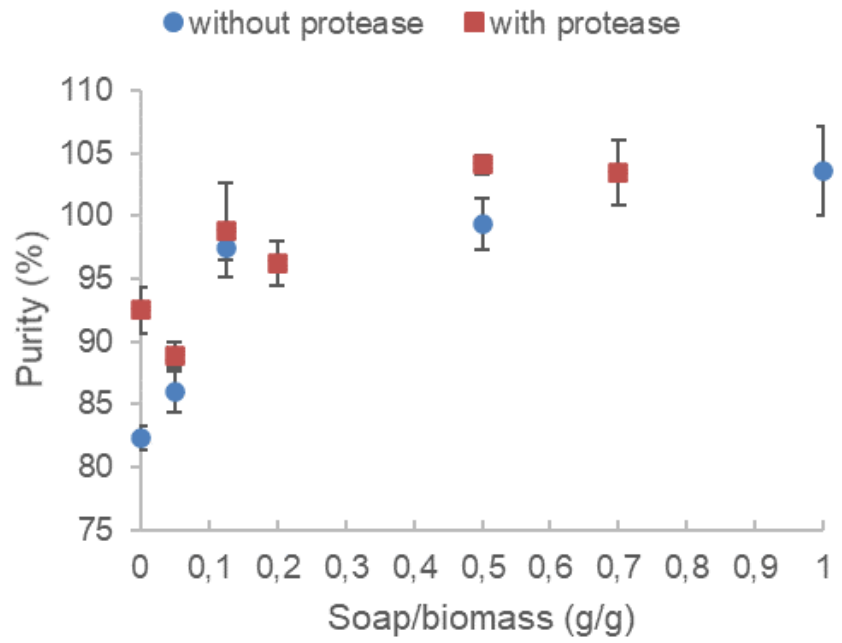

Fig. 4.11: Purity of PHB obtained by soap-based digestion [11]

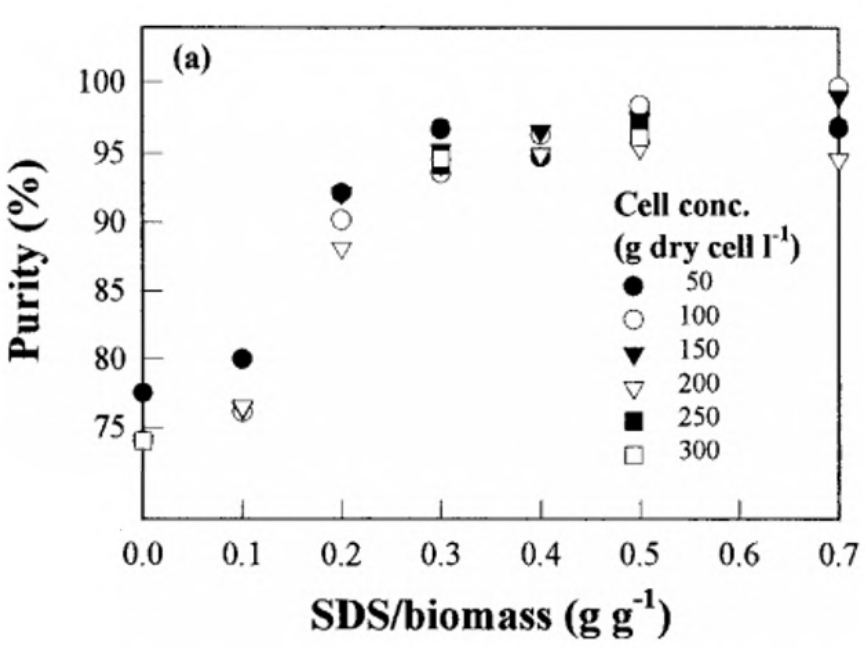

Fig. 4.12: Purity of PHB from SDS-based method. Obtained from previous publication.[22]

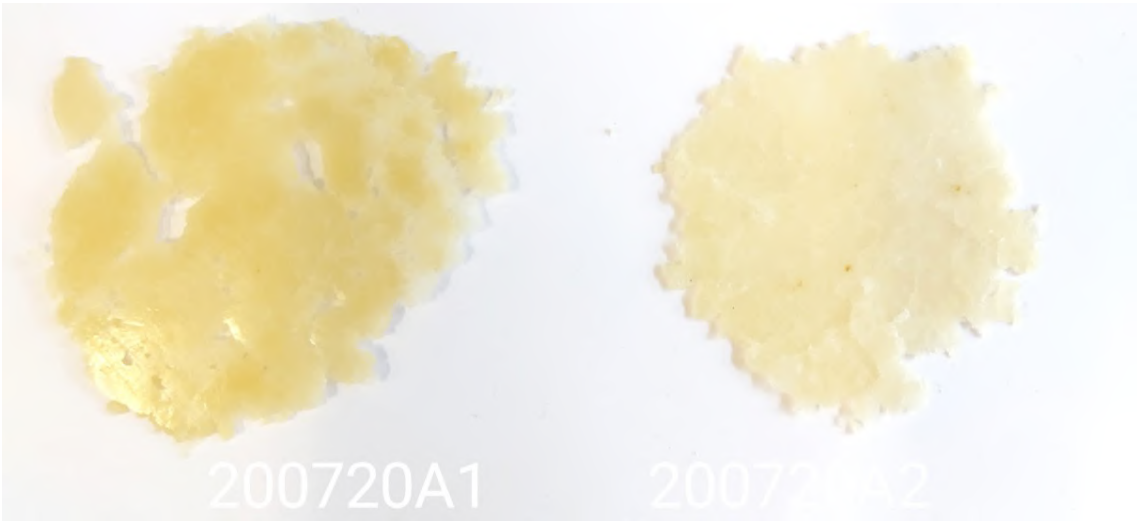

Fig. 4.13: PHB from soap-based digestion with 0.25 g soap/g CDW and protease. Left: soap was added in one dose, according to General procedure 3.3.3.2. Right: first half of soap was used for pre-purification, then the mixture was centrifuged, pallet was made up to the original volume and reacted with second half of soap and enzyme.

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. 4.13).

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. 4.14)
- (GC-FID): 96 %
- M_w (GPC): 389 000 g/mol
- M_n (GPC): 167 000 g/mol
- FTIR: matches PHB standard

Microstructure of the product (fig. 4.15) was different from the PHB obtained by acid digestion (fig. 4.10). 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

Fig. 4.14: PHB isolated by soap-based digestion

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. Both components precipitate upon acidification, and were suitable as co-substrates in subsequent fermentation, as described in detail in a recent paper [11]. Therefore, we expect that the precipitate could be recycled this way in the process and save costs and environmental impact.

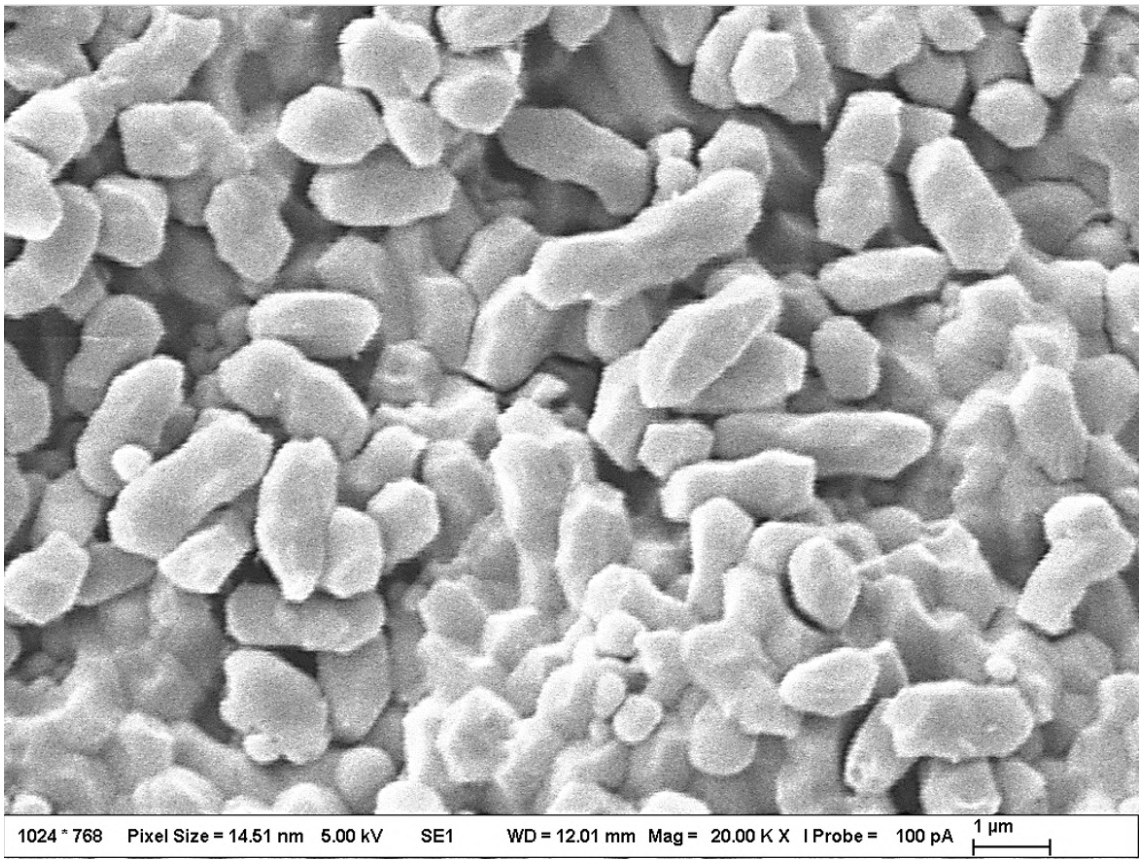

Fig. 4.15: SEM image of PHB isolated by soap-based digestion

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 inevitably 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. Data were scraped, analyzed and processed using dedicated Python scripts that are described in detail in a full version of this thesis. As an output, the scripts gave three .csv files containing dimensions of product and microplastics together with a fact table relating the above. The data model is given in fig. 4.16.

The data were visualized in PowerBI Desktop. 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. 4.17. 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

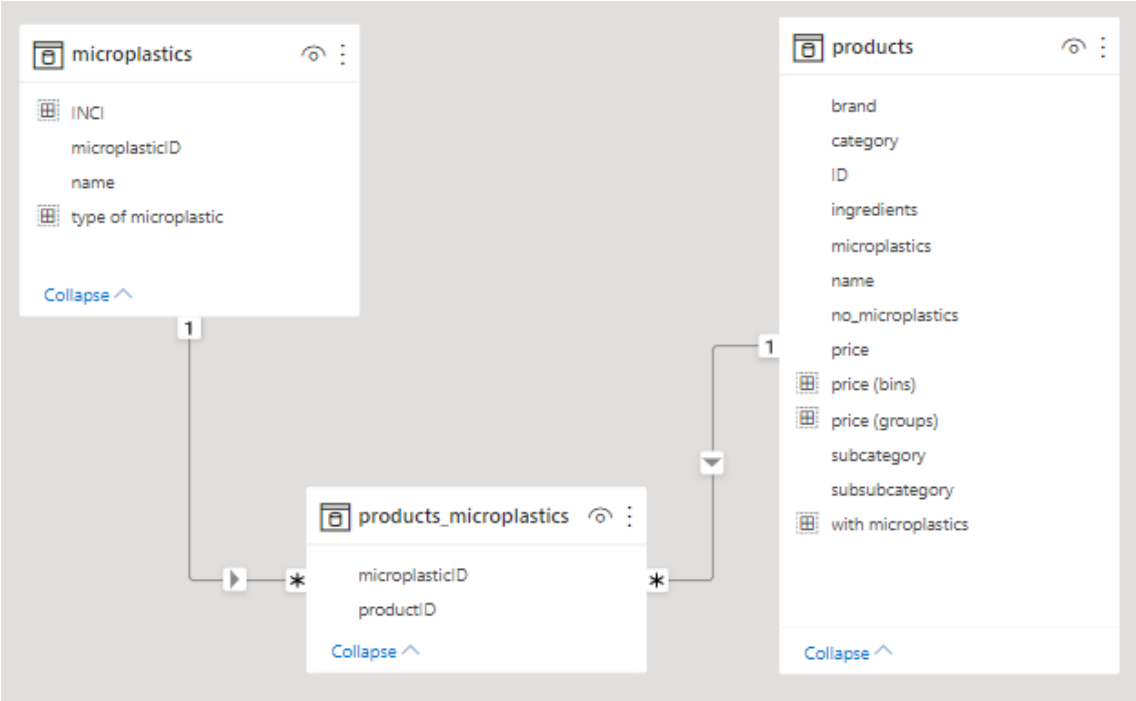

Fig. 4.16: Data model

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

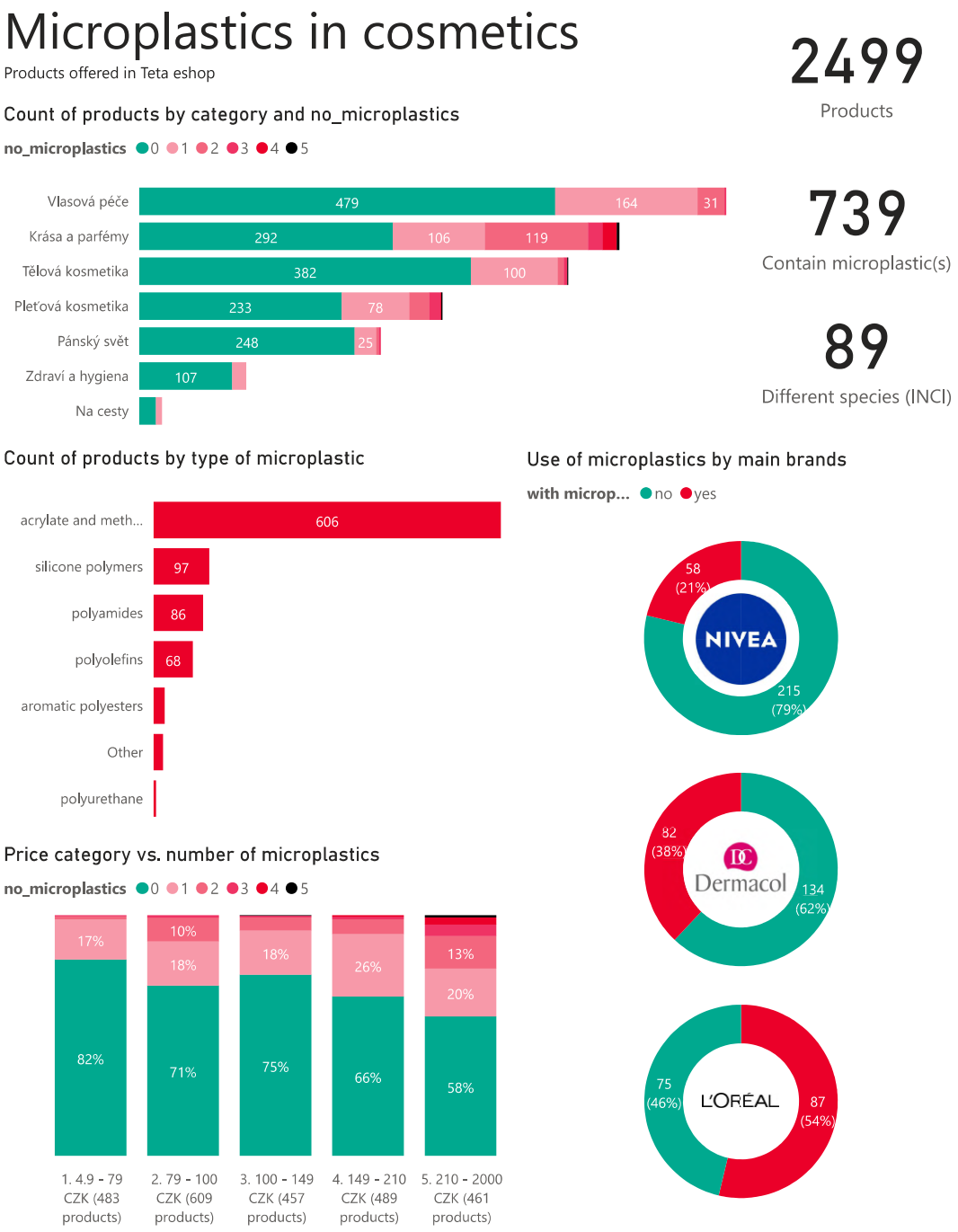

Fig. 4.17: Report on microplastics in cosmetic products

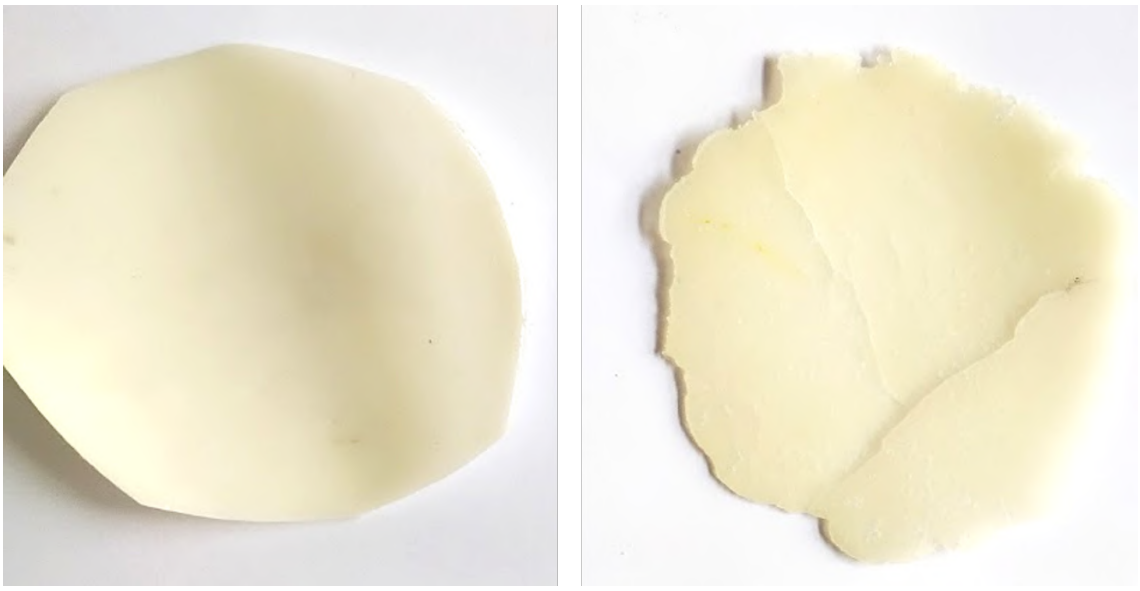

Fig. 4.18: PHB samples after melting at 190 °C/5 min and cooling. Left: commercial PHB, right: PHB obtained by soap-based digestion. The sample spontaneously cracked upon cooling.

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 3.2.1.4, chloroform extraction (3.2.1.5), 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. 4.18).

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

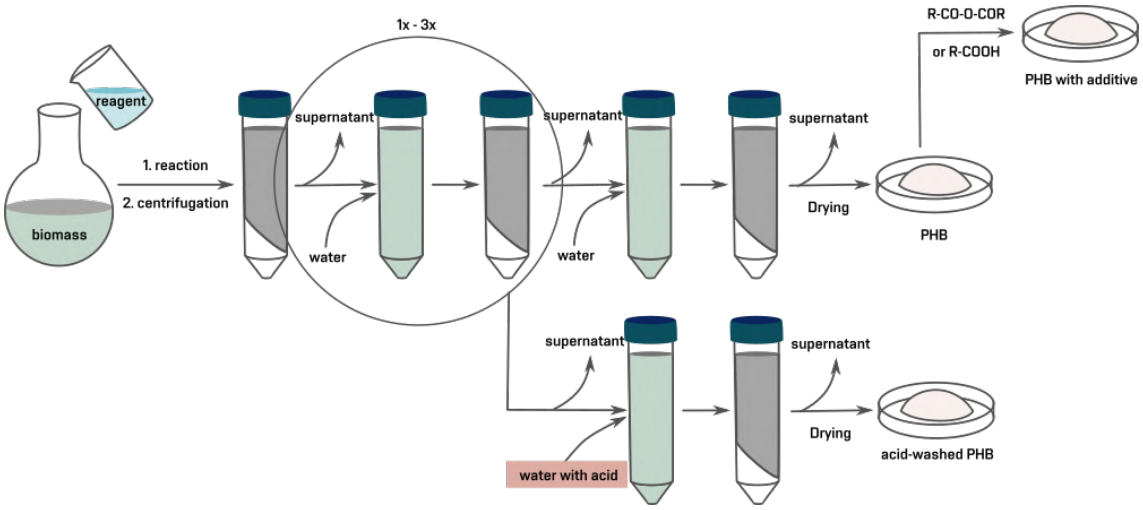

Fig. 4.19: Schematic representation of PHB isolation by a digestion method and incorporation of methods for thermal stabilization.

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) were tested. These steps are simple and do not complicate the potential production process (fig. 4.19). 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, CH3COOH, 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. 4.20). The same phenomenon could be seen in DSC (fig. 4.21). 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. 4.22). 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.

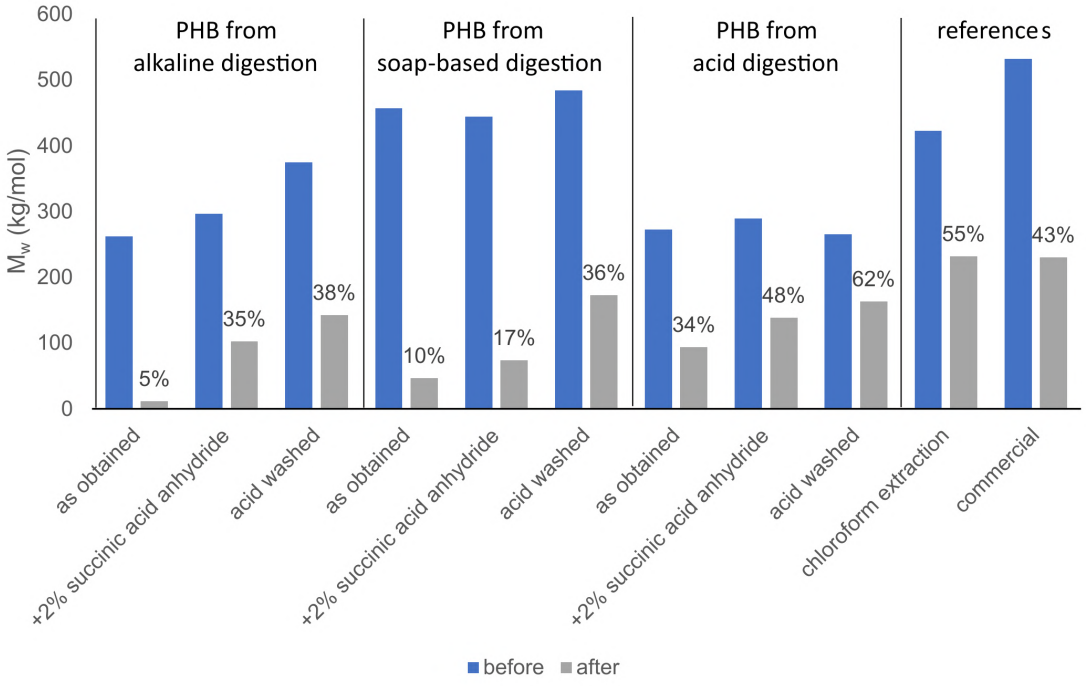

Fig. 4.20: Molecular weight of the polymer before and after thermal treatment Numbers above columns represent percentage of original molecular weight [12]

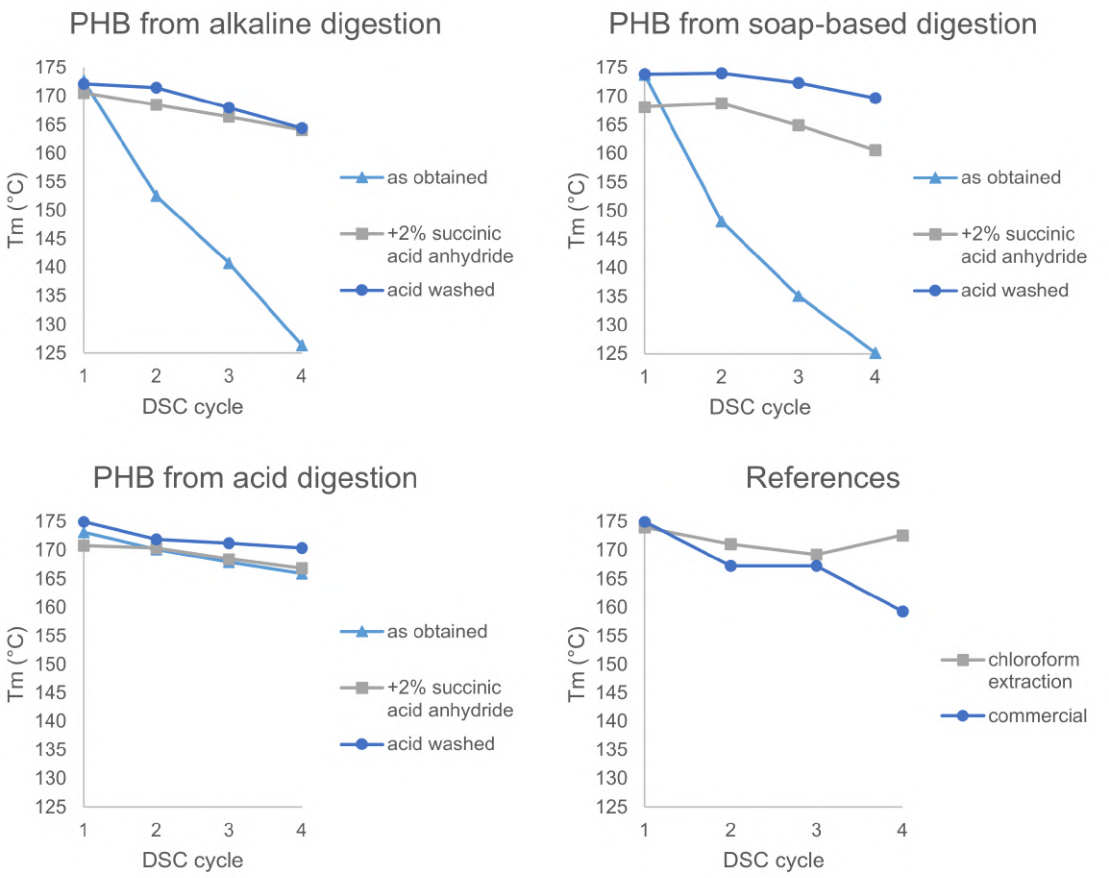

Fig. 4.21: Melting point of PHB after several cycles of differential scanning calorimetry. Each cycle consisted of heating to 190 °C, annealing at the temperature for 5 min and cooling. [12]

Fig. 4.22: 3D printed object made from our 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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