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Isolation of poly(3-hydroxybutyrate) from bacterial biomass using soap made of waste cooking oil

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Abstract

The aim of this work was to develop a soap-based method for the isolation of poly(3-hydroxybutyrate) from bacterial biomass. The method consisted of adding soap derived from waste cooking oil to a concentrated (25%) biomass suspension, heating and centrifugal separation. Purity above 95% could be achieved with soap:cell dry mass ratios at least 0.125 g/g, making the method comparable to other surfactant-based protocols. Molecular weights M_w of products from all experiments were between 350 - 450 kDa, being high enough for future material applications. Addition of hydrochloric acid to the wastewater led to the precipitation of soap and part of non-P3HB cell mass. The resulting precipitate was utilized as a carbon source in biomass production and increased substrate-to-P3HB conversion.

Keywords: PHA, P3HB, recovery, downstream process, isolation

1. Introduction

Poly(3-hydroxybutyrate) (P3HB) is the best known natural polyhydroxyalkanoate (PHA). It is produced by many organisms (Koller et al., 2017), out of which the bacterium *Cupriavidus necator* is the most significant in industrial practice. It can be grown on primary feedstock (cane sugar, vegetable oil...) or, preferably, secondary feedstock (molasses, whey...).(Choi and Lee,

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1997; Koller et al., 2017) P3HB is considered a possible alternative to commonly used plastics, especially for material applications where the biodegradability is required (Sirohi et al., 2020). For a viable industrial production of P3HB, the cost-effective and environmentally friendly isolation method is a key step. Furthermore, the isolation method should lead to high purity of the material and minimal decrease of molecular weight.(Koller, 2020)

Currently, two main strategies are used for the P3HB isolation – extraction and digestion. Extraction of P3HB usually employs halogenated solvents, mainly chloroform and dichloromethane. Resulting polymer is reportedly very pure and has high molecular weight, but the environmental and occupational hazards associated with these solvents are high. (Koller, 2020; López-Abelairas et al., 2015) Several non-halogenated solvents can also be used. (Jacquel et al., 2008) Their main disadvantages are high cost and complicated recycling.

The digestion methods may employ different agents (or their mixtures) able to disrupt the cell membranes and to dissolve or solubilize non-P3HB cell mass (NPCM). Main advantages of the digestion methods are relative simplicity and less hazardous operation. Quality of the product and economy of operation depends on the reagent used - methods with acids, bases, and oxidants are reportedly economical and have low carbon footprint, but considerably decrease the molecular weight of the polymer.(López-Abelairas et al., 2015; Jiang et al., 2015; Kim et al., 2003) In contrast, highly selective methods with surfactants, enzymes, or other organisms cause negligible degradation of the polymer, but are still rather uneconomical.(Jacquel et al., 2008; Gonzalez et al., 2020) In all digestion methods, liquid waste containing NPCM is formed, leading to difficult wastewater treatment.(Jacquel et al., 2008) Methods for upcycling this waste stream are under development. (Yadav et al., 2020)

In this study, the feasibility of P3HB recovery from *C. necator* by non-traditional surfactant was explored. The surfactant, fatty acid sodium salts ("soap") was prepared from waste cooking oil which is a cheap and sustainable starting material.Dependence of P3HB purity on soap:cell dry mass (CDM) ratio was determined and the effectiveness of additional digestion agent protease

was tested. Molecular weight was monitored to ensure that the product would be suitable for material applications in the future. Another goal was to efficiently utilize wastewater resulting from digestion. The advantage of soap over conventional surfactants is that it precipitates by acidification to form free fatty acids,(Samorì et al., 2015; Kaoru, 2019) which were reported to be a suitable substrates for *C. necator*.(Sharma et al., 2016) Experiments with acidification of wastewater and the use of a precipitate were performed.

2. Materials and Methods

2.1. Soap preparation

Waste cooking oil was obtained from Nafigate Corporation (Czech Republic). According to the supplier, it was a mixture of used oils, collected for the sake of recycling, having saponification value 186 mg KOH/g, acid value 5 mg KOH/g and containing mainly oleic, linoleic, palmitic and stearic acid triacylglyceroles, diacylglyceroles, free fatty acids and oxidised forms of the above. Iodine value and peroxide value were analyzed iodometrically according to AOAC methods.(AOAC, a,b)

NaOH (26.5 g, 0.663 mol) was dissolved in water (76 g) and mixed with 200 g of waste cooking oil (0.663 mol of saponifiable groups; calculated from saponification value). The mixture was vigorously stirred at 80 °C. The conversion of NaOH was monitored acidimetrically (1 M HCl, phenolphthalein) in 0.5 h intervals and the reaction was considered completed when the conversion was >98%. Then the mixture was left to cool at room temperature.

2.2. Digestion of biomass using soap

Biomass was produced by Nafigate Corporation via the fermentation of waste cooking oil by *C. necator* H16. Specific procedure for biomass production is described in patent application (Marova et al., 2014). The biomass was shipped and stored frozen at -20 °C. It contained 50% CDM, out of which 75% was P3HB. The P3HB in biomass had M_w = 541 000 g/mol.

Two series of experiments with different doses of soap were carried out. The first series used a protease (Sigma-Aldrich; from *Bacillus licheniformis* $\geq$ 2.4 U/g) as an additional digestion agent, the second did not. The ratio (w/w) of soap to CDM ranged from 0 to 1.

For each experiment, the biomass was diluted with water to contain 25% CDM. The mixture was heated to 95 °C for 0.5 h and cooled to 80°C. Soap was dissolved in the mixture and pH was measured. If pH was <9, it was increased to 9-9.5 by the addition of 20% NaOH. The mixture was stirred for 1 h at 80 °C and then cooled to 55°C. For experiments involving protease, the enzyme (0.01 ml/g CDM) was added in this stage. Then the mixture was stirred at 55 °C for 1 h and pH was measured. If pH was <9, it was increased to 9-9.5 with 20% NaOH solution. 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 the 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 2.3). The pellet was dried at 105 °C to constant weight.

2.3. Precipitation of supernatant

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 the transparent yellow liquid. Dry weight of the precipitate was measured thermogravimetrically. The precipitate was stored at 4°C prior to use (see Section 2.4).

2.4. Use of the precipitate for biomass production

Cultivations of inoculum of bacterial strain *C. necator* H16 (CCM 3726) were performed in 100 ml Erlenmeyer flasks with 50 ml of the Nutrient Broth medium (peptone 10.0 g/l, beef extract 10.0 g/l, NaCl 5.0 g/l) during 24 hours at 30°C with constant shaking at 180 rpm, when culture was inoculated from cryoculture.

Subsequent cultivations focused on comparison of growth potential and PHB producing capability using different substrates 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). As carbon sources, waste cooking oil, precipitate, and their mixture were used. Final concentrations of substrates in the cultivation media were 20 g/l for the sole ones and 10 + 10 g/l for mixture. All cultivations were performed in two parallel 250 ml Erlenmeyer flasks with 100 ml media (inoculation ratio 10 %) under permanent shaking at 180 rpm at 30°C for 72 hours, when the cultivations were stopped, cells were harvested and analysed. All substrates (sole ones or the mixture) were prepared twice to obtain statistically more valuable results. To determine the content of biomass gravimetrically, 10 ml of cultures in duplicates from each flask were centrifuged (6 000 rpm; 5 min), washed with distilled water and centrifuged once more. Then the pellets were dried to constant weight and the concentration of biomass was expressed in g/l as CDM. The data for every type of substrate were obtained from two parallel cultivations as mentioned previously, when two values for each Erlenmeyer flask were determined, i.e. averages were taken from four individual values.

2.5. Analysis

Purity of P3HB was analysed as methyl esters of 3-hydroxybutyric acid by gas chromatography as reported previously.(Obruca et al., 2014) Molecular weight was measured by GPC using 185 Agilent HPLC series 1100 chromatograph with PLgel mixed-c 5 µm, 7.5x300 mm column, with chloroform as the mobile phase. 12 polystyrene standards (0.2 - 2000 kDa) were used for calibration.

3. Results and Discussion

3.1. Soap preparation

The main feedstock for the preparation of soap was waste cooking oil. High degree of deterioration was evident mainly sensorially (dark color, odor) and by a higher acid value (5 mg KOH/g). The oil was rich in unsaturated fatty acids (iodine value 96 g/100 g), and partially oxidized, as indicated by the peroxide value 20 meq/kg.

The soap was prepared by ordinary saponification of the oil with one equivalent of NaOH at 80 °C. Time needed for the reaction was 2.5 h, after which the soap was obtained as a pale yellow waxy solid. Neither the purification of the oil nor the purification of generated soap was necessary. Despite being contaminated with frying and saponification byproducts, the soap performed comparably to pure commercially available surfactant (see Section 3.2).

3.2. Digestion of biomass using soap

To quantify the influence of soap concentration and protease addition on the purity of P3HB, experiments with different doses of soap were performed. The experiments were carried out at higher temperatures than usual for surfactant-based methods. At low temperatures, the mixtures tended to be very viscous, which disabled efficient stirring and centrifugal separation. pH was maintained alkaline because soap is fully efficient only at higher pH. However, the susceptibility of P3HB to alkaline hydrolysis had to be considered.(Burniol-Figols et al., 2020) pH between 9 and 9.5 led to optimal performance and minimal molecular weight loss.

As expected, the purity of P3HB increased with increasing amount of soap and with protease as can be seen in Fig. 1. Plotted values follow a similar trend as in SDS-based method, published previously by Kim et al. (2003) who achieved >95% purity if the SDS:CDM ratio was at least 0.3. Soap was seemingly more effective, leading to >95% with soap:CDM at least 0.125. The result was surprising because soap is generally considered inferior in terms of solubilization

and denaturation power.(Holmberg, 2019) Probable explanation is that alkaline pH and high temperature in the soap-based method enhanced the digestion.

In addition to purity, other important parameters were monitored - molecular weight, recovery, and color of the product. Molecular weight M_w of all products was between 350 000 - 450 000 Da (65-83% of the original value in biomass) and the recovery was 80 - 100 %. No relationship between the dose of soap or protease addition was found. The small differences in M_w could be explained by little differences in pH and by the experimental error of the GPC method. Differences in recovery were probably caused by the experimental error - with a viscous supernatant and a rather soft pellet, decantation occasionally led to product loss. Dose of soap and especially the presence of the enzyme affected color - with increasing amount of soap and with the protease, the product became significantly paler.

Based on the experiments, soap:CDM ratio between 0.125 - 0.5, preferably 0.2, combined with the protease was evaluated as the most promising experimental setup. The product obtained at these conditions had high enough purity and beige color, making it together with high molecular weight suitable for material applications.

3.3. Precipitation of supernatant

Supernatants were acidified to convert soap to free fatty acids. Principally similar reaction was previously described by Samorì et al. (2015) whose digestion method was based on high doses of a "switchable anionic surfactant" ammonium laurate (surfactant:CDM up to 2). In Samori's method, acidification of the supernatant produced lauric acid in 98% yield that could be used for the preparation of the surfactant again. Supernatants from soap-based digestion reacted differently. The titration curve had a practically flat shape (Fig. 2), indicating the acid-base reaction of more compounds with different pK_A values. Dry weights of the precipitates were significantly higher than the theoretical yields of fatty acids, indicating that a considerable portion of NPCM precipitated together with the fatty acids. Relative content of NPCM in precipitate

was dependent on soap:CDM ratio - the more soap used for digestion, the lower content of NPCM in the precipitate. This may explain the sufficient purity of Samori's fatty acid – with higher amount of the surfactant, the amount of NPCM in the precipitate could be negligible. Precipitates from soap-based digestion with lower doses of soap (soap:CDM 0.125 - 0.5) contained 48 - 26% of NPCM as impurity. Due to the complex and diverse character of NPCM, attempts to remove it were not performed. Instead, the precipitate was used as a substrate for fermentation (Section 3.4.

3.4. Use of precipitate for biomass production

Laboratory-scale fermentation tests showed that the precipitate or its mixture with a standard substrate (waste cooking oil) could be readily used as a substrate for bacteria to produce bacterial biomass. For reference, the standard substrate was fermented in the same manner. Results are in Table 1.

As the table shows, there was a synergy between precipitate and waste cooking oil – more substrate was converted to P3HB if a mixture of the above was used as a carbon source. The reason could be the emulsifying properties of the precipitate which made the oil more bioavailable. Pure precipitate did not perform so well as the mixture or oil alone. High amount of hydrolyzed protein in the precipitate could be the reason. *C. necator* accumulates P3HB faster in N-deficient environment (Ahn et al., 2015) and thus nitrogen-rich precipitate may slow down the process.

Based on these results, the process shown in Fig. 3 was designed, the material flows were calculated (counting with soap:CDM ratio 0.2) and compared with the SDS-based method published previously (Chen, 2009).

4. Conclusions

Soap-based isolation method produced a high-molecular-weight P3HB, potentially suitable for material applications. Soap:CDM ratio at least 0.125 was needed to reach >95% purity of the product and the relationship between the

soap dose and purity showed a similar trend as in the method with the standard surfactant (SDS). Effect of protease on analytical purity was less pronounced but positively affected the product's color. After digestion, the soap was precipitated from the wastewater and the precipitate was advantageously utilized in biomass production. Future research will focus on upscale, optimization of the separation step and material research of the P3HB thus obtained.

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6. Figure Captions

Fig. 1. Purity of P3HB from soap-based method. Error bars represent 95% confidence intervals.

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

Fig. 3. Comparison of SDS-based biomass digestion described previously(5) and soap-based biomass digestion (this work). Line widths are proportional to weights.

Table 1. Results of laboratory-scale cultivation. WCO stands for waste cooking oil, CDM stands for cell dry mass, S.D. stands for standard deviation.

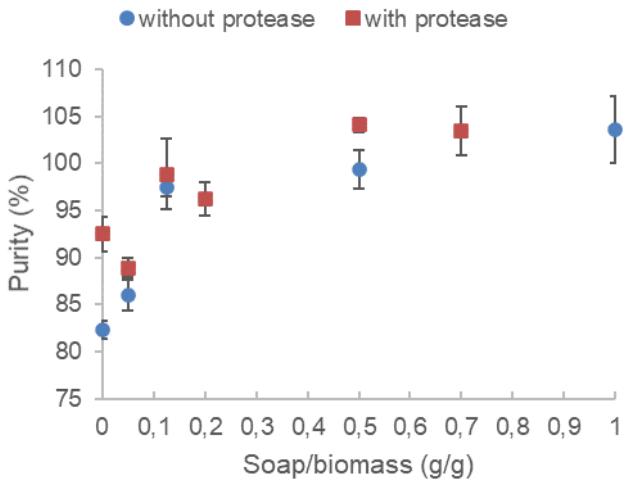
without protease
with protease
110
105
100
95
90
85
80
75
Purity (%)
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1
Soap/biomass (g/g)

Figure 1:

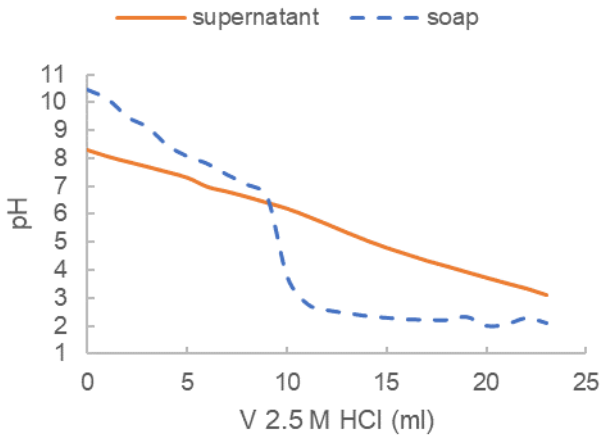

Figure 2:

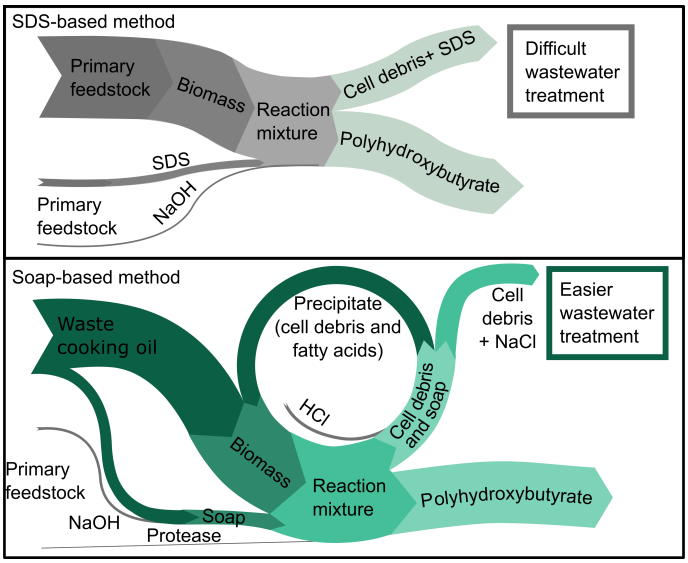
SDS-based method
Primary feedstock
Biomass
Reaction mixture
Cell debris+ SDS
Difficult wastewater treatment
Polyhydroxybutyrate
SDS
Primary feedstock
NaOH
Soap-based method
Waste cooking oil
Precipitate (cell debris and fatty acids)
Cell debris + NaCl
Easier wastewater treatment
HCl
Cell debris and soap
Biomass
Primary feedstock
NaOH
Soap
Protease
Reaction mixture
Polyhydroxybutyrate

Figure 3:

Substrate	Substrate dry weight (g/l)	CDM ±S.D. (g/l)	P3HB ±S.D. (%)	P3HB ±S.D. (g/l)	**Substrate conversion (%)**
20 g/l WCO (control)	20	10.6±0.3	80.9±2.7	8.6±0.2	**43**
10 g/l WCO + 10 g/l precipitate	14.5	12.5±0.2	70.6±2.3	8.8±0.2	**61**
20 g/l precipitate	9	7.5±0.2	42.8±1.6	3.2±0.1	**36**

Table 1: