Production of PHB from Chicory Roots – Comparison of Three *Cupriavidus necator* Strains

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doi: 10.15255/CABEQ.2014.2250

Original scientific paper
Received: August 18, 2014
Accepted: June 3, 2015

Chicory roots from hydroponic salad cultivation are an abundant food residue in Navarra (Spain) that are underutilized to date. Aiming at a holistic utilization of resources, we report here the first process using chicory root hydrolysate for the production of poly([R]-3-hydroxybutyrate) (PHB). The polymer can be used for packaging material made for the locally produced vegetables.

In the first step, we developed a pre-treatment process to obtain a hydrolysate, which contained 34 g L^{-1} sugars and 0.7 g L^{-1} total Kjeldahl nitrogen. This hydrolysate was used as fermentation substrate for three PHB-producing strains. *Cupriavidus necator* DSM 428 reached a dry biomass concentration of 11.3 g L^{-1} with a PHB content of 66 % in dry mass within 5 days. *C. necator* DSM 531 yielded 3.5 g L^{-1} dry biomass containing 46 % PHB within the same period. *C. necator* DSM 545 was superior over the other two in that 14.0 g L^{-1} of biomass containing 78 % PHB after only 3 days were obtained. These results show that even within the same species, the productivities on natural substrates are very different. The produced polymers were extracted using chloroform, and several thermo-physical parameters are in good accordance with published data. Overall, our holistic approach and the encouraging results prove that chicory roots are a viable fermentation substrate for PHB-production.

Key words

Cichorium intybus, fructose, inulin, by-product, food residue, pre-treatment, hydrolysate, batch fermentation, *Cupriavidus necator*, P3HB, polymer characterisation

Introduction

Renewable resources constitute a 'limited infinity', meaning that a limited amount is being produced, but for an infinite time.[1] One of the most promising approaches to reduce the resource intensity is to use the existing biomass more efficiently: the biorefinery concept. It aims to use the complete biomass and all by-products emitted by the processes. Particularly the by-products of established processes have been disregarded in the past and are receiving more attention now.[2]

Chicory (*Cichorium intybus*) is a very versatile plant as its varieties are either grown for salad, inulin or coffee-substitute production.[3] The leafy salad (also called Belgian endive, chicon or witloof) is grown predominantly in Belgium, the Netherlands, France and Spain.[4] In Spain, the major growing region is Navarra, where 13,000 tons of chicory are produced by hydroponic cultivation per year.[5] The production is not seasonal as the salad is consumed throughout the year. Alongside the chicory production, 10000 tons of roots accumulate, which are presently used as animal feed or are composted. However, the nutritional value of the roots after hydroponic cultivation is limited because inulin, a fructose polymer that is the main carbon- and energy-storage of the plant, is not metabolised during the intrinsic mammalian digestion process. It is only metabolised by the colon microflora to gases and organic acids.[6,7]

Hydroponic cultivation, growing plants using aqueous mineral nutrient solutions instead of soil, is carried out in complete darkness. In order to provide the energy required for the salad (chicon) formation, the polymeric inulin, stored in the roots, is depolymerised into mono- and oligosaccharides. This renders the classical inulin extraction and utilization pathways not economically feasible.[8] An integrated process in which the inulin is extracted/hydrolysed and the hydrolysate is directly used for fermentations is economically more viable.[9] However, there have been no investigations into such a process until now.

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Polyhydroxyalkanoates (PHAs) are a family of fully biobased, biodegradable, and biocompatible polyesters. Currently, PHAs are a niche material in the bioplastics sector, but they exhibit one of the fastest growing markets.[10] The most common PHA is poly([R]-3-hydroxybutyrate) (PHB) and its copolyester with ([R]-3-hydroxyvalerate) (PHBHV), which is well suited for food packaging.[11] It is accumulated in intracellular granules by a wide range of bacteria. As PHAs function as carbon and energy storage, most of the time they are produced in the presence of excess carbon source and under limitation of another nutrient, such as nitrogen or phosphorous. An intracellular PHB-load of 60–90 % is typically attained by optimized processes. In order to increase productivity, fermentations are done in fed-batch, or in a continuous mode.[12,13,14] On an industrial scale, production costs are still not competitive compared to petrochemical plastics, mainly due to the high cost of raw materials and downstream processing.[15]

Typical carbon sources for the PHA fermentations are sugars and fatty acids, most of the time in mineral salt media (minimal media).[13,16,17] Aside from refined fructose or fructose-containing sugars in minimal media, complex fructose-containing carbon sources have also been used for fermentations and are discussed below (Table 1). The reported results are typically not as good as is the case with minimal media, but medium costs are significantly lower.

The best results with complex fructose-containing carbon sources were obtained by supplementation of minimal media (Table 1). This was done by Kulpreecha *et al.* with a newly isolated *Bacillus megaterium* strain grown on diluted sugarcane molasses in a medium containing urea as nitrogen source.[18] In batch mode, they reached 8.8 g L^{-1} dry biomass containing 62 % PHB in the dry biomass. The optimized fermentation was done in a fed-batch mode, feeding molasses, urea and minerals into the bioreactor. Beaulieu *et al.* added varying amounts of sugarcane molasses to a minimal medium containing glucose in order to cultivate *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*) DSM 545.[19] Various nitrogen sources were studied, but ammonium sulphate proved to be the best. Depending on the amount of molasses supplementation (0–5 g L^{-1}), cell densities of 17–23 g L^{-1} with a PHB content of 31–44 %

Table 1 – *Fructose or fructose-containing carbohydrates used for PHA fermentations using (a) refined sugars in minimal media, (b) complex carbohydrates in minimal media, (c) complex carbohydrates with addition of only certain elements/minerals, and (d) complex carbohydrates without supplements. *Calculated. n.a.: not available. Ref.: Reference number*

	Organism	Fermentation type	PHA type	Carbon Source. (Sugars obtained by hydrolysis are in brackets)	Biomass concentration (g L^{-1})	PHA (% in dry mass)	Yield (g PHB g^{-1} substrate)	Volumetric productivity (g L^{-1} h^{-1})	Ref.
(c)	*Azohydromonas australica* DSM 1124	Fed-batch	PHB	Soya milk waste acid hydrolysate (Fructose, Lactose, Maltose) with a Sucrose feed	18	33	n.a.	0.11	22
(b)	*Bacillus megaterium* BA-019 (new isolate)	Batch	PHB	Cane molasses (Saccharose)	8.8	62	0.37	0.45	18
(b)	*Bacillus megaterium* BA-019 (new isolate)	Fed-batch	PHB	Cane molasses (Saccharose)	72	42	n.a.	1.27	18
(a)	*Cupriavidus necator H16* DSM 428	Batch	PHB	Fructose	8	45*	0.21*	0.12*	35
(a)	*Cupriavidus necator H16* DSM 428	Batch	PHBHV-block-HB	Fructose and valerate	0.4	66	n.a.	0.03 (PHBHV), 0.033 (PHB)	36
(a)	*Cupriavidus necator* DSM 531	Batch	PHB	Fructose	21	45	0.31	0.15	29
(b)	*Cupriavidus necator* DSM 545	Shake flasks	PHB	Glucose and cane molasses (Saccharose)	17–23	31–44	n.a.	0.08–0.12	19
(b)	*Cupriavidus necator* DSM 545	Shake flasks	PHB	Cane molasses acid hydrolysate (Fructose and glucose)	9.5	14*	0.06	0.02*	20
(a)	*Cupriavidus necator* DSM 545	Fed-batch	PHB	Sucrose hydrolysate	125–150	65–70	0.32	1.44	37
(d)	*Cupriavidus necator* strain not specified	n.a.	PHB	Jerusalem artichoke hydrolysate (Fructose)	6–7	70	n.a.	n.a.	23
(c)	*Pseudomonas fluorescens* A2a5 (new isolate)	Batch	PHB	Sugarcane liquor (Saccharose)	32	70	n.a.	0.23	21

were reached. The same organism was also grown by Sharifzadeh *et al.* on sugarcane molasses as the only carbon source, again in a minimal medium.[20] Molasses was pre-treated with sulphuric acid at high temperatures to hydrolyse sucrose into glucose and fructose.

Jiang *et al.* supplemented sugarcane liquor only with monosodium glutamate as nitrogen source in a phosphate buffer. From the sugarcane liquor, PHB was produced using a new isolate of *Pseudomonas fluorescens*.[21] Yu *et al.* subjected soya waste from a soya milk dairy to an acidic hydrolysis, producing a fermentation substrate containing fructose, lactose and maltose.[22] Upon supplementation with K_2HPO_4, Fe(III)citrate and $MgSO_4$, *Azohydromonas australica* DSM 1124 (formerly known as *Alcaligenes latus*) accumulated PHB. After the total organic carbon dropped below 15 g L^{-1} a sucrose-feed was added, but biomass and product concentrations did not increase again.

Emelina *et al.* are to our knowledge, the only researchers having published experimental data on the production of PHB from inulin in a conference abstract.[23] They extracted and hydrolysed inulin from Jerusalem artichoke (topinambour) into its monomers with diluted acid and obtained a hydrolysate with 27 g L^{-1} of fructose. This hydrolysate was used without supplementations as a fermentation broth and was converted by *Cupriavidus necator* (strain not specified) into 6–7 g L^{-1} of biomass containing up to 70 % of PHB.

The novelty of the study described in this paper is that we cover a more holistic approach. To our knowledge, we are the first researchers having investigated the waste valorization of chicory roots after hydroponic cultivation to produce PHB for packaging material. The produced packaging material is envisaged to be used for the locally produced vegetables, such as chicory or asparagus, which are usually sold pre-packaged to the consumer. We optimized an extraction and hydrolysis protocol for inulin from chicory roots. The obtained hydrolysate was used without supplementation of nutrients for PHB production. Three production strains, *Cupriavidus necator* DSM 428, 531 and 545, were compared. To our knowledge, this study is also the first to compare these three strains in a complex medium. As pre-treatments frequently extract/create compounds toxic to microorganisms, the level of hydroxymethylfurfural and lignin degradation products (phenols) were quantified. Two detoxification methods were tested on the hydrolysate in order to reduce the level of lignin degradation products. Furthermore, the produced PHB was extracted with chloroform and several physico-chemical parameters characterised.

Materials and methods

Pre-treatment of chicory roots

Pre-treatment protocol development

All chemicals used in the experiments were of analytical grade and commercially available from the major suppliers unless stated otherwise. Chicory roots obtained from Tratamiento Subproductos Agroalimentarios S.L. (San Adriàn, Spain) were cut with an ILC.2 cutter (FAM, Kotnich, Belgium), dried with a rotating drum dryer (Vandenboek, Waddinxveen, Netherlands), and milled with a DFZC hammer mill (Bühler, Uzwil, Switzerland). The structural carbohydrates were determined according to the NREL/TP-510–42618 method.[24]

When the roots are grown industrially for inulin production, inulin is extracted with water at 70–80 °C for 1.5–2 h.[25] Accordingly, the following conditions were tested: solid fraction (1:3, 1:5, 1:10 w/v), pH (4, 5, 7, adjusted with H_2SO_4 or NaOH), temperature (50, 60, 70, 80, 95 °C), autoclaving (yes/no), Viscozyme® and inulinase addition (0, 0.2 and 5 multiples of the concentration recommended by the supplier). The pre-treatment experiments were done in 5 mL aqueous suspension in sealed falcon tubes. All reactions were done in duplicate in a temperature-controlled water bath. The pH was adjusted with 1 mol L^{-1} NaOH or HCl. Viscozyme® L, containing carbohydrases such as arabanase, cellulase, beta-glucanase, hemicellulase, xylanase, and pectinase, was obtained from Novozymes (Bagsværd, Denmark). No enzyme activities were specified, but a working concentration of 0.2 to 1 g enzyme solution per kg of raw material was stated in the product sheet. For ease of representation in the manuscript, a normative concentration of 1 g enzyme per kg of raw material was defined for the experiments. Inulin was hydrolysed by the liquid Fructanase Mixture from Megazyme (Bray, Ireland), containing 2000 U mL^{-1} exo-inulinase and ~ 200 U mL^{-1} endo-inulinase. A normative inulinase concentration was calculated which is required to break down the amount of inulin in the hydrolysate within 1 h. Both enzymes were only tested at 50 °C and pH 5, which are in the supplier-specified optimal range of both enzyme cocktails.

Samples (0.5 g) were drawn in duplicate at specified intervals for up to 24 h. The liquid phase was separated by centrifugation (2000x g for 10 min) and the following two parameters quantified therein. The extraction of inulin was monitored by quantifying the sum of the oligomeric and monomeric carbohydrates and the inulin hydrolysis was monitored by quantifying the monomeric sugars. The concentrations are given in g L^{-1} in the hydrolysate.

The differences in the duplicates were quantified as standard deviations. In the final hydrolysate, the mineral composition and total Kjeldahl nitrogen, as well as free amino nitrogen were determined in the liquid supernatant as described in the analytical methods section.

Preparation of the fermentation broth

Preparative pre-treatments according to the developed protocol were done first at 3 L and then at 60 L scale in temperature-controlled vessels. The dried milled roots were suspended in water (1:10 w/v) at a temperature of 50 °C. The pH was approximately 5. Inulinases were added amounting to 3600 U exo-inulinase kg^{-1} dry roots and 360 U endo-inulinase kg^{-1} dry roots. After approximately 6 h of reaction time, the solids were removed by a 30 L capacity Impos X4 press (Lagerhaus, Wien, Austria), followed by centrifugation of the suspension at 4000x g for 30 min, and vacuum filtration through paper filters (grade 595, Schleicher & Schuell, Dassel, Germany). The hydrolysate was autoclaved for 20 minutes at 121 °C and the pH adjusted to 7.0 with sterile 1 mol L^{-1} NaOH. Samples were drawn in duplicate at regular intervals and the differences between the duplicates were quantified as standard deviations.

Detoxification of the fermentation broth

Activated charcoal is one of the most widely applied detoxification methods and is very useful for adsorbing acidic compounds i.e. phenols (lignin degradation products) or carboxylic acids in an acidic medium.[26] The activated charcoal (2 %) was stirred in the hydrolysate for 1 h at room temperature. Afterwards, the hydrolysate was centrifuged, filtered and autoclaved as described in the section on the preparation of the fermentation broth.

Oxidases, such as laccases, oxidize phenols to radicals that undergo coupling to larger molecules that are less toxic.[27] Laccase from *Trametes hirsuta* was added to the chicory hydrolysate (2.9 U mL^{-1}) in an aerated bioreactor (0.38 vvm). The reaction was followed by monitoring consumption of dissolved oxygen, during 1 h incubation time. Afterwards, the hydrolysate was autoclaved as described in the section on the preparation of the fermentation broth. This enzyme was produced as reported by Almansa *et al*. [28] The activity was determined by adding 50 μL of 10 mmol L^{-1} ABTS (2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonic acid)) to 170 μL of diluted enzyme solution in a succinic acid buffer pH 4.5. The reaction was followed by immediately determining the slope of the absorbance increase at 420 nm in a Tecan Reader infinite M200Pro (Männedorf, Switzerland) and calculating the activity from the slope (273 U mL^{-1}, 8.06 mg mL^{-1} protein).

The inhibitor concentration was determined by downscaling the total phenol estimation procedure published by Areskogh *et al.*[29] For the determination, 20 μL standard or diluted hydrolysate, 60 μL Folin Ciocalteu-reagent and 600 μL bidest water were mixed and incubated for 5–8 minutes at room temperature. Afterwards, 120 μL sodium carbonate 20 % w/v and 200 μL bidest water were added, and the samples incubated for 2 h at 800 rpm. The absorbance was measured at 760 nm with the Tecan Reader as specified above. Vanillin was used as standard for generating a calibration curve. Triplicates were performed for all preparations.

Fermentation procedures

Microorganisms

Cupriavidus necator DSM 428 and 531 were obtained from DSMZ (Braunschweig, Germany). The long-term storage was in glycerol stocks at –80 °C in nutrient broth. *C. necator* DSM 545 was kindly provided by Martin Koller (Univ. Graz, Austria). The long-term storage was in glycerol stocks at –80 °C in minimal medium owing to low survival in nutrient broth after freezing. The genus *Cupriavidus* was chosen because it proved to be an excellent PHB-producer in a number of relevant studies and several strains are available. *Cupriavidus necator* DSM 428, commonly known as strain H16, is probably the best-studied strain for PHB production.[30] Strain 545 is a glucose-utilizing mutant of strain DSM 529, which also gave very good results, and strain 531 has recently received more attention.[31]

Shake-flask cultivation

Shake flask experiments were conducted to check whether the microorganisms were able to utilize inulin as carbon source in a reasonable amount of time. For the shake-flask experiments, cultures from nutrient-broth plates were grown in 50 mL of nutrient broth in 300 mL baffled conical flasks rotating at 150 rpm and 30 °C overnight. After harvesting the cells at 2000x g for 15 minutes, they were resuspended in 10 mL of sterile hydrolysate and shaken again at 150 rpm and 30 °C. One sample (1 mL) was taken every day and the following parameters determined: the pH and the concentrations of the dry biomass, fructose, glucose, and PHB. If necessary, the pH was adjusted with sterile 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl to 6.8. All strains switched from cell growth to PHB production upon

limitation of an essential nutrient, which was endogenous nitrogen in the hydrolysate.

Bioreactor cultivation

The strain comparison as well as the fermentations on the detoxified substrate were conducted in bioreactors. The inoculation procedure from the shake-flask culture was changed from using nutrient broth to minimal medium for the bioreactors because it was found that this adapted strategy leads to a denser and stronger inoculum.

The minimal medium for the preculture was prepared according to Koller *et al.*[32] and contained 4.8 g L^{-1} Na_2HPO_4, 2.0 g L^{-1} KH_2PO_4, 3.0 g L^{-1} $(NH_4)_2SO_4$, 0.8 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 1.0 g L^{-1} NaCl, 0.02 g L^{-1} $CaCl_2 \cdot 2\ H_2O$, 0.05 g L^{-1} NH_4Fe(III)citrate, 10 g L^{-1} fructose, and 5 mL L^{-1} SL6 trace element solution (prepared according to DSMZ Medium No. 27). The iron citrate and the sugar solution were prepared as 10x stock and each autoclaved separately. For solid media, 14 g L^{-1} agar was added.

Prior to cultivation, the strains were conditioned on minimal medium plates for at least 2 weeks at 30 °C, sub-culturing the strains every 2–3 days on a new plate. Minimal medium (250 mL) in 1 L baffled conical flasks was inoculated with cells from the plates and shaken at 150 rpm in an incubation shaker at 30 °C for 48 h. Towards the end of this period, the cells are in the late exponential phase and exhibit a relatively narrow biomass distribution (1.7 to 2.1 g L^{-1}). The preculture was harvested at 2000x g for 20 minutes. The cell pellet was used to inoculate 500 mL sterile hydrolysate (pH 7.0) in a DASGIP bioreactor (Jülich, Germany). The bioreactor control system kept the pH at 7.0 (using 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl), the dissolved oxygen above 20 % (adjusting the stirrer speed), and the temperature at 30 °C. Sterile pressured air was supplied at 0.38 vvm (volumes of air per volume of liquid per minute). If foam was generated during the fermentations, it was led into a foam trap containing 2 mol L^{-1} NaOH. The system monitored the dissolved oxygen, stirrer speed, consumption of acid/base, and temperature in real time. The strains switched from cell growth to PHB production upon limitation of endogenous nitrogen in the hydrolysate.

Samples (5 mL) were taken at regular intervals. The cells were separated from the supernatant (2500x g for 7 min) and the latter taken for determination of the fructose and glucose concentration, as well as the total Kjeldahl nitrogen. From the cell pellet, the dry biomass (g L^{-1}) and PHB (% in dry mass) were determined. The yield was calculated as g PHB g^{-1} consumed sugar. The productivity given in g PHB L^{-1} h^{-1} was calculated as the quantity of produced PHB between the inoculation time (time = 0) and the time at which the highest PHB-concentration was reached. All fermentations were done in duplicate and the individual duplicates lead to the same conclusions.

Extraction and characterisation

Extraction

After the fermentation, the biomass was centrifuged (3500x g, 60 min), frozen, and lyophilized (Beta 1–16 Lyophilisator, Christ, Osterode am Harz, Germany). The dried biomass was stirred in a 10-fold mass of 96 % ethanol overnight, in order to remove fatty acids. Next, the biomass was vacuum filtered and air dried. PHB was extracted in a sealed flask into a 20-fold mass of chloroform for 24 h at 4 °C. The cell debris was removed by vacuum filtration and the filtrate poured into 4x surplus of cold 96 % ethanol. The precipitated polymer suspension was vacuum filtered and dried to remove solvent residues. From the extracted polymer, the PHB content, molecular weight, polydispersity, as well as the melting and glass transition temperatures were determined.

Molecular weight and polydispersity

The number- and weight-average molecular weights were determined with a gel permeation chromatography (PL-GPC50, Agilent Technologies, Santa Clara, USA), equipped with an IR detector. The system contained a styrene-divinylbenzene column (RESIPORE PL1113–6300, Agilent Technologies, Santa Clara, USA) heated to 25 °C, and used chloroform as mobile phase (flow rate 1 mL min^{-1}). The standard EasiCal ps-2 (Agilent Technologies, Santa Clara, USA) was used. Polydispersity was calculated as the ratio between the weight- and number-average molecular weights.

Differential scanning calorimetry (DSC)

Samples (3–4 mg) were weighed into aluminium pans. They were sealed, and then heated in a DSC calorimeter (TA Instruments, New Castle, Delaware, USA) at a constant rate of 10 °C min^{-1} from 20 °C to 220 °C, cooled down to –50 °C, and heated up again to 220 °C. An empty pan was used as a reference. Dry nitrogen was used as a purge gas at 50 mL h^{-1}.

Analytical methods

Carbohydrates

Monomeric sugars were determined after a Carrez precipitation on a HPLC system with an

ion exchange column ION 300 (Transgenomic, Omaha, USA) at 45 °C, and a refractive index detector (Agilent 1100, Santa Clara, USA). The mobile phase was 0.005 mol L^{-1} sulphuric acid at a flow rate of 0.325 mL min^{-1} For the determination of the oligomeric carbohydrates, an overnight treatment in 4 % sulphuric acid prior to the HPLC analysis was used to hydrolyse the glycosidic bonds.

Biomass and PHB

PHB was determined by a modified method of Karr and coworkers.[33] The cell pellet was dried at 105 °C for at least 24 h and subsequently weighed to determine the dry biomass concentration. The pellet was then submerged in 1 mL conc. sulphuric acid and heated to 90 °C for 30 minutes in air-tight Pyrex glass tubes. During this step, PHB is hydrolysed and the monomers are oxidised to crotonic acid. After cooling down, the samples were diluted and subjected to a Carrez precipitation using zinc sulphate and potassium hexacyanoferrate(II) at a molar ratio of 4:1. Crotonic acid was quantified by an Agilent series 1100 HPLC system equipped with a CARBOSep CORGEL 87H column (Transgenomic, Omaha USA) at 65 °C and the corresponding RI detector (Agilent Technologies, Santa Clara, USA). The mobile phase was 0.005 mol L^{-1} sulphuric acid at a flow rate of 0.9 mL min^{-1}. The biomass and PHB concentration was calculated as g L^{-1} of fermentation broth, the PHB content as % w/w in dry biomass.

Total Kjeldahl nitrogen (TKN) and free ammonium nitrogen

The total Kjeldahl nitrogen was determined by degrading the organic nitrogen of 2 mL fermentation supernatant suspended in 20 mL sulphuric acid, and 1 Kjeltab (Thomson & Capper, Cheshire, UK) at elevated temperatures in a Digest Automat K-438 (Buechi, Flawil, Switzerland). Next, the nitrogen concentration was determined by titration with an AutoKjeldahl Unit K-370 (Buechi, Flawil, Switzerland). In order to determine the free ammonium nitrogen, 10 mL of fermentation supernatant were directly analysed with the AutoKjeldahl Unit.

Mineral composition

The hydrolysate was pre-treated with nitric acid (0.65 % w/v) and any solids spun down (3500x g for 15 min). An OES-ICP spectrometer (HORIBA Jobin Yvon, Bensheim, Germany) was used. The quantification of the analysed elements was done using Plasma Emission Standards.

Results and discussion

Pre-treatment of chicory roots

Chicory roots contain a total of 4.6 % w/w hydrolysable sugars in fresh weight (37 % w/w in dry weight). The major constituent is fructose (3.2 %), followed by glucose (0.74 %), coming from chemical hydrolysis of the storage polymer inulin. Van den Ende *et al.* determined the levels of free fructose, glucose and sucrose, as well as 1-kestose and 1,1-nystose in chicory roots after hydroponic cultivation.[8] Depending on the harvest date, the quantified sugars amounted to 1.5–3.2 % in fresh weight. They also detected inulin molecules with higher degrees of polymerisation, but did not quantify them, thereby reaching a lower total concentration of sugars compared to our results. Before the hydroponic cultivation of chicory, the total sugar concentration in the roots was reported to be much higher (15.3–20.1 % of fresh weight).[34]

For the development of the pre-treatment protocol, several conditions were studied. In order to follow the extraction progress, the oligomeric carbohydrates were determined. The hydrolysis process of oligo- and polymeric carbohydrates was followed separately by determining the monomeric sugars. The studied solid fractions influenced strongly all carbohydrate concentrations (Figure 1a), but the amounts of carbohydrates extracted relative to the dry roots were the same (data not shown). A reasonable amount of hydrolysate was only retrievable with a solid fraction of 1:10, which was used in all experiments thereafter. Between 60 and 95 °C there was no effect on the extraction and hydrolysis of inulin (Figure 1b). At 50 °C the extraction of inulin was lower for the 0.5 h sampling point, the hydrolysis yield did not differ from the higher temperatures. Upon longer incubation, the extraction efficiency at 50 °C increased to the same level, which was reached at higher temperatures. Increasing the pH had no effect on the extraction and hydrolysis prior to autoclaving (Figure 1c). Autoclaving the liquid supernatant of the hydrolysis or the liquid-solid suspension did support the hydrolysis at pH 4, but not at pH 7. As the effect was equally pronounced with and without solids present during autoclaving, the solids could be separated before autoclaving the hydrolysate. The extraction was not affected by autoclaving at both tested pH values. Enzyme additions were only tested at 50 °C and pH 5, which are in the supplier-specified optimal range of both enzyme cocktails. The addition of Viscozyme® released some galacturonic acid from the roots, but too little to be of significance. Under the conditions used, no monomers of hemicellulose (xylose) or cellulose (glucose) were found. The glucose found originated from inulin, which contains a

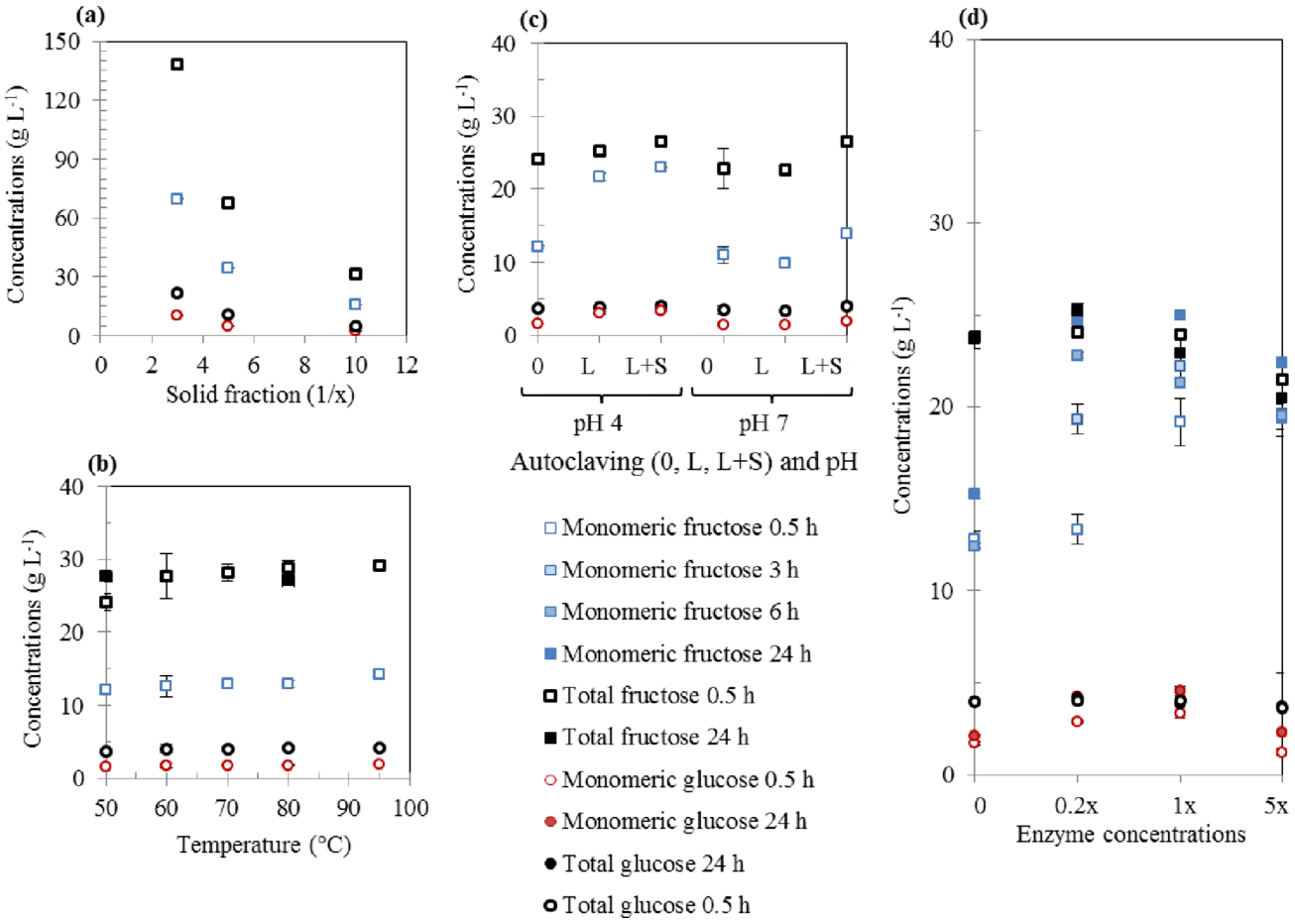

Fig. 1 – *Pre-treatment of chicory roots. (a) Effect of solid fraction at 80 °C and pH 4. (b) Effect of temperature at pH 4 and a solid fraction of 1:10. (c) Comparison of no autoclaving (0), autoclaving only the liquid after solid-liquid separation (L) or autoclaving the solid and liquid suspension together (L+S). Experiments were done at pH 4 and 7 at 80 °C and a solid fraction of 1:10. (d) Effect of Viscozyme® and inulinase mix addition at 50 °C, pH 5, and a solid fraction of 1:10. Both enzyme mixtures were also tested separately. Using the inulinase mix only, gave the same results as here shown, using Viscozyme® only had no effect on the levels of glucose and fructose.*

terminal glucose residue, but was never released in measurable quantities under cellulose-degrading conditions (Viscozyme®-addition or when doing the carbohydrate compositional analysis with the NREL-method). Inulinases did not aid in the extraction of inulin, but were necessary for the hydrolysis (Figure 1d). The reaction was faster when using more enzyme. With 5, 1 or 0.2 multiples of the reference concentration, the reaction was over after 0.5, 1, or 6 h. Two *Cupriavidus necator* strains (DSM 428 and 531) were tested for their ability to degrade inulin, but both were not able to utilize the oligomeric fructose in the fermentation broth within a reasonable time, as shown in Figure 2.

From the data presented, the overall optimum conditions for the extraction and hydrolysis of the chicory roots were obtained with a solid fraction 1:10, pH of 5, temperature of 50 °C, and 6 h incubation with the inulinase mix (0.2 multiples of the recommended concentration = 3600 U exo-inulinase kg^{-1} dry roots and 360 U endo-inulinase kg^{-1} dry roots). Autoclaving promoted the hydrolysis of remaining oligomeric carbohydrates. Tests showed that autoclaving alone has only a minor effect on the hydrolysis of inulin and cannot replace the addition of inulinases (data not shown).

The course of the extraction using the designed protocol is depicted in Figure 3. The extraction of carbohydrates is complete after 2 h, as can be seen in the constant level of total fructose and glucose. The hydrolysis of carbohydrates is completed later while autoclaving the hydrolysate, as can be seen in the level of free fructose and glucose approaching the level of total fructose and glucose.

In the final hydrolysate there are 27.6 g L^{-1} fructose and 4.8 g L^{-1} of glucose, as shown in Table 2a. This corresponds to a recovery rate of 86 % of the total fructose and 65 % of the total glucose from chicory roots determined with the NREL-method.[24] Only minor amounts of organic acids or sugar decomposition products, such as hydroxymethylfurfural are present. The hydrolysate has an intensely brown colour and it is not viscous. The intense co-

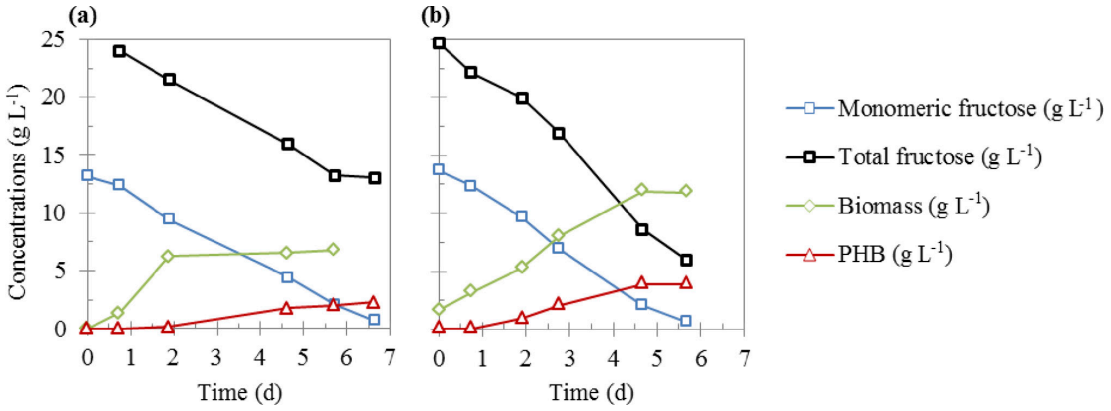

Fig. 2 – *Shake flask culture of Cupriavidus necator DSM 428 and 531 on hydrolysate containing free fructose as well as inulin (measured as total fructose)*

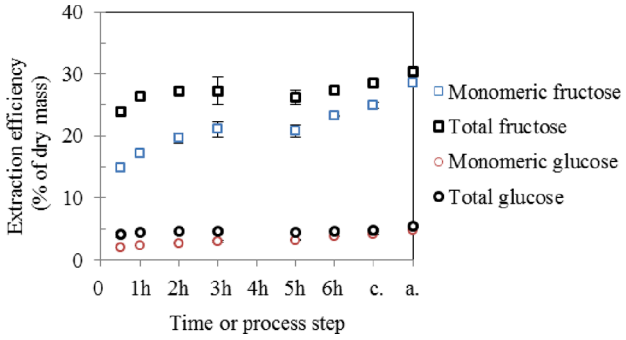

Fig. 3 – *Time course of the hydrolysis and extraction using the optimized protocol. c.: after centrifugation (removal of solids). a.: after autoclaving the liquid hydrolysate.*

lour indicates lignin or degradation products thereof. These phenolic compounds are often toxic to microorganisms, and were found in the hydrolysate at a concentration of 180.8 mg L^{-1}. In order to reduce the phenol content, two detoxification procedures were tested on the hydrolysate, as detailed in the following section on fermentation studies. There is essentially no free amino nitrogen in the hydrolysate, apart from some nitrogen (0.69 g L^{-1}) in the form of extracted proteins (and possibly some other reduced nitrogen compound/s).

When comparing the mineral composition and concentration of the hydrolysate to the minimal medium, some differences were observed (Table 2b). The concentrations of cobalt, iron, sodium and phosphate are less than half the respective concentrations in the minimal medium (Co: in the hydrolysate below the quantification limit, Fe: 0.12x, Na: 0.45x, P: 0.38x). The high concentrations of sodium and phosphate in the minimal medium come from the sodium-potassium phosphate buffer and are not necessary in these quantities for bacterial growth. Six elements are present in the hydrolysate in more than twice the concentration found in the minimal medium (B: 4.3x, Ca: 68x, Mg: 2.2x,

Table 2 – *(a) Macronutrient-composition of the hydrolysate after autoclaving. (b) Mineral composition of the minimal medium (calculated) as well as the hydrolysate (measured by ICP).*

(a) Component	Concentration (g L^{-1})
Fructose	27.6
Glucose	4.8
Arabinose	0.27
Xylose	0.28
Total Kjeldahl nitrogen	0.69
Free amino nitrogen	0.01
Hydroxymethylfurfural	0.30
Total phenols	0.18

(b) Element	Minimal medium (mg L^{-1})	Hydrolysate (mg L^{-1})
Al	0	1.07
B	0.261	1.13
Ca	2.72	186
Co	0.248	<0.01
Cr	0	0.064
Cu	0.186	0.311
Fe	10.5	1.33
K	660	1307
Mg	49.3	109
Mn	0.042	1.53
Mo	0.060	n.a.
Na	375	168
Ni	0.025	0.059
P	1030	394
S	65.0	127
Zn	0.113	1.18

Mn: 37x, Ni: 2.4x, Zn: 10x). Two elements were completely absent in the minimal medium, but present at very low concentrations in the hydrolysate (Al, Cr). An addition of minerals was not thoroughly investigated, as initial studies showed no stimulating effect on PHB-production (data not shown).

Fermentation studies

All three *Cupriavidus necator* strains are able to grow and produce PHB on the chicory root hydrolysate as shown in Figure 4. Strain 428 fully consumes the available fructose within 5.0 days, but does not utilize glucose. The fructose consumption rate increases for 1.6 days after the inoculation and falls off as the level of fructose drops below 12 g L^{-1}. Biomass formation of *Cupriavidus necator* DSM 428 starts after a lag phase of 11 h and the residual biomass (biomass without intracellular PHB) reaches a plateau at 2.8 g L^{-1} after 19 h. The dissolved oxygen drops with increased residual biomass formation and then increases again slowly during PHB formation. During growth, i.e. fructose oxidation to carbon dioxide, oxygen is required to keep the redox balance. The PHB-formation process from fructose (or any other sugar) is stochiometrically also an oxidation process, making oxygen essential for the reaction, but not to the same extent as is the case for a complete oxidation.[35] PHB formation starts after 19 h, proceeds rapidly for about a day, and then slowly levels off until all fructose is consumed. The highest biomass concentration (11.0 g L^{-1}) and PHB content (66 %) are reached after 5.0 days at the end of the fermentation, after all the fructose is consumed (Table 3). Overall, the yield is 0.32 g PHB g^{-1} consumed sugar and PHB is produced with a productivity of 0.062 g PHB L^{-1} h^{-1}.

Strain DSM 531 has a lag phase of 12 h after which it accumulates biomass, while the dissolved oxygen is low, reaching 2.6 g L^{-1} residual biomass. After 21 h, PHB accumulation starts and has a peak after 2.0 days (3.5 g L^{-1} biomass, 45 % PHB), after which it is slowly consumed again. Overall, only 35 % of the fructose and no glucose is consumed. The reason for the partial fructose consumption is unclear, but was already reported for this strain in other batch fermentations.[31] The yield and productivity of the fermentation are 0.15 g g^{-1} and 0.016 g L^{-1} h^{-1}, respectively.

Strain DSM 545 consumes both sugars much more rapidly than the other two tested strains. Both sugars are consumed immediately after inoculation and in parallel. Glucose is depleted earlier than fructose (1.3 vs. 3.0 days). This is in line with earlier reports describing *C. necator* DSM 545 as glucose-utilizing mutant of *C. necator* DSM 529. In accordance with the sugar consumption, strain DSM 545 starts biomass accumulation straightaway. Most of the residual biomass is accumulated during the first 8 h, reaching a value of 2.7 g L^{-1} residual biomass, while the dissolved oxygen is used. After that, the dissolved oxygen increases again and the residual biomass accumulation rate is only marginal for the remaining fermentation time. PHB production is not strictly separated from residual biomass production, i.e. some PHB is already formed during cell growth. PHB production also starts straightaway and reaches the maximum rate after 19 h, after which it slows down and ceases after 3.0 days when fructose is depleted. At this point, a biomass concentration of 14.0 g L^{-1} containing 78 % PHB is reached. This corresponds to a yield of 0.38 g g^{-1} and a productivity of 0.15 g L^{-1} h^{-1}.

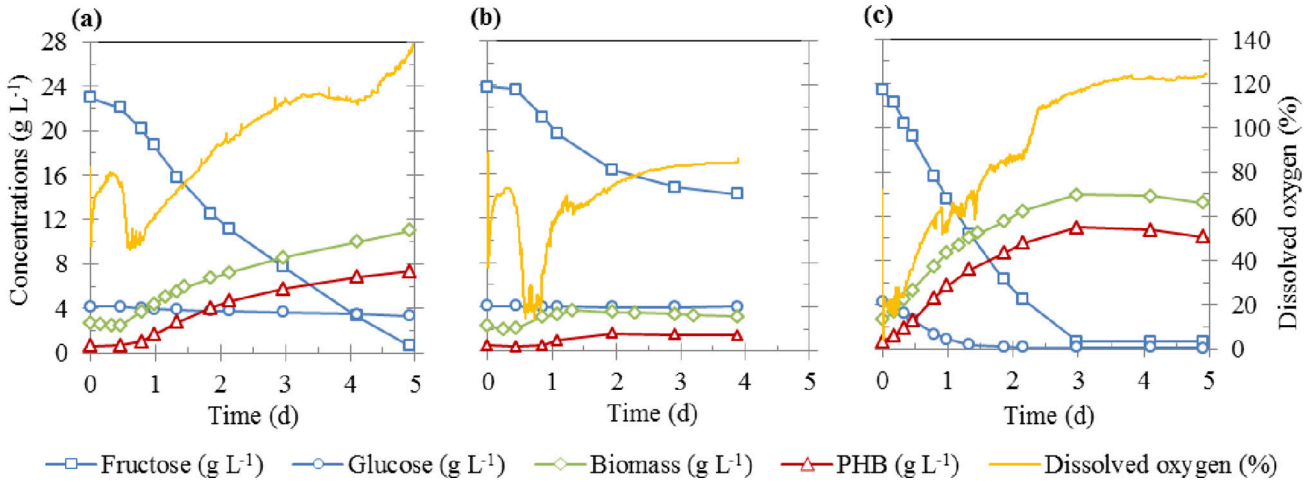

Fig. 4 – *Bioreactor fermentations using Cupriavidus necator (a) DSM 428, (b) DSM 531, and (c) DSM 545 in the obtained chicory root hydrolysate. On the left axes are the concentrations of the substrates and products, and on the right axes is the dissolved oxygen.*

Only one other research group reported a PHB production from fructose-based hydrolysates using *Cupriavidus necator*. However, they specify neither the strain nor the fermentation mode used during the experiments.[23] At the end of the experiments, they reached a biomass concentration of 6–7 g L^{-1} with a PHB content of 70 %.

There have been batch fermentations on refined fructose in minimal media. Strain DSM 428 was grown by Franz *et al.* on minimal medium to a cell density of 8 g L^{-1} and a PHB content of 45 % (Table 1).[36] The yield was 0.21 g g^{-1} and the productivity was 0.12 g L^{-1} h^{-1}. The same strain was also grown by Kelley *et al.* to a cell density of 0.4 g L^{-1} (66 % PHBHV-block-HB) with a productivity of 0.03 g L^{-1} h^{-1}.[37] Our results for DSM 428 are more comparable to the first study. However, in the second study, the emphasis was on producing a block copolymer rather than obtaining high yields.

Strain DSM 531 has also been used for fructose-based batch fermentations, but again only in minimal medium. Khanna *et al.* report biomass concentrations of 21 g L^{-1} (45 % PHB), a yield of 0.31 g g^{-1}, and a productivity of 0.15 g L^{-1} h^{-1}.[31] These findings are contrary to ours, but can be attributed to the minimal medium used by the authors of the study. When we tested the strain in minimal medium, the strain also performed significantly better (data not shown). However, upon cultivation in chicory root hydrolysate, strain DSM 531 is less efficient, and therefore less desirable for PHB production.

In the case of strain DSM 545, no batch fermentations based on fructose were found in the literature. However, the results are superior when compared to shake flask experiments using sugarcane molasses (Table 1).[19,20] Results from fed-batch fermentations report significantly higher cell densities and productivities. For example, an industrial process using strain DSM 545, reaches 125–150 g L^{-1} of biomass (65–70 % PHB), a yield of 0.32 g g^{-1}, and a productivity of 1.44 g L^{-1} h^{-1}.[38]

Kulpreecha *et al.* did batch fermentations on fructose with a new isolate of *Bacillus megaterium* and applied the optimized protocol to a fed-batch fermentation strategy.[18] In their fermentations, the cell density increased by a factor of eight (8.8 vs. 72 g L^{-1}) and the productivity almost tripled (0.45 vs. 1.27 g L^{-1} h^{-1}). However, applying a fed-batch strategy to the chicory root hydrolysate, keeping the process economics in mind, needs additional process steps. The sugar concentration in the hydrolysate could be increased by altering the solid fraction during the hydrolysis. This also decreases the obtained amount of liquid hydrolysate compared to solid press cake. In turn, this means that more sugars end up in the solid press cake and are lost for the fermentation. Another option would be to concentrate the carbohydrates, by either evaporation or membrane technology.[39]

The total Kjeldahl nitrogen of the hydrolysate (0.69 g nitrogen L^{-1}) drops during biomass formation of all three strains to 0.53 g nitrogen L^{-1} and stays constant thereafter. All three strains also form the same amount of biomass during the biomass accumulation phase from the consumed nitrogen (2.6–2.8 g biomass L^{-1} from 0.16 g nitrogen L^{-1}). The nitrogen not consumed by the bacteria indicates that only a part of the nitrogen, which is determined with the Kjeldahl method, is accessible to the microorganisms. It could be either that a part of the proteins and peptides form polymeric maillard-reaction products with fructose during autoclaving, or a part of the proteins denatures into a protease-resistant form during autoclaving. Bioavailable nitrogen is nonetheless the limiting nutrient and the trigger for bacteria to switch to PHB formation, as nitrogen addition to fermentations (ammonium sulphate or peptone) results in the formation of more residual biomass (data not shown).

During growth, all strains require the addition of acid to the fermentation medium in order to keep the pH constant (data not shown). This finding is opposite to fermentations in minimal medium, where the addition of base replaces the ammonium taken up by the cells. The utilization of amino acids or proteins is expected to cause a release of basic nitrogen compounds, and increase the pH during growth.

Two detoxification methods were tested on the hydrolysate prior to fermentations with *Cupriavidus necator* DSM 545. The first detoxification treatment was done with activated charcoal (adsorption of phenols) and the second with laccases (polymerisation of phenolic compounds). Both methods decreased the total phenol content (180.8 mg L^{-1}) by 16.8 % or 35.8 % in the case of the laccase or charcoal treatment. The charcoal treatment also reduced the sugar content and total Kjeldahl nitrogen by 6 and 14 %, respectively. However, both fermentations are very similar to each other (Table 3). When comparing the two fermentations to the fermentation without detoxification, the productivity is only slightly lower and presumably not significant. The yields are similar in all cases.

Researchers working on acid hydrolysates of different origins did report positive effects of the detoxification treatments. Radhika *et al.* showed that activated charcoal treatment (2 %) decreased the total phenol content of an acid hydrolysate of water hyacinths by 78 %.[40] When *C. necator* MTCC-1472 was grown on the detoxified substrate, the PHB concentration increased from 2.0 g L^{-1} to 4.3 g L^{-1}. Pan *et al.* prepared a hemicellulosic hy-

Table 3 – *Bioreactor fermentation results of the different Cupriavidus necator strains tested*

	DSM 428	DSM 531	DSM 545	DSM545 + laccase treatment	DSM 545 + charcoal treatment
Biomass (g L^{-1})	11.0	3.5	14.0	14.2	13.7
PHB content (%)	66	45	78	71	75
Yield (g PHB g^{-1} fructose)	0.32	0.15	0.38	0.36	0.38
Volumetric productivity (g PHB L^{-1} h^{-1})	0.062	0.016	0.15	0.11	0.11

drolysate from sugar maple and used it as a fermentation substrate for *Burkholderia cepacia* ATCC 17759.[41] Several detoxification methods, including overliming, activated charcoal (5 %), cation exchange resin, and low-temperature sterilization were compared. Overliming combined with low-temperature sterilization resulted in the highest xylose consumption during the fermentation process. Silva *et al.* compared the treatment of bagasse acid hydrolysate with/without overliming and activated charcoal in addition to overliming.[42] They concluded that a new strain of *Burkholderia cepacia* grows and produces more PHB when subjected to both treatments instead of overliming alone or no detoxification treatment.

Contrarily, the productivities of *C. necator* DSM 545 on the hydrolysate of this study are not improved upon detoxification. There are two possible explanations for this: First, the reduction in phenolic compounds was too small. Second, the enzymatic hydrolysis procedure probably does not release inhibitors that can be removed by either charcoal or laccase treatment. Low concentrations of phenolic degradation products have been reported to even have a stimulating effect on the growth of *C. necator* NCIMB 11599.[43]

Extraction and characterisation of PHB

PHB from all three *Cupriavidus necator* strains was extracted and tested to be pure. The glass transition temperature, melting temperature, and enthalpy of melting are in good accordance with results from the literature (Table 4).[44,45] The number and weight average molecular weight, as well as the polydispersity are shown in Table 4. Strain DSM 545 seems to make longer chains compared to the other two strains in the chicory root medium. In any case, they are in the typically reported range, indicating that only negligible degradation occurred during the extraction.[13,44,46]

Conclusions

A pre-treatment protocol for chicory roots was developed, extracting inulin and hydrolysing it under mild conditions. The resulting hydrolysate contained fructose (27.6 g L^{-1}), glucose (4.8 g L^{-1}) and nitrogen (0.7 g L^{-1}), and was a viable fermentation substrate without nutrient supplementation. Three *Cupriavidus necator* strains (DSM 428, 531 and 545) grew and produced PHB from the sugars in the hydrolysate. Strain DSM 545 gave the best results (yield: 0.38 g PHB g^{-1} sugar, productivity: 0.15 g PHB L^{-1} h^{-1}), which is comparatively high for batch fermentations. Detoxification of the hydrolysate using charcoal or laccases did not improve the fermentation results. The thermo-physical parameters determined for the chloroform-extracted PHB are in good accordance with PHB produced in minimal media.

With the current process characteristics, 11,250 t chicory hydrolysate could be annually produced in Navarra, containing 547 t sugars, which would yield 208 t PHB per year prior to purification. For most applications, PHB has to be blended with other

Table 4 – *Thermo-physical parameters of the extracted polymers in comparison to published data for Cupriavidus necator. n.d.: not determined*

Parameter	Own results	Own results	Own results	Ref.[44]	Ref.[45]	Ref.[13]	Ref. [46]
	DSM 428	DSM 531	DSM 545	strain n.a.	new isolate	DSM 428	DSM 428
Glass transition temperature (°C)	4.6	4.4	6.2	4	2.4	n.d.	n.d.
Melting temperature (°C)	174	176	164	179	178	n.d.	n.d.
Enthalpy of melting (J g^{-1})	92.4	86.5	76.3	n.d.	98	n.d.	n.d.
Number average polymer weight (Da)	$4.55 \cdot 10^5$	$3.65 \cdot 10^5$	$5.11 \cdot 10^5$	$0.1–30 \cdot 10^5$	n.d.	$1.70 \cdot 10^5$	$3.2 \cdot 10^5$
Polydispersity	1.82	1.90	1.32	~2	n.d.	3.2	2.5

polymers or plasticizers.[47] The amount of plastics that could be produced from chicory roots is still marginal compared to the plastic consumption in Spain (3,500,000 t per year[48]). In addition, the production volume would be too small for an economy-of-scale benefit, making competition difficult with bulk petrochemical plastics produced at full scale. However, European legislation is pushing corporate social and ecological responsibility measures.[49] Furthermore, consumer awareness regarding bio-based and biodegradable products is rising. This opens opportunities particularly for tangible materials, such as packaging materials. PHB from chicory roots combine several advantages. It is bio-based, but does not interfere with the food production directly as well as on the level of land use. PHB is biodegradable, also under non-composting conditions. A process using chicory roots can be operated throughout the year, as the roots are continuously available in roughly equal quantities. The PHB yield from chicory roots is good and the fermentations are stable. Due to the high water content of the roots, it has to be produced locally, reducing transport requirements. Summing up, chicory roots are an excellent substrate for PHB production and might contribute to a green economy in the future.

ACKNOWLEDGEMENT

This work was conducted as a part of the LEAD-ERA Project CARBIO, which was financed by the Federal State of Lower Austria and co-financed by the European Regional Development Fund (ERDF) of the European Union.

The authors would like to thank Irantzu Goenaga from Tratamiento Subproductos Agroalimentarios S.L. for her input on chicory roots, and Gibson S. Nyanhongo as well as Andreas Ortner for providing the laccase.

List of symbols and names with units

C. necator – *Cupriavidus necator*

CARBIO – CARbohydrate derived BIOpolymers

DSC – differential scanning calorimetry

DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen

PHA – Polyhydroxyalkanoate

PHB – poly([R]-3-hydroxybutyrate)

PHBHV – poly([R]-3-hydroxybutyrate-co-[R]-3-hydro--xyvalerate)

PHBHV-block-HB – poly([R]-3-hydroxybutyrate-co--[R]-3-hydroxyvalerate)-block--([R]-3-hydroxybutyrate)

TKN – total Kjeldahl nitrogen

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