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PRODUCTION OF BIOPLASTICS FROM AGRO-RESIDUES

Dissertation

zur Erlangung des Doktorgrades

an der Universität für Bodenkultur Wien

Eingereicht von

Cornelia Haas, MSc

Wien, Oktober 2016

PREAMBLE

This dissertation was conducted at the Institute of Environmental Biotechnology in Tulln from March 2013 until September 2016 in the working group of Markus Neureiter under the supervision of Georg Gübitz. The scientific output generated during this time are two first-author publications (see chapter 3 and 5) and two co-authorships (see chapter 7.1) in peer-reviewed SCI-listed Journals. Furthermore, several oral as well as poster presentations (see chapter 4, 7.4 and 7.5) were held and one article in a governmental news bulletin (see chapter 7.2) was published. All of this combined form the basis of the quest for obtaining a doctoral degree from the University of Natural Resources and Life Sciences (BOKU) by the author.

The scientific output of this cumulative dissertation is completed by an introduction to the topic, equipping the reader with the essential background and directing him/her to the necessity of the research conducted by the author (chapter 1). In chapter 2 the objectives of the research conducted by the author are specified. A general conclusion connects the obtained results and highlights how these results have led to the successful acquisition of a follow-up project (chapter 6).

ACKNOWLEDGEMENT

First of all I would like to thank my direct supervisor Markus Neureiter for his support concerning the practical and theoretical work (including the creative freedom I got to develop my own ideas). I learned a lot from him in these three years!

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I have gained a lot of insights from Martin Koller and his expertise in PHAs in the numerous discussions we had in the last couple of years. Thanks for that!

My thank also goes to Marina Smerilli for the good time we had together in the lab and at summer schools/conferences! This PhD thesis would not have been possible without Valentin Steinwandter, Lukas Burgstaller, Tarek El-Najjar, Nikolaus Virgolini, Patrick Schögl, Annika Putz, Flavius Farcas and Hyunjeong Song. They generated lots of data, had good ideas and were essential for the positive and nice working environment in the Nährbodenküche as well as the Fermenterlab. I am proud of having worked together with so many talented students! As it is time to say good bye for me soon, I am happy that the PHA work has been taken over by Maximilian Schmid and Milos Kacanski, two smart and enthusiastic PhD students.

Markus Sadler and Wilhelm Müllner were invaluable for me when it came to technical/electronic problems. The analytical instruments were the pets of Marcus Pruckner, who often had to care intensively for these demanding objects. Sabine was important for keeping everything in order and preventing rank growth among all students.

Furthermore, I would like to thank the cooking group (old and new!) for providing the nutritional "input" and for being a positive and enriching checkpoint throughout the working day.

Next to mention are Michael and Valentin for their great companionship in finding our ways through life. I hope that we still have a looong way to go.

ABSTRACT

In this thesis an alternative production mode for producing the bioplastics poly[(R)-3-hydroxybutyrate] (P3HB) from low-cost agro-industrial residues was developed. The use of low-cost by-products as raw materials is expected to decrease the price of this polymer and pave the way for the commercialisation of this promising material.

The first part of this thesis was conducted within the scope of the CARBIO project (LEAD-ERA project nr. WST3-T-95/011-2010), a project on the valorization of residues from the fruit and vegetable processing industry in northern Spain. In this project, chicory roots from hydroponic salad cultivation were identified as an abundant food reside which has been underutilized to date. A pre-treatment process was successfully developed leading to a hydrolysate, containing 34 g/L sugars. This hydrolysate was then used as a fermentation substrate for three P3HB-producing *Cupriavidus necator* strains. Strain DSM 545 was superior over strain DSM 428 and 531, as a dry biomass concentration of 14.0 g/L was reached containing 78 % P3HB. The polymers produced by all three species were extracted and several thermo-mechanical parameters determined. They were in good accordance with literature data for P3HB from organisms grown on synthetic substrates and showed that this polymer can be used as blend component for packaging material made for the locally produced vegetables.

The main drawback for the process is, that the productivity (0.15 g P3HB/L/h) is too low for industrial fermentations. This is inherently linked to the concentration of the carbon source in the hydrolysate. In order to make this substrate, as well as many other cheap agro-industrial by-products with a low carbon content amenable for P3HB production, a membrane bioreactor was developed during the second phase of the thesis. In this setup, the cells were retained in a constant volume inside the bioreactor during the fermentation using an external microfiltration module. A synthetic medium containing a low glucose concentration was continuously fed to *Cupriavidus necator* DSM 545, which converted the sugar to P3HB. With the optimized setup a high productivity (3.10 g P3HB/L/h), high cell density (148 g/L) containing 76% P3HB, and a good yield was reached.

This approach creates the possibility for bioplastic production from a range of cheap and easily available substrates, for which only anaerobic digestion was cost-competitive until now.

KURZFASSUNG

Diese Dissertation beschäftigt sich mit der Produktion des Biokunststoffs Poly[(R)-3-Hydroxybutyrat] (P3HB) aus agro-industriellen Nebenprodukten. Durch die Nutzung dieser günstigen Rohstoffe soll der Preis des Polymers gesenkt werden und so diese vielversprechenden Materialien wieder einen Schritt weiter in Richtung kommerzieller Produktion gebracht werden.

Der erste Projektteil wurde im Rahmen des CARBIO Projekts (LEAD-ERA Projekt Nr. WST3-T-95/011-2010) zur wertsteigernden Nutzung von Reststoffen der obst- und gemüseverarbeitenden Industrie in Nordspanien durchgeführt. In diesem Projekt wurden Zichorienwurzeln aus der hydroponischen Kultivierung als chancenreichstes Nebenprodukt identifiziert. Es wurde ein Vorbehandlungsprozess entwickelt und ein Hydrolysat mit 34 g/L Zucker hergestellt. Dieses Hydrolysat wurde als Fermentationssubstrat zur P3HB Produktion eingesetzt. *Cupriavidus necator* DSM 545 lieferte im Vergleich zu *Cupriavidus necator* DSM 428 und DSM 531 die besten Ergebnisse, da er eine Biomassekonzentration von 14 g/L mit einem P3HB-Anteil von 78% erreichte. Die produzierten Polymere wurden extrahiert und charakterisiert. Die untersuchten thermo-mechanischen Eigenschaften von P3HB aus Zichorienwurzeln sind vergleichbar mit jenen von P3HB aus raffinierten Zuckern. Daher können die Polymere als Blendkomponenten zur Produktion von Verpackungsmaterial der lokal produzierten Gemüseprodukte genutzt werden.

Der Nachteil an diesem Prozess ist eine für industrielle Maßstäbe zu niedrige Produktivität (0.15 g P3HB/L/h), die durch die niedrige Konzentration der Kohlenstoffquelle bedingt wird. Im zweiten Projetteil wurde daher ein Membranbioreaktor entwickelt, mit welchem dieses Substrat, wie auch viele andere kostengünstige Nebenströme mit niedrigen Kohlenstoffkonzentrationen, als Rohstoffe für P3HB Produktion nutzbar gemacht werden können. In diesem Fermentationssystem werden die Zellen während der Fermentation durch ein externes Membranmodul zurück- und das Volumen konstant gehalten. Synthetisches Medium mit einer niedrigen Glukosekonzentration wurde kontinuierlich zugefüttert und durch *Cupriavidus necator* zu P3HB umgesetzt. Mit dem optimierten Setup, wurden eine sehr hohe Produktivität (3.1 g P3HB/L/h), eine hohe Zelldichte (148 g/L) mit 76% P3HB und eine gute Ausbeute erreicht.

Diese Ergebnisse eröffnen die Möglichkeit der industriellen Biokunststoffproduktion aus günstigen, leicht verfügbaren Nebenströmen, für die bis dato lediglich die Verwertung in Biogasanlagen möglich war.

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1 INTRODUCTION

Plastic materials have shaped the previous decades like no other substance. Due to their diversity of properties and versatility of applications, the global production capacity has increased from $1.5*10^6$ t/a in 1950 ($0.35*10^6$ t/a in Europe) to $299*10^6$ t/a in 2013 ($57*10^6$ t/a in Europe).[1] In the last 15 years the increase in global production capacities was decoupled from the European production capacity, as the latter is relatively constant and has not significantly increased. Also in the future, no major changes are expected in this business sector.

On the other hand, the European sector for biodegradable plastics, which is currently a niche market compared to conventional plastic production, is expected to witness a tremendous increase by 94% from $663*10^3$ t/a in 2013 to $1286*10^3$ t/a in 2019.[2]

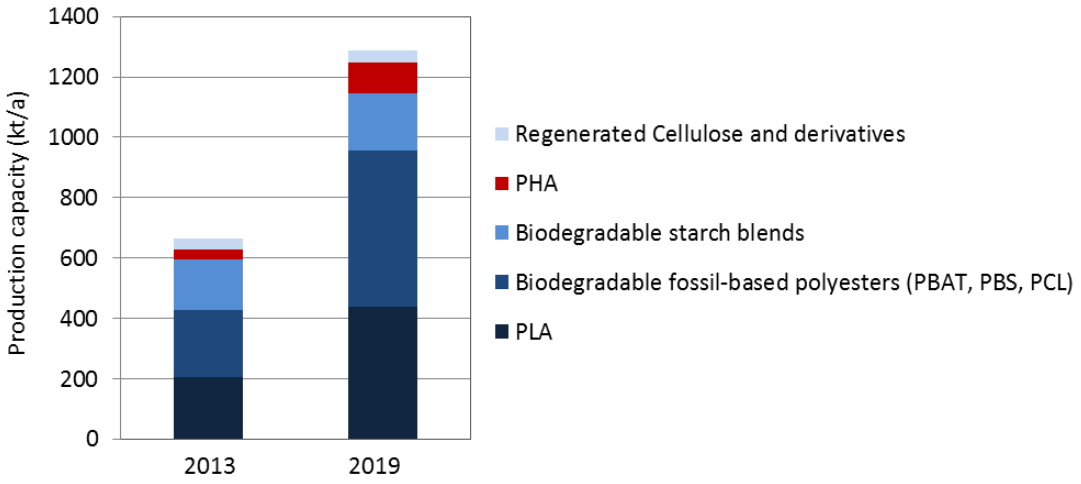

Figure 1.1. Existing production capacities for biodegradable plastics in 2013 and expected production capacities for 2019.[2] PBAT: polybutylene adipate-co-terephtalate, PBS: polybutylene succinate, PCL: polycaprolactone.

The market segment for biodegradable plastics at the moment is dominated by four materials: polylactic acid (PLA), fossil-based polyesters, starch blends and polyhydroxyalkanoates (PHA).

PLA is produced via polycondensation of lactic acid, which is in turn produced by fermentation of carbohydrates. The properties are variable. They depend primarily on the ratio of the lactic acid enantiomers (D- vs. L-form), as well as the molecular weight and the crystallinity of the polymer. It is applied for injection molding (e.g. cups, trays, cutlery), as well as for fibers and films.[3] With a price well below 2 €/kg it is the cheapest among the three biobased and biodegradable plastic materials. At the moment the market is dominated by the company NatureWorks, operating a $140*10^3$ t/a facility in Nebraska (USA).[4] Although the biodegradability is advertised strongly, in fact it is only biodegradable under industrial composting conditions.

1 INTRODUCTION

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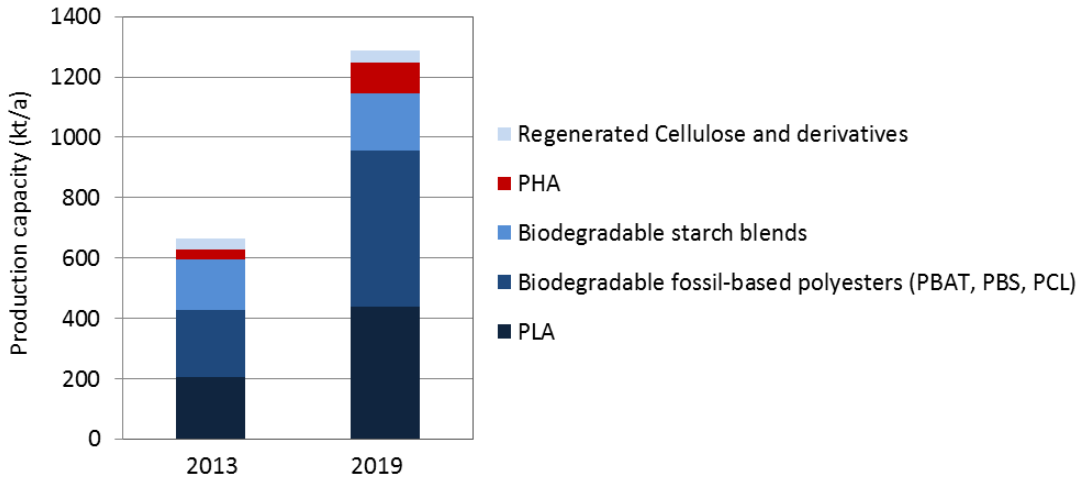

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Biodegradable polyesters, such as polybutylene adipate-co-terephtalate (PBAT), polybutylene succinate (PBS), polycaprolactone (PCL) are made from aromatic and aliphatic dicarboxylic acids and diols (especially glycol). All dicarboxylic acids (e.g. phtalic acid) and most of the diols are fossil-based, although the fraction of biobased diols in these polymers is increasing. The properties are very diverse, making them applicable for injection moulding, foil and fiber applications. A rather high price of 3.5 to 6.5 €/kg, limits their applications to technically challenging applications.

Starch blends consist of thermoplastic starch and a roughly equal amount of a hydrophobic and ductile polymer (e.g. polycapolactone or polyvinylalcohol (PVAL)).[3] Thermoplastic starch is made by extrusion of native starch, which leads to a thermo-mechanical destruction of the starch grains. The starch inside the final blend increases the overall strength and biodegradability. Due to the hygroscopic nature of starch, only short-lived products in the packaging sector (e.g. shopping bags) are produced from starch blends. The most important producer in Europe is the Italian company Novamont ($120*10^3$/a production capacity), selling starch blends for 2.5 to 5.5 €/kg. The second polymer besides starch in the blends is most of the time equally well biodegradable. However, these blend components are based on petrochemical resources. Accordingly, the overall starch blend is biodegradable but only partially biobased.

The fourth group of materials are polyhydroxyalkanoates (PHA). Compared to the other three competitors, this family of polyesters is fully biobased as well as biodegradable. Their properties can be modulated over a wide spectrum, depending on the monomeric composition and degree of polymerisation. Due to their good processability and excellent performance in diverse applications, they are at the verge of emanating from being a nice material to gaining industrial significance. In line with this, these materials are at the moment heavily researched by public as well as private institutions.

1.1 Polyhydroxyalkanoates as Bioplastics

Polyhydroxyalkanoates (PHA) are microbial storage materials, which can be applied – upon extraction, compounding and extrusion – as plastics for numerous applications. The carbon sequestered in the materials originates from the biosphere, closing the carbon cycle of this material. After the utilization of the product, the polymer can be recycled like other plastic materials. In addition to most plastic materials, undesirable littering into the environment is less problematic as PHA can be degraded by aerobic as well as anaerobic microorganisms in soil or aquatic ecosystems. In line with the transition to a sustainable post-fossile carbon society, PHA may play a proactive role as excellent alternative to conventional plastics.

A range of bacteria are able to synthesize intracellular PHA-granules, which serve them as carbon and energy source. Typically, they are produced under unbalanced nutrient conditions – if an excess of carbon is available concurrently to the limitation of one or more nutrients. For polymer production, nitrogen or phosphorous are most frequently limited in the medium. This limitation causes a shift of metabolism from growth to PHA-accumulation.

Polyhydroxyalkanoates are linear polyesters made of hydroxy-alkanoic acids. The polymers are divided into three groups, depending on the number of carbon atoms in the monomer units. Short-, medium-, and long-chain length PHA are made of 3-5, 6-12, and 12-18 carbon atoms, respectively (see Figure 1.2). Most organisms reported to date synthesize short-chain length PHA (scl-PHA), either as homopolymer (e.g. poly[(R)-3-hydroxybutyrate], P3HB) or as co-polymer (e.g. poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate], P3HB3HV). The exact type of polymer synthesized depends on the genetic background of the organism, as well as the nature of the carbon source. The microorganisms synthesizing scl-PHA are phylogenetically diverse and belong to the groups of Gram-negative Eubacteria (e.g. *Cupriavidus necator, Azohydromonas lata, Burkholderia cepacia, Halomonas boliviensis*), Gram-positive Eubacteria (e.g. *Bacillus megaterium*) as well as Archaea (e.g. *Haloferax mediterranei*) and microalgae (e.g. *Synechocystis* sp.).[5, 6] Medium-chain length PHA (mcl-PHA) are only synthesized by the genus *Pseudomonas*.[7]

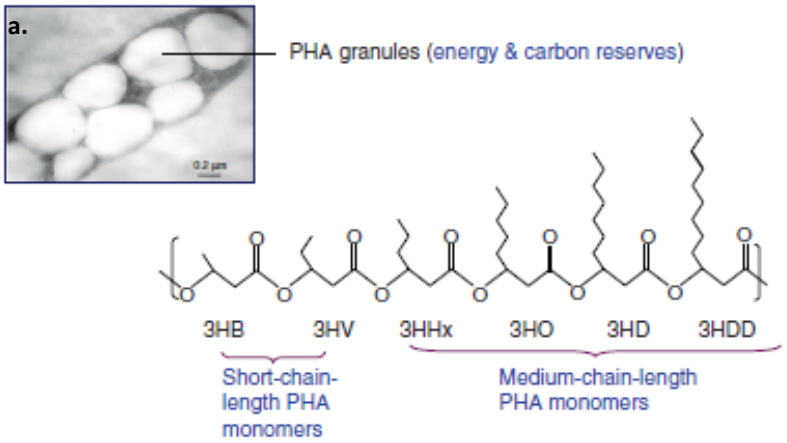

Figure 1.2 (a). Electron micrograph of intracellular PHA granules.
(b) Structural formula of the most frequent monomer units in the linear polyester. 3HB: 3-hydroxybutyric acid, 3HV: 3-hydroxyvaleric acid, 3HHx: 3-hydroxyhexanoic acid, 3HO: 3-hydroxyoctanoic acid, 3HD: 3-hydroxydecanoic acid, 3HDD: 3-hydroxydocecanoic acid. Modified from [8]

Acetyl-CoA is the central point of the PHA metabolism. PHA-producing bacteria build it from various sugars, fatty acids, lipids, and even carbon dioxide. In three steps two molecules of acetyl-CoA are conjugated to acetoacetly-CoA, then reduced to 3-hydroxybutryryl-CoA and polymerized to P3HB (see Figure 1.3). The three genes conducting these steps have been transferred numerous times to other hosts, enabling well studied strains such as *E. coli* to produce PHA.[9]

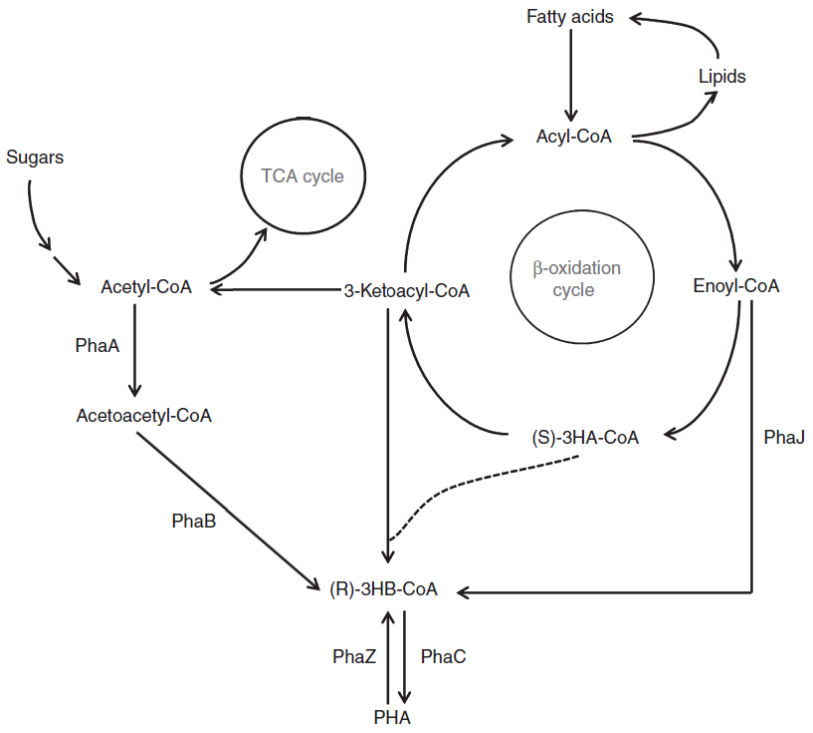

Figure 1.3. Metabolic pathways of PHA accumulation.
PhaA: β-ketothiolase,
PhaB: NADPH-dependent acetoacetyl-CoA reductase,
PhaC: PHA polymerase,
PhaZ: PHA depolymeras;
PhaJ, enoyl-CoA hydratase;
Acc, acetyl-CoA carboxylase.
Modifed from [10]

Depending on the cultivation conditions, the polymers can consist of 100-30 000 monomers and have a molecular weight distribution of 50 to 1 000 kDa. Due to their apolar nature, PHA molecules agglomerate to granules inside the cells. Every cell possesses at least one granule which is inherited in a DNA-associated manner. In total the granules can make up in very high cellular polymer loads (e.g. 60-90% for *Cupriavidus necator*, 40-70% by *Haloferax mediterranei*).

Once the cells are filled up with PHA, the polymer only needs to be extracted in order to get a processable plastic. This extraction is usually conducted with organic solvents, as this method separates PHA from the residual biomass with a high recovery rate and purity, as well as low costs. In the literature, there are also alternative approaches discussed in order to circumvent the utilization of organic solvents.[11]

1.1.1 Properties and Applications of PHA

The properties of PHA depend primarily on the monomeric composition. In addition to that, the distribution of the different monomers in the polymer chain (random vs. block-type), as well as the degree of polymerisation (molecular weight), play a role. All these factors combined open up a huge design space for PHA materials, extending the material property combinations currently used by the plastics industry (see Figure 1.4).[12]

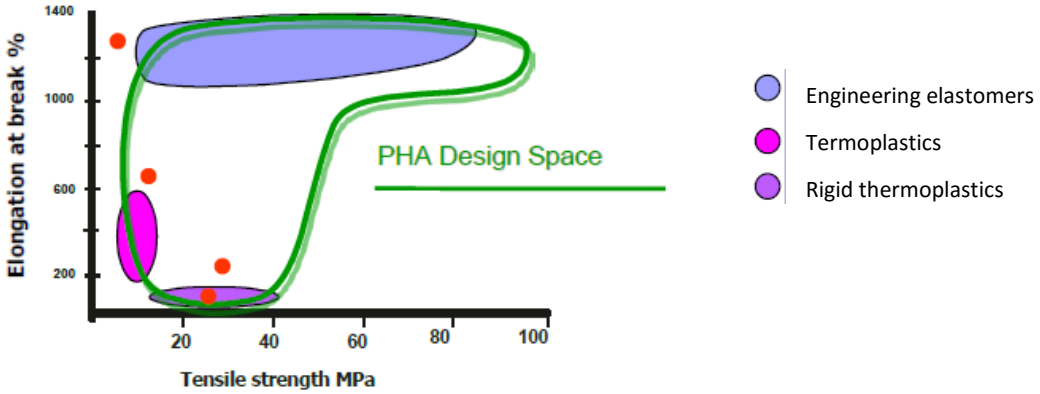

Figure 1.4. Design space for PHA polymers.[13]

Most PHA are made from 3-hydroxy acids, resulting in a uniform polymer backbone, from which the carbon atoms anterior to the hydroxyl group emanate as little side-chains (see Figure 1.2.b.). Pure P3HB is due to its short methyl side-chain highly crystalline (>70%), rather stiff and brittle, making it not the easiest to process. The temperature range feasible for processing is rather low but nonetheless workable. As the chain length of the monomers gets longer than butyrate, the crystallinity of the material is decreased and it gets more ductile. This effect can be scaled by incorporating only certain fractions of monomers with a longer chain length. The incorporation of only 10 mol% of the C5 acid hydroxyvalerate results in an easily processable material (P3HB3HV), well suitable for extrusion in conventional plastic manufacturing equipment (see Figure 1.4).[3] Even longer side chains, e.g. in mcl-PHA, cause the material to behave like rubber or other elastomers.

The properties may be modified even further by chemical/physical modifications of the bulk material or just on the surface of the products after extrusion/foil production. Compounding with other (bio-) polymers or fillers offers another route for tailoring the desired properties.

Excellent gas barrier properties (especially for oxygen) and good printability make P3HB3HV an excellent material for food packaging applications, incl. bottles. Due to the low melt-viscosity, it is possible to apply thin layers on e.g. beverage cartons. [3]

In the medical field, PHA are a promising material owing to their biocompatibility as well as biodegradability. The US American FDA already approved certain PHA materials (TephaFLEX®) for sutures, surgical meshes and films. [14] In addition to that, PHA are discussed for use as bone plates,

osteosynthesis and stitching materials. They are also well suited for embedding drugs and facilitating the long-term release of pharmaceuticals or hormones. [15]

1.1.2 Current Status and Challenges for Market Penetration

In 2009, the production capacities of PHA worldwide were $76*10^3$ t/a, distributed over 5 companies (see Table 1.1). Until 2015 the existing producers as well as new market entrants announced a cumulative production capacity of $900*10^3$ t/a. However, in reality only about $1*10^3$ t/a of PHA materials was sold in 2013.[13]

Table 1.1. PHA producers with existing production capacities in 2009. The polymers as composed of the following monomers: 3HB: 3-hydroxybutyric acid, 4HB: 4-hydroxybutyric acid, 3HV: 3-hydroxyvaleric acid, 3HHx: 3-hydroxyhexanoic acid, 3HO: 3-hydroxyoctanoic acid, 3HD: 3-hydroxydecanoic acid. Modified from [3, 12, 16]

Company	Polymer	Tradename	Production capacity 2009 (10^3t/a)	Raw materials
Meredian, now MHG (US)	P3HB3HHx, P3HB3HO, P3HB3HD	Nodax®	13.5	fatty acids (cannola)
Metabolix (US)	P3HB, P3HB3HV, P3HB4HB	Mirel®	50	glucose, glucose + 1,4-butanediol
PHB Industrial SA (Brazil)	P3HB	Biocycle®	0.05	sucrose (molasses)
Tianan Biologic Materials (China)	P3HB3HV	ENMAT®	2	glucose
Tianjin Green Biomaterials (China)	P3HB4HB	Sogreen®	10	glucose + 1,4-butanediol

The major reason for PHA not taking a bigger market share is its price. Compared to other (bio-)polymers, PHA are significantly more expensive (see Figure 1.2). The price range in 2014 was 3.8 - 4.5 €/kg, i.e. well above the other established biodegradable and biobased polymers.[13] Due to economy of scale benefits, the price is expected to decrease once the amounts produced are beyond pilot productions. The other factors driving the costs of PHA are the costs for the raw materials as well as the extraction process. The price for raw materials is thereby of central importance as it can make up to 50% of the entire production costs.[17] This is caused by the fact that PHA is (in contrast to lactic acid for PLA) produced under aerobic conditions, where a part of the carbon source is lost during intracellular respiration. This loss is reflected in lower yields, which are discussed in more detail in chapter 1.2.2 on page 21.

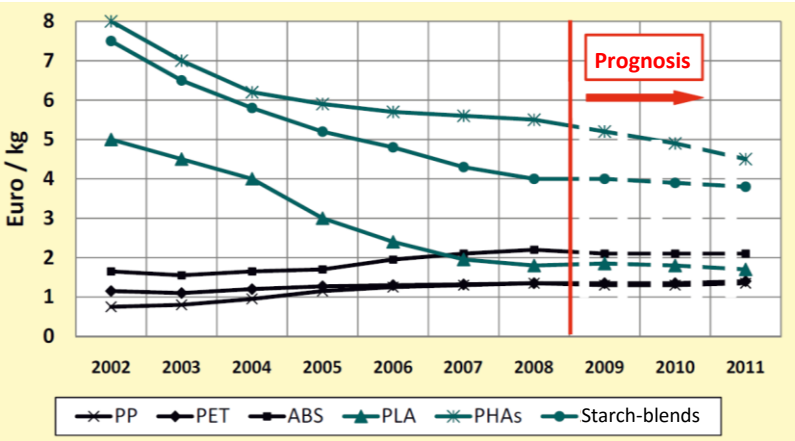

Figure 1.5. Price development of petrochemical and biobased polymers. PP: polypropylene, PET: polyethylene terephthalate, ABS: acrylonitrile butadiene styrene, PLA: polylactic acid. [3]

The raw material costs are not only a major cost factor, the prices for refined sugars or fatty acids/lipids, currently employed in industrial processes, are also very volatile. They strongly depend on the oil price and have witnessed a long and steady increase over the past decades. In order to get (more) independent of this big cost factor, a quest for cheap raw materials for PHA production has been set off. Several research groups as well as research-oriented companies have focussed on that strategy, creating a new and vibrant field of research.

1.2 Criteria for Cost-Competitive PHA Production

All potential advantages of PHA are of little value if it cannot be produced at competitive prices. Switching from refined sugars or fatty acids/lipids to surplus or low-cost processing streams is envisaged to reduce costs and thereby aid in the market penetration of PHA materials. However, care has to be taken with the choice of residue used as raw material, the technical layout of the process and superordinate factors in order to actually achieve the goal: a price reduction of the final product.

1.2.1 By-Products as Raw Materials

By-products or waste streams containing fermentable carbon and/or other nutrients emanate from various product routes at every stage of the supply chain, starting from production, transport and storage, up to industrial processing, retail and the end user. They may accrue during food or feed production and consumption (incl. sewage and municipal solid waste), chemical or material production (e.g. biodiesel or wood products), as well as incineration (carbon dioxide).
When evaluating these materials, two characteristics are of central importance: the availability and the composition, both of which will be discussed in the text below.

Availability

It is difficult to identify and quantify the occurrence, composition and amount of these potential low-cost raw materials on a larger scale, as they are neither shown in trade nor in waste statistics. Currently, there is no cross-sectoral reliable data available. Trade statistics available from the FAO only include the primary product of interest, such as sugarbeet, olive oil, and milk.[18] The type and amount of by-products generated throughout the supply chain can only be extrapolated from site-specific data. Waste on the other hand has a strict definition in the EU ("substances or objects that the holder discards or intends or is required to discard" [19]) and is tracked on a regional basis. In Austria, the Federal Ministry of Agriculture, Forestry, Environment and Water Management (BMLFUW) monitors the data on waste accruing in the country according to the "Abfallwirtschaftsgesetz" (BGBl. I Nr. 102/2002) and presents every six years the results in the "Bundesabfallwirtschaftsplan"[20]. In order to identify potentially interesting streams this data is also of limited use, as most by-products have some sort of low-value utilization (spread on land, composting, animal feed) and are therefore do not end up in this statistics. In the case of food waste

for example studies from the UK Department of Environmental, Food and Rural Affairs (DEFRA) showed that over 90% of food waste from DEFRA member manufacturing sites in the UK was utilised in some form. The by-products were used as land spread agents (75%), for animal feed (10%), they were incinerated (4%) or anaerobically digested (1%). They found that only 9% was "waste" and ended in landfill sites.[21]

The food sector is the probably the sector in which most information has been gathered to date. When only taking this single sector in consideration, the amounts of by-products generated are huge. For example, approximately $1.3*10^9$ t of food waste are generated annually across the globe.[22] In comparison, "only" $5.9*10^8$ t of petroleum are used as raw material for the chemical and material industry ("non-energy use" of oil in 2013).[23] Direct comparison is of course misleading as the carbon content is considerably lower in food waste, but the sheer order of magnitudes are astonishing. Figure 1.6 exemplifies the amounts of by-products produced by the food supply chain.

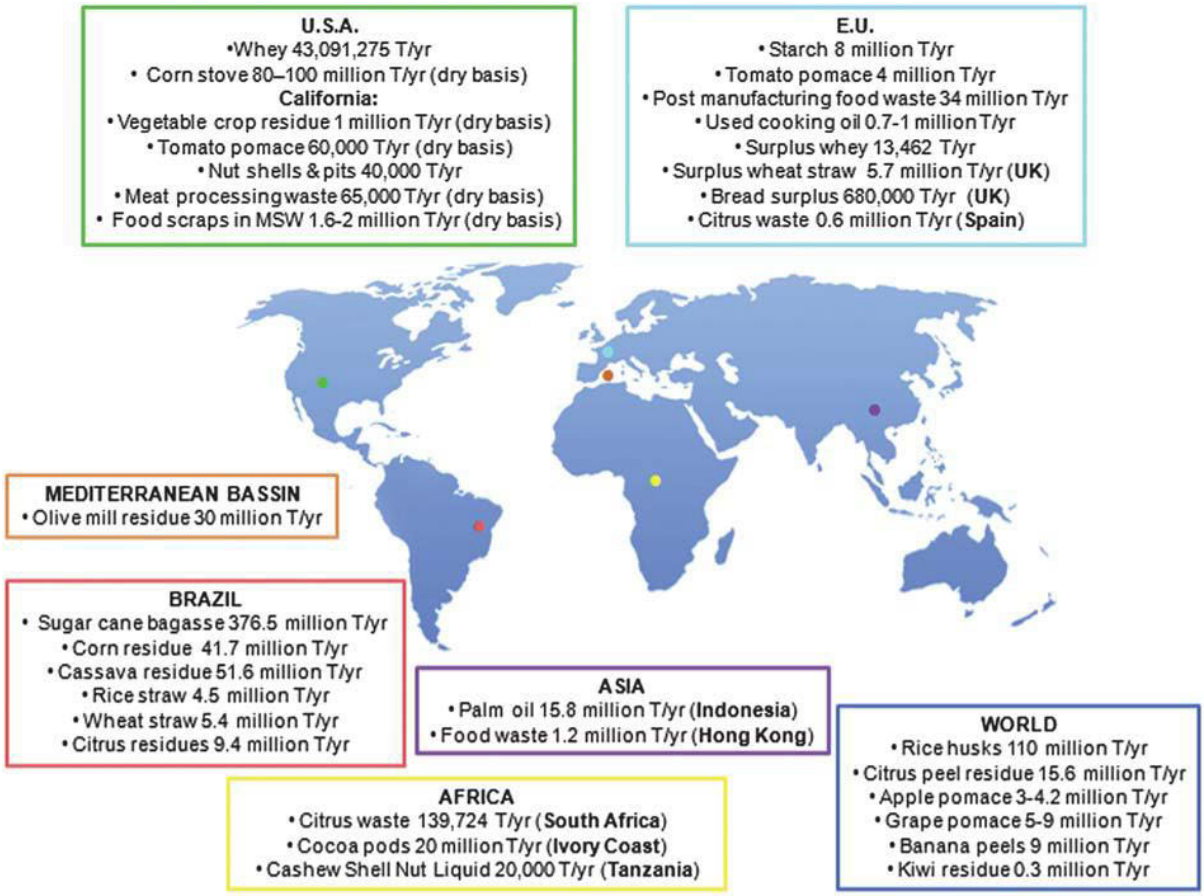

Figure 1.6. By-products of the food supply across the globe.[24]

The potential of by-products as a raw material is tackled in a recent communication of the European Commission. This "EU Action Plan for the Circular Economy" promotes re-use and intends to stimulate industrial symbiosis – turning one industry's by-product into another industry's raw

material. [25] In line with this, in the future more light should be shed on the "grey area" of by-product valorization in order to make them competitive as raw material for manufacturing.[24]

If PHA are to be applied as cost-competitive bulk material, only raw materials which are available in large quantities can be considered. This is due to the economy of scale. In the process industry, the equipment-related capital costs for each unit of product drop dramatically for larger scale operations. It can be described by the power law shown in Equation 1.1, which on average has an exponent of 0.6. In the case of PHA production, economic calculations by Choi *et al*. were done with a targeted amount of $100*10^3$ t/a, although they claim that above a production amount of $15*10^3$ t/a the scaling benefit levels off.[26]

$$New\ Cost = Original\ Cost\ \left(\frac{New\ Size^*}{Original\ Size^*}\right)^{exp}$$

Equation 1.1. Relation of capital costs for scale-up. *: Size or other characteristic linearly related to size. exp: empirical scaling exponent.[27]

The capital cost savings for larger production capacities are counteracted by the increased costs for feedstock transportation, as not enough material can be sourced in the vicinity of the plant. If the raw material has to be obtained farther from the production site, the expenditures for the transport of the raw materials will eventually offset the savings resulting from economies of scale. Agro-Industrial by-products typically have a relatively high water content (e.g. whey) or low density (straw). Unfortunately, there are no model calculations for the economics of transport distances to PHA facilities operating on by-products available. However, they were done for lignocellulosic ethanol plants in the US, where this maximum distance was found to be quite low. The National Renewable Energy Laboratory calculated that a facility requiring 2000 t corn stover per day should only source from a radius of 50 miles (80 km).[27] Consequently, the PHA plant needs to be located in the vicinity to the production sites from which the by-product emerges.

In order to save storage costs, a non-seasonal occurrence (e.g. most animal-derived by-products), is preferable over fluctuating volumes in seasons (e.g. most vegetable-derived by-products). Particularly in the latter case, the by-product needs to be stable so that it does not degrade until it is processed further.

Composition

For fermentation, primarily the carbon – preferrably in the form of carbohydrates or fatty acids – is of interest (see

Table 1.2). To a lower extent also nitrogen or phosphorous in the by-products are relevant too. Other (trace) elements or vitamins may be beneficial for the microbial productivity. Components missing for an efficient metabolism, must be substituted either by other residues [28, 29] or synthetic chemicals.[30, 31] Inhibitors may pose a problem for fermentation, as substances such as furfural, plant alkaloids or phenolics restrain microorganisms. This effect may be relieved by diluting the by-product, using a large inoculum, or adapting the strain to the inhibitory components. The dilution of the by-product has a two-fold benefit for the microorganism: Inhibitor concentrations are lowered and beneficial nutrients may be added to the fermentation broth. A large inoculum increases the initial number of active cells in the fermentation broth which are not inactivated by the first contact with the by-product. Upon subculturing the cells for a long time in the by-product, the microorganisms will adapt to the conditions and exhibit a metabolism which is less disturbed by inhibitors. If only one or more of these strategies are followed, depends on the nature and concentration of the inhibitors. Yu and Stahl tried all three approaches and found that the dry biomass obtained by the using a large inoculum in bagasse hydrolysate is the same as using an inoculum which was adapted in the laboratory to the bagasse hydrolysate. If the hydrolysate was diluted in several steps with mineral medium, the dry biomass concentration reached was lower by 27%.[32] For the long run, the tolerant inoculum is in this case most promising. Nonetheless, for each microorganism and by-product the best method has to be chosen on an individual basis.

Table 1.2. Composition of major by-products interesting for PHA fermentation. Ref: reference number

By-product	Water content (%)	Carbohydrate content (%)	Oil/fat content (%)	Other carbon sources (%)	Protein content (%)	Ref.
Whey	94-95	4.5-5	0.4-0.5	-	0.6-0.8	[33]
Crude glycerol	3-10	-	-	60-90 (glycerol, methanol)	-	[34]
Potato residues	77-85	7-12	0.08-0.6	-	1.2-3.7	[35]
Chicory roots	88	4.6	-	-	0.5	Own data
Wheat straw	6.9-15	56-67	-	-	-	[36, 37]
Woody biomass	20	42-65	-	-	-	[22, 38]

The by-products are often composed of several carbon sources, e.g. mixtures of different sugars or fatty acids. If they are not utilized at the same conversion rate, only the compound with the fastest conversion rate will be fully consumed, whereas the other(s) will accumulate in the reactor. In addition to that, most microorganisms consume different carbon sources in a hierarchical, sequential

manner (catabolite repression, diauxia). Both phenomena lead to a build-up of the less favourable carbon sources. High concentrations of these less favoured carbon sources might in turn inhibit the overall metabolism. Many organisms, such as *Burkholderia sacchari*, preferentially consume glucose, compared to xylose or other pentoses. In order to overcome this issue, induced mutations (by UV or genetic engineering)[39] or the addition of certain trace elements might be a by-pass.[40]

Another issue of by-products as raw materials which requires attention, is that physical properties and chemical composition fluctuate depending on the origin of the raw material, as well as the exact process conditions during which the by-product is generated. This varying nature of the raw material may have a significant influence on the produced polymer and its properties. To date this issue has not been covered well in the body of literature and needs to get better investigated.[41]

Last but not least, the harvest practices may significantly reduce the amounts available for bioprocessing. In the case of lignocellulosic raw materials, 50% or more of the lignocellulosic wheat straw residues are estimated to be required for soil conservation. Only the remainder may be available for industrial purposes.[36]

1.2.2 Upstream and Downstream Processes

Effective microbial production of PHA is strongly influenced by potential pre-treatment processes of the feedstock and the PHA-production process itself. Furthermore, the isolation and purification of the polymer also play a very important role. All three steps are important for a robust large scale, consistent industrial processes and are discussed below.

Pre-treatment

Some by-products, such as whey, may be used directly for PHA production.[29] Others, particularly lignocellulosic feedstocks, require pre-treatments in order to extract and break down the carbohydrates.[42, 43] The development of pre-treatment methods has been pressed ahead by the second-generation biofuel initiatives. In the last decade many processes have been developed and improved. Physical disintegration, such as milling [32] is used to increase the surface area of the substrate. Chemical and biochemical treatments break down carbohydrate polymers and release mono- or oligomeric sugars. Examples are ammonia fiber expansion (AFEX) [39], dilute acid [32] or enzymatic hydrolysis [39]. Often several technologies are combined in consecutive steps. The choice of pre-treatment has significant effects on all downstream processes. Consequently there is not a single "perfect" pre-treatment process for all types of feedstock and end products. It has to be chosen case-by-case.

Conversion yields reduce the available carbon. For example, a dilute acid pre-treatment of the hemicellulosic fraction of corn stover yields 90% of the original sugars.[27]

PHA Fermentation

In order to get to an industrially feasible PHA fermentation process, high yield, high productivity, and high intracellular polymer content are crucial.

A high yield primarily affects the costs of the substrate, as more carbon is consumed and not wasted. The PHA production itself has a theoretical yield of 0.48 g P3HB/g glucose (or similar carbohydrates) as two carbon dioxide molecules are lost upon the formation of the intermediate acetyl-CoA.[44] In the literature, practically attained yields of 0.3-0.4 g scl-PHA/g hexose are reported, due to the formation of non-PHA cell mass.[45, 46] If hemicellulosic sugars are used for PHA production, practically attained yields are lower (0.2-0.3 g scl-PHA/g sugar), i.e. more raw material is required to generate the same amount of polymer.[39, 47]

Substrates, which are more reduced than carbohydrates, such as triglycerides or fatty acids, have higher yields. They do not lose CO_2 upon formation of acetyl-CoA, but retain all carbon of the starting material in the final polymer. The theoretical yield of a C18 fatty acid is 1.38 g P3HB/g carbon source.[48] In practice, yields of 0.6-0.8 g PHA/g fatty acid or triglyceride have been reported in the literature.[49, 50] From this perspective, reduced substrates have a higher potential for utilization in PHA production processes. There are attempts to use fat-derived raw materials. However, carbohydrate-based by-products, are often much more abundant and therefore more readily available for bioprocessing.

The productivity has the largest effect on the equipment related-costs, which is the second large cost factor besides expenses for raw materials.[26] In order to achieve high productivities, an efficient strain grown to high cell densities is necessary. In the literature productivities in the range of 2-5 g/L/h are regarded as high. Four organisms are amongst this premium league of PHA production. All of them, except *Escherichia coli* are intrinsic PHA producers and do not need genetic modification, although in some cases also recombinant versions of *Cupriavidus necator* are used (see Table 1.3).

Concerning process control, the batch process is the simplest and most robust setup. However, the productivities reached are inherently low as only the carbon from the batch medium is converted into product. In order to supply more carbon, fed-batch or continuous process modes need to be employed. Fed-batch processes are the most frequent mode of highly productive PHA fermentations. They require highly concentrated feed streams which is often not the case for agro-residues. Upon (thermal) up-concentration, inhibitors which have been present beforehand are up-concentrated too (e.g. phenolic compounds) as well as new inhibitors are formed (e.g. sugar decomposition products such as hydroxymethylfurfural).[39, 51] Continuous processes can be run at high productivities too, but

they also require quite high carbon concentrations in the feed. Furthermore, it is not possible to ensure distinct nutrient conditions, which are necessary during the two phases of growth and PHA production in a continuous stirred tank reactor (CSTR).

Most highly productive PHA fermentations are still conducted with synthetic media. However, due to research efforts which are detailed in chapter 1.2.3 on page 24, the productivities achieved with industrial by-products have been increasing in the last decade. Nonetheless, in the case of by-products productivities above 1 g/L/h can be regarded as high, as they have been reached only a couple of times yet.

In contrast to the other two factors yield and productivity, the PHA content has multiple effects on the process economics.[26] At a high PHA content, the portion of residual cell mass that is formed from the raw materials is low, thereby positively affecting the yield. Furthermore, the PHA content strongly influences the polymer isolation and purification steps. The reason for this is that at higher intracellular polymer loads the extraction efficiency is increased, which in turn reduces the amount of chemicals necessary for the extraction.

The intracellular polymer content depends primarily on the type of organism used. Some organisms stop producing the polymer already at rather low contents, other reach very high polymer contents. For example *Burkholderia sacchari* reaches 40-70% of polymer content.[39, 52] Intracellular polymer contents of 70-90% are typically attained using *Cupriavidus necator* or recombinant *Escherichia coli*, the best established strain in the literature.[53, 54]

Product Isolation and Purification

After the polymer is produced, it needs to be isolated and purified. Several methods are available for this purpose, such as solvent extraction by halosolvents and nonhalosolvents or digestion of non-polyhydroxyalkanoate cell material involving surfactants, sodium hypochlorite or enzymes.[11, 55] The established methods give good purity (>90%) and yield (>90%), but differ in their effect on the polymer (e.g. molecular weight). The halosolvent extraction is the oldest and best established method. Environmentally it is worrisome, particularly at industrial production facilities.

Table 1.3. Production of PHA with different microorganisms. Only publications with productivities higher than 2 g/L/h are listed when synthetic media are used as carbon source. For the relevant by-products, only the best fermentation done on this by-product is listed. The by-products are marked in red. **Bold font** highlights the publications of the author. (a) During this batch fermentation no feed was added, so the concentration in the hydrolysate is given.

Organism	Fermentation strategy	PHA type	C-source	Concentration of carbon source in feed (g/L)	Cells (g/L)	PHA (g/L)	PHA (%)	Productivity (g/L/h)	Yield (g PHB /g carbon source)	Reference
Azohydromonas lata	fed-batch	P3HB	sucrose	900	143	71	50	3.97	0.17	[56]
Azohydromonas lata	fed-batch	P3HB	sucrose	900	111	99	88	4.94	n.a.	[57]
Burkholderia cepacia	fed-batch	P3HB	sugar maple hemicellulosic hydrolysate (xylose)	72	17	9	51	0.09	0.19	[58]
Burkholderia sacchari	fed-batch	P3HB	saccharified concentrated straw hydrolysate (glucose, xylose)	840	146	105	72	1.60	0.22	[39]
Cupriavidus necator	**batch**	**P3HB**	**saccharified chicory root hydrolysate**	**34** [a]	**14**	**11**	**78**	**0.15**	**0.38**	**[59]**
Cupriavidus necator	fed-batch	P3HB	crude glycerol	1174	69	26	38	0.84	0.34	[60]
Cupriavidus necator	fed-batch	P3HB	saccharified waste potatoe starch hydrolysate	550-650	179	94	55	1.47	0.22	[61]
Cupriavidus necator	fed-batch	P3HB	glucose	650	164	125	76	2.03	n.a.	[62]
Cupriavidus necator	fed-batch	P3HB	glucose/propionic acid	700	158	117	74	2.55	n.a.	[63]
Cupriavidus necator	fed-batch	P3HB	glucose	700	208	139	67	3.10	n.a.	[64]
Cupriavidus necator	**cell recycle fed-batch**	**P3HB**	**glucose**	**50**	**148**	**113**	**76**	**3.10**	**0.33**	**[65]**
Cupriavidus necator	fed-batch	P3HB	glucose	800	281	232	82	3.14	0.38	[46]
Escherichia coli	fed-batch	P3HB	glucose/thiamine	700	156	89	57	2.40	n.a.	[66]
Escherichia coli	feed-batch	P3HB	concentrated whey solution	280	120	96		2.57	n.a.	[51]
Escherichia coli	fed-batch	P3HB	glucose/thiamine	700	204	157	77	3.20	0.28	[67]
Escherichia coli	cell recycle fed-batch	P3HB	concentrated whey solution	280	194	168	87	4.60	n.a.	[54]
Escherichia coli	fed-batch	P3HB	glucose/thiamine	700	194	141	73	4.63	n.a.	[68]
Methylobacterium organophilum	fed-batch	P3HB	methanol	conc.	250	130	52-56	1.8-2.0	0.19	[69]

1.2.3 Integration into the Techno-Economic Environment

In order to push PHA production towards economic viability, it cannot be industrialised without giving close attention to the techno-economic environment, e.g. competitive uses of raw materials or resources such as land.

About a decade ago the intensification of the biofuel production triggered a discussion of land usage ("food vs. feed"). Since then, every new biobased product has to put its land usage into a global perspective. In the case of bioplastics currently only about 0.01 percent of the global agricultural area is required to grow sufficient feedstock for today's bioplastics production (see Figure 1.7). This current land use for the bioplastic production is not significant. However, upon the transition to a non-fossil based economy in the future, bioplastics needs to replace petroleum derived plastics which increases this number 500-fold. This corresponds to a land use of 5% of the global agricultural area or 15% of the global arable land, which is inacceptable from an ethical point of view. In line with this, the substitution of petrochemical derived plastics by bioplastics is only feasible if significantly less land is required. If by-products are used as carbon source, in fact no additional land is required. Only indirect effects have to be considered. For example, if all straw was diverted to bioplastics production, other products would be required as stable litter. With these products also land usage might be connected, e.g. forest area in the case of wood chips.

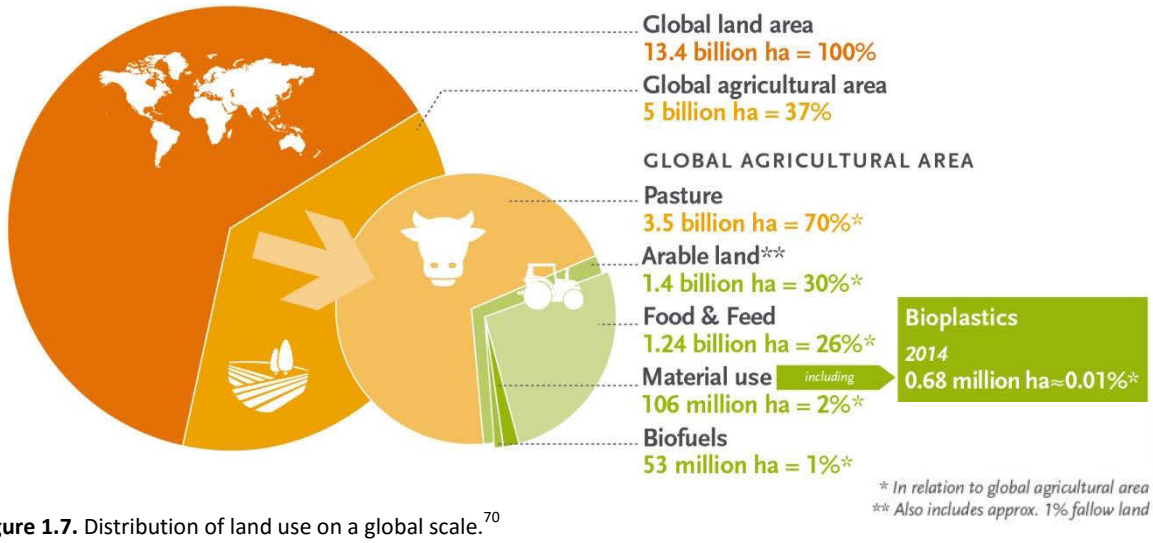

Figure 1.7. Distribution of land use on a global scale.[70]

By-products are a resource that can be converted to all kinds of different products. In fact, industrial by-products are seen by a growing body of stakeholders as platform resources for a biobased society.[41] This increases the competition for the by-products and favors the most efficient utilization strategy. For the long-term success of PHA from such by-products, these by-products need to be the most competitive utilization strategy. The most important concept regarding the use of by-products

as well as virgin biomass is cascadic use. This means that several (bio)processes are integrated into a holistic utilization of biomass, a so-called biorefinery. The biorefinery is described as "integrated biobased industry using a variety of technologies to make products such as chemicals, biofuels, food and feed ingredients, biomaterials, fibres and heat and power, aiming at maximizing the added value along the three pillars of sustainability (Environment, Economy and Society)".[71] In line with this, PHA production would be one step in the cascadic utilization of biomass, just as it is the case for the current plastic manufacturing in the oil refinery. For example, the BIOCYCLE biorefinery in Serrana (Brazil) shown in Figure 1.8 converts sugar cane into sugar (sucrose), ethanol and P3HB.[45] In this biorefinery the by-product molasses, accruing at the sugar crystallization step, is converted into P3HB. The polymer is extracted from the cells using a by-product from the ethanol distillation: long-chain alcohols. The process energy is provided by burning the by-product bagasse. In this optimized system, the polymer can be produced at low costs and adequate purity.

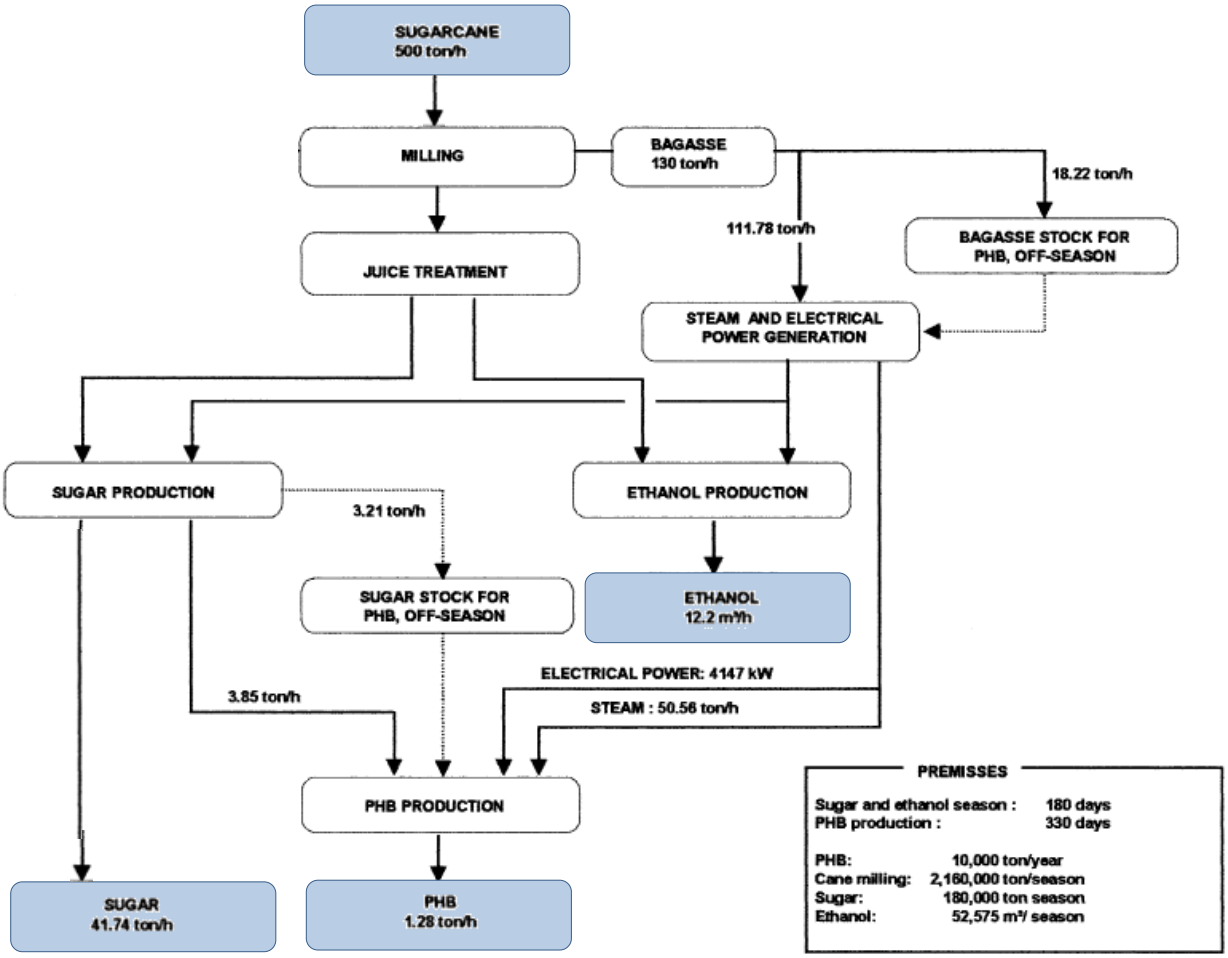

Figure 1.8. Example of a biorefinery: the BIOCYCLE biorefinery in Serrana (Brasil). The raw material sugar cane is processed completely to obtain sugar, ethanol as well as PHB. Bagasse is used for the generation of process energy. Modified from [45]

Biobased products have – particularly in Europe – a very positive image among end-consumers, who are most of the time also willing to pay a "green premium".[72] However, at the current development stage of PHA production, most biorefineries need some legislative and/or infrastructure support. Biofuels were the first biobased product which received very strong political support in the early 2000s. However, the expected ecological benefits of biofuels, are still debated in the scientific community.[73] This led to a reluctance of political support for biobased products without sound evidence of an improved environmental performance. Policy makers seem to get more in favour of quantifying the environmental sustainability.

The methodologies for the assessment of the environmental sustainability are still under development and heavily debated in the community.[74] Nonetheless, the European Union is pushing forward the development of a "product environmental footprint" PEF, a harmonised methodology for the calculation of the environmental footprint of products. [75]

One of the parameters assessed in PEF is the emissions of greenhouse gases, which is also one of the most important parameters investigated by sustainability benchmarking in general. Surprisingly, when comparing the greenhouse gas emissions, Endres *et al*. found that most plastic materials (biobased as well as conventional petrochemical plastics) have similar carbon dioxide emissions.[3] At first glance this finding is unexpected, because – unlike oil-derived carbon – carbon originating from renewable sources does not add to the carbon footprint. However, this comparison is based on data from vastly different levels of industrialisation. The data for conventional petrochemical plastics is derived from full scale industrial plants, whereas all data for bioplastics is at the moment only available for semi-industrial or laboratory-scale experiments. Upon scaling up, the greenhouse gas emissions are expected to decrease significantly.

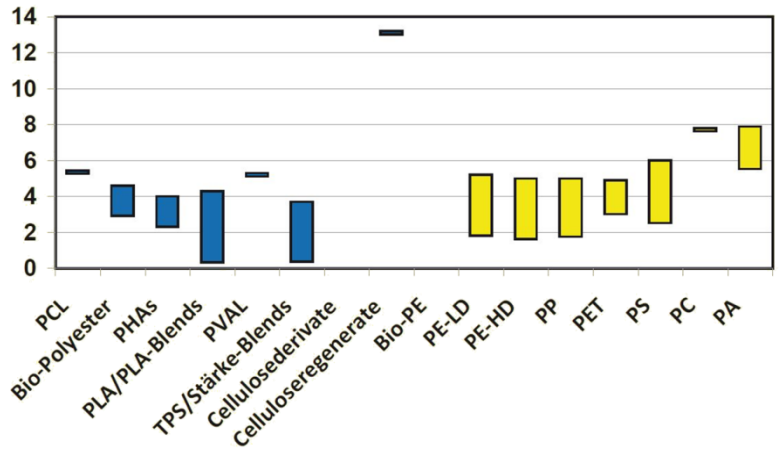

Figure 1.9. Greenhouse gas emissions (kg CO_2 eq./kg polymer) of biobased or biodegradable plastics (blue) vs. conventional petrochemical plastics (yellow). The data for the conventional oil based plastics is from full-scale industrial sites. The data for the biobased or biodegradable plastics is mainly from semi-industrial or laboratory-scale experiments.[3]

The current perception of two stakeholders, the production industry itself and the public, are also of key importance. The industry shows a strong affinity towards “drop-in” solutions, i.e. exchanging petroleum based polyethylene against biobased polyethylene which both have exactly the same properties and applications. In line with this, bio-polyethylene is biobased, but like the petrochemical polymer not biodegradable. A new feature, such as biodegradability seems to be distrusted by industrial stakeholders. In this case, the push for a more complete picture given by sustainability indexing is expected to aid in the market penetration of truly biobased, biodegradable and overall sustainable plastic materials.

The second stakeholder, the public, shows a volatile attitude towards using by-products or “waste” as a resource for products applied in their vicinity. The properties associated with the by-products, such as disgust, might get associated also with the new product. Many consumers do not understand, that (bio)chemical conversion changes the properties of the raw materials beyond these associations. For example, the food ingredient Vanillin is produced from wood as well as recycled paper.[76, 77] For many consumers it is difficult to understand, that both processes yield pure vanilla flavour, which is neither ligneous nor dirty. The most promising solution to this issue is proactive marketing, which needs to be done on the product-level by the producing companies or professional interest groups. In addition to that, governments need to provoke a more positive image on the recycling of by-products in order to eventually head for a so-called “circular economy”.

1.3 PHA from Agro-Industrial By-Products

As discussed, the production of PHA from by-products is a viable option to enhance the sustainability as well as economics of plastics. In this chapter, more detailed data on specific residues are provided. The by-products used for PHA production can be grouped into four categories: carbon dioxide, sewage/sewage sludge, lignocellulosic biomass and agro-industrial processing residues. The first two are interesting as they come at no costs (apart from possible transport costs). In the case of sewage/sewage sludge, the concentration of utilizable carbon is in the order of up to several grams per litre, i.e. very low. In order to treat this waste and concomitantly produce PHA, there are special PHA production techniques being developed. In these processes, natural PHA producers, which are already present in sewage, are selected by nutrient conditions in an unsterile open fermentation process. A completely different approach is the use of carbon dioxide. Carbon dioxide is in a very deep energetic valley, from which it can be lifted by the action of sunlight, i.e. by photosynthesis, or energy-rich chemicals such as hydrogen. Both strategies are investigated by several research groups around the world, but these approaches are at early basic research stages. Accordingly, they are far from being commercially feasible and are discussed in specialized reviews.[6, 78]

Lignocellulosic by-products and agro-industrial processing residues do have a price, but due to their surplus availability, this price is comparably low. Furthermore, their carbon concentration is high enough to be suitable for pure-culture heterotrophic PHA fermentation conditions. This PHA fermentation strategy is efficient and well established for the use of refined, highly concentrated, carbon sources as detailed in chapter 1.2.2 on page 20. The by-products offer advantages like easy processing (whey), high carbon content (glycerol) or vast untapped availability (straw). This study and the conducted research focuses on the use of such promising agro-industrial processing residues for the production of PHA. In order to provide the reader with the status quo on the productions of PHA from these by-products, the properties of selected by-products and results from PHA fermentations are discussed in the following sections. Interested readers may also have a look at other recent reviews on the topic.[41, 79, 80]

1.3.1 Lignocellulosic Raw Materials

Straw originating from Wheat, Rice or other Grasses

In 2013 about $713*10^6$ t of wheat and $745*10^6$ t of paddy rice were produced globally.[18] Around 150% of this weight accrue as straw,[42] of which 15-40% are not required for soil conservation or livestock feed.[81] This corresponds to 328 - $874*10^6$ t/a of straw which could enter (bio)chemical production processes. The dry straw of both plants is composed of cellulose, hemicellulose, lignin and ash, i.e. silica as shown in Figure 1.10.[36, 37] In order to extract and break down the carbohydrates, extensive pre-treatments are necessary. Along with the advent of second generation biofuels, a lot of effort was put into the development of pre-treatment methods. In 2013, a crucial milestone was achieved, as the first commercial ethanol plant based on wheat straw and giant reed started operating in Crescentino, Italy, ($56*10^3$/a ethanol).[82] Nonetheless, the pre-treatments are still a difficult issue for the biotechnological utilization of straw as well as any other lignocellulosic feedstock.

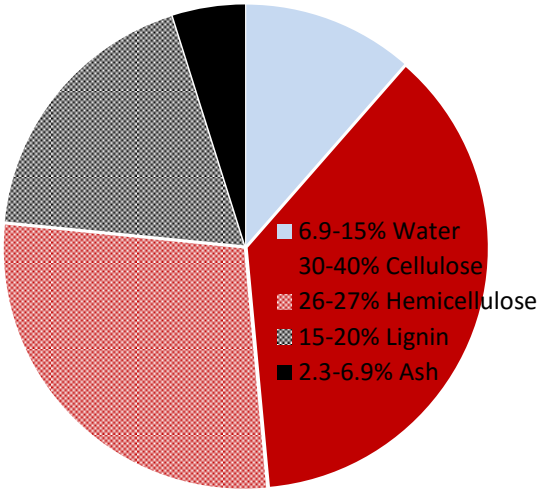

Figure 1.1. Composition of wheat straw.[36, 37]

In the scientific literature, Cesario *et al*. obtained the best results on concentrated wheat straw hydrolysate. Using *Burkholderia sacchari* they were able to reach a biomass concentration of 146 g/L containing 72% P3HB at a productivity of 1.6 g/L/h (see Table 1.3).

In the patent literature only two expired patent applications were found producing PHA from straw. In the first one, cellulose from crop straw residues is hydrolysed and converted to PHA by *Cupriavidus necator* (CN101812482 (A) filed on the 25th of August 2010 [i]).[83] In the second patent, rice straw, corn stover or distillers grains are used as carbon source (US2015018573 (A1) filed on the 15th of January 2015).[84] They claim the use of mixed microbial communities for the production of high-value specialty chemicals, such as PHA, in conjunction with lower value fuel compounds in order to improve the overall economics of the biorefinery unit.

[i] The letter "A" in brackets after the patent number designates the document as patent application. After the filing date (25th of August 2010), the applicant has one year to complete the patent in order to get full patent protection for his/her invention.
In this thesis, preferentially patent applications which are still under preliminary protection and full patents (letter "B" or "C" in brackets) are cited. Preliminarily protected, but expired applications are omitted if full patents are available.

Wood Biomass

According to the FAO, 3 700*10^6 m^3 of round wood have been produced worldwide in 2014, of which 6% (223*10^6 m^3) end up as wood residues.[85] These by-products are sawmill rejects, slabs, edgings and trimmings, veneer log cores, veneer rejects, sawdust, residues from carpentry and joinery production. The amount of wood chips and bark are not included. The major utilisation of wood residues is heat and power generation either on-site or in other plants as solid fuel. Besides this, they are used for the production of particle boards, fiberboards and wood pellets.[22] Wood residues have similar composition as the inner part of a tree, i.e. consist of cellulose, hemicelluloses, and lignin as shown in Figure 1.11.[22, 38] The structure of cellulose and lignin are species-independent, whereas the monomeric sugars in hemicellulose differ between hard and soft wood. Wood residues have only a low water content and are generally, a clean solid uniform material without impurities. Along with the advent of second generation biofuels, the utilization of this enormous biomass reservoir was investigated. However, the higher lignin content and lower surface area compared to straw residues, led to difficulties in the extraction and hydrolysis of fermentable carbohydrates.[36] Despite a lot of research efforts in the field of pre-treatments, to date the conversion of woody biomass into cost-competitive products such as ethanol is an economically challenging process.

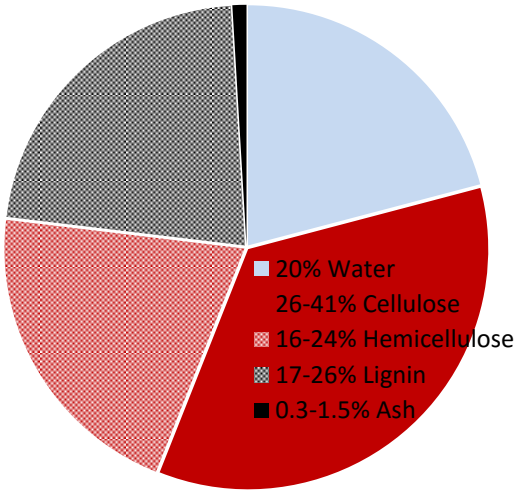

Figure 1.2. Composition of round wood.[22, 38]

The knowledge and innovation gap on the pre-treatment is also reflected by the literature on PHA production from woody biomass.[43] The best results so far have been obtained by Pan *et al.* who generated a maple hemicellulosic hydrolysate (containing 72 g/L xylose), which was utilized as feedstock for PHA production (see Table 1.3).[58] In order to increase the fermentability, several detoxification methods were tested. The highest removal of total inhibitory phenolics, arising from lignin breakdown, was achieved with overliming combined with low-temperature sterilization. In a fed-batch fermentation using *Burkholderia cepacia*, a biomass concentration of 17 g/L containing 51% PHA was reached. A productivity of 0.09 g/L/h is low, but could be improved by optimization of the feeding strategy, according to the authors.

In the patent literature one active patent was found, describing the use of saccharified wood for the production of PHA by *Halomonas* sp KM-1 (JP5299969 (B2)).[86]

1.3.2 Agro-industrial processing residues

Whey

Cheese whey is the liquid remaining after the precipitation and removal of milk casein during cheese-making. This by-product represents about 85-95% of the milk volume used for making cheese, i.e. around $185*10^6$ t/a whey are generated globally (according to the FAO $20.6*10^6$ t/a cheese were produced worldwide in 2012 [18]). The majority of nutrients (55%) are retained in the whey. It contains lactose, soluble proteins, lipids and mineral salts as shown in Figure 1.12.[33] The low concentration of sugar in whey makes a concentrating step necessary in order to get a feasible feed solution for highly productive fermentations.

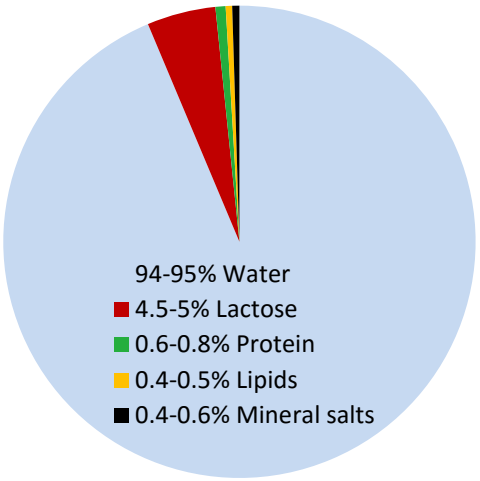

Figure 1.3. Composition of Whey.[33]

Currently about half of the total whey produced is transformed into value-added products such as whey powder, whey protein, lactose for food and feed purposes.[87] The remaining half is directly disposed, which is of environmental concern as well as questionable from an economic perspective. In line with this, whey is a prime target for developing new applications such as serving as a feedstock for fermentative processes.

This residue has been used in 110 research publications[ii] and was also used in the scientific reports achieving the highest PHA productivities done with by-products. *Eschericia coli* is an excellent lactose utilizer and when equipped with the correct genes (from *Azohydromonas lata*) a productivity of 2.6 g/L/h was reached in fed-batch experiments by Ahn *et al.*[51] (see Table 1.3). They tried to increase the cell concentration, but did not succeed due to the dilution effect caused by nutrient feeding. Lactose in whey has a maximum solubility of 280 g/L, causing a certain dilution upon addition to the bioreactor. In order to overcome this issue, the same authors applied a cell-recycle fed-batch fermentation strategy.[53] Using this cell-recycle approach, they almost doubled the biomass concentration (194 g/L) which contained 73% P3HB. Overall, a remarkable productivity of 4.6 g P3HB/L/h was reached, the highest reported to date using industrial by-products. These two publications clearly demonstrate a great potential for this by-product as PHA fermentation substrate. The method for doing so was patented by the group author in two patents (KR100241183 (B1)[88] and KR100199993 (B1)[89]).

[ii] Keywords "whey" and "polyhydroxy* OR PHA OR P3HA OR poly3* OR poly(hydroxy*)") were searched in the Web of Science search engine. All documents except research articles were excluded from the search done on the 5th of August 2016.

Recently, two other patents were filed for PHA production from lactose and / or whey. The first outlines the use of a recombinant strain of *Haloferax mediterranei*, not able to synthesize exopolysaccharides, for the production of P3HB3HV (CN103451201 (B)).[90] The carbon sources for the organism are specified as glucose, starch or whey. The second patent deals with an hitherto unknown PHA producer (*Caulobacter segnis*), which produces PHA from lactose (EP2987862 (A1)).[91]

Crude Glycerol

Transesterifications of triglycerides during the soap and biodiesel production release the by-product glycerol. Since the early 2000s the global production of biodiesel has witnessed a tremendous increase in volumes ($0.7*10^6$ t/a in 2002 to $1.5*10^6$ t/a in 2011)[34] and thereby generated a lot of surplus glycerol. This "crude glycerol" contains mainly glycerol, but also significant amounts of methanol, salts and water (see Figure 1.13). It is sold at significantly lower prices than refined glycerol (100 vs. 750 US$/t in 2011).[34] The concentration of glycerol, the main carbon source, is high enough in order to serve as feed in fed-batch experiments.

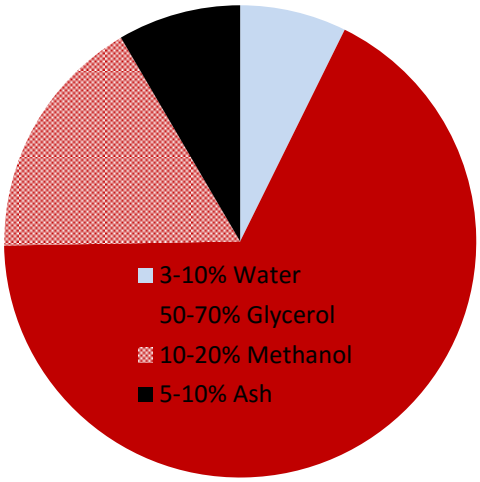

Figure 1.4. Composition of crude glycerol.[34]

Crude glycerol can be refined and used in many different products in the food and personal hygiene sector.[92] However, all current applications are rather low in volume and cannot valorise all the surplus glycerol. In line with this, new valorisations routes – particularly as raw material for (bio)chemistry – are under development.

In the scientific literature, refined glycerol has been used several times as carbon source for PHA production.[62, 93] The PHA productivities obtained with waste glycerol tend to be lower than when using refined glycerol. Cavalheiro *et al.* compared both carbon sources using *Cupriavidus necator* DSM 545 in fed-batch processes (see Table 1.3).[60] They achieved to date the best PHA process parameters for glycerol in the published literature. With pure glycerol, they obtained a maximum dry biomass concentration of 82.5 g/L containing 62% P3HB (productivity 1.52 g P3HB/L/h). When crude glycerol was used, less biomass (68.8 g/L) with a lower P3HB content (38%) was reached (productivity 0.84 g P3HB/L/h). This corresponds to a decrease in productivity by 45% if crude glycerol is used as carbon source. An inhibition due to methanol can be excluded, as it was removed prior to the fermentations. The authors speculate that sodium ions from the crude glycerol slowly accumulate throughout the fermentation and inhibit the process.

In the patent literature, 7 full patents or active patent applications have been found using glycerol as carbon source for PHA production. One of them specifically mentions the use of crude glycerol as carbon source (US8956835 (B2)).[94] Another three patents use glycerol as carbon source but do not specifically mention the use of crude glycerol (DE19633858 (C2)[95], US9169501 (B2)[96], and WO2016021604 (A1) [97] filed on the 11th of February 2016). Three patents generate recombinant strains which are able to use glycerol for biomass and PHA formation (CN103497922 (B)[98], US8609378 (B2)[99], and KR100454250 (B1)[100]).

Fruit and Vegetable Processing Residues

This group of residues is very heterogeneous in terms of composition and availability, so that in most cases the specific residues have to be discussed separately. Two promising residues, potato processing by-products and chicory roots have been chosen and will be discussed below.

Potatoes are one of the most commonly consumed vegetables. In Europe they are also grown for the production of food as well as non-food starch. During the processing of potatoes large amounts of by-products are generated due to sorting out, washing, peeling or size reduction, resulting in residual streams with a high organic load. On average 80% of the original potato are found in the by-products, such as potato peels and, pulp and slurry.[22] Their primary constituent (apart from water) is starch, which is easily fermentable (see Figure 1.14).[35] A disadvantage of this residue is the seasonal occurrence and that it can only be stored if the water is removed.

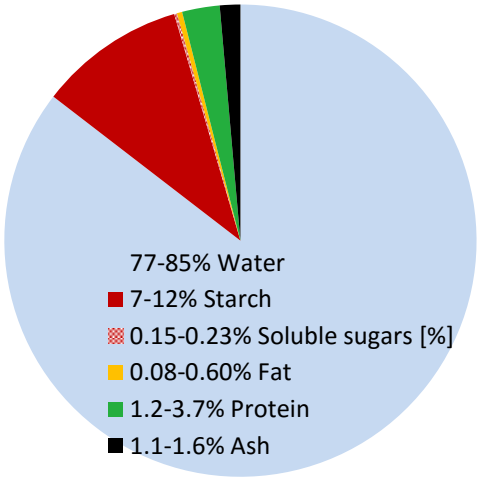

Figure 1.5. Composition of potato peel residues.[35]

The group of Florian Zepf was able to convert waste potato starch into PHA using *Cupriavidus necator*. They obtained a dry biomass concentration of 179 g/L containing 55% P3HB.[61] This concentration was reached within a short time, giving an excellent productivity (1.47 g P3HB/L/h) and a reasonable yield (0.22 g P3HB/g starch) (see Table 1.3).

No patents on the use of potato processing residues has been found in the literature.

In the course of the CARBIO project, the underlying project for this thesis, potato residues were identified as promising substrate for the production of lactic acid. The two publications emanating from these results were done in close cooperation between the author of this thesis and another PhD student in the same working group (Marina Smerilli). These publications are co-authoships and

are therefore not discussed in detail in the following chapters, but are listed in the publication enumeration in chapter 7.1 on page 82.

Chicory (*Cichorium intybus*) is a very versatile plant as its varieties are either grown for the production of salad, inulin or coffee-substitute.[101] The leafy salad (also called Belgian endive, chicon or witloof) is grown in large quantities in Belgium, the Netherlands, France and Spain.[18] Alongside the chicory production, the same amount of roots accumulate, which are currently used as animal feed or are composted. The composition is shown in Figure 1.15 However, the nutritional value of the roots after the witloof production is limited because inulin, 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.[102, 103] However, the fructose polymer inulin can be degraded and used as carbon and energy source in fermentations. The roots after the hydroponic cultivation are not stable when stored at ambient temperatures, but they are produced continuously throughout the year and consequently could be fed continuously into a biotech process.

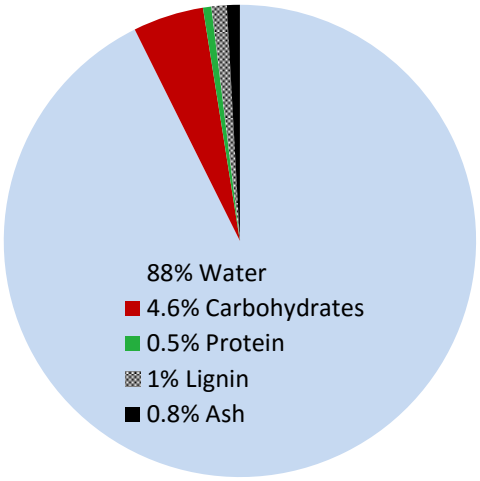

Figure 1.6. Composition of chicory roots after hydroponic cultivation. Own data

Although the material is promising, there has been no report in the literature on the utilization of the roots for PHA production. In order to fill this gap in knowledge and make use of their favourable composition, spatial and temporal abundance, they were chosen as ideal substrate for PHA production in the CARBIO project (see chapter 3).

No patents have been filed yet on the utilization of chicory roots in the corresponding literature.

2 OBJECTIVES

The work within this thesis is divided into two phases and two general objectives. In the first phase, the aim was to evaluate and establish a laboratory-scale process for the production of PHA from chicory roots which can be applied by the Spanish waste company TRASA (tratamientos subproductos agroalimentarios S.L.). The company manages and treats by-products from the fruit and vegetable processing industry in the Ebro valley in northern Spain. Chicory roots are there an abundant by-product (10 000 t/a), which contain a reasonable amount of easily degradable carbohydrates (3.3 % w/w inulin in fresh weight). The roots degrade within weeks, but accrue throughout the year and can be processed continuously. In order to do so, the following scientific objectives had to be tackled (see Figure 2.1):

- Establish a pre-treatment protocol to extract and hydrolyse inulin from the chicory roots.
- Test the ability of several PHA-producing microorganisms to degrade inulin as well as its degradation products glucose and fructose, since little knowledge is available comparing different strains.
- Develop a fermentation process for the production of PHA. Investigate the effect of detoxification on the fermentation performance.
- Evaluate the produced polymer by extracting and characterizing it in relation to physical and thermomechanical properties.

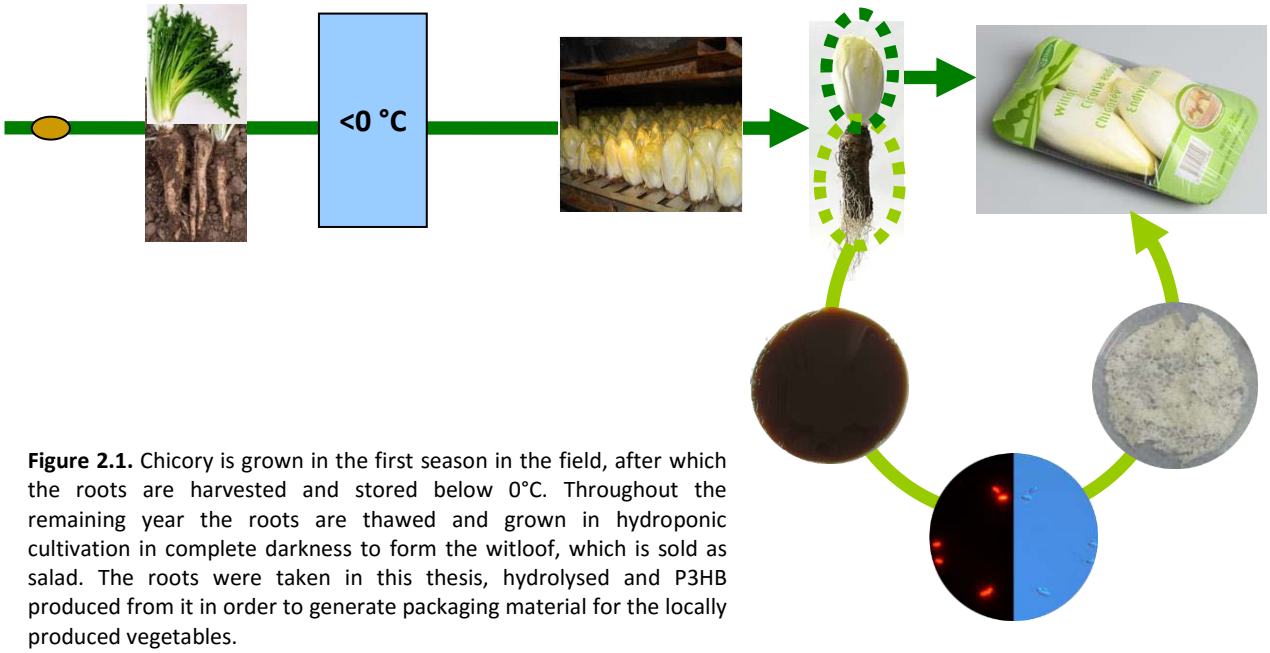

Figure 2.1. Chicory is grown in the first season in the field, after which the roots are harvested and stored below 0°C. Throughout the remaining year the roots are thawed and grown in hydroponic cultivation in complete darkness to form the witloof, which is sold as salad. The roots were taken in this thesis, hydrolysed and P3HB produced from it in order to generate packaging material for the locally produced vegetables.

With the experience of this process development in mind, the main limitation of this by-product – a low concentration of fermentable carbohydrates – was tackled in the second part of the PhD project. This low concentration of carbon is a common feature of many by-products, such as whey and straw hydrolysate. However, it renders the classical fed-batch processes unfeasible. In the literature there are reports on concentrating the carbon by the evaporation of water in order to regain the ability for fed-batch processes.[39, 51] However, these approaches suffer from the co-concentration of inhibitors, such as small organic acids or aldehydes which decrease the fermentation performance. In line with this, the aim was to develop a highly productive P3HB production process using a model substrate with a low carbon concentration. This was done via a membrane bioreactor, though which the by-product is passed through without previous up-concentration steps. This process development was done with a model substrate as the aim was the layout of a generic process, which is in the future applicable for real by-products. In order to do so, the following objectives were targeted:

- Develop the technical setup of a membrane bioreactor. This includes the evaluation of different membranes and cross-flow systems as well as operational parameters.
- Establish a highly productive fermentation procedure for the production of P3HB in the bioreactor.

3 PRODUCTION OF P3HB FROM CHICORY ROOTS

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Contribution of the author:

The author conducted all hydrolysis experiments as well as the shaking flask experiments herself. The experiments in the bioreactor as well as the polymer extraction were conducted in part by the author. The author was supported by a masters student (Valentin Steinwandter) concerning the practical work for the bioreactor experiments, whom she supervised for these experiments. All conceptual work for the experiments was done by the author. Furthermore, the publication was written and revised by the author.

3.1 Abstract

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) (P3HB). 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 sugars and 0.7 g/L total Kjeldahl nitrogen. This hydrolysate was used as fermentation substrate for three P3HB-producing strains. Cupriavidus necator DSM 428 reached a dry biomass concentration of 11.3 g/L with a P3HB content of 66 % in dry mass within 5 days. C. necator DSM 531, yielded 3.5 g/L dry biomass containing 46 % P3HB within the same period. C. necator DSM 545 was superior over the other two in that 14.0 g/L of biomass containing 78 % P3HB 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 P3HB-production.

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

3.2 Introduction

Renewable resources constitute a 'limited infinity', meaning that a limited amount is being produced, but for an infinite time.[104] 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.[22]

Chicory (*Cichorium intybus*) is a very versatile plant as its varieties are either grown for salad, inulin or coffee-substitute production.[101] The leafy salad (also called Belgian endive, chicon or witloof) is predominantly grown in Belgium, the Netherlands, France and Spain.[18] In Spain the major growing region is Navarra, where 13 000 tons of chicory are produced by hydroponic cultivation per year.[105] The production is not seasonal as the salad is consumed throughout the year. Alongside the chicory production, 10 000 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 which 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 [102, 103]

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.[106] An integrated process in which the inulin is extracted/hydrolysed and the hydrolysate is directly used for fermentations is economically more viable.[107] However, there have been no investigations into such a process until now.

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.[16] The most common PHA is poly([R]-3-hydroxybutyrate) (P3HB) and its copolyester with ([R]-3-hydroxyvalerate) (P3HB3HV), which is well suited for food packaging.[108] It is accumulated in intracellular granules by a wide range of bacteria. As PHAs function as carbon and energy storage, they are most of the time produced in the presence of excess carbon source and under limitation of another nutrient, such as nitrogen or phosphorous. An intracellular P3HB-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.[57, 109, 110] On an industrial scale, production costs are still not competitive compared to petrochemical plastics, mainly due to a high cost of raw materials and downstream processing.[12]

Table 3.1. 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)	PHA (% in dry mass)	Yield (g P3HB/ g substrate)	Volumetric productivity (g/L/h)	Ref.
(c) *Azohydromonas australica* DSM 1124	Fed-batch	P3HB	Soya milk waste acid hydrolysate (Fructose, Lactose, Maltose) with a Sucrose feed	18	33	n.a.	0.11	[111]
(b) *Bacillus megaterium* BA-019 (new isolate)	Batch	P3HB	Cane molasses (Sucrose)	8.8	62	0.37	0.45	[112]
(b) *Bacillus megaterium* BA-019 (new isolate)	Fed-batch	P3HB	Cane molasses (Sucrose)	72	42	n.a.	1.27	[112]
(a) *Cupriavidus necator H16* DSM 428	Batch	P3HB	Fructose	8	45*	0.21*	0.12*	[113]
(a) *Cupriavidus necator H16* DSM 428	Batch	P3HB3HV-block-HB	Fructose and valerate	0.4	66	n.a.	0.03 (P3HB3HV), 0.033 (P3HB)	[114]
(a) *Cupriavidus necator* DSM 531	Batch	P3HB	Fructose	21	45	0.31	0.15	[115]
(b) *Cupriavidus necator* DSM 545	Shake flasks	P3HB	Glucose and cane molasses (Sucrose)	17-23	31-44	n.a.	0.08-0.12	[116]
(b) *Cupriavidus necator* DSM 545	Shake flasks	P3HB	Cane molasses acid hydrolysate (Fructose and glucose)	9.5	14*	0.06	0.02*	[117]
(a) *Cupriavidus necator* DSM 545	Fed-batch	P3HB	Sucrose hydrolysate	125-150	65-70	0.32	1.44	[118]
(d) *Cupriavidus necator* strain not specified	n.a.	P3HB	Jerusalem artichoke hydrolysate (Fructose)	6-7	70	n.a.	n.a.	[119]
(c) *Pseudomonas fluorescens* A2a5 (new isolate)	Batch	P3HB	Sugarcane liquor (Sucrose)	32	70	n.a.	0.23	[31]

Typical carbon sources for the PHA fermentations are sugars and fatty acids, most of the time in mineral salt media (minimal media).[120, 52, 109] 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 (see Table 3.1). The reported results are typically not as good as it 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 (see Table 3.1). This was done by Kulpreecha *et al.* with a newly isolated *Bacillus megaterium* strain grown on diluted sugar cane molasses in a medium containing urea as nitrogen source.[112] In batch mode they reached 8.8 g/L dry biomass containing 62 % P3HB 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 cane molasses to a minimal medium containing glucose in order to cultivate *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*) DSM 545.[116] Various nitrogen sources were studied, but ammonium sulphate proved to be the best. Depending on the amount of molasses supplementation (0-5 g/L), cell densities of 17-23 g/L with a P3HB content of 31-44 % were reached. The same organism was also grown by Sharifzadeh *et al.* on cane molasses as the only carbon source, again in a minimal medium. [117] 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, P3HB was produced using a new isolate of *Pseudomonas fluorescens*.[31] Yu *et al.* subjected soya waste from a soya milk dairy to an acidic hydrolysis, producing a fermentation substrate containing fructose, lactose and maltose.[111] Upon supplementation with K_2HPO_4, Fe(III)citrate and $MgSO_4$, *Azohydromonas australica* DSM 1124 (formerly known as *Alcaligenes latus*) accumulated P3HB. After the total organic carbon dropped below 15 g/L 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 P3HB from inulin in a conference abstract.[121] They extracted and hydrolysed inulin from Jerusalem artichoke (topinambour) into its monomers with diluted acid and obtained a hydrolysate with 27 g/L of fructose. This hydrolysate was used without any supplementations as a fermentation broth and was converted by *Cupriavidus necator* (strain not specified) into 6-7 g/L of biomass containing up to 70 % of P3HB.

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 P3HB 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 any supplementation of nutrients for P3HB production. Three production strains, *Cupriavidus necator* DSM 428, 531 and 545, were compared. To our knowledge, this study is also the first study 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 P3HB was extracted with chloroform and several physico-chemical parameters characterised.

3.3 Materials and Methods

3.3.1 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 compositional analysis of all carbohydrates was done according to the NREL/TP-510-42618 method.[119]

When the roots are grown industrially for inulin production, inulin is extracted with water at 70-80 °C for 1.5-2 h.[122] 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 recommended concentration by the supplier). The pre-treatment experiments were done in 5 mL aqueous suspension in sealed falcon tubes. All reactions were done in duplicates in a temperature-controlled water bath. The pH was adjusted with 1 M 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 2 000 U/mL exo-inulinase and ~200 U/mL 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 duplicates at specified intervals for up to 24 h. The liquid phase was separated by centrifugation (2 000x 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 in the hydrolysate.

The difference of the duplicates was quantified as standard deviation. 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 dry roots and 360 U endo-inulinase/kg 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 4 000x g for 30 min, and vacuum filtration through paper filters (grade 595, Schleicher & Schuell, Dassel, Germany). The hydrolysate was autoclaved for 20 min at 121 °C and the pH was adjusted to 7.0 with sterile 1 M NaOH. Samples were drawn in duplicate at regular intervals and the difference between the duplicates was quantified as standard deviation.

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.[123] 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.[124] Laccase from *Trametes hirsuta* was added to the chicory hydrolysate (2.9 U/mL) 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*.[125] The activity was determined by adding 50 µl of 10 mM 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, 8.06 mg/mL protein).

The inhibitor concentration was determined by downscaling the total phenol estimation procedure published by Areskogh *et al.*[115] 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.

3.3.2 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 P3HB-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 P3HB production.[126] 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.[127]

Shake Flask Cultivation

Shake flask experiments were conducted to check whether the microorganisms are 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 2 000x g for 15 min, 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 P3HB. If necessary, the pH was adjusted with sterile 1 M NaOH or 1 M HCl to 6.8. All strains switched from cell growth to P3HB 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 an inoculum that is denser and stronger.

The minimal medium for the preculture was prepared according to Koller *et al.*[128] and contained 4.8 g/L Na_2HPO_4, 2.0 g/L KH_2PO_4, 3.0 g/L $(NH_4)_2SO_4$, 0.8 g/L $MgSO_4$*7 H_2O, 1.0 g/L NaCl, 0.02 g/L $CaCl_2$*2H_2O, 0.05 g/L NH_4Fe(III)citrate*, 10 g/L fructose and 5 mL/L 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 agar was added.

Prior to cultivation, the strains were conditioned on minimal medium plates at least for 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). The preculture was harvested at 2 000x 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 M NaOH or 1 M 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 M 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 P3HB production upon limitation of endogenous nitrogen in the hydrolysate.

Samples (5 mL) were taken at regular intervals. The cells were separated from the supernatant (2 500x 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) and P3HB (% in dry mass) was determined. The yield was calculated as g P3HB/g consumed sugar. The productivity given in g P3HB/L/h was calculated as the quantity of produced P3HB between the inoculation time (time = 0) and the time at which the highest P3HB-concentration was reached. All fermentations were done in duplicates and the individual duplicates lead to the same conclusions.

3.3.3 Extraction and characterisation

Extraction

After the fermentation, the biomass was centrifuged (3500 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. P3HB 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 P3HB 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). 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 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.

3.3.4 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 M sulphuric acid at a flow rate of

0.325 ml/minutes. 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 P3HB

P3HB was determined by a modified method of Karr and coworkers.[129] 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 min in air-tight Pyrex glass tubes. During this step, P3HB 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 M sulphuric acid at a flow rate of 0.9 ml/minutes. The biomass and P3HB concentration was calculated as g/L of fermentation broth, the P3HB 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 (3 500x 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.

3.4 Results and discussion

3.4.1 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.[106] 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).[130]

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 (see Figure 3.1.a.), but the amounts of carbohydrates extracted relative to the dry roots was 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 (see Figure 3.1.c.). 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 (see Figure 3.1.b.). 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 can 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 originates from inulin, which contains a 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 (see Figure 3.1.d.). 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 3.2.

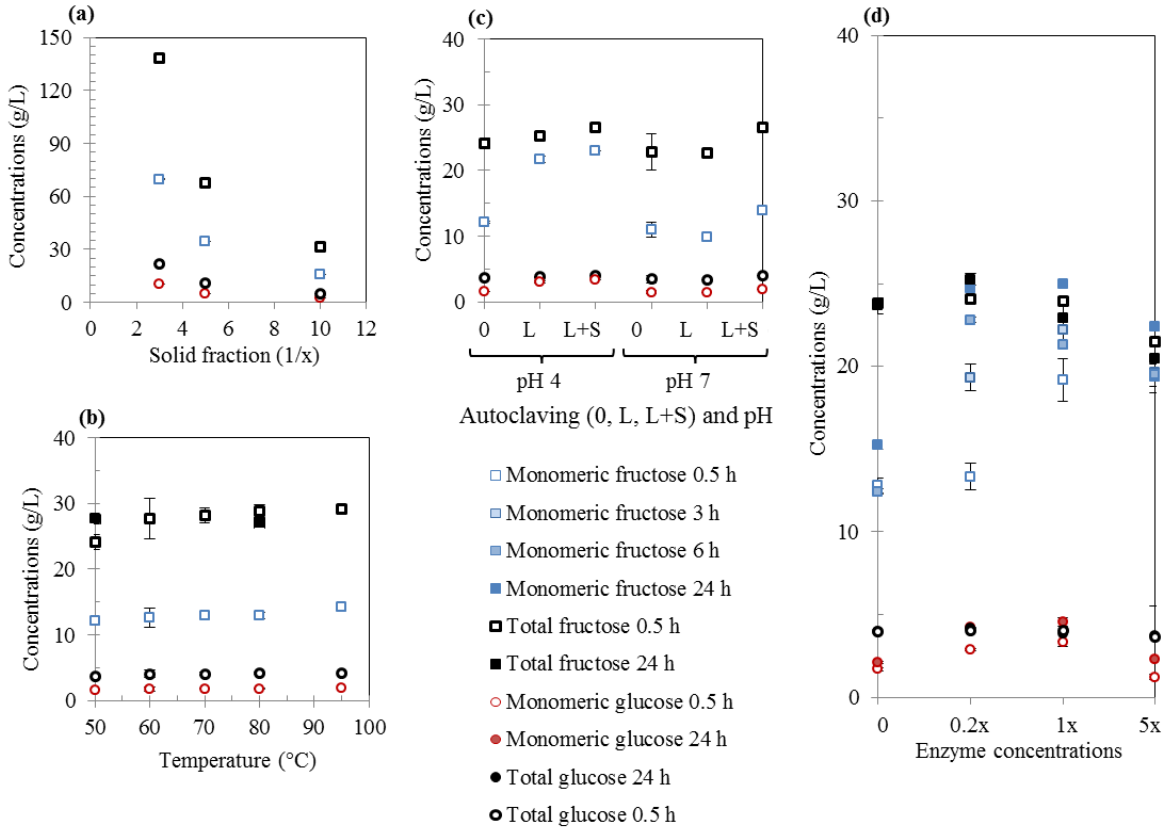

Figure 3.1. Pre-treatment of chicory roots. **(a)** Effect of solid fraction at 80 °C and pH 4. **(b)** Comparison of no autoclaving (0), autoclaving only the liquid after solid-liquid separation (L) or autoclaving the solid and liquid suspension together (L+S). Experiments were done at pH 4 and 7 at 80 °C and a solid fraction of 1:10. **(c)** Effect of Temperature at pH 4 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 only the inulinase mix, gave the same results as shown here, using Viscozyme® only had no effect on the levels of glucose and fructose.

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, a pH of 5, a temperature of 50 °C and 6 h incubation with the inulinase mix (0.2 multiples of the recommended concentration = 3 600 U exo-inulinase/kg dry roots and 360 U endo-inulinase/kg dry roots). Autoclaving promoted the hydrolysis of remaining oligomeric carbohydrates. Test showed that autoclaving alone has only a small effect on the hydrolysis of inulin and cannot replace the addition of inulinases (data not shown).

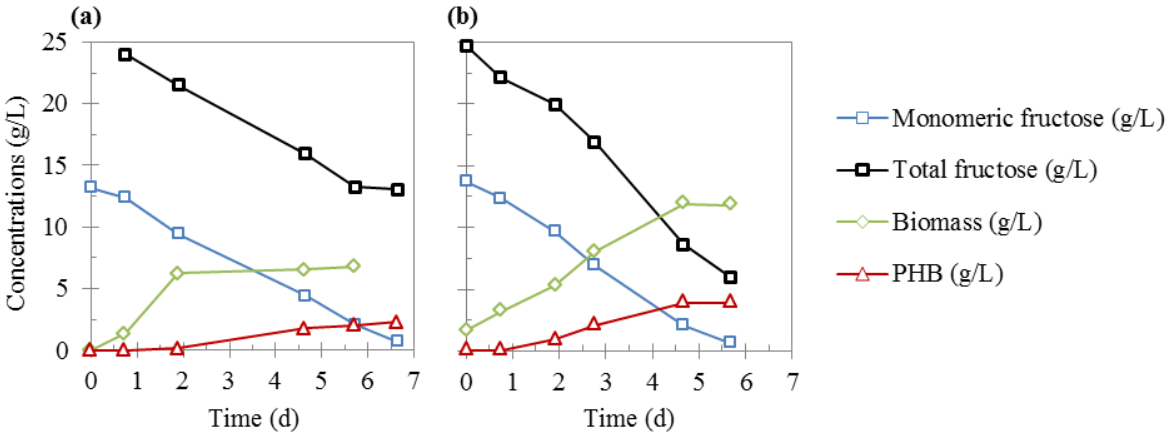

Figure 3.2. Shake flaks culture of Cupriavidus necator DSM 428 **(a)** and 531 **(b)** on hydrolysate containing free fructose as well as inulin (measured as total fructose).

The course of the extraction using the designed protocol is depicted in Figure 3.3. 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.

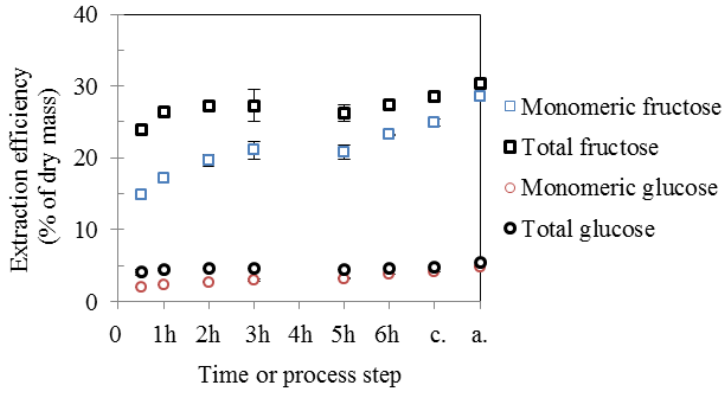

Figure 3.3. Time course of the hydrolysis and extraction using the optimized protocol. c.: after centrifugation (removal of solids). a.: after autoclaving the liquid hydrolysate.

In the final hydrolysate there are 27.6 g/L fructose and 4.8 g/L of glucose as shown in Table 3.2.a. 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.[119] 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 colour 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. 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, but some nitrogen (0.69 g/L) in 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 (see Table 3.2.b). 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, 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 P3HB-production (data not shown).

Table 3.2. (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)
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)	Hydrolysate (mg/L)
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

3.4.2 Fermentation studies

All three *Cupriavidus necator* strains are able to grow and produce P3HB on the chicory root hydrolysate as shown in Figure 3.4. 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. Biomass formation of *Cupriavidus necator* DSM 428 starts after a lag phase of 11 h and the residual biomass (biomass without intracellular P3HB) reaches a plateau at 2.8 g/L after 19 h. The dissolved oxygen drops with increased residual biomass formation and then increases again slowly during P3HB formation. During growth, i.e. fructose oxidation to carbon dioxide, oxygen is required to keep the redox balance. The P3HB-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 it is the case for a complete oxidation. [113] P3HB 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) and P3HB content (66 %) are reached after 5.0 days at the end of the fermentation, after all the fructose has been consumed (see Table 3.3). Overall the yield is 0.32 g P3HB / g consumed sugar and P3HB is produced with a productivity of 0.062 g P3HB/L/h.

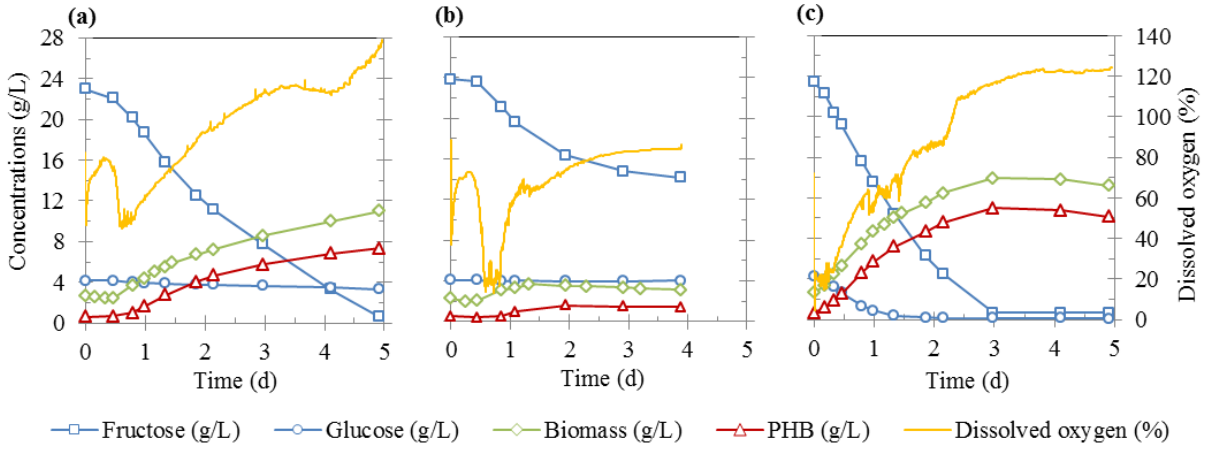

Figure 3.4. Bioreactor fermentations using Cupriavidus necator **(a)** DSM 428, **(b)** DSM 531, and **(c)** DSM 545 in the obtained chicory root hydrolysate. On the left axes are the concentrations of the substrates and products and on the right axes is the dissolved oxygen.

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 residual biomass. After 21 h, P3HB accumulation starts and has a peak after 2.0 days (3.5 g/L biomass, 45 % P3HB), 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.[127] The yield and productivity of the fermentation are 0.15 g/g and 0.016 g/L/h, respectively.

Strain DSM 545 consumes both sugars and does so 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 straight away. Most of the residual biomass is accumulated during the first 8 h, reaching a value of 2.7 g/L 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. P3HB production is not strictly separated from residual biomass production, i.e. some P3HB is already formed during cell growth. P3HB production also starts straight away 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 containing 78 % P3HB is reached. This corresponds to a yield of 0.38 g/g and a productivity of 0.15 g/L/h.

Table 3.3. 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)	11.0	3.5	14.0	14.2	13.7
P3HB content (%)	66	45	78	71	75
Yield (g P3HB/g fructose)	0.32	0.15	0.38	0.36	0.38
Volumetric productivity (g P3HB/L/h)	0.062	0.016	0.15	0.11	0.11

Only one other research group reported a P3HB production from fructose-based hydrolysates using *Cupriavidus necator*. However, they neither specify the strain nor the fermentation mode used during the experiments.[121] At the end of the experiments, they reached a biomass concentration of 6-7 g/L with a P3HB 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 and a P3HB content of 45 % (see Table 3.1).[114] The yield was 0.21 g/g and the productivity was 0.12 g/L/h. The same strain was also grown by Kelley *et al.* to a cell density of 0.4 g/L (66 % P3HB3HV-block-HB) with a productivity of 0.03 g/L/h.[118] 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 (45 % P3HB), a yield of 0.31 g/g and a productivity of 0.15 g/L/h.[127] 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 is less desirable for P3HB 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 cane molasses (see Table 3.1).[116, 117] 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 of biomass (65-70 % P3HB), a yield of 0.32 g/g and a productivity of 1.44 g/L/h.[45]

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.[112] In their fermentations, the cell density increased by a factor of eight (8.8 vs. 72 g/L) and the productivity almost tripled (0.45 vs.

1.27 g/L/h). 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, either by evaporation or membrane technology.[131]

The total Kjeldahl nitrogen of the hydrolysate (0.69 g nitrogen/L) drops during biomass formation of all three strains to 0.53 g nitrogen/L 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 from 0.16 g nitrogen/L). 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 that either a part of the proteins and peptides form polymeric maillard-reaction products with fructose during autoclaving or that 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 P3HB 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 one with laccases (polymerisation of phenolic compounds). Both methods decreased the total phenol content (180.8 mg/L) by 16.8 % or 35.8 % in the case of the laccase or charcoal treatment. The charcoal treatment reduced also the sugar content and total Kjeldahl nitrogen by 6 and 14 %, respectively. However, both fermentations are very similar to each other (see Table 3.3). 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 %.[132] When *C. necator*

MTCC-1472 was grown on the detoxified substrate, the P3HB concentration increased from 2.0 g/L to 4.3 g/L. Pan *et al.* prepared a hemicellulosic hydrolysate from sugar maple and used it as a fermentation substrate for *Burkholderia cepacia* ATCC 17759.[58] 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.[47] They came to the conclusion that a new strain of *Burkholderia cepacia* grows and produces more P3HB 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.[133]

3.4.3 Extraction and characterisation of P3HB

P3HB from all three *Cupriavidus necator* strains was extracted and was tested to be pure. The glass transition temperature, melting temperature and enthalpy of melting are in good accordance with results from the literature (see Table 3.4).[134, 135] The number and weight average molecular weight as well as the polydispersity are shown in Table 3.4. 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 range typically reported, indicating that only negligible degradation occurred during the extraction.[109, 134, 136]

Table 3.4. Thermo-physical parameters of the extracted polymers in comparison to published data for *Cupriavidus necator*. n.d.: not determined

Parameter	own results DSM 428	own results DSM 531	own results DSM 545	Ref. [42] strain n.a.	Ref. [43] new isolate	Ref. [13] DSM 428	Ref. [44] 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)	92.4	86.5	76.3	n.d.	98	n.d.	n.d.
Number average polymer weight (Da)	$4.55*10^5$	$3.65*10^5$	$5.11*10^5$	$0.1\text{-}30*10^5$	n.d.	$1.70*10^5$	$3.2*10^5$
Polydispersity	1.82	1.90	1.32	~2	n.d.	3.2	2.5

3.5 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), glucose (4.8 g/L) and nitrogen (0.7 g/L) and was a viable fermentation substrate without any nutrient supplementation. Three *Cupriavidus necator* strains (DSM 428, 531 and 545) grew and produced P3HB from the sugars in the hydrolysate. Strain DSM 545 gave the best results (yield: 0.38 g P3HB/g sugar, productivity: 0.15 g P3HB/L/h), 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 P3HB are in good accordance with P3HB produced in minimal media.

With the current process characteristics, 11250 tpa chicory hydrolysate could be produced in Navarra, containing 547 tpa sugars which would yield 208 tpa P3HB prior to purification. For most applications, P3HB has to be blended with other polymers or with plasticizers.[137] The amount of plastics which could be produced from chicory roots is still marginal compared to the plastic consumption in Spain (3 500 000 tpa [138]). Also, 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.[139] Furthermore, consumer awareness for bio-based and biodegradable products is rising. This opens opportunities particularly for tangible materials, such as packaging materials. P3HB 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. P3HB 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 P3HB 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 P3HB production and might contribute to a green economy in the future.

3.6 Acknowledgement

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4 DEVELOPMENT OF A MEMBRANE BIOREACTOR FOR P3HB PRODUCTION

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Oral presentation at the

- Global Cleaner Production in Barcelona (1.-4.11.2015)

Contribution of the author:

The author planned, supervised as well as aided in the practical implementation of all experiments. In the course of the development, the author was in charge of one masters' students (Lukas Burgstaller) who did most of the practical work. Furthermore, the poster was designed and written by the author.

4.1 Abstract

A cell-recycle membrane bioreactor for the high-cell-density production of poly(3-hydroxybutyrate) (PHB) was developed. This fermentation strategy is particularly suitable for low carbon concentrations in the feed stream, as it is often found in agricultural residues.

Cupriavidus necator DSM 545 was continuously supplied with synthetic medium containing 50 g/L of glucose. If the continuous supply was not sufficient, an automatic DO and pH-triggered increase in the pump rates was executed. A constant working volume of 0.5 L inside the bioreactor was maintained by an external polysulfone microfiltration membrane module. The PHB-production phase was started after 8 hours by supplying nitrogen-free medium. After another 32 hours, 52 g/L dry biomass was accumulated containing 92 % PHB. The process is characterised by a high average productivity of 1.2 g PHB/Lh.

4.2 Introduction

Agricultural residues with a high carbon content such as molasses are already intensively used as fermentation substrates. Residues with a lower carbon content, such as whey, stillage, wastewater from plant oil mills, etc. are not suitable for fed-batch fermentations, the most frequent fermentation mode for PHB-production. This problem can be circumvented by either concentrating the carbon in the feed stream or retaining the cells during the fermentation in the bioreactor. The latter strategy has the advantage that it can also be used for substrates containing low concentrations of inhibitors, which would get co-concentrated along the desired carbon source. Excretion products during the fermentation are continuously removed from the bioreactor. Furthermore, as PHB is accumulates intracellularly, the product is up-concentrated during the fermentation.

4.3 Experimental Setup

A 0.5 L bioreactor (dasgip®, Eppendorff) was equipped with a 420 cm^2 polysulfone hollow fibre membrane cartridge (GE Healthcare). Membrane pore sizes of 0.2 µm (microfiltration) and 750 kDa (ultrafiltration) were tested and compared. The cross-flow was established with a diaphragm pump, which produced an alternating tangential flow in the membrane of 1.2 L/min (Refine technologies, see Figure 4.1).

Two types of feed solution were fed to the bioreactor. The first contained glucose (50 g/L) and ammonium sulphate (3 g/L) in a mineral salts medium (for exact composition of all media see chapter 5.3.1 on page 70). It was fed (40 mL/L/h) at rates of 1.25 g glucose/L/h and 0.06 g ammonium sulphate/L/h to the bioreactor. The second feed was installed once the OD reached ~20 - 30, which corresponds to a residual biomass concentration of 5 - 8 g/L. It did not contain nitrogen in order to induce the nitrogen limitation. In the basic setup, which was used for comparing the microfiltration and ultrafiltration membranes, the same feed rate was used. The microfiltration membrane was also tested at an increased constant feed rate. Inside the bioreactor *Cupriavidus necator* DSM 545 converted the glucose into residual biomass and P3HB. The glucose concentration in the bioreactor was not monitored and the base for pH control was 1 M NaOH. The other procedures are the same as detailed in chapter 5.3.

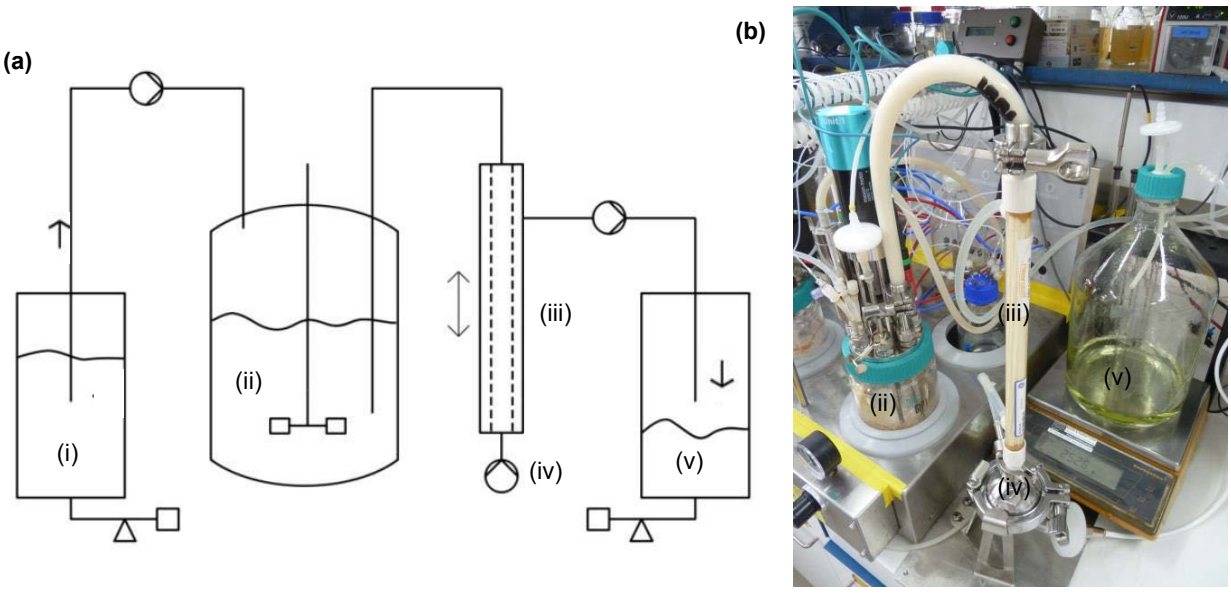

Figure 4.1 (a). Schematic drawing of the membrane bioreactor. From the feed bottle (i), the feed solution was supplied via a controlled pump into the fermenter (ii). In the fermenter the microorganisms utilized the supplied glucose and converted it into residual biomass or P3HB. The fermenter was in equilibrium with a cross-flow type microfiltration membrane (iii), from which a cell-free permeate (v) was removed by a controlled pump into the permeate bottle. The volume inside the bioreactor was kept constant by matching the amount of feed added and permeate removed. A diaphragm pump (iv) created an alternating flow in order to reduce fouling and keep the shear stress low. **(b)** Picture of the reactor setup with the same indices.

4.4 Results and Discussion

In order to efficiently convert substrates containing only a moderate to low concentration of carbon into P3HB, different membrane bioreactor setups were tested. In the first setup, a microfiltration unit was employed and a constant feed stream pumped through the system. This feed stream supplied 1.25 g glucose/L/h to the microorganisms and was first converted into residual biomass and later to P3HB. Nitrogen became already limiting before the feed change as the C:N ratio was slightly too high in the feed. This resulted in a low residual biomass concentration (5 g/L). However, the growth and P3HB production was fast (see Figure 4.2.a.) and lead to a good productivity (0.85 g P3HB/L/h, see Table 4.1). During the experiment, not all glucose supplied to the bioreactor was utilized. Particularly at the beginning, the equilibrium concentration of glucose is relatively high (up to 35 g/L), resulting in significant amounts of glucose being swept out of the system. This is reflected in the yield (0.28 g P3HB/g supplied sugar), which is lower than usually reported in the literature (0.30 - 0.38 g P3HB/g sugar).[46, 63]

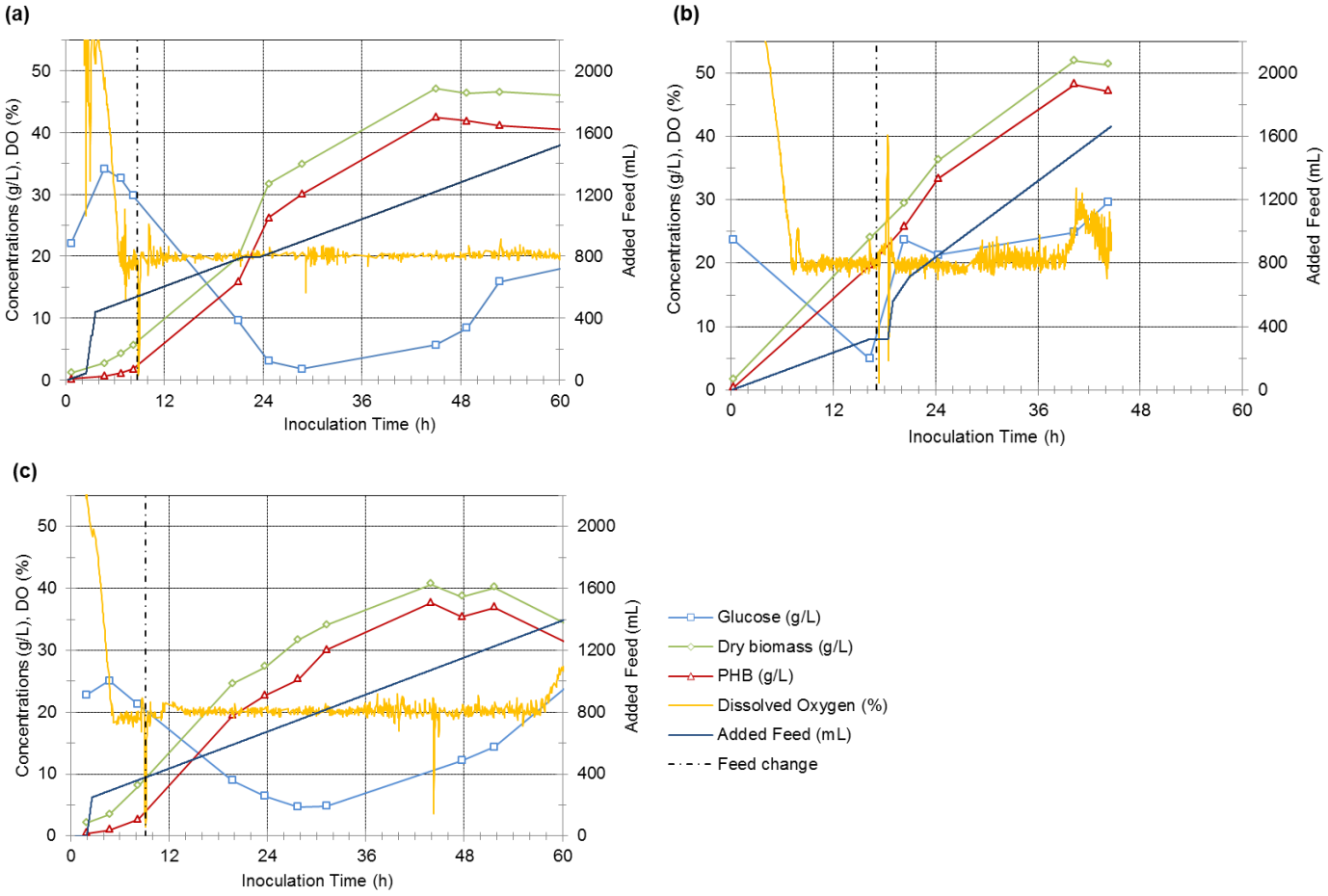

Figure 4.2. Time profiles of fermentations employing **(a, b)** microfiltration and **(c)** ultrafiltration membranes for cell-recycle. The second microfiltration experiment **(b)** was conducted with an increased feed rate for PHB-production.

Ultrafiltration membranes have lower fouling propensities, which is particularly interesting for the long-term operation of the membrane. Upon implementation of an ultrafiltration membrane, the same fermentation performance was achieved. This indicates that fouling did not play a role in the fermentation performance of the first setup with the microfiltration membrane. However, in later experiments described in chapter 5, which employed much higher flow rates, fouling eventually blocked the microfiltration membrane. At this point, the ultrafiltration membrane was tested again, but the trans-membrane pressure which could be established by the system was too low to maintain such high flow rates. In line with this, the ultrafiltration membrane did not bring added benefit to the fermentations conducted at rather low trans-membrane flux, whereas the setup itself (silicone instead of metal tubes) was not feasible for the ultrafiltration membrane at high trans-membrane flux.

In a third setup, the flow rate of the feed was increased in order to increase the productivity. By doubling the feed flow rate, the productivity could be increased by 41% to 1.2 g P3HB/L/h. However, in this setup, the average glucose concentration throughout the experiment was around 20 g/L, so that even more sugar left the system without being consumed (yield: 0.27 g P3HB/g supplied sugar).

Table 4.1. Process data of the microfiltration and ultrafiltration membrane bioreactor experiments.

	Microfiltration (max @ 44 h)	Microfiltration at increased pump rate (max @ 40 h)	Ultrafiltration (max @ 45 h)
Biomass (g/L)	40.6	51.9	47.5
P3HB (%)	92	92	90
Av. Productivity (g/Lh)	0.85	1.2	0.95
Yield (g P3HB/g sugar)	0.28	0.27	0.27

4.5 Conclusions and Basic Considerations for Subsequent Experiments

With the described setup of the membrane bioreactor system good P3HB productivities were reached. At the given trans-membrane flux, microfiltration and ultrafiltration membranes performed equally well. The concentration of the carbon source in the bioreactor during the cell recycling phase is of utmost importance in order to get reasonable yields. However, during these experiments it was not possible to monitor the carbon concentration inside the bioreactor online. Furthermore, the cell recycling phase was started soon after inoculation when batch carbon was still present in the system, which decreased the yield. In the subsequent experiments discussed in chapter 5, the system was improved in that the cell recycling phase was started upon exhaustion of the batch glucose. A feed rate was chosen at which the microorganisms could instantly consume the supplied carbon. Test strips for monitoring the glucose concentration were obtained in order to attain also a high yield. Furthermore, the volumetric feed rate was increased in order to raise the productivity.

The pump used in the initial experiments for establishing the alternating cross-flow is designed to impose very low shear forces on the microbes. It worked very well, but upon comparison with a cheaper conventional peristaltic pump in subsequent experiments, it was found that the shear forces of the conventional pump do not have adverse effects on the microorganisms.

In the following experiments, a larger bioreactor was used (3 L instead of 0.5 L). The bigger bioreactor offered the advantage of having a more versatile control over the fermentation via a customizable digital control unit (DCU). This was particularly of advantage for the programming of triggers. In addition to that, the specific membrane area of the larger bioreactor is closer to industrial process conditions (140 cm^2/L instead 840 cm^2/L).

The experiments as well as the gained experience presented in this chapter formed the basis for the subsequent experimental development which cumulated in the publication described in the following chapter.

5 HIGH CELL-DENSITY PRODUCTION OF P3HB IN A MEMBRANE BIOREACTOR

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Contribution of the author:

The author planned, supervised as well as aided in the practical implementation of all experiments. In the course of the development, the author was in charge of one masters' students (Tarek El-Najjar) and one Bachelor student (Nikolaus Virgolini) who did most of the practical work. Furthermore, the publication was written and revised by the author.

5.1 Abstract

Agro-industrial residues with a low carbon content, such as whey, stillage or wastewater from plant oil mills are abundant and cheap. However, they cannot be directly used in highly productive industrial poly(3-hydroxybutyrate) (P3HB) production, as the classical fed-batch fermentation strategy requires highly concentrated feed streams. This problem was circumvented in this report by retaining the cells during the fermentation in the bioreactor using an external microfiltration module. Synthetic media containing a glucose concentration of 50 g/L was continuously fed to *Cupriavidus necator,* which converted the sugar to P3HB. With our setup we were able to achieve high productivities (3.10 g P3HB/L/h), reach high cell densities (148 g/L) containing 76% P3HB, and obtained good yields (0.33 g P3HB/g added glucose). The added sugar from the feed was instantly consumed by the bacteria, resulting in a negligible loss of sugar to the permeate. This approach creates the possibility for polyhydroxyalkanoate production from a range of cheap and easily available substrates, for which only waste water treatment or biogas production were cost-competitive until now.

Highlights

Successful high cell-density fermentation with low carbon feed solution

High PHA productivity and good yield attained

Very low carbon concentration in permeate is crucial aspect in process

Process enables novel biotechnological utilization route for cheap agro-residues

Keywords

low carbon substrate, waste material*, Cupriavidus necator*, polyhydroxyalkanoate production, high productivity, cell recycle fed-batch

5.2 Introduction

Polyhydroxyalkanoic acids are a family of polyesters, formed by a number of microbes as intracellular carbon and energy storage. Their accumulation is enhanced under conditions in which the microbes cannot divide and grow, *i.e.* the limitation of one or more essential nutrient, except carbon. The most commonly produced PHA are poly[(*R*)-3-hydroxybutyrate] (P3HB) and its copolyester with (*R*)-3-hydroxyvalerate (P3HB3HV), which are well suited for packaging, as well as medical applications.[140] Currently, PHA are still a niche material in the bioplastics sector, but exhibit one of the fastest growing markets.[16]

In order to get to an industrially feasible PHA fermentation process, high productivity, high yield, and high intracellular polymer content are crucial.[26] The yield is mainly limited by the theoretical yield of 0.48 g P3HB/g glucose, as two carbons of each sugar molecule are lost in the form of CO_2.[44] Typical values reached in practice are between 0.3 and 0.4 g P3HB/g sugar. The productivity is governed by the microorganism involved in P3HB synthesis and the number of cells in the fermentation broth. Accordingly, it is essential to reach higher cell densities prior to the onset of the nutrient limitation (*i.e.* residual cell dry mass), upon which proliferation ceases and the cells enhance polymer accumulation. High cell-density cultivation (> 100 g/L total cell dry mass (CDM)) is therefore a prerequisite for high-productivity fermentations. In optimized processes, an intracellular polymer content of 70-90 % is typically attained.[53, 54]

The best established mode of fermentation to obtain high productivities (2-5 g P3HB/L/h) is fed-batch. *Cupriavidus necator* [46, 64], recombinant *Escherichia coli* [53, 68], and *Burkholderia sacchari* [141] are the organisms most frequently used for this purpose. In fed-batch culture, the addition of feed increases the fermentation volume, causing a concomitant dilution of the fermentation broth. In order to keep this dilution low, highly concentrated feed solutions containing several hundred grams of carbon source per litre are used. At the end of the cultivation the cells are harvested and the polymer can be extracted. Recently, also the production of PHA in continuous reactor systems has been explored and optimized for high productivities.[110, 142]

Besides the process-issues yield and productivity, the main cost-drivers are substrate acquisition and downstream processing. In line with the EU flagship initiative on a resource-efficient Europe, the use of industrial by-products for PHA-fermentation is in the focus of research and industry.[17, 41, 143] By-products from the agro-industrial food and materials production chain, such as crude glycerol [144], starch residues [61], molasses [112], and waste lipids [50] are in the focus of researchers. Due to the high concentrations of carbon in these residues, conventional fed-batch and continuous fermentation modes can be applied. However, there are already utilization strategies to various products established, increasing the price of these raw materials. On the other hand, many abundant agro-

residues are very low-cost substrates, but contain only low concentrations of carbon. Examples are: whey (45 g/L lactose, [53]), thin stillage (47 g/L carbohydrates, [145]) or plant hydrolysates (28 g/L fructose, [59]). Such low carbohydrate concentrations prohibit a direct use due to the low cell-densities that can be achieved in fermentations. As such, only biogas production or a disposal in waste water treatment plants may be economically feasible.

A potential way out is concentrating the substrate by evaporation or membrane processes. However, these processes are energy and resource intense. Furthermore, toxic small molecules (e.g. hydroxymethylfurfural or furfural, which are formed during thermal processing) might be co-concentrated and affect fermentation performance. Some sugars, such as lactose also exhibit solubility problems.[53]

Another strategy is to couple a membrane process directly with the bioreactor and operate the bioprocess in a cell recycling mode. In the literature, the cell recycle approach has been described a few times for PHA production. Ahn *et al.* [53] fermented concentrated whey at lactose concentrations of 280 g/L. They were able to reach a CDM of 194 g/L with a P3HB content of 87%, resulting in a remarkable productivity of 4.6 g/L/h. Ienczak *et al.* [146] aimed at high cell-density cultivation using low carbon feed solutions (90 g/L glucose), without preliminary concentrating steps. With their repeated batch strategy in synthetic medium, they reached a CDM of 61.6 g/L with a P3HB content of 69%, corresponding to a productivity of 1.0 g/L/h. Du *et al.* [54] implemented the continuous addition of anaerobically digested food scraps and removal of cell-free permeate. A CDM of 22.7 g/L/h containing 72.6% P3HB3HV was reached within 73 h. Recently, archea have been also cultivated in cell-recycle fermentations. Lorantfy *et al.* [147] cultivated *Haloferax mediterranei* in a synthetic medium containing lactate, glycerol, acetate, and/or ethanol. They employed cell recycling in order to get a cell-free harvest stream low in total organic carbon (99.6% reduction). None of these approaches was able to combine high productivity with low carbon concentrations in the feed stream.

Reducing the total organic carbon in the feed stream along with a concomitant production of PHA is mainly implemented in mixed microbial cultures.[78] The aim is to establish a value-added waste-water treatment. In mixed microbial cultures, genetically diverse PHA-accumulating organisms are selected by the operating conditions imposed to the reactor. However, in these processes biomass concentrations of only several g/L have been obtained so far, resulting in a very low volumetric productivity.

There is a gap in research on establishing high PHA productivities based on residues with low carbon concentration. Closing this gap is of industrial importance, as these residues are a cheap raw material, which could be converted into a higher value bioplastics. We aim to close this gap and generate the proof of concept by utilizing an external microfiltration membrane for full cell-recycling in pure-culture fermentations. After a batch phase, a synthetic medium feed containing 50 g/L of

glucose is continuously supplied to the bioreactor. Inside the bioreactor, *Cupriavidus necator* is grown to high cell densities in order to achieve high P3HB productivities. Simultaneously to the feed addition, permeate with a very low glucose concentration is removed to achieve a high yield.

5.3 Materials and Methods

5.3.1 Microorganism and Media

Cupriavidus necator DSM 545 was kindly provided by Martin Koller from the University of Graz. The long-term storage was in synthetic medium glycerol stocks at -80 °C. For the individual experiments the culture was sub-cultivated several times on agar plates with synthetic medium. The synthetic medium for the frozen stocks, agar plates and pre-culture contained 4.8 g/L Na_2HPO_4, 2.0 g/L KH_2PO_4, 3.0 g/L $(NH_4)_2SO_4$, 0.8 g/L $MgSO_4 \cdot 7\ H_2O$, 1.0 g/L NaCl, 0.02 g/L $CaCl_2 \cdot 2\ H_2O$, 0.05 g/L NH_4Fe(III)citrate, 10 g/L glucose, and 25 mL/L SL6 trace element solution (prepared according to DSMZ Medium No. 27).[128] The iron citrate, sugar solution and magnesium sulphate were prepared as 10x stock and each autoclaved separately. For solid media, 14 g/L agar was added. The pH-value was adjusted to 7.0 for all media components prior to autoclaving. Autoclaving was conducted at 121°C for 15 minutes.

The batch medium was adapted to high cell-density cultivation and contained a higher amount of glucose (30 g/L) and magnesium sulphate heptahydrate (1.2 g/L). Furthermore, 2.5 mL/L of a different trace element solution, prepared according to Mozumder *et al*. [62], was filter-sterilized and added after autoclaving the other medium constituents. This trace element solution also contained calcium and iron and substituted these elements in the inoculum medium. The medium fed to the bioreactor after the batch phase had the same composition as the batch medium, except for glucose (50 g/L) and ammonium sulphate (0 g/L). As the feed solution was typically prepared in 12 L batches, the autoclaving time was prolonged to 90 minutes in order to reach and keep 121°C for at least 10 minutes in the core.

5.3.2 Cultivation

Baffled shake flasks containing 200 mL of pre-culture medium were inoculated in an orbital shaker at 37°C for 24 h, reaching the late exponential growth phase (OD_{600} 10-12). Two flasks were used as inoculum in a 5 L fermenter (B. Braun Biotech International GmbH, Germany) operating at a working volume of 3 L (see Figure 5.1). We modified the fermenter in order to have customizable process control options and operated it with a 1769 CompactLogix Controller (Rockwell Automation,

Wisconsin, USA). The dissolved oxygen concentration (DO) was controlled to 20% saturation by adjusting the stirrer speed with two six-bladed Rushton turbine impellers (300-800 rpm) as well as the air flow (1-4 vvm). The pH-value was regulated to 7.0 by 12.5% w/v ammonia. Addition of the antifoaming agent Glanapon DG 160 in an aqueous 1:10 dilution was controlled by a foam probe. The trans-membrane pressure was calculated as the difference between the pressure in the cross-flow before the membrane and in the permeate.

The batch mode took typically 14-18 h. A decrease in stirrer speed below 600 rpm, due to the complete consumption of glucose in the fermentation broth, automatically triggered the cell recycle mode. During this cell recycle mode, feed/permeate were simultaneously added/removed at a flow rate of 720 g/h (12 g/L/h glucose). The complete retention of cells was accomplished by a 0.02 µm pore size microfiltration membrane (CFP-2-E-4MA, 420 cm^2 membrane area, GE healthcare, UK). After reaching a certain CDM, which was estimated by the optical density (OD_{600}), P3HB production was triggered by switching to 2 M NaOH for the pH control. The base change was conducted at OD_{600} 80 or 140, corresponding to a CDM of 20 or 35 g/L, respectively. In the second setup, the feed supply and permeate removal were increased to 1080 g/h at optical densities above 140. The feed rate was decreased, when the sugar concentration increased above 2.5 g/L or the permeate removal rate could not keep up with the feed addition.

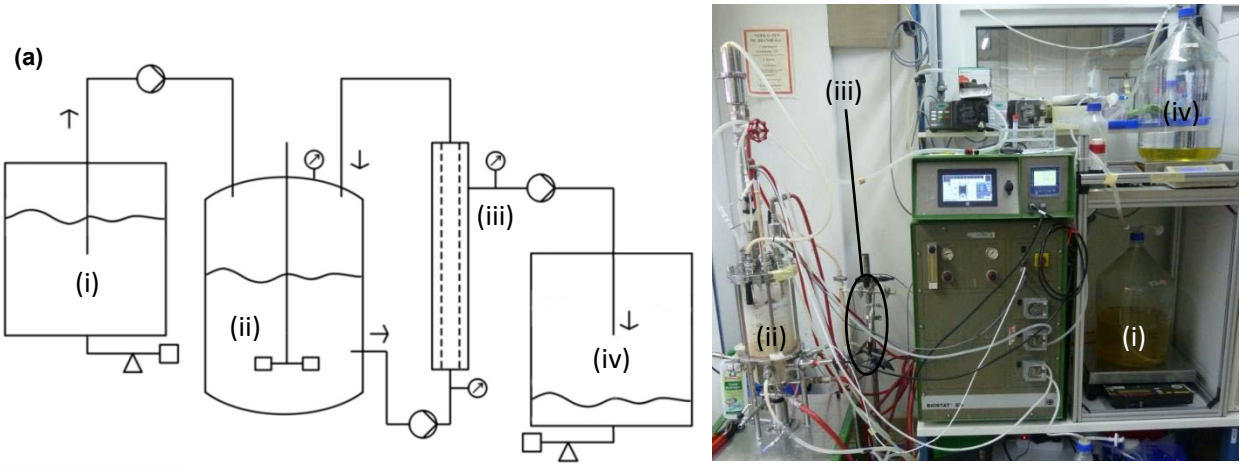

Figure 5.1. (a). Schematic diagram of the membrane bioreactor. From the feed bottle (i), the feed solution was supplied via a controlled pump into the fermenter (ii). In the fermenter the microorganisms utilized the supplied glucose and converted it into residual biomass or P3HB. The fermenter was in equilibrium with a cross-flow type microfiltration membrane (iii), from which a cell-free permeate was removed by a controlled pump into the permeate bottle (iv). The simultaneous addition/removal of feed/permeate according to the set-point was regulated via the weight of the two bottles. The cross-flow pump operated at a constant speed of 1750 mL/min, corresponding to a shear rate of ~6000 1/s as recommended by the membrane supplier. The pressure before and after the membrane, as well as on the permeate side were monitored. If the inlet pressure of the membrane exceeded 1.3 barg or the permeate removal lagged 1 kg behind the expected value, the digital control unit (DCU) automatically turned off all pumps. In order to prohibit fouling, the cross-flow and the permeate pumps automatically reversed the flow rate every hour for 1 min. If required, this interval was increased manually. Lorantfy *et al.* [147] pointed out that the temperature loss in the external loop leads to decreased metabolic activity of *Haloferax mediterranei* in cell-recycle fermentations. In the setup reported here, the temperature loss (0.6°C) in the external loop was no problem due to the fast cross-flow rate. **(b)** Picture of the reactor setup.

Samples were withdrawn periodically in duplicates and tested immediately for their optical density and approximate sugar concentration (MQant glucose test strips, Merck KGaA, Germany). The cells were separated from the supernatant (2500x g for 7 min). The duplicates of the supernatant were pooled and then taken for determination of the glucose and ammonium nitrogen concentration. From the cell pellet, the CDM and P3HB concentrations were determined. The deviations between the duplicates from the biomass and P3HB quantification are depicted as standard deviation in the graphs. The complete fermentations were also performed in duplicates and the individual duplicates lead to the same conclusions (data not shown).

The membranes were used multiple times and did not exhibit a significant reduction in the clean water flux (at least 450 L/m^2/h at a trans-membrane pressure of 0.2 barg). In some cases, the supplier-recommended cleaning procedure (50°C, 0.5 M NaOH for 1 h) had to be conducted twice to regain the water flux level.

5.3.3 Analysis

Biomass and P3HB

P3HB was determined by a modified method of Karr and coworkers.[129] The cell pellet was dried at 105 °C for at least 24 h and subsequently weighed to determine the CDM 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, P3HB is hydrolysed and the monomers are oxidized to crotonic acid. After cooling down, crotonic acid was quantified by an Agilent series 1100 HPLC system equipped with a CARBOSep CORGEL 87H column (Transgenomic, Nebraska, USA) at 65 °C and the corresponding RI detector (Agilent Technologies, California, USA). The mobile phase was 0.005 mol/L sulphuric acid at a flow rate of 0.9 mL/min. The CDM and P3HB concentration were calculated as g/L of fermentation broth, the P3HB content as % w/w in the CDM. The yield was calculated as g P3HB/g supplied sugar. The productivity given in g P3HB/L/h was calculated as the quantity of produced P3HB between the inoculation time (time = 0) and the time at which the highest productivity was reached.

Glucose

The exact glucose concentration was determined, on an Agilent series 1100 HPLC system with an ion exchange column ION 300 (Transgenomic, Nebraska, USA) at 45 °C, and a refractive index detector (Agilent Technologies, California, USA). The mobile phase was 0.005 mol/L sulphuric acid at a flow rate of 0.325 mL/min. Prior to the HPLC, the samples were clarified by a Carrez precipitation using

zinc sulphate and potassium hexacyanoferrate(II) at a molar ratio of 4:1. The loss of glucose to the permeate was estimated from the average glucose concentration in the bioreactor during the cell recycling phase and the total added glucose during the experiment.

Ammonia nitrogen

The ammonia nitrogen concentration was determined by steam distillation of fermentation supernatant and titration against 0.05 mol/L hydrochloric acid with an AutoKjeldahl Unit K-370 (Buechi, Flawil, Switzerland).

Microbial contamination

The absence or presence of microbial contamination was examined by staining the P3HB granules inside the cells at the end of each fermentation with aqueous 1% nile blue A and observing the stained cells under an OLYMPUS Vanox AHBT3 research fluorescence microscope (Olympus Corporation, Tokyo, Japan). The staining procedure was conducted according to Ostle *et al.*[148] We used a 480-550 nm filter for excitation and a 590-820 nm emission filter. If every cell observed contained PHA granules, the reactor was considered free of contamination.

5.4 Results and Discussion

In order to achieve high PHA productivities based on residues with a low carbon concentration, we implemented a membrane bioreactor system with a continuous flow of synthetic medium through the system. The reactor volume was kept constant while operating the bioreactor in a cell-recycle mode. This was done by using an external microfiltration module, from which a cell-free permeate devoid/low in carbon was withdrawn and discarded (see Figure 5.1). Two experimental setups were tested. In the first one a constant feed flow rate was employed. In the second setup an advanced feeding strategy was employed in order to increase the productivity.

In the first setup, the batch phase lasted 16 h, during which all glucose in the reactor was consumed. This resulted in the drop of stirrer speed below 600 rpm (see Figure 5.2), triggering the continuous supply of 720 g/h feed medium (12 g/L/h glucose). The rate of feed supply was kept constant throughout the remaining experiment. A trans-membrane pressure of 0.1 bar was established and did not increase during the experiment. The supply of nitrogen was stopped at an OD_{600} of 80 and nitrogen became limiting after 20 h, triggering the enhanced P3HB production phase. After 29 h, P3HB made up 73% of the CDM (65.4 g/L P3HB in 89.8 g/L total CDM). At this point a productivity of 2.27 g/L/h and a yield of 0.38 g P3HB/g glucose were reached. The fermentation was monitored also beyond this point, but due to the rapid decline in P3HB accumulation and reduced substrate

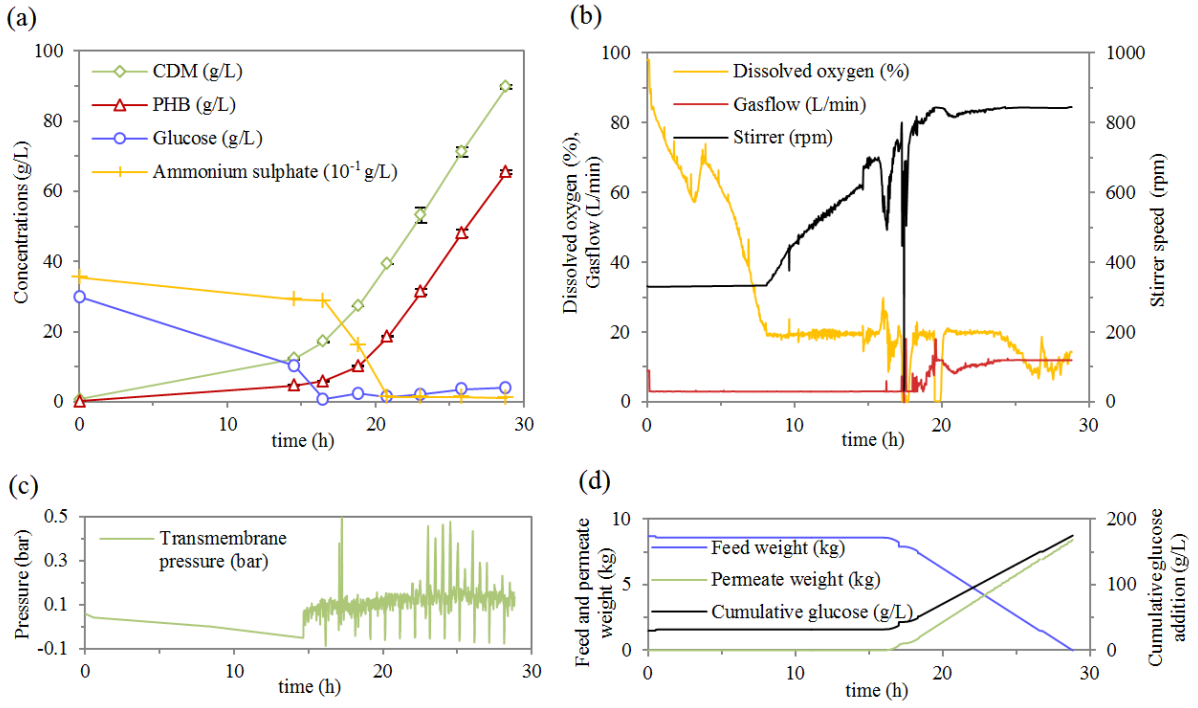

Figure 5.2 (a-d). Time profiles of a fermentation at a constant feed/permeate flow rate (720 g/h). After 29 h the PHA accumulation slowed down and the fermentation was stopped. The irregularities in stirrer speed, dissolved oxygen, trans-membrane pressure and feed/permeate weights after 18 h are due to control issues at this time point. The spikes in the pressure displayed are due to pre-scheduled changes in the direction of the cross-flow and permeate.

conversion the data is not of relevance. The sugar concentration in the bioreactor throughout the cell-recycling phase was always below 3.9 g/L, resulting in the withdrawal of a permeate very low in sugar.

Based on this experience, a second modified setup was investigated in order to further increase the productivity. The supply of nitrogen was stopped later (at an OD_{600} of 140), and during intervals of fast glucose consumption the feed supply was even further increased to 1080 g/h (19-24 h, 29-33 h, see Figure 5.3). The feed/permeate flow rates were decreased again or the membrane was exchanged, if one of the following issues occurred: Adequate permeate removal could not keep up with the target values (24 h). Glucose concentration in the bioreactor increased due to a decrease in the consumption (33 h). After 36 h a CDM of 148 g/L, containing 113 g/L (76%) P3HB was attained. This corresponds to a productivity of 3.10 g/L/h P3HB and a yield of 0.33 g P3HB/g glucose. The sugar concentration throughout the cell recycling phase was below 4.5 g/L, except for the sample point at 33 h (6.6 g/L).

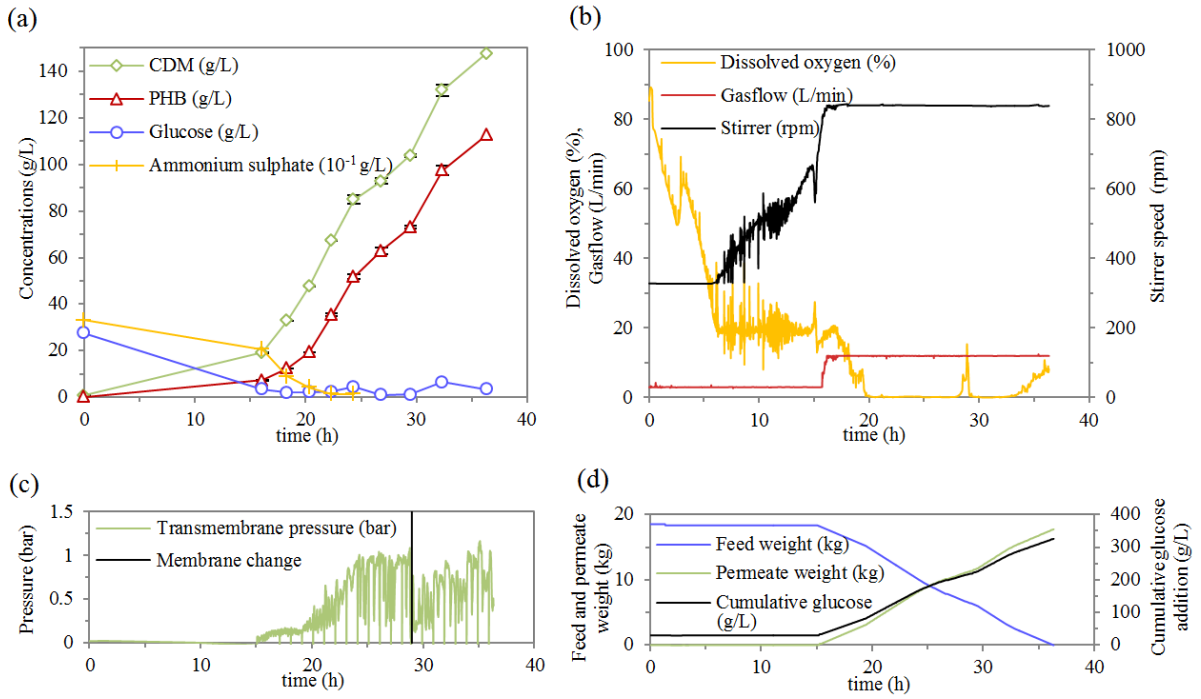

Figure 5.3 (a-d). Time profiles of a fermentation at an increased feed/permeate flow rate (720-1080 g/h). After 20 h the dissolved oxygen dropped to 0% and remained low for most of the production phase. It was not possible to raise the dissolved oxygen concentration to 20%, even at the highest stirring and aeration rates. Possibly this partial oxygen limitation was even beneficial for P3HB production as has been reported by others.[141]

Compared to the first setup, the second experimental setup achieved an increase in productivity by 36% to 3.1 g/L/h. This productivity is comparable to the best published results in the field. So far, only a few times productivities above 3 g/L/h have been described in literature. Amongst these publications, only two wild type organisms were applied: *Cupriavidus necator* (<3.1 g/L/h [46], [64]) and

Azohydromonas lata (<5.0 g/L/h [57], [56]). In the other cases, recombinant *Escherichia coli* (<4.6 g/L/h [67], [53], [68]) was used. All of these fermentations employ the classical fed-batch approach with a highly concentrated feed stream. There is only one exception: Ahn *et al.* [53] reached a productivity of 4.6 g/L/h with a recombinant *Escherichia coli* by fermenting concentrated whey in a membrane bioreactor at concentrations of 280 g/L lactose. A part of the fermentation broth was removed each time the lactose was depleted. The removed broth was replaced with the same volume of concentrated whey feeding solution. However, for this process, the whey had to be concentrated already before the application in the membrane bioreactor, complicating the overall process. The setup described in the current study, circumvents the first concentrating step by directly using the low-carbon feed stream for polymer production.

In the PHA literature, there seems to be a dogma of keeping the carbon concentration around 10-20 g/L during fermentations.[63] However, it is important to keep in mind that this optimal concentration range was developed for fed-batch fermentations, where no carbon is lost from the bioreactor. In the case of membrane bioreactors, an increase in the equilibrium carbon concentration tends to increase the productivity. However, higher equilibrium carbon concentrations in the feed come at the cost of losing carbon, as well as other medium components, to the permeate stream. Accordingly, there is an optimal feeding regime, depending on the carbon concentration in the feed, as well as the bioreactor. If the feed solution has a high carbon concentration, the volume change upon addition is small and it is not necessary to remove large amounts of fermentation broth from the bioreactor during each cell recycle phase.[53] In this setup, the actual concentration of carbon during the intermittent cell recycle phases does not have a big impact on the yield. If the feed stream is already quite low in carbon, conducting the cell recycle intermittently results in significant losses of carbon to the permeate stream. Ienczak *et al*. utilized a synthetic medium (90 g/L glucose) and partially withdrew the fermentation broth (25% v/v) each time the glucose concentration dropped to 10 g/L. Immediately afterwards, the same amount of reaction medium was fed to the bioreactor. As the fermentation broth was removed while it still contained more than 10 g/L of the initial sugar quantity, the yield (which was not specified) is presumably comparably low.[146] For our study, we chose a narrow glucose concentration range in the bioreactor of 1-5 g/L in order to obtain a high yield. This ensures a sufficient supply of carbon for the microorganisms, but does not waste the carbon to the permeate. Only a continuous supply and withdrawal of feed and permeate – as implemented in this study – are able to fulfil this criterion. We calculate that the amount of sugar lost to the permeate was 3.7 and 5.8% of the added glucose in setup one and two, respectively. As these losses are quite low, they have only a small effect on the yield. Both setups give good yields, which are comparable to classic fed-batch processes (0.38 and 0.33 g /L calculated as grams P3HB per gram added sugar in setup one and two, respectively). The effluent carbon from this process is too low to

be reused as feed solution in the process and may only be used in biogas production, or directly enter a waste water treatment facility.

With the second setup the long-term limits of the membrane permeate flux (1080 g/h) were approached. An ultrafiltration membrane from the same supplier was subsequently tested, as this type of membrane tends to have lower fouling propensity at high flux rates.[149] However, the trans-membrane flux of the fermentation broth in the applicable trans-membrane pressure range (< 1.0 barg) was significantly lower than in the case of the microfiltration unit (data not shown). Accordingly, a microfiltration membrane is the best choice for high flux rates in this setup.

Besides the substrate costs, the presented setup also offers advantages in another cost-relevant area: the costs for sterilization. According to Akiyama *et al.* [48] sterilization energy for P3HB fermentations in a fed-batch mode based on glucose are 8.9% (1.5 MJ/kg P3HB) of the total energy required for the fermentation utilities electricity, steam and cooling water. If significantly higher amounts of medium require sterilization, the additional energy required has negative effects for the economics of the overall process. The solution of this problem could be to sterilize only the medium components required during the growth phase. In the PHA production phase, growth of unwanted microorganisms is suppressed due to the lack of one essential nutrient. During this phase also the major part of the feed is consumed. In the current setup, the feed was prepared in 7-10 L batches. Only the first feed bottle of each fermentation experiment was autoclaved, as the second feed solution was used after the end of the growth phase. In the cases where the membrane was exchanged, the second membrane was not sterilized either. In all experiments which were done for this paper, we never encountered any contamination, supporting the argument for a partial feed sterilization.

The membrane bioreactor described here was run with synthetic medium to generate the proof of principle for highly productive fermentations with a low carbon concentration in the feed. Currently the organic carbon in these fractions can be merely used for biogas production or poses a problem for waste water treatment. Our process removes the organic carbon from the waste stream and converts it to a valuable product at a high efficiency.

5.5 Conclusions

We demonstrated the highly efficient production of P3HB by wild-type *Cupriavidus necator* in a membrane bioreactor. Despite the low concentration (50 g/L glucose), the carbon delivered with the feed solution was almost completely consumed (94%) in the bioreactor and a permeate very low in carbon was drawn. This is critical for the efficiency in terms of substrate exploitation, which is also expressed by the good yield (0.33 g P3HB/g supplied sugar). Most importantly, with the given setup, a productivity higher than most of the available literature values to date (3.10 g P3HB/L/h) was reached. Important for the success of this strategy were a high cell concentration prior to the onset of the nutrient limitation, faster feed/permeate flow rates coupled with a low concentration of carbon in the permeate as well as an efficient microbial strain.

We were able to simultaneously achieve high PHA productivities and good yields using substrates with a low carbon concentration. A range of very cheap raw materials is made available for industrial PHA production. This setup is the proof of principle for a cheap and efficient conversion of waste materials to a higher value biobased and biodegradable polymer.

5.6 Acknowldegements

The authors would like to express gratitude to Martin Koller from the University of Graz for supplying the *Cupriavidus necator* strain. Wilhelm Müllner made invaluable contributions by customizing the process control system according to the needs of the experimental setups. Furthermore, the assistance in proof-reading by Werner Fuchs from the University of Natural Resources and Life Sciences is appreciated.

6 GENERAL CONCLUSIONS

In the frame of this PhD project it was demonstrated that chicory roots can be efficiently converted to the bioplastics PHA. The developed protocol for inulin extraction and hydrolysis gave a respectable yield of monomeric sugars (86 % of fructose and 65 % of glucose content). With this procedure, a final hydrolysate containing primarily fructose (27.6 g/L) and glucose (4.8 g/L), as well as some nitrogen and essential minerals was obtained.

All three tested *Cupriavidus necator* strains grew and produced P3HB from the chicory root hydrolysate. However, their performance was quite different, as considerably different dry biomass concentrations were reached (DSM 428: 11.0 g/L, DSM 531: 3.5 g/L, DSM 545: 14.0 g/L). This was surprising as they belong to the same species and demonstrate that careful strain selection is a critical factor. *Cupriavidus necator* DSM 545 did not only give the highest biomass concentration, it also had the highest P3HB content, yield and productivity.

To check the properties of the polymer produced in this thesis, it was extracted with chloroform and several physico-mechanical characteristics were determined. The glass transition temperature, melting temperature and the molecular weight, are in good accordance with published data from cultivations on synthetic medium.

Although chicory roots are an overlooked by-product resource, no other research group reported yet on the utilization of inulin from chicory roots for the production of PHA. One report was found using another inulin-storing plant: Jerusalem artichoke.[121] Nonetheless, the results presented in this thesis are superior by a factor of two over the published data.

The results obtained as part of this thesis demonstrate that the polymer produced from chicory roots is a highly viable substitute for PHA produced from refined sugars. Due to the scientific novelty of the work, the results have been published in the Journal “Chemical and Biochemical Engineering Quarterly”.

The chicory root process in this first publication is a batch process. In order to reach higher productivities with the chicory root hydrolysate as well as other abundant agro-residues containing a low carbon concentration, a membrane bioreactor setup was developed.

During the elaborate development of different membrane bioreactor setups, the effects of various parameters including pumps, membranes, and actions against fouling were tested. In the final setup, it was possible to reach a very high productivity (3.1 g/L/h). Very little carbon passed through the

reactor into the permeate (6%), giving a good yield (0.33 g P3HB/g supplied sugar), ensuring an efficient utilization of the carbon supplied by the feed.

A similar membrane bioreactor was published a couple of months ago.[146] The productivity (1.0 g/L/h) as well as biomass concentration and yield reported by Ienczak *et al.* are well below the values reported in this thesis. The following considerations, which were implemented in the setup described in this thesis, are presumed to be responsible for the improved performance obtained here:

1. A high cell concentration before the onset of the nutrient limitation ensured a fast polymer production.
2. An optimal feed/permeate pump rate supplied enough carbon for a high biomass or polymer conversion rate. It is important that the feed rate is not too high so that surplus carbon is not lost to the permeate.
3. A membrane suitable to provide the high flux rates necessary for the process.
4. A strain producing efficiently PHA, which is not affected by the shear forces in the pump and has a low fouling propensity.

The results for the second setup are accepted for publication in the Journal "New Biotechnology". Currently, the article is in press.

The next step which is beyond this dissertation is the process transfer of the developed membrane bioreactor setup to agro-industrial by-products. Unfortunately, the chicory roots from northern Spain are no longer available, as the farmers do not dispose them any more at the waste collector. It is supposed that they have found a different route of valorization, possibly the extraction of "second"-grade inulin.

However, the author has identified two by-products from a local sugar mill which are promising substrates: pressed beet pulp and desugarized molasses. Both streams accrue in large quantities at sugar mills and have no commercial value at the moment.

The first one, pressed beet pulp, contains residual concentration of 1.5 - 2.5 % sucrose, as most of it is extracted during the sugar separation. Besides sucrose, its dry matter contains high amounts of structural carbohydrates (pectin, cellulose, hemicellulose) which might be utilized as substrate for PHA fermentation. At the moment this by-product stream is dried and fed to animals, which can only partly metabolize the constituents. In a valorizing step, the simple as well as complex carbohydrates could get converted to volatile fatty acids (acetic acid, propionic acid, …) by acidogenic microorganisms. The obtained hydrolysate typically contains 20 - 25 g/L total volatile fatty acids, which could serve as a substrate for PHA production in the developed membrane bioreactor.[150]

The second by-product, desugarized molasses, accrues at the chromatographic separation of sucrose from molasses. It is relatively high in salt (15 g/L ash) and quite low in carbohydrates (10 g/L sucrose).

Currently, no valorization is in place for this by-product. This stream could enter a membrane bioreactor directly and serve there as substrate for PHA production.
In line with these considerations, the author of this dissertation identified and contacted project partners and wrote a research proposal on the conversion of these two by-products to PHA using the membrane bioreactor. The main industrial partners are the sugar mill R&D facility AGRANA Research and Innovation Center and IM Polymers, which supplies and characterized the raw materials and does the up-scaling of the process. The process itself is developed in the working group to which the author belongs (Microbial Production Technology, led by Markus Neureiter). Two other project partners work on specific tasks of the project in their respective field of expertise (Institute for chemistry and technology of materials at the TU Graz and Bioenergy2020+). The three-year project was submitted as cooperative research and development project to the Austrian "Forschungsförderungsgesellschaft" (FFG). It was accepted for funding, and is meanwhile running since March 2016. The overall aim is to produce PHA test batches from the two raw materials and to identify areas of polymer application. The results obtained should aid the sugar mill in the decision to venture into the polymer-production market.

Besides the mentioned first-author publications, the author was also involved in two other conceptually similar publications, raising the scientific output of this thesis. In these two publications, another promising agro-industrial by-product: potato processing residue was utilized for making the polymer precursor lactic acid. The results show that lactic acid from these by-products can be generated at high productivities, and thereby contribute in paving the way to an economic and sustainable biopolymer production.

In conclusion, the findings generated with the by-product chicory roots showed that the roots can be converted to PHB. However, the PHA fermentation process strategies published at that time were not feasible for the highly efficient utilization of such low-carbon agro-industrial by-products. In order to overcome this issue, a membrane bioreactor process was designed and successfully implemented. With this setup, highly productive PHA fermentations based on substrates with a low carbon concentration were accomplished. The obtained results are highly promising also with respect to work published in the scientific literature. Moreover, the developed concepts and ideas have led to the acquisition of funding for the further evaluation and eventually establishment of a commercial PHA production process based on these low-cost by-products.

7 List of Publications

7.1 Scientific Publications

- Chemical and Biochemical Engineering Quarterly
  Haas, C.; Steinwandter, V.; Diaz De Apodaca, E.; Maestro Madurga, B.; Smerilli, M.; Dietrich, T.; Neureiter, M., Production of PHB from Chicory Roots – Comparison of Three Cupriavidus necator Strains. **2015,** *29* (2), 99-112.
- New Biotechnology
  Haas, C.; El-Najjar, T.; Virgolini, N.; Smerilli, M.; Neureiter, M., High Cell-Density Production of Poly(3-hydroxybutyrate) in a Membrane Bioreactor. *(accepted)* **2016**.

Parallel to the work described above, a second PhD project dealing with a closely related issue: the production of lactic acid from agro-industrial residues as precursor for the polymer polylactic acid (PLA), was conducted by Marina Smerilli. A close co-operation was carried in terms of experimental design, joint development of methods, practical implementation and interpretation of results. With respect to the mutual support and contributions Marina Smerilli co-authors the publications developed within the frame of this thesis and vice versa.

- Journal of Chemical Technology and Biotechnology
  Smerilli, M.; Neureiter, M.; Wurz, S.; Haas, C.; Fruhauf, S.; Fuchs, W., Direct fermentation of potato starch and potato residues to lactic acid by Geobacillus stearothermophilus under non-sterile conditions. **2015,** *90* (4), 648-657.
- Chemical and Biochemical Engineering Quarterly
  Smerilli, M.; Neureiter, M.; Haas, C.; Frühauf, S.; Fuchs, W., Valorization of Potato-processing Residues for the Production of Lactic Acid. **2016,** *30* (2), 255-263.

7.2 Other Printmedium Contribution

- Biobased Future – Mitteilungsblatt über Biomasse für Energie und Industrie in einer nachhaltigen Wirtschaft
  Haas, C.; Neureiter, M., Biokunststoffe aus agro-industriellen Nebenströmen, **7/2016**, (6), 14

7.3 Research Proposal

- Valorisierung von Restströmen der Zuckerindustrie zur Biokunststoffproduktion – ValorPlast
  Kooperative F&E Projekte der österreichischen Forschungsförderungsgesellschaft (FFG), FTI-Initiative: Produktion der Zukunft, Total buget 624 072 €, Start of project 3/2016

7.4 List of Oral Presentations

- Vienna Workshop on Sustainability for PhD students
  Microbial production of PHAs from selected food residuals, 11/2013, Vienna
- Renewable Resources and Biorefineries Conference (RRB)
  Production of PHB from Chicory Roots, 06/2014, Valladolid, Spain
- Global Cleaner Production Conference (GCP)
  Utilizing Agro-Industrial Residues with a low Carbon Content for Bioplastic Production, 10/2015, Barcelona, Spain
- Europan Congress on Biotechnology (ECB)
  High Cell-Density P3HB Production in a Membrane Bioreactor, 7/2016, Krakow, Poland
- International Symposium on Biopolymers (ISBP)
  High Cell-Density P3HB Production in a Membrane Bioreactor, 9/2016, Madrid, Spain

7.5 List of Poster Presentations

- European Biorefining Training School in Budapest
  Production of PHB from Chicory Roots, 7/2014, Budapest, Hungary
- Ghent Bio-Economy Summer School
  Production of PHB from Chicory Roots, 8/2014, Ghent, Belgium
- Biopolymer materials and engineering (bimate)
  PHB Production in a Membrane Bioreactor by *Cupriavidus necator*, 4/2015, Slovenj Gradec, Slovenia
- International Conference and Exhibition on Biopolymers and Bioplastics
  PHB Production in a Membrane Bioreactor by *Cupriavidus necator*, 4/2015, Slovenj Gradec, Slovenia
- European Congress of Applied Biotechnology (ECAB)
  Haas, C.; Neureiter, M., Enhanced production of PHB by cell recycling, 9/2015, Nice, France
- European Symposium on Biopolymers (ESBP)
  Development of a high-cell density PHB-production process suitable for agro-residues, 9/2015, Italy, Rome
- Renewable Resources and Biorefineries
  High Cell-Density P3HB Production in a Membrane Bioreactor, 6/2016, Ghent, Belgium

8 DECLARATION OF AUTHORSHIP

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9 GLOSSARY

C. necator	*Cupriavidus necator*
CARBIO	CARbohydrate derived BIOpolymers
DSC	differential scanning calorimetry
DSM, DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
mcl	medium-chain length
PHA	Polyhydroxyalkanoic acids
P3HB	poly[(R)-3-hydroxybutyrate]
P3HB3HV	poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate]
P3HB3HV-block-HB	poly[(R)-3-hydroxybutyrate-co-(R)-3-hydroxyvalerate]-block-[(R)-3-hydroxybutyrate]
PLA	Polylactic acid
scl	short-chain length
TKN	total kjeldahl nitrogen

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Acetyl-CoA is the central point of the PHA metabolism. PHA-producing bacteria build it from various sugars, fatty acids, lipids, and even carbon dioxide. In three steps two molecules of acetyl-CoA are conjugated to acetoacetly-CoA, then reduced to 3-hydroxybutryryl-CoA and polymerized to P3HB (see Figure 1.3). The three genes conducting these steps have been transferred numerous times to other hosts, enabling well studied strains such as *E. coli* to produce PHA.[9]

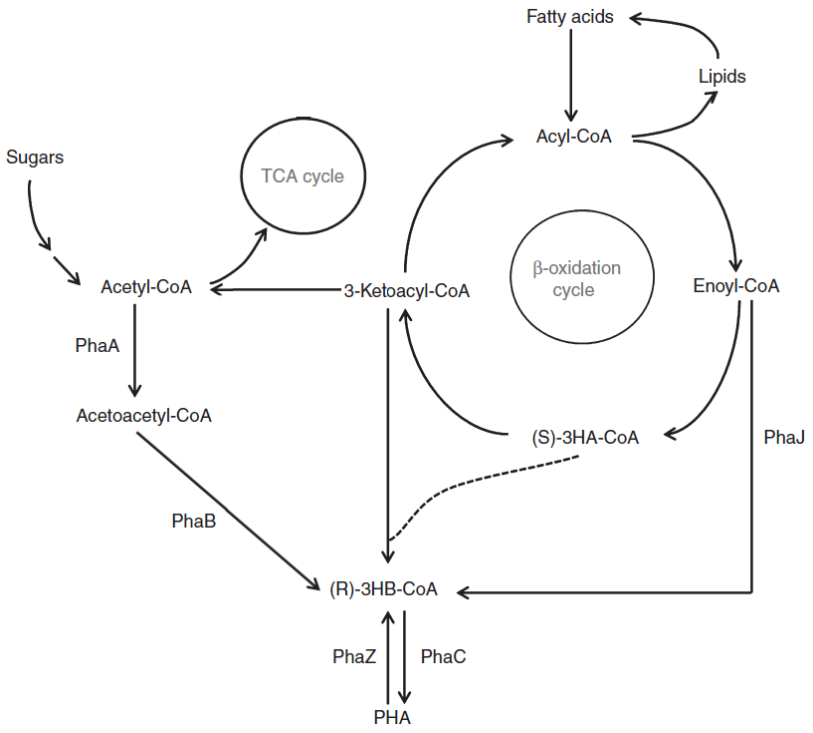


Figure 1.3. Metabolic pathways of PHA accumulation.
PhaA: β-ketothiolase,
PhaB: NADPH-dependent acetoacetyl-CoA reductase,
PhaC: PHA polymerase,
PhaZ: PHA depolymeras;
PhaJ, enoyl-CoA hydratase;
Acc, acetyl-CoA carboxylase.
Modifed from [10]

Depending on the cultivation conditions, the polymers can consist of 100-30 000 monomers and have a molecular weight distribution of 50 to 1 000 kDa. Due to their apolar nature, PHA molecules agglomerate to granules inside the cells. Every cell possesses at least one granule which is inherited in a DNA-associated manner. In total the granules can make up in very high cellular polymer loads (e.g. 60-90% for *Cupriavidus necator*, 40-70% by *Haloferax mediterranei*).

Once the cells are filled up with PHA, the polymer only needs to be extracted in order to get a processable plastic. This extraction is usually conducted with organic solvents, as this method separates PHA from the residual biomass with a high recovery rate and purity, as well as low costs. In the literature, there are also alternative approaches discussed in order to circumvent the utilization of organic solvents.[11]

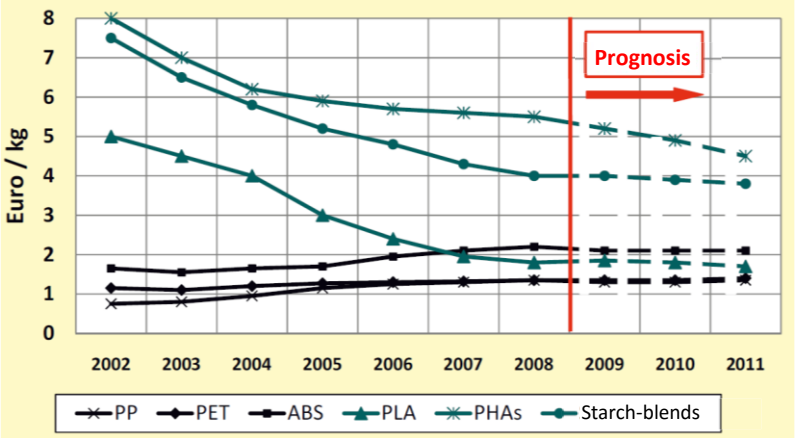


Figure 1.5. Price development of petrochemical and biobased polymers. PP: polypropylene, PET: polyethylene terephthalate, ABS: acrylonitrile butadiene styrene, PLA: polylactic acid. [3]

The raw material costs are not only a major cost factor, the prices for refined sugars or fatty acids/lipids, currently employed in industrial processes, are also very volatile. They strongly depend on the oil price and have witnessed a long and steady increase over the past decades. In order to get (more) independent of this big cost factor, a quest for cheap raw materials for PHA production has been set off. Several research groups as well as research-oriented companies have focussed on that strategy, creating a new and vibrant field of research.

as well as virgin biomass is cascadic use. This means that several (bio)processes are integrated into a holistic utilization of biomass, a so-called biorefinery. The biorefinery is described as "integrated biobased industry using a variety of technologies to make products such as chemicals, biofuels, food and feed ingredients, biomaterials, fibres and heat and power, aiming at maximizing the added value along the three pillars of sustainability (Environment, Economy and Society)".[71] In line with this, PHA production would be one step in the cascadic utilization of biomass, just as it is the case for the current plastic manufacturing in the oil refinery. For example, the BIOCYCLE biorefinery in Serrana (Brazil) shown in Figure 1.8 converts sugar cane into sugar (sucrose), ethanol and P3HB.[45] In this biorefinery the by-product molasses, accruing at the sugar crystallization step, is converted into P3HB. The polymer is extracted from the cells using a by-product from the ethanol distillation: long-chain alcohols. The process energy is provided by burning the by-product bagasse. In this optimized system, the polymer can be produced at low costs and adequate purity.

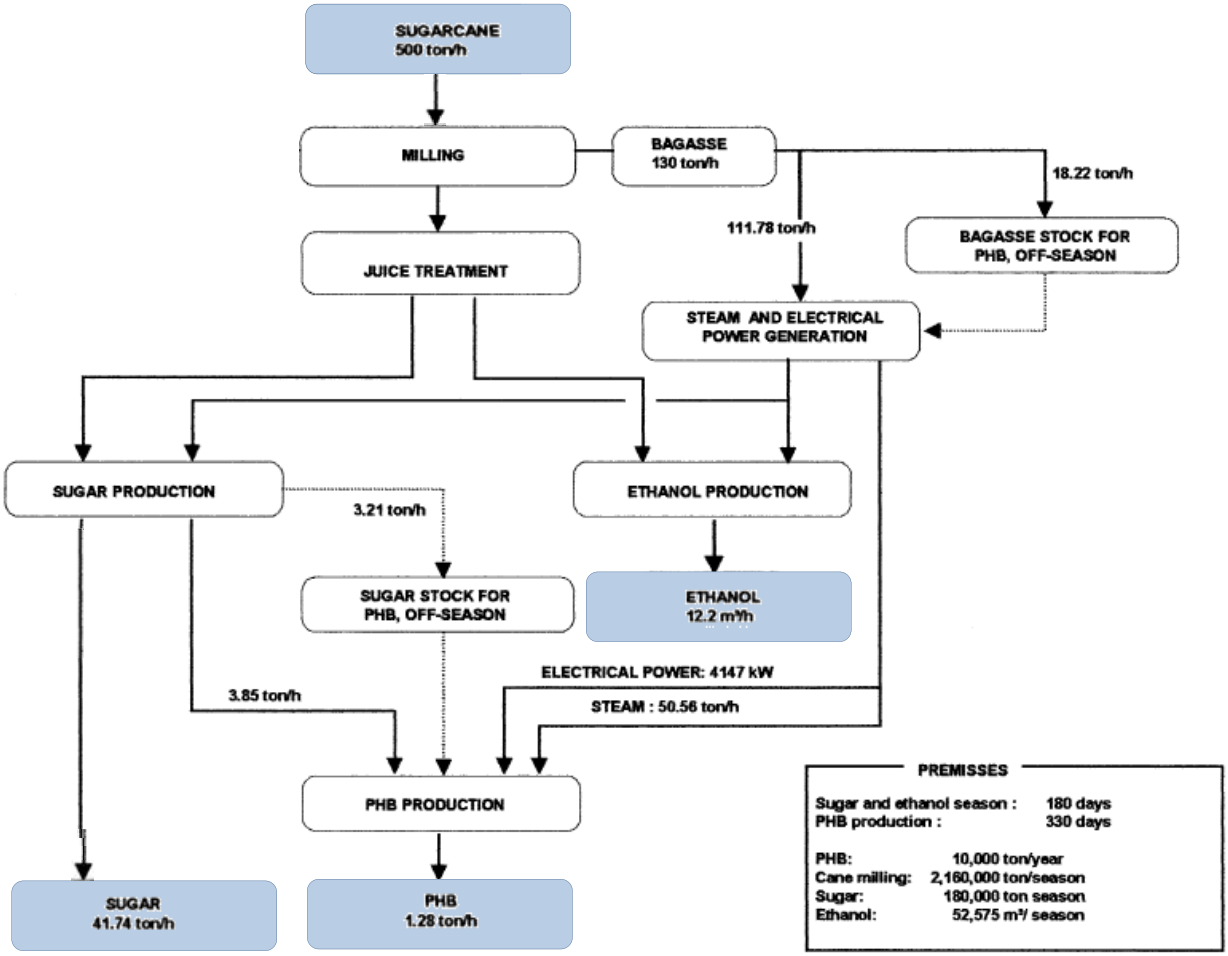


Figure 1.8. Example of a biorefinery: the BIOCYCLE biorefinery in Serrana (Brasil). The raw material sugar cane is processed completely to obtain sugar, ethanol as well as PHB. Bagasse is used for the generation of process energy. Modified from [45]

Biobased products have – particularly in Europe – a very positive image among end-consumers, who are most of the time also willing to pay a "green premium".[72] However, at the current development stage of PHA production, most biorefineries need some legislative and/or infrastructure support. Biofuels were the first biobased product which received very strong political support in the early 2000s. However, the expected ecological benefits of biofuels, are still debated in the scientific community.[73] This led to a reluctance of political support for biobased products without sound evidence of an improved environmental performance. Policy makers seem to get more in favour of quantifying the environmental sustainability.

The methodologies for the assessment of the environmental sustainability are still under development and heavily debated in the community.[74] Nonetheless, the European Union is pushing forward the development of a "product environmental footprint" PEF, a harmonised methodology for the calculation of the environmental footprint of products. [75]

One of the parameters assessed in PEF is the emissions of greenhouse gases, which is also one of the most important parameters investigated by sustainability benchmarking in general. Surprisingly, when comparing the greenhouse gas emissions, Endres *et al*. found that most plastic materials (biobased as well as conventional petrochemical plastics) have similar carbon dioxide emissions.[3] At first glance this finding is unexpected, because – unlike oil-derived carbon – carbon originating from renewable sources does not add to the carbon footprint. However, this comparison is based on data from vastly different levels of industrialisation. The data for conventional petrochemical plastics is derived from full scale industrial plants, whereas all data for bioplastics is at the moment only available for semi-industrial or laboratory-scale experiments. Upon scaling up, the greenhouse gas emissions are expected to decrease significantly.

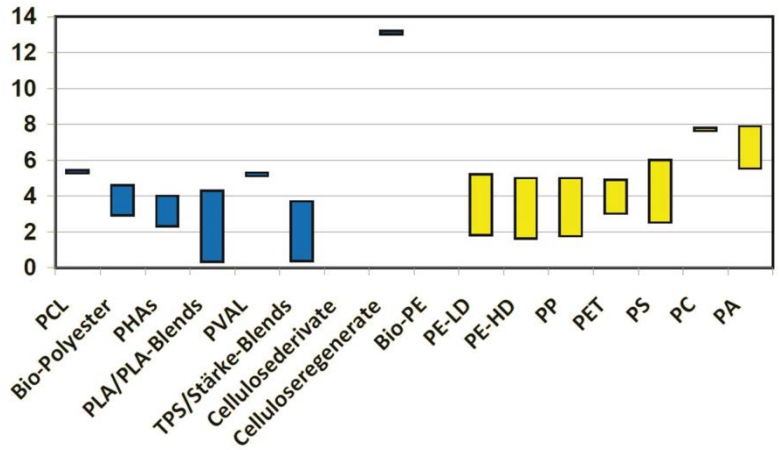


Figure 1.9. Greenhouse gas emissions (kg CO_2 eq./kg polymer) of biobased or biodegradable plastics (blue) vs. conventional petrochemical plastics (yellow). The data for the conventional oil based plastics is from full-scale industrial sites. The data for the biobased or biodegradable plastics is mainly from semi-industrial or laboratory-scale experiments.[3]

2 OBJECTIVES

The work within this thesis is divided into two phases and two general objectives. In the first phase, the aim was to evaluate and establish a laboratory-scale process for the production of PHA from chicory roots which can be applied by the Spanish waste company TRASA (tratamientos subproductos agroalimentarios S.L.). The company manages and treats by-products from the fruit and vegetable processing industry in the Ebro valley in northern Spain. Chicory roots are there an abundant by-product (10 000 t/a), which contain a reasonable amount of easily degradable carbohydrates (3.3 % w/w inulin in fresh weight). The roots degrade within weeks, but accrue throughout the year and can be processed continuously. In order to do so, the following scientific objectives had to be tackled (see Figure 2.1):

- Establish a pre-treatment protocol to extract and hydrolyse inulin from the chicory roots.
- Test the ability of several PHA-producing microorganisms to degrade inulin as well as its degradation products glucose and fructose, since little knowledge is available comparing different strains.
- Develop a fermentation process for the production of PHA. Investigate the effect of detoxification on the fermentation performance.
- Evaluate the produced polymer by extracting and characterizing it in relation to physical and thermomechanical properties.

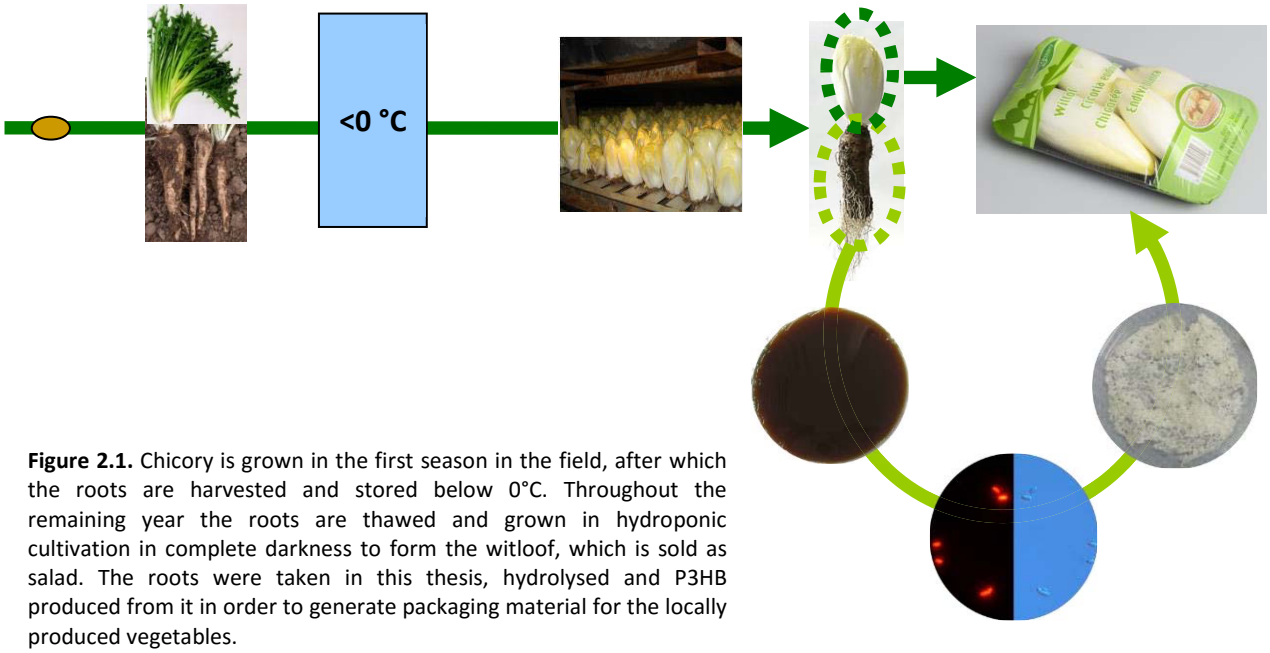


Figure 2.1. Chicory is grown in the first season in the field, after which the roots are harvested and stored below 0°C. Throughout the remaining year the roots are thawed and grown in hydroponic cultivation in complete darkness to form the witloof, which is sold as salad. The roots were taken in this thesis, hydrolysed and P3HB produced from it in order to generate packaging material for the locally produced vegetables.

making oxygen essential for the reaction, but not to the same extent as it is the case for a complete oxidation. [113] P3HB 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) and P3HB content (66 %) are reached after 5.0 days at the end of the fermentation, after all the fructose has been consumed (see Table 3.3). Overall the yield is 0.32 g P3HB / g consumed sugar and P3HB is produced with a productivity of 0.062 g P3HB/L/h.

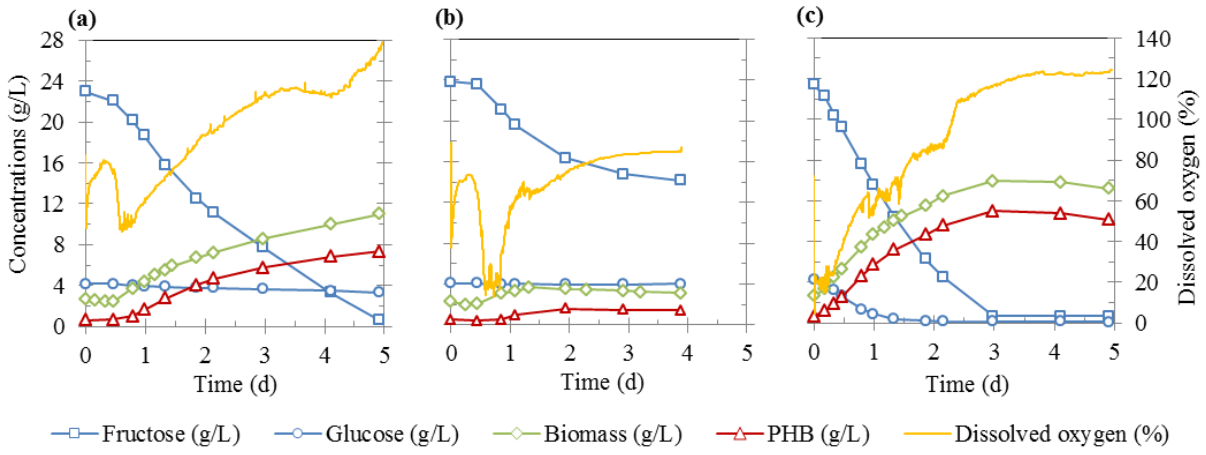


Figure 3.4. Bioreactor fermentations using Cupriavidus necator **(a)** DSM 428, **(b)** DSM 531, and **(c)** DSM 545 in the obtained chicory root hydrolysate. On the left axes are the concentrations of the substrates and products and on the right axes is the dissolved oxygen.

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 residual biomass. After 21 h, P3HB accumulation starts and has a peak after 2.0 days (3.5 g/L biomass, 45 % P3HB), 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.[127] The yield and productivity of the fermentation are 0.15 g/g and 0.016 g/L/h, respectively.

Strain DSM 545 consumes both sugars and does so 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 straight away. Most of the residual biomass is accumulated during the first 8 h, reaching a value of 2.7 g/L 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. P3HB production is not strictly separated from residual biomass production, i.e. some P3HB is already formed during cell growth. P3HB production also starts straight away and reaches the maximum rate after 19 h, after

4.3 Experimental Setup

A 0.5 L bioreactor (dasgip®, Eppendorff) was equipped with a 420 cm^2 polysulfone hollow fibre membrane cartridge (GE Healthcare). Membrane pore sizes of 0.2 µm (microfiltration) and 750 kDa (ultrafiltration) were tested and compared. The cross-flow was established with a diaphragm pump, which produced an alternating tangential flow in the membrane of 1.2 L/min (Refine technologies, see Figure 4.1).

Two types of feed solution were fed to the bioreactor. The first contained glucose (50 g/L) and ammonium sulphate (3 g/L) in a mineral salts medium (for exact composition of all media see chapter 5.3.1 on page 70). It was fed (40 mL/L/h) at rates of 1.25 g glucose/L/h and 0.06 g ammonium sulphate/L/h to the bioreactor. The second feed was installed once the OD reached ~20 - 30, which corresponds to a residual biomass concentration of 5 - 8 g/L. It did not contain nitrogen in order to induce the nitrogen limitation. In the basic setup, which was used for comparing the microfiltration and ultrafiltration membranes, the same feed rate was used. The microfiltration membrane was also tested at an increased constant feed rate. Inside the bioreactor *Cupriavidus necator* DSM 545 converted the glucose into residual biomass and P3HB. The glucose concentration in the bioreactor was not monitored and the base for pH control was 1 M NaOH. The other procedures are the same as detailed in chapter 5.3.

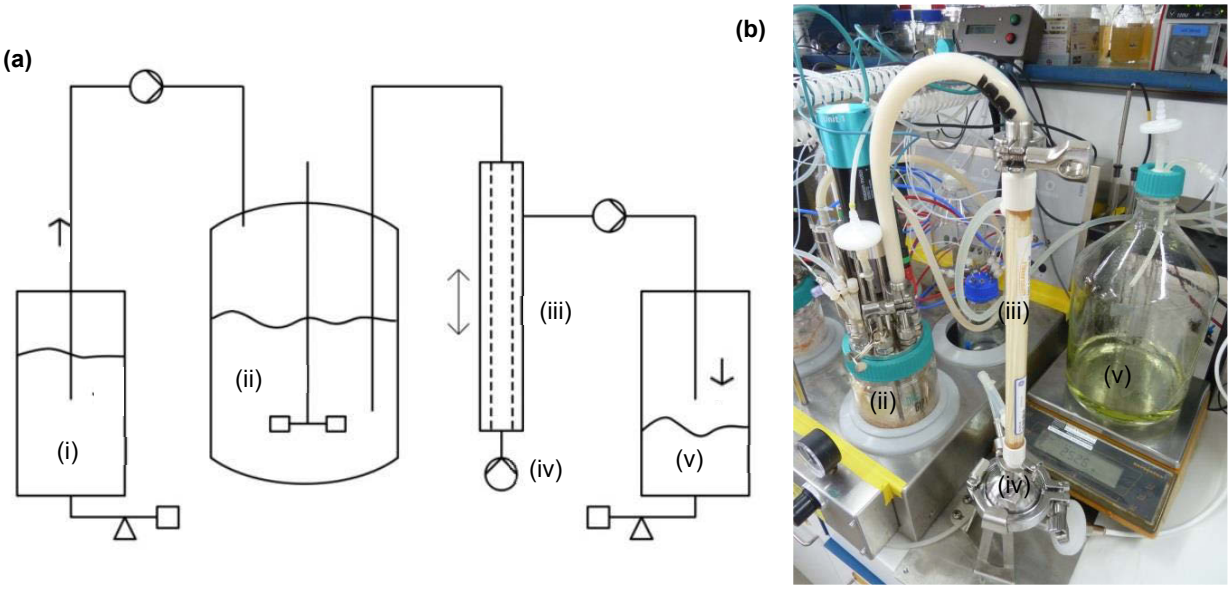
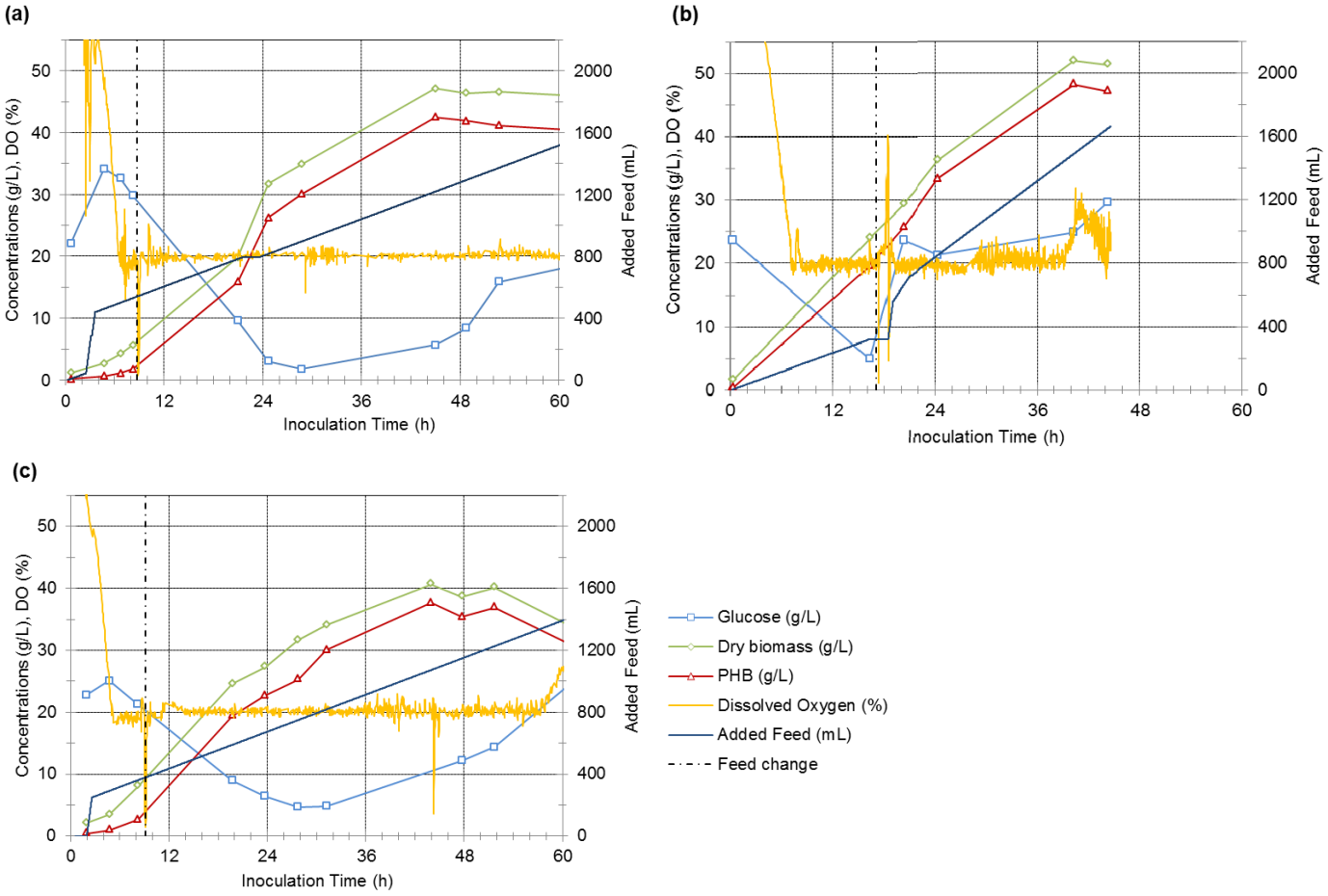


Figure 4.1 (a). Schematic drawing of the membrane bioreactor. From the feed bottle (i), the feed solution was supplied via a controlled pump into the fermenter (ii). In the fermenter the microorganisms utilized the supplied glucose and converted it into residual biomass or P3HB. The fermenter was in equilibrium with a cross-flow type microfiltration membrane (iii), from which a cell-free permeate (v) was removed by a controlled pump into the permeate bottle. The volume inside the bioreactor was kept constant by matching the amount of feed added and permeate removed. A diaphragm pump (iv) created an alternating flow in order to reduce fouling and keep the shear stress low. **(b)** Picture of the reactor setup with the same indices.

4.4 Results and Discussion

In order to efficiently convert substrates containing only a moderate to low concentration of carbon into P3HB, different membrane bioreactor setups were tested. In the first setup, a microfiltration unit was employed and a constant feed stream pumped through the system. This feed stream supplied 1.25 g glucose/L/h to the microorganisms and was first converted into residual biomass and later to P3HB. Nitrogen became already limiting before the feed change as the C:N ratio was slightly too high in the feed. This resulted in a low residual biomass concentration (5 g/L). However, the growth and P3HB production was fast (see Figure 4.2.a.) and lead to a good productivity (0.85 g P3HB/L/h, see Table 4.1). During the experiment, not all glucose supplied to the bioreactor was utilized. Particularly at the beginning, the equilibrium concentration of glucose is relatively high (up to 35 g/L), resulting in significant amounts of glucose being swept out of the system. This is reflected in the yield (0.28 g P3HB/g supplied sugar), which is lower than usually reported in the literature (0.30 - 0.38 g P3HB/g sugar).[46, 63]



Wisconsin, USA). The dissolved oxygen concentration (DO) was controlled to 20% saturation by adjusting the stirrer speed with two six-bladed Rushton turbine impellers (300-800 rpm) as well as the air flow (1-4 vvm). The pH-value was regulated to 7.0 by 12.5% w/v ammonia. Addition of the antifoaming agent Glanapon DG 160 in an aqueous 1:10 dilution was controlled by a foam probe. The trans-membrane pressure was calculated as the difference between the pressure in the cross-flow before the membrane and in the permeate.

The batch mode took typically 14-18 h. A decrease in stirrer speed below 600 rpm, due to the complete consumption of glucose in the fermentation broth, automatically triggered the cell recycle mode. During this cell recycle mode, feed/permeate were simultaneously added/removed at a flow rate of 720 g/h (12 g/L/h glucose). The complete retention of cells was accomplished by a 0.02 µm pore size microfiltration membrane (CFP-2-E-4MA, 420 cm^2 membrane area, GE healthcare, UK). After reaching a certain CDM, which was estimated by the optical density (OD_{600}), P3HB production was triggered by switching to 2 M NaOH for the pH control. The base change was conducted at OD_{600} 80 or 140, corresponding to a CDM of 20 or 35 g/L, respectively. In the second setup, the feed supply and permeate removal were increased to 1080 g/h at optical densities above 140. The feed rate was decreased, when the sugar concentration increased above 2.5 g/L or the permeate removal rate could not keep up with the feed addition.

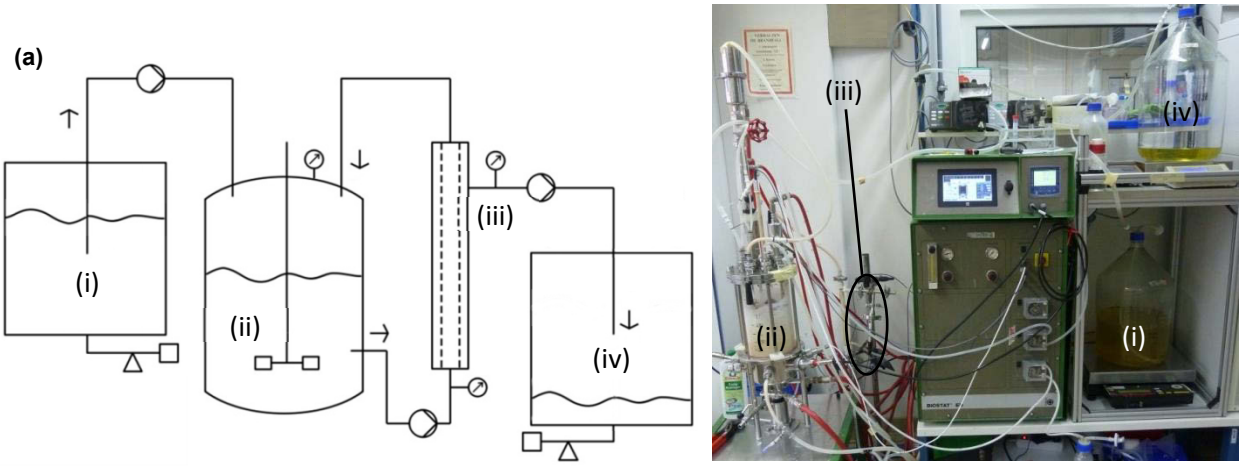


Figure 5.1. (a). Schematic diagram of the membrane bioreactor. From the feed bottle (i), the feed solution was supplied via a controlled pump into the fermenter (ii). In the fermenter the microorganisms utilized the supplied glucose and converted it into residual biomass or P3HB. The fermenter was in equilibrium with a cross-flow type microfiltration membrane (iii), from which a cell-free permeate was removed by a controlled pump into the permeate bottle (iv). The simultaneous addition/removal of feed/permeate according to the set-point was regulated via the weight of the two bottles. The cross-flow pump operated at a constant speed of 1750 mL/min, corresponding to a shear rate of ~6000 1/s as recommended by the membrane supplier. The pressure before and after the membrane, as well as on the permeate side were monitored. If the inlet pressure of the membrane exceeded 1.3 barg or the permeate removal lagged 1 kg behind the expected value, the digital control unit (DCU) automatically turned off all pumps. In order to prohibit fouling, the cross-flow and the permeate pumps automatically reversed the flow rate every hour for 1 min. If required, this interval was increased manually. Lorantfy *et al.* [147] pointed out that the temperature loss in the external loop leads to decreased metabolic activity of *Haloferax mediterranei* in cell-recycle fermentations. In the setup reported here, the temperature loss (0.6°C) in the external loop was no problem due to the fast cross-flow rate. **(b)** Picture of the reactor setup.