Production of polyhydroxyalkanoate from high cell density fermentations using palm oil and waste animal fats as carbon feedstocks and their recovery with non-halogenated solvents

vorgelegt von

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Preamble

This commutative PhD thesis is based on six (four 1st author; two 2nd author) peer reviewed publications in the journals: *Applied Microbiology and Biotechnology*, *Applied and Environmental Microbiology*, *Biotechnology and Bioengineering* and *Journal of Biotechnology*, as indicated in front of each chapter.

The 1st part of the experiments for this thesis (Chapter II–IV; Chapter VI), were performed at the Massachusetts Institute of Technology in the Sinskey laboratory (Department of Biology) between 04/10–06/12. This contribution was also part of the Malaysian-MIT Biotechnology Partnership Programme bioplastics, funded by the Ministry of Science, Technology and Innovation, Malaysia.

The 2nd part of the experiments (Chapter V), were performed at the Technische Universität Berlin in the Stahl laboratory (Institute of Biotechnolgy) between 07/12–06/13. This contribution was funded by the Research and Teaching Institute for Brewing in Berlin VLB.

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John Quimby, I thank you for the invention of the “cable tie foam breakers”, which were critical for reaching the high cell density cultures, in laboratory fermenters, shown in this thesis.

Charles, thank you for your initial support during lab life at MIT, especially for showing me all the lab methods as HPLC, GC and GPC. TDB, thank you for showing me your TLC method. Dr. Nils Rettberg, thank you for PHA-GC analyses at VLB.

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Finally I want to thank all Sinskey, Rha and Stahl lab members for the great time I had in the labs.

Contents

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters that have similar properties to conventional plastic materials. Many microorganisms synthesize versatile PHAs as carbon and energy storage under nutrient limitation and excess carbon. PHAs can be defined as "bioplastics", which are biodegradable and produced from renewable carbon sources (C-sources). *Ralstonia eutropha,* a Gram-negative soil bacterium of the β-Proteobacteria class, is the model organism for PHA homeostasis. The wild type *R. eutropha* strain, H16, is able to store up to 90% of its cell dry weight as polyhydroxybutyrate (PHB) from various C-sources. This thesis focuses on the production of PHA polymers from plant oil and animal waste fat as principal C-sources. These are favorable C-sources, since they are available in large quantities, have a high carbon content and are comparatively inexpensive compared to other feedstocks (*e.g.* corn syrup) based on the carbon content. Methods have been developed to make these hydrophobic C-sources bioavailable for growth and PHA production. These methods are necessary to shorten the lag phase of growth and are essential when using fat/oils with high melting temperatures (T_m) as C-sources. Batch fermentation methods were developed, using engineered *R. eutropha* strains from MIT (Sinskey laboratory), to select a robust PHA production strain. Different fed-batch fermentation strategies where developed for the chose PHA production strain with palm oil as C-source. The optimal strategy, using a total palm oil concentration of 170 g/L, led into a PHA production of >100 g/L with a space time yield of >1 g/L/h of a versatile PHA copolymer. This is the highest reported yield in the literature for this kind of copolymer. Following the palm oil fermentation strategy, various animal waste fats were screened for best PHA productivity. The animal waste fat with the lowest quality was chosen, since it is by far the cheapest available oil/fat worldwide (100–130 USD/ton), as compared to higher quality animal waste fats (500–1,000 USD/ton) or palm oil (700–1000 USD/ton). The high content of free fatty acids (>40%) makes low quality animal waste fats unattractive for other industries as *e.g.* the biodiesel industry. A mixed feeding strategy of C-sources was developed in order to use directly animal waste fats with high T_m (up to 55°C). Initial fed-batch fermentations resulted in a production of 26 g PHA/L from a total of 60 g/L fat added to the culture. Finally, a PHA recovery strategy with non-halogenated solvents was developed from dry and wet biomass which led into polymer purities of >99%. During this development a unique separation technique of PHA copolymers based on their monomer concentration was discovered.

Zusammenfassung

Polyhydroxyalkanoate (PHA) sind Polyester, die ähnliche Eigenschaften wie konventionelles Plastik aufweisen. Eine Vielzahl von Mikroorganismen synthetisiert PHA als Kohlenstoff- und Energiespeicher unter Nährstofflimitierung und gleichzeitigem Vorhandensein einer C-Quelle. PHA ist definiert als „Bioplastik“, welches biologisch abbaubar ist und aus nachwachsenden Rohstoffen gebildet werden kann. *Ralstonia eutropha*, ein gramnegatives Bakterium aus der Klasse der β-Proteobakterien, ist der Modelorganismus für die PHA Homöostase. Der Wildtyp *R. eutropha* H16 ist in der Lage, eine Vielzahl von C-Quellen zu verwerten und bis zu 90 % seines Zelltrockengewichts als Polyhydroxybutyrat (PHB) zu speichern. Diese Arbeit befasst sich mit der Produktion von PHA, die aus Pflanzenöl und tierischen Abfallfetten als C-Quelle gewonnen wurden. Diese C-Quellen sind zu bevorzugen, da sie relativ günstig im Vergleich zu anderen Ausgangsmaterialien (z.B. Maissirup), basierend auf den C-Gehalt sind. Sie sind in großen Mengen verfügbar und weisen eine hohe Konzentration an verwertbarer C-Quelle auf. Es wurden Methoden entwickelt, die die „Bioverfügbarkeit“ von hydrophoben C-Quellen für das Zellwachstum und die PHA Produktion ermöglichen. Dies ist notwendig, um die lag Phase zu verkürzen und essentiell beim Einsatz von Fetten/Ölen, die einen hohen Schmelzpunkt aufweisen. Batch Fermentationsbedingungen wurden unter der Verwendung von Palmöl, für genetisch veränderte *R. eutropha* Stämme vom Massachussets Institute of Technology (MIT, Sinskey Laboratorium), entwickelt um einen PHA Produktion Stamm auszuwählen. Anschließend wurden verschiedene Fed-batch Fermentationsstrategien mit dem ausgewähltem Stamm entwickelt. Die beste Strategie, mit einer totalen Palmölkonzentration von 170 g/L, resultierte in einer PHA Produktion von >100 g/L und einer spezifischen Produktleistung von >1 g PHA/L/h des neuartigen PHA Copolymers. Das ist eine der höchsten publizierten Ausbeuten dieses Polymers. Anschließend wurden verschiedene tierische Abfallfette für die beste PHA Produktion hin untersucht. Für weiterführende Arbeiten wurde das tierische Abfallfett mit der niedrigsten Qualität ausgesucht, da es mit Abstand das günstigste verfügbare Öl/Fett weltweit ist (100–130 USD/t). Diese C-Quelle wurde mit tierischen Abfallfetten mit einer guten Qualität (500–1000 USD/t) oder Palmöl (700–1000 USD/t) verglichen. Der hohe Anteil an freien Fettsäuren (>40 %) macht tierische Abfallfette einer niedrigen Qualität unattraktiv für die Nutzung in anderen Industrien wie z.B. der Biodieselindustrie. Eine Mischfütterungsstrategie wurde entwickelt, um tierische Abfallfette, die einen hohen Schmelzpunkt aufweisen (bis zu 55 °C), direkt als C-Quelle einsetzen zu

können. Erste Fed-batch Fermentationen erzielten 26 g PHA/L mit einer eingesetzten totalen Fettkonzentration von 60 g/L. Abschließend wurde eine PHA Aufarbeitungsstrategie mit nicht-halogenen Lösungsmitteln, ausgehend von trockener und feuchter Biomasse, entwickelt. Damit wurde eine Reinheit von >99 % erreicht. Während der Entwicklung dieser Methode wurde eine neuartige Separationstechnik von PHA-Copolymeren, basierend auf ihrer Monomerkonzentration, entdeckt.

Aim of Thesis

The aim of this thesis was a high yield (>50 g/L) production and recovery of the novel polyhydroxyalkanoate (PHA) biopolymer poly(hydroxybutyrate-*co*-hydroxyhexanoate) [P(HB-*co*-HHx)], with a high HHx-monomer concentration (>10 mol%), from plant oil and animal waste fat feedstocks. *Ralstonia eutropha* H16, the model organism of PHA homeostasis and engineered derivatives[1], were used as biocatalysts for PHA production.
In order to achieve the overall goals, the following objectives had to be accomplished:

i. Development of growth culture conditions using the hydrophobic carbon sources
ii. Selection of a PHA production strain
iii. Development of a fed-batch fermentation process for high yield PHA production
iv. Determination of the effects that influence the HHx content of the polymer
v. Development of a PHA recovery process for purifying polymers from dry and wet biomass

Structure of thesis

The thesis is separated into seven chapters. The 1st chapter, the introduction, is partly adapted from peer reviewed publications, including a literature review, which is discussing lipid and fatty acid metabolism in *R. eutropha,* to summarize the relevance of *R. eutropha* for the biotechnological production of value added products, mainly PHA. The 2nd chapter, a peer reviewed publication, shows the development of culture conditions for *R. eutropha* for the consumption of hydrophobic carbon sources (for example, palm oil) and also provides methods for monitoring of the lipid consumption over the course of experiment. The 3rd chapter, a peer reviewed publication, includes the selection of the engineered PHA production strain. The 4th, a peer reviewed publication, shows the development of a fed-batch fermentation process for high yield PHA production from palm oil. 5th chapter shows the screening of various animal waste fats for PHA production, followed by the upscale to 5-L lab fermenters with low quality animal waste fats. The 6th chapter, a peer reviewed publication, shows the development of a PHA recovery process, with non-halogenated solvents, from wet and dry biomass and a separation technique for PHA polymers based on the monomer composition. Effects that influence the HHx concentration in the polymer were determined in Chapters III through VI. The 7th and final chapter contains the conclusions and future planned work.

[1] By Charles Budde (Sinskey laboratory, MIT)

CHAPTER I

Introduction

Parts of this chapter were modified from previously published articles:

- Applied Microbiology and Biotechnology, 2014. 98: 1469-1483 'Lipid and fatty acid metabolism in *Ralstonia eutropha*: relevance for the biotechnological production of value-added products' Sebastian L. Riedel, Jingnan Lu, Ulf Stahl and Christopher J. Brigham © Springer-Verlag Berlin Heidelberg.
- Biotechnology and Bioengineering, 2013. 110(2): 74-83 'Recovery of Poly (3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) From *Ralstonia eutropha* Cultures with Nom-Halogenated Solvents' Sebastian L. Riedel, Christopher J. Brigham, Charles F. Budde, Johannes Bader, ChoKyun Rha, Ulf Stahl and Anthony J. Sinskey © Wiley Periodicals, Inc.

Bioplastics

The worldwide plastic production increased from 204 Mt in 2002 to 299 Mt in 2013. Since 2011 the annual production is increasing about 4% (Plastic Europe 2015). Today, the majority of industrial plastic production is dependent on fossil–based petroleum. To reduce this dependency, new polymer production processes based on alternative substrates must be developed. One key motivation for the migration to biodegradable plastic is the increasing accumulation of non-biodegradable waste in the environment, which has recently been reviewed (Barnes et al. 2009; Ryan et al. 2009). The negative environmental effects of plastic debris, *e.g*. through consumption of marine life or sea birds was reviewed by Gregory (2009). Ericson et al (2014) calculated that a minimum of 5.25 trillion plastic particles with a total weight of 270,000 tons are polluting our seas. All these numbers are beyond imagination, and this underlines the urgent needs of alternative, biodegradable materials. At least 40% of the produced plastic materials are only used in short-term applications and could be substituted easily by society (Chen and Patel 2011). The classic demonstrative example, even if their plastic production portion is negligible. (*e.g.* less than 1% in Germany 2011), are single-use bags that have only a very short consumer time period before they are disposed of in the trash or in the environment (Umweltbundesamt 2013).

The term "bioplastics" is widely spread in society and literature. Endres and Siebert-Raths (2011), describing all polymers that fulfill one of the following statements as biopolymers: (a) consists (partly) of bio-based (renewable) raw materials and/or (b) is in some way biodegradable. After this definition, there are three basic groups of biopolymers: (i) non-degradable bio-based biopolymers, (ii) degradable (mainly) bio-based biopolymers and (iii) degradable petro-based biopolymers, as shown in Figure 1.1.

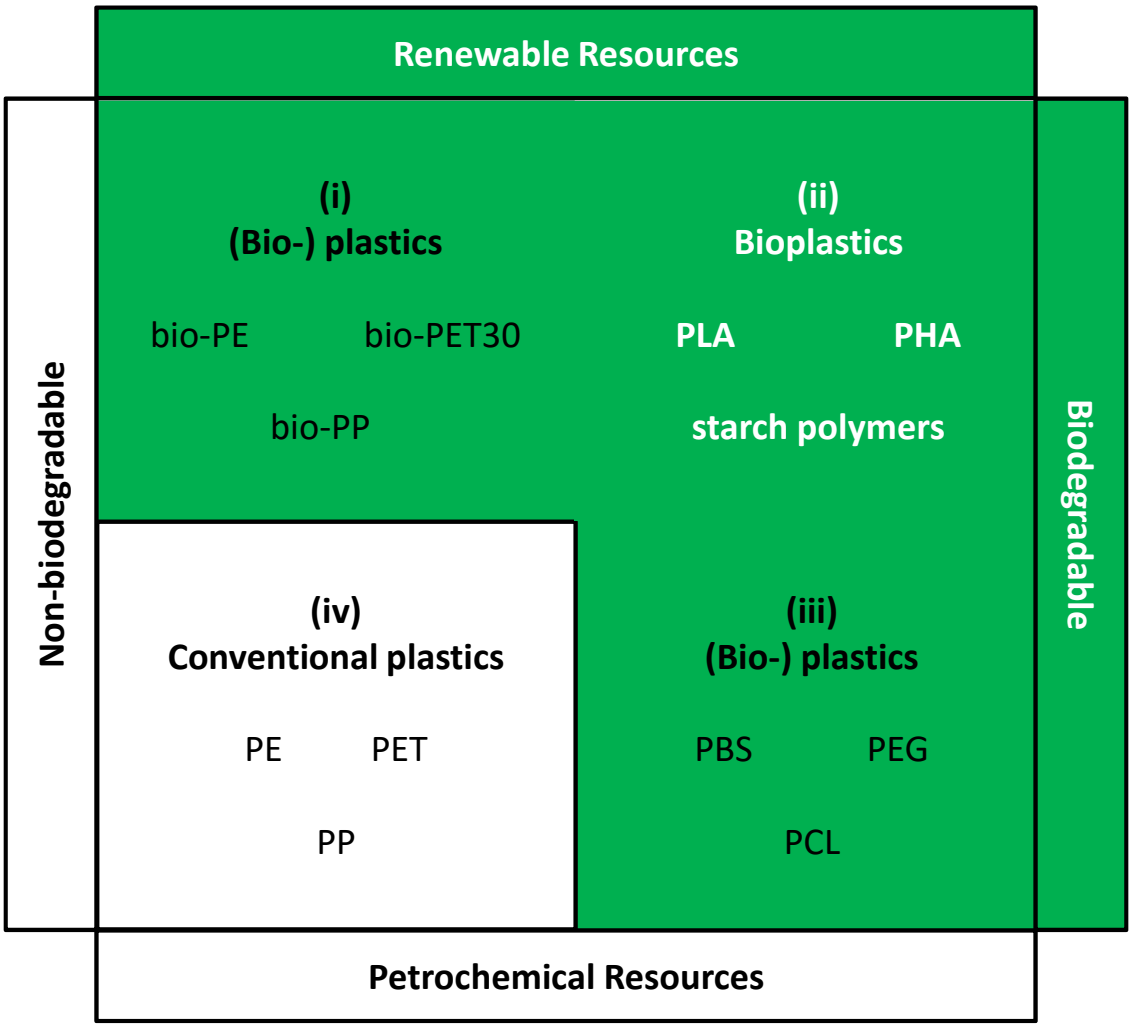


Figure 1.1: Classification of biopolymers with selected examples: (i) non-degradable bio-based biopolymers, (ii) degradable (mainly) bio-based biopolymers and (iii) degradable petro-based biopolymers compared to (iv) conventional plastics. PE = polyethylene, PET = polyethylene terephthalate, PP = polypropylene, PHA = polyhydroxyalkanoate, PLA = polylactic acid, PBS = polybutylene succinate, PCL = polycaprolactone, PEG = Polyethylene glycol, Bio-PET30 = PET material consist to 30% of PET which is made from renewable resources.

Non-degradable bio based polymers

Using renewable materials, as *e.g.* natural latex and cellulose, for the production of engineered polymers was standard from the late 19th century. Celluloid was the first commercial produced material, a thermoplastic, which is produced from cellulose nitrate and camphor. It usage was common until the 1950s for various items of daily use (Reilly 1991). One major concern is the easy flammability of celluloid. However, even until 2014, the use of celluloid ping pong balls was standard in tournaments organized by the International Table Tennis Federation.

Nowadays, the production of plastic materials, starting from biotechnologically produced monomers, which normally had been produced from petrochemical raw materials have come

into focus. The most common polymers of this group are bio-polyethylene (bio-PE) and bio-polyethylene terephthalate (bio-PET). Bio-PE can be completely synthesized from bioethanol, which is already microbially produced at the multimillion ton scale mostly from corn starch and sugar cane for biofuel production (Chen and Patel 2012). Whereas commercial PET, at the moment, can only be produced (up to 30%) from renewable feedstocks. This PET is than called bio-PET30. PET consists of the two precursor molecules monoethylene glycol (MAG, 30%) and purified terephthalic acid (PTA, 70%). Right now only MAG can be synthesized from bioethanol at industrial scale. Coca Cola produces since 2015 their bio-PET30 based "plantbottle™" with bio-MAG which is synthesized from sugarcane based on bioethanol from Brazil. Coca Cola is working to replace the petroleum based PTA by 2020 at industrial scale (Coca Cola 2015). Promising raw materials for bio-PTA are cellulosic based feedstocks. Cellulose can be converted to the platform chemical hydroxylmethylfurfural (Caes et al. 2013), which than can be converted to PTA (Pacheco and Davis 2014).

Indeed, the production of bio-based conventional plastics reduces the dependency on crude oil and has a much better CO_2-footprint as compared to petrochemical based plastics. However, it does not address the environmental problems caused by plastic.

Degradable petrochemical based polymers

Petrochemical based polymers are synthesized from hydrocarbon monomers that are produced from crude oil, natural gas or coal raw materials. The biodegradability of these materials comes from integrated heteroatoms, such as oxygen or nitrogen. Besides polyvinyl alcohols, where only some are biodegradable, polyglycols and polycaprolactone (PCL), belong to the most important group of petrochemical based polymers. The most important polymers within the group of polyglycols are polyethylene glycols, which are often used as antifoam agents in biotechnological processes. PCLs are produced by a ring opening polymerization of ε-caprolactone and have in general similar mechanical properties to conventional plastics. However, its low melting point of around 60°C is limiting their application window. Even besides their high price of 4.5–6 €/kg (Figure 1.2), they can be seen as the favorite petro based biopolymer because of its rapid and complete degradation behavior. PCL is fully biodegradable under aerobic conditions to capric acid and apidic acid.

Degradable (mainly) bio-based biopolymers

The main biological raw materials for biodegradable polymers are cellulose, sugar, starch, proteins, lignin, fats and oils (Stevens and Verhé 2004). The main polymer groups are cellulose polymers, starch polymers, partly microbially synthesized polymers, such as

polylactic acid (PLA), and fully microbially synthesized polymers, such as polyhydroxyalkanoate (PHA). Also biopolymers that are directly synthesized by animals have a promising future, such as chitosan, made from chitin recovered from shrimps (Inamdar and Mourya 2014) and other crustaceans; qmilk a biopolymer from casein (Domaske 2011, US20130256942 A1) and silk made by silkworms (Kundu et al. 2014).

Cellulose acetate polymers

Cellulose polymers are the oldest known biopolymers of this group. They can be divided into regenerated cellulose, such as cellophane or viscose, and cellulose derivate with the main groups of cellulose ester and cellulose ether, such as cellulose acetate (CA), and carboxyl methyl cellulose. The properties of the derivate mainly depend on the type of substituent and its degree of substitutions.

Starch based polymers

Starch based polymers can be divided into the three main groups: starch-filled composites, thermoplastic starch and starch blends. To generate starch-filled composites, other polymers like CA, PCL or PE are each extruded with starch granulate with contents up to 50%. It is important to note that the starch should not be denatured during the extrusion process. The starch additives improve mechanical properties and accelerate biodegradation in the composites. When starch is used as additive for petrochemical based polymers as PE, it will help to accelerate break down of the polymer from the macro stage. However, the further degradation process of PE will not change. To generate a starch polymer with thermoplastic properties, the starch will be denatured and supplied with additives like water or glycerin and/or further blended with other (bio)-polymers.

Poly lactic acid

Poly lactic acids (PLA) are chemically synthesized polyesters based on lactic acid (LA), which have thermoplastic properties similar to petroleum based plastics. PLA have been used to substitute petroleum based plastics in many consumables (Nampoothiri et al. 2010). The backbone of PLA, the LA, can be produced biotechnologically via fermentation with lactobacilli using a wide range of *e.g.* carbon sources including sugars, starch or lignocellulosic feedstocks as molasses, corn or corn stover (Ghaffar et al. 2014). A current European research project focuses on the PLA production from waste products of the bakery industry (Bread4PLA). However, after the fermentative production of the LA, a more or less complex recovery/purification process, depending on the feedstock used, is necessary to purify the LA before the chemical synthesis to PLA can be conducted (Abdel-Rahman et al.

2011). The mechanical properties of basic PLA are between crystal polystyrene and PET (Lunt 1998). The (bio)-degradation of the aliphatic polyester PLA takes place in two steps. First a hydrolyses at high temperatures (55–70°C) and high humidity takes between 7–14 days until the polymer is broken down to an average molecular weight of 10,000 Da. After that, soil microorganisms can perform the final biodegradation to produce CO_2 and water. The initial need of high temperature for degradation made common PLA poorly bio-degradable in common habitats. Blending PLA with other better biodegradable polymers, as *e.g.* PHA, enhances its biodegradation (Rasal et al. 2010).

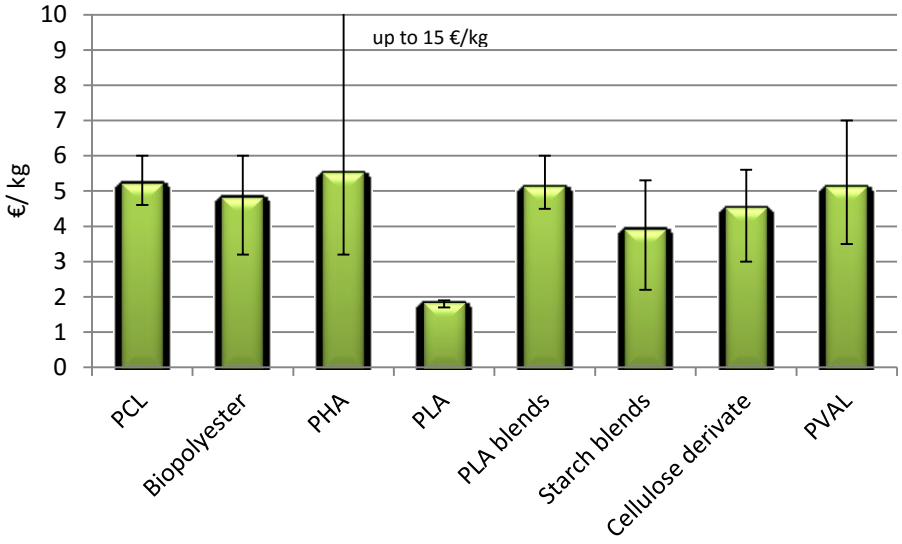
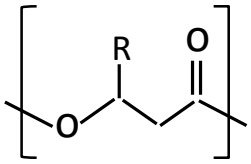


Figure 1.2: Material prices of various biopolymers (adapted from Endres and Siebert-Raths 2011).

Polyhydroxyalkanoate

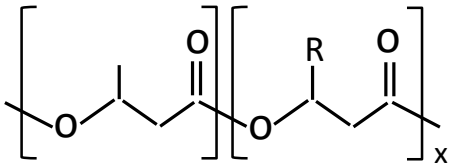
PHA are microbially synthesized polyesters, which are stored in the form of intracellular granules. Microorganisms are using PHA for energy and carbon storage (Steinbüchel 1991). However, PHA can be used effectively as a biodegradable and biocompatible alternative to petroleum-based plastic (Anderson and Dawes 1990; Sudesh et al. 2000). Lemoigne (1926) discovered in *Bacillus megaterium* the parent member of PHA, polyhydroxybutyrate (PHB), in the 1920s. Most PHAs contain a three-carbon backbone structure (Figure 1.3) and have analogous manufacturing characteristics to conventional plastics (Braunegg et al. 1998). The huge variety of PHAs depends on different side chains and the configuration of the polyester units (Lee 1996). The components of PHA are mainly divided in short chain length (*scl*) monomers with 3–5 carbon atoms and medium chain length (*mcl*) monomers with 6 or more carbon atoms (Rehm 2003; Figure 1.3).

a



R	PHA	Abbr.	Cluster
Methyl	Polyhydroxybutyrate	PHB	*scl*-PHA

b



R	x-monomer	Abbr.	Cluster
Ethyl	Hydroxyvalerate	P(HB-*co*-HV)	*scl*-PHA
Propyl	Hydroxyhexanoate	P(HB-*co*-HHx)	*mcl*-PHA
Pentyl	Hydroxyoctanoate	P(HB-*co*-HO)	*mcl*-PHA

Figure 1.3: Basic structure of polyhydroxyalkanoates (a) and structure of poly (hydroxybutyrate-*co*-hydroxyalkanoates) (b) are presented. "R" is depending on organism and carbon source. *scl* = short chain length, 3–5 carbon, *mcl* = medium chain length, 6–4 carbon.

The different side chains affect the properties of the polymer (Chen and Wu 2005). Over 150 different hydroxyalcanoic acids are known to occur as constituents of PHA (Rehm 2003). A comparison of physical properties of chosen PHA with polypropylene is shown in Table 1.1.

Table 1.1: Properties of polyhydroxyalkanoates compared to polypropylene and low-density polyethylene. Table modified from Tsuge et al. 2002.

Polymer	T_m[f] (°C)	T_g[g] (°C)	Crystallinity (%)	Tensile strength (MPa)	Extension to break (%)	Ref
PHB	177	4	60	43	5	h
PHB(uhmw)[a]	185	4	80	400	35	h
P(HB-*co*-20mol%HV)	145	-1	56	20	50	h
P(HBHVHHx)[b]	140	-4		20	321	j
P(HB-*co*-10mol%HHx)	127	-1	34	21	400	h
P(HB-*co*-17mol%HHx)	120	-2			850	i
P(HBHHxHO)[c]	126	-4		22	15	k
P(HBHHxHOHD)[d]	111	-6	50–70	10	188	k
Polypropylene	176	-10		38	400	h
LDPE[e]	130	-36	20–50	10	620	h

[a] Ultra-high-molecular-weight PHB; [b] P(HB-*co*-16mol%HV-*co*-4mol%HHx); [c] P(HB-*co*-3mol%HHx-*co*-3mol%HO); [d] P(HB-*co*-6mol%HHx-*co*-4mol%HO-*co*-1mol%HD); [e] Low-density polyethylene; [f] Melting temperature; [g] Glass transition temperature; [h] Tsuge et al. 2002; [i] Doi et al. 1995; [j] Bhubalan et al. 2008; [k] Tsuge et al. 2009.

PHB has similar properties to polypropylene, but due to the lower extension to break of 5% (polypropylene extension to break 400%) it is a very brittle and stiff plastic material. Therefore PHB is not a favorite for industrial applications. PHB properties can be improved by producing PHB with an ultra-high-molecular-weight of 3–20 x10^6 Da with recombinant

Escherichia coli harboring the PHB biosynthesis genes from *Ralstonia eutropha* (Tsuge et al. 2002). Another way is to insert hydroxyalkanoates (HA) units with longer side chains into the PHB polymer. These P(HB-*co*-HA) copolymers have a variety of different properties, depending on the type of HA, the content of HA, and the numbers of different HA units in the copolymer (Verlinden et al. 2007). The low extension to break of PHB could be enhances 10 fold to 50% by producing poly(hydroxybutyrate-*co*-hydroxyvalerate) [P(HB-*co*-HV)] with a content of 20mol% HV (Table 1.1). Nodax™ PHA copolymers consist mainly of HB units with a relatively small amount of *mcl*-PHA (Noda et al. 2005b). They have much more commercially useful properties than PHB or P(HB-*co*-HV) (Noda et al. 2005a). The simplest copolymer of this class is P(HB-*co*-HHx). A copolymer of P(HB-*co*-HV-*co*-HHx) containing 16 mol% HV and 4 mol% HHx had 6 fold more extension to break of 321% compared to 50% of the *scl*-PHA copolymer P(HB-*co*-HV) containing 20 mol% HV. This shows how important PHB copolymers with *mcl*-HA units are, even in low concentrations.

The *mcl*-HA content regulates the T_m and crystallinity almost independently of the *mcl*-PHA size (Noda et al. 2005b). This is shown by the comparison of P(HB-*co*-HHx) copolymer containing 10 and 17 mol% HHx. The increasing HHx level decreases T_m and crystallinity, increases flexibility, and has a significant positive effect on the elongation to break (similar properties to low-density polyethylene are shown). To carefully control the *mcl*-HA level such as HHx in P(HB-*co*-HHx) is one opportunity to reach specific desired properties. Another way is to introduce different *mcl*-HA units in the PHB polymer with various side chains. The side group chain length has profound effect on the flexibility of the copolymer (Noda et al. 2004). The insertion of only 1 mol% hydroxydecanoate (HD) to the copolymer poly(HB-*co*-HHx-*co*-HO) reaches a 15x fold elongation to break. It is also decreases the T_m more than 10%.

PHA already has a wide variety of applications. Traditionally, PHA has been used to produce everyday items such as packing material or containers for storage of household products (Philip et al. 2007). Because of its biocompatibility and ability to degrade to non-toxic compounds in the human body, PHA is used in tissue engineering as a scaffold material (Chen and Wu 2005; Williams et al. 1999; Zhao et al. 2002). As a biofuel, methyl-esters derived from PHA monomers have been shown to have potential as transportation fuels (Zhang et al. 2009).

Ralstonia eutropha relevance for production of polyhydroxyalkanoate

R. eutropha, a Gram-negative β-proteobacterium, is an important organism in biotechnology, due to its native ability to produce large quantities of intracellular PHA biopolymer. *R. eutropha* is often referred to as the model organism for PHA production (Reinecke and Steinbüchel 2009) and is well-studied in terms of biopolymer homeostasis. In nature, *R. eutropha* accumulates PHB (Figure 1.4) up to 90% per cell dry weight (CDW), as a means of carbon and energy storage under stress conditions (Steinbüchel 1991).

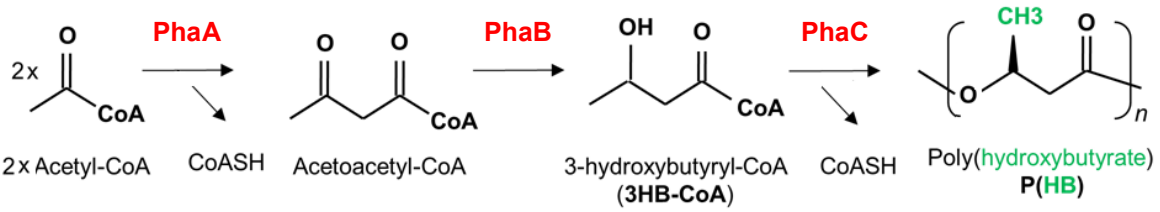


Figure 1.4: Polyhydroxybutyrate (PHB) biosynthesis in wild-type *R. eutropha* proceeds by the formation of acetoacetyl-CoA from two molecules of acetyl-CoA, and subsequent reduction to (R)-3-hydroxybutyryl-CoA. The (R)-3-hydroxybutyryl-CoA is polymerized by a PHA synthase (PhaC) to PHB. Enzymes: PHA synthase (PhaC), β-ketothiolase (PhaA) and acetoacetyl-CoA reductase (PhaB).

Because of the bacterium's genetic tractability, many researchers have engineered *R. eutropha* to produce different, and potentially more valuable and versatile, types of PHA (Kahar et al. 2004; Sudesh et al. 2011; Budde et al. 2011b).

Another characteristic that underpins the importance of *R. eutropha* in biotechnology is the bacterium's ability to utilize a multitude of carbon sources for growth and PHA biosynthesis. It has been documented in the scientific literature that sugars (Lutke-Eversloh et al. 2002; Brigham et al. 2012), amino sugars (Holder et al. 2011), carbon dioxide (Ishizaki et al. 2001; Volova et al. 2002; Cramm 2009), short-chain fatty acids (Yang et al. 2010), phenolic compounds (Nickzad et al. 2012), plant oils (Sudesh et al. 2011; Riedel et al. 2012), animal fats (Taniguchi et al. 2003), fatty acids (Brigham et al. 2010) and glycerol (Cavalheiro et al. 2009; Cavalheiro et al. 2012, Tanadchangsaeng and Yu 2012) act as sources of carbon for the production of biomass and of polymer. The use of fatty acids and lipids for microbial production of value-added products has gained popularity, because synthesis of products like PHA is observed to be more efficient when these carbon sources are used, due in part to the high carbon content per mol of these carbon sources (Kahar et al. 2004; Riedel et al. 2012; Tsuge et al. 2013). Also, using engineered strains, PHA incorporating longer chain length monomers (>C6) can be produced using lipids and fatty acids as a carbon source.

In order to utilize lipids as carbon source *R. eutropha* excretes an extracellular lipase (LipA, locus tag H16_A1322) (Brigham et al. 2010; Lu et al. 2013). The Lipases play the crucial role in lipid metabolism by catalyzing the hydrolysis of triacylglycerol (TAG) into diacylglycerol (DAG), monoglycerol (MAG), glycerol, and free fatty acids (FFA) at the interface of lipid and water. The TAG molecules, together with their cleavage products, form an emulsion within the aqueous media and therefore become bioavailable for cell growth (Lu et al. 2013).

Polyhydroxyalkanoate production from plant oils

Plant oils have been shown to be excellent carbon sources for PHA production using *R. eutropha* as the biocatalyst. Efficient P(HB-*co*-HHx) accumulation using recombinant *R. eutropha* strains, between 72–87% per CDW, from various plant oils (soybean oil, olive oil, corn oil, jatropha oil, palm oil, and palm kernel oil) was shown by several groups (Fukui and Doi 1998; Tsuge et al. 2004; Loo et al. 2005; Mifune et al. 2008; Ng et al. 2010). The PHA synthases used in these studies are enzymes with broad substrate specificity that can incorporate both *scl* and *mcl* monomers into the final polymer. However, the HHx concentration of the stored polymer was low, only 2–5 mol% from these experiments. Mifune et al. (2010) were able to produce this copolymer containing a higher HHx level (up to 9.9 mol%) together with a high PHA content of 79% per CDW from soybean oil, through insertion of a *phaJ* from *Aeromonas caviae* into the PHA biosynthesis operon (MF03). Higher 3-HHx concentrations could only be reached by feeding MCFA, although this would be undesired due to the cost of these carbon substrates. These authors were also able to produce copolymers with high HHx levels of up to 26 mol% from soybean oil using a strain containing a *phaB1* deletion (NSDGΔB) but at the cost of overall PHA accumulation (max 26% per CDW). It is interesting to compare the PHA accumulation behaviors of the *ΔphaB1* strains during cultivation on octanoate (representing MCFA) or soybean oil (representing LCFA). These strains produce PHA with higher 3HHx content when grown on soybean oil compared to octanoate, but accumulate much less PHA per CDW (Mifune et al. 2010). The HHx integration in PHA from this work is the opposite of expected results, based on studies discussed above. In another study, strain Re2160/pCB113, produced P(HB-*co*-HHx) containing extremely high HHx levels (up to 62 mol%) in the resulting copolymer when cells were grown on the plant oils mentioned above and additionally with coconut oil, crude palm oil and palm olein as sole carbon source (Wong et al. 2012).

Besides efficient PHA accumulation per CDW as described above, a high total PHA production per liter is desired for an economical PHA production process. Kahar et al. (2004)

produced 95.5 g/L P(HB-*co*-HHx) from soybean oil under phosphate limitation (Table 1.2). The fermentation was initiated with 20 g/L soybean oil and 0.4% NH_4Cl. Soybean oil and NH_4Cl were fed in pulses, shortly before limitation back to initial concentrations. The culture medium included the antibiotic kanamycin to stably maintain the plasmid containing the gene encoding a PHA synthase (*phaCAc*) with broad substrate specificity from *A. caviae*.

R. eutropha PHA production from lipid waste streams

Lipid waste streams have also been a focus for PHA production with *R. eutropha*, because of their low price and their availability in relatively large quantities. Efficient PHB accumulation per CDW using waste frying oil as the sole carbon source in flask cultures of wild-type *R. eutropha* was shown by both Taniguchi et al. (2003) (waste sesame oil, 63% PHB per CDW) and Obruca et al. (2013) (waste rapeseed oil, 62% PHB per CDW). Using random chemical mutagenesis on the wild-type strain, a mutant (strain E01) was isolated that was able to produce 87.9% PHB per CDW from waste rapeseed oil. High production of P(HB-*co*-HV) from waste rapeseed oil, using propanol as the HV precursor, was shown by Obruca et al. (2010). A final copolymer concentration of 105 g/L with a HV content of 8 mol% was produced. The authors demonstrated a yield of 0.83 g PHA/g oil (Table 1.2). A P(HB-*co*-HV) content of 80% per CDW, with a low HV level (1 mol%), was obtained in flask cultures of wild-type *R. eutropha* when grown using tallow as the sole carbon source (Taniguchi et al. 2003). Tallow has a high T_m (40–45°C) and was solid under the cultivation conditions, but it was fully degraded and consumed during cultivation. The T_m of a fat is dependent on the chain lengths of the fatty acids and the portion of unsaturated fatty acids in the individual TAG molecules (*e.g.*, longer chain lengths and fewer double bonds in the requisite fatty acids increases T_m). Fats with a higher T_m are harder to emulsify for use in *R. eutropha* cultures. This can increase the lag phase or even lead to a lack of cell growth, depending on the type of cultivation method (shaking flask culture or fermentation).

Table 1.2: PHA production with *R. eutropha* H16 or its recombinant strains (m) from different C-sources, from shaking flask (SF), batch (b), extended-batch (eb) or fed-batch (fb) fermentations (F). Palm oil, palm kernel oil, soy bean oil or waste rapeseed oil (WRO) with propanol (PrOH) as HV precursor where used as TAG feedstocks. As VFA were used: Acetic acid (AA), propionic acid (PA), and butric acid (BA). Lactic acid (LA), Glycerol (Gly) and waste glycerol (wGly), where also used for PHA production. n shows numbers of replications. Nitrogen (N) or phosphate (PO_4) limitation. n.a. indicates that data were not available.

Carbon	Strain	Scale	*n*	Limi-tation	N or PO_4 (mM)	C (%)	PHA/CDW (%)	PHA (g/L)	STY (g/L/h)	Yield (g/g C)	HB (mol%)	HV (mol%)	HHx (mol%)	Ref.
Palm oil	H16	F (b)	6	N^h	19	2	79	8	0.1	0.61	100			m[#]
	m[a]	SF	1	N^h	9	1	81	3			96		4	n
	m[b]●	SF	3	N^h	9	1	73	3			87		13	o
	m[b]●	F (b)	3	N^h	75	4	71	18	0.2		83		17	o
	m[c]●	F (b)	3	N^h	75	4	66	11	0.1		70		30	o
	m[b]●	F (eb)	2	N^i	150	6	72	33	0.3	0.52	83		18	p
	m[b]●	F (fb)	2	N^j	480	17	70	69	0.7		76		24	p
	m[b]●	F (fb)	3	N^i	480	17	74	102	1.1	0.63	81		19	p
Palm kernel oil	m[d]	SF	n.a.	n.a.	n.a.	0.5	87	4			95		5	q
	m[d]	SF	n.a.	n.a.	n.a.	0.5	70	2			95		5	q
	m[e]●	F (fb)	2	N^k	n.a.	n.a.	76	126	1.9	n.a.	98		3	r
	H16	F (fb)	2	N^k	n.a.	n.a.	72	118	1.8	n.a.	100			r
Soy bean oil	H16	F (fb)	2	PO_4	80	n.a.	74	90	0.9	0.74	100			s
	m[d]	F (fb)	2	PO_4	80	n.a.	73	97	1.0	0.73	95		5	s
	H16	F (eb)	1	PO_4[**]	n.a.	8	81	67	2.5	0.85	100			t
WRO/PrOH	H16	F (fb)	1	N^l	n.a.	n.a.	76	138	1.5	0.83	92	8		u
LA[*]	H16	F (fb)	1	low N^k	n.a.	n.a.	58	59	1.2	0.17	100			v
PA[*]	H16	F (fb)	1	low N^k	n.a.	n.a.	58	37	0.7	n.a.	n.a.	n.a.		w
AA/LA[*]	H16	F (fb)	1	low N^k	n.a.	n.a.	73	55	1.3	0	100			x
BA	m[g]	F (fb)	1	PO_4	n.a	6.4	82	38	0.57	0.61	100			y
Gly	m[f]	F (fb)	1	N^k	n.a.	26.5	71	53	0.9	0.2	100			z
Gly	m[g]	F (fb)	1	N^k	n.a.	24.9	62	51	1.5	0.2	100			za
wGly	m[g]	F (fb)	1	N^k	n.a.	n.a.	50	38	1.1	n.a.	100			za

a = PHB-4/PJRDEE32d13, b = Re2058/pCB113, c = Re2160/pCB113, d = PHB-4/pBBREE32d13, e = CNPCN, f = laboratory mutant of H16, g = DSM 545, h = NH_4Cl, i = urea, j = NH_4Cl/NH_4OH - pH controlled, k = $(NH_4)_2SO_4/NH_4OH$ - pH controlled, l = $(NH_4)_2SO_4$, m# = Budde et al. 2011; n = Fukui and Doi 1998; o = Budde et al. 2011; p = Riedel et al. 2012; q = Loo et al. 2005; r = Sato et al. 2013, s = Kahar et al. 2004; t = de la Cruz Pradella et al. 2012; u = Obruca et al. 2010; v = Tsuge et al. 1999; w = Kobayashi et al. 2000; x = Tsuge et al. 2001; y = Grousseau et al. 2013 z = Tanadchangsaeng and Yu 2012; za = Cavalheiro et al. 2009; * = C-source mixed with NaOH and fed over base control in two stage fed-batch: 1st stage C/N = 10, 2nd stage C/N ratio = 23v or 50w,x, resulting in low N levels. Acid concentration controlled at around 2-3 g/L. ** = may also be Cu-, Ca-, and/or Fe-limited. ● = no antibiotic additions were necessary for maintenance of plasmid stability

PHA from short-chain fatty acids

Short-chain fatty acids, or volatile fatty acids (VFA), are an auspicious carbon feedstock for PHA production, since they are inexpensive and widely available in large quantities. VFA are produced via microbial acidogenesis from organic waste streams *e.g.* from anaerobically treated palm oil mill effluent (Yee et al. 2003; Mumtaz et al. 2008), sludge (Elefsiniotis and Oldham 1993) or food scraps (Digman and Kim 2008). VFA, individually or in mixtures, are suitable as carbon sources for *R. eutropha*. PHA accumulation has been demonstrated, using strain H16, from acetic acid [PHB, (Wang and Yu 2000)], propionic acid [P(HB-*co*-HV), (Kobayashi et al. 2000)], butyric acid [PHB, (Kawaguchi and Doi 1992; Grousseau et al. 2013)] and valeric acid [P(HB-*co*-HV), (Khanna and Srivastava 2007; Lindenkamp et al. 2013)], or from mixed VFA cultures of acetic, propionic and butyric acid [PHB, P(HB-*co*-HV), (Yu et al. 2002; Yang et al. 2010)]. Hassan et al. (2002) also showed the direct use of treated palm oil mill effluent, which contains three of the abovementioned (acetate, propionate and butyrate) VFA in a ratio of 3:1:1, respectively (Yee et al. 2003). Lactic acid, which can also be produced during microbial acidogenesis (Zhao et al. 2006) or in large quantities from renewable carbon sources (Datta and Henry 2006), can be used for PHB production (Tsuge et al. 1999). Lindenkamp et al. (2012) was able to produce P(HB-*co*-HV), with an extremely high HV content of 99 mol%, but with low PHA accumulation per CDW (25%), using a mutant *R. eutropha* strain that was missing 9 out of 15 β-ketothiolase gene homologues with valerate as the sole carbon source.

VFA and lactic acid are inhibitory or toxic for bacterial cell growth in large quantities, depending on culture pH and acid concentration of the feedstock, also because undissociated lipophilic molecules attack the cell membranes, resulting in cell morphology and growth defects (Salmond et al. 1984; Lawford and Rousseau 1993; Roe et al. 1998). These toxic effects occur at very low concentrations of VFA in *R. eutropha* cultures (*e.g*. Wang and Yu 2000). It has been demonstrated that an initial concentration of only >0.3% acetic acid results in significant growth inhibition, and with an initial concentration of up to 0.6%, no cell growth was observed. In order to reach high cell densities using VFA as a carbon source, a sensitive feeding strategy that keeps VFA concentrations at low levels in the culture media is necessary for an effective PHA production process. Cell densities between 64–103 g/L using strain H16 with final PHA contents of 58–73% per CDW have been reached with a pH-controlled two-stage feeding strategy (Tsuge et al. 1999; Tsuge et al. 2001; Kobayashi et al. 2005; Table 1.2). The VFA were mixed with ammonium hydroxide solution

and potassium phosphate and fed over the base reservoir by keeping the pH at initial values. In this way, VFAs were kept at the low concentrations of ~0.3%. In the first stage of the culture, VFA was fed in a nitrogen-rich C/N ratio of 10 for the first 12–24 h. In the second stage, the nitrogen content in the feeding solution was decreased, thus increasing the C/N ratio up to 50 for greatest PHA production. The highest PHA accumulation per CDW was reached when the feeding solution was changed during the PHA production phase, where the residual cell dry weight [rCDW = CDW (g/L) - PHA (g/L)] is constant (Table 1.2). Besides nitrogen, none of these fermentations were knowingly limited for other nutrients.

PHA recovery

Efficient recovery and purification of PHA from cells is required for industrial polymer production. The recovery process is one of the most costly steps in PHA production, and several different strategies have been reported in the literature. Comprehensive reviews and comparisons of published recovery strategies are presented by Kunasundari and Sudesh (2011), Jacquel et al. (2008), Koller et al. (2013) and Madkour et al. (2013). For example, different chemical-based digestion methods have been developed. In the these processes, surfactants like sodium dodecyl sulfate (SDS) and Triton X-100 are combined with sodium hydroxide, sodium hypochlorite or sulfuric acid to digest proteins, nucleic acids, peptidoglycan and other "non-PHA" parts of the bacterial biomass. One critical limitation of these processes, is that the harsh chemical treatment required to achieve high purities may lead to a reduction of the molecular weight of the polymer (Ramsay et al. 1994). A further issue of sodium hypochlorite as a polymer recovery agent is its strong oxidizing effect, potentially damaging the stainless steel equipment of a production plant. Yang et al. (2011) developed a strategy for the P(HB-*co*-HV) recovery using linear alkyl benzene sulfonic acid LAS-99 as an alternative to the commonly used SDS. In this method, only 20% of the surfactant was required, compared to previous SDS-based methods. A yield of 86% and purity of 88% were achieved when polymer was recovered under acidic pH. The main disadvantages of these chemical based strategies are the large amount of salt produced as a byproduct and the amount of surfactant-containing wastewater generated from the process, potentially resulting in high costs for wastewater treatment.

There are also alternative digestion-based recovery strategies that utilize enzymatic treatment of cellular components to release PHA. Kapritchkoff et al. (2006) investigated the utilization of different enzymes (bovine trypsin (EC 3.4.21.4), bromelain (EC 3.4.22.32), lysozyme (EC 3.2.1.17), and others) for the recovery of PHA from *R. eutropha* and selected proteases

suitable for this process. Additional treatments with detergents and chelating agents can be beneficial to these approaches. However, compared to solvent-extracted PHA, the molecular weight may be lower following enzymatic recovery methods despite the mild reaction conditions (Kathiraser et al. 2007). To prevent the high costs incurred for using purified enzymes in this process, live bacterial cells could be utilized for the degradation of the undesired components of the PHA producing microorganisms, followed by liberation of the PHA (Lakshman and Shamala 2006). The digestion of cellular components of the PHA-producing microorganisms through a secondary fermentation product is inherently time consuming, and still requires a second treatment like solvent extraction following digestion to increase the purity of the recovered PHA.

Mechanical methods have been combined with chemical treatments for cell disruption during PHB recovery, including the use of bead mills or high pressure homogenization, along with sodium hypochlorite treatment (Tamer et al. 1998). However, the viability of this procedure as a scalable process is unknown, given the lack of follow-up works in the literature. After disruption of the cells, a separation of PHA from cell debris must still be achieved. Separation methods are typically dependent on the type of polymer extraction method used. Such separation methods include centrifugation, filtration, floatation or aqueous two-phase systems (ATPS). Ibrahim and Steinbüchel (2009) have demonstrated simple separation of a PHB/chloroform-solution through self-floatation of the residual biomass, which replaced centrifugation and simplified filtration before polymer precipitation. PHA purity of 98% was reached by the aforementioned authors. Using ATPS, Divyashree et al. separated P(HB-*co*-HV) from a *Bacillus flexus* cell lysate with high recovery yield (92%) and high purity of 97% (Divyashree et al. 2009). However, currently ATPS systems are not often industrially applied due to their tremendous complexity (Bensch et al. 2007).

Rapid and straightforward recovery of PHA from bacterial cells using organic solvents is often applied in industrial processes due to its recovery efficiency, polymer purity obtained, and the possible removal of endotoxins from the recovered polymer, which is important for medical applications (Lee et al. 1999; Sevastianov et al. 2003). In a first step, the PHA is extracted from biomass with a suitable solvent (*e.g.* chloroform) and then separated from the residual biomass, *e.g.* through centrifugation and filtration. Polymer precipitation is then conducted with the addition of a non-PHA-dissolving solvent (*e.g.* methanol) or the polymer is recovered through cooling the solution or by solvent evaporation. Chloroform was the first solvent used to extract PHB from cells (Lemoigne 1927) followed by 1,4-dioxane, pyridine, and dichloromethane (Baptist 1962a; Baptist 1962b). For the recovery of *mcl*-PHAs, a greater

range of solvents is potentially usable, including a variety of ketones, esters, and alcohols (Kinoshida et al. 2006; Noda 1998; VanWalsem et al. 2007). For PHA production and purification, application of toxic halogenated solvents should be avoided to protect operators, customers, and the environment.

Biodegradation of PHA

Biological degradation depends on several surrounding ambient conditions including: temperature, pH, moisture, aerobic or anaerobic conditions and presents of microorganism. In the primary degradation (macromolecule splitting) the polymer gets degraded into smaller chains, respective its monomers. The primary degradation usually takes place through extracellular enzymes, since the large, water-insoluble polymer cannot be taken up by the cell. The extracellular enzymes therefore attack the covalent, non-carbon bonds on the surface of the polymer, *e.g.*, ester bonds. High amounts of non-carbon atoms in the backbone of a polymer, enhances in general its biodegradability. Degradation products of the polymer will then usually be water soluble and then be absorbed by the cell, followed by a further metabolization to water, carbon dioxide, methane (during anaerobic conditions) and biomass during the final degradation process (Müller 2006, Endres and Siebert-Raths 2011). The general mechanism of a biodegradation by microorganisms is shown below (Figure 1.5).

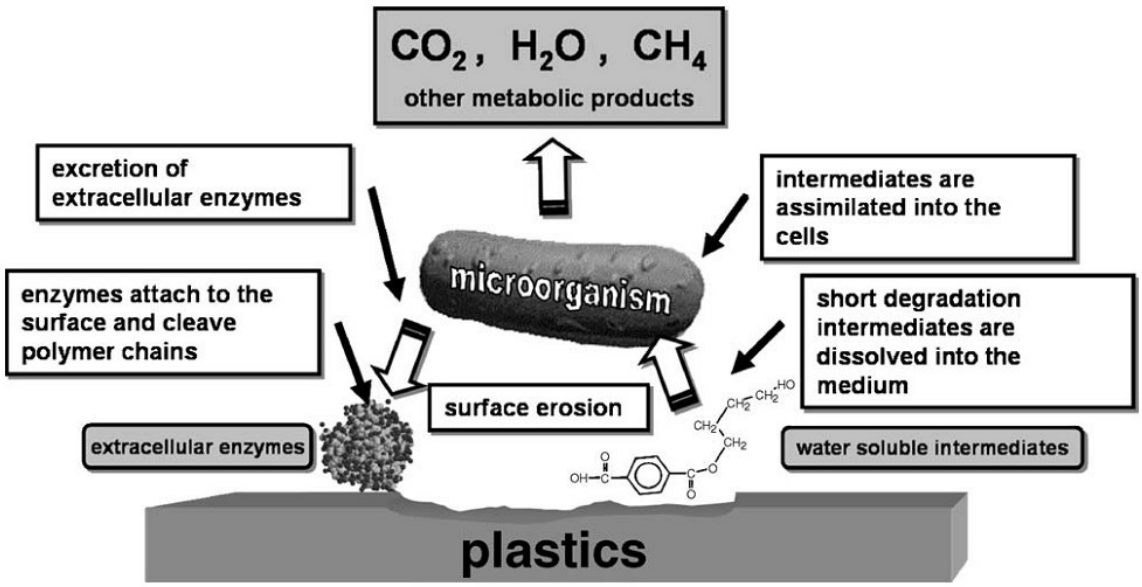


Figure 1.5: Scheme of general mechanism of enzymatic catalyzed hydrolytic polymer degradation (Mueller 2006).

PHA polymers in particular are degraded by extracellular enzymes, so called PHA depolymerases, which are common in many bacteria and fungi (Jendrossek et al. 1996;

Matavulj and Malitoris 1992). Even though PHA polymers possess the same backbone, there are different PHA depolymerases classes for the degradation of *scl*- or *mcl*-PHA polymers, with a pH optimum generally between pH 7.5–10 (Jendrossek 1998). During the macromolecule splitting, PHA polymers get degraded into their monomers, which are water soluble: PHB into *R*-3-hydroxybutyric acid (Doi et al. 1992), P(HB-*co*-HV) into 3-hydroxybutyrate and 3-hydroxyvalerate (Luzier 1992) and P(HB-*co*-HHx) into 3-hydroxybutyrate and 3-hydroxyhexanoate (Wang et al. 2004). The PHA monomers are then metabolized in the cell via β-oxidation and then the TCA cycle to form carbon dioxide and water under aerob conditions (Scott 1999) and additionally to methane under anaerobic conditions (Luzier 1992). There have not been any known toxic degradation products of PHA. In fact, 3-hydroxybutyrate is naturally present in the blood of humans (Lee 1996). This favors the application of PHA products in the medical products (Brigham and Sinskey 2012).

Wang et al. (2004) studied the degradation of P(HB-*co*-HHx) (Figure 1.6) with different HHx contents in activated sludge. P(HB-*co*-HHx) degraded faster than PHB, basically due to its lower molecular weight (the polymer chains are more rapidly degradable to their monomers). They also discovered that the surface properties and crystallinity of the polymer have a much bigger influence on the degradation process as the HHx content. The P(HB-*co*-12mol%HHx) sample degraded faster than the P(HB-*co*-5mol%HHx) sample with a lower crystallinity and slower than the P(HB-*co*-20mol%HHx) sample, which had a much smoother surface compared to the copolymer with 12 mol% HHx.

The different surface and crystallinity properties of the different samples were based on different origins of the PHA samples. For a better determination of the effect of the HHx concentration on the biodegradation, PHA materials produced through identical processes should be used.

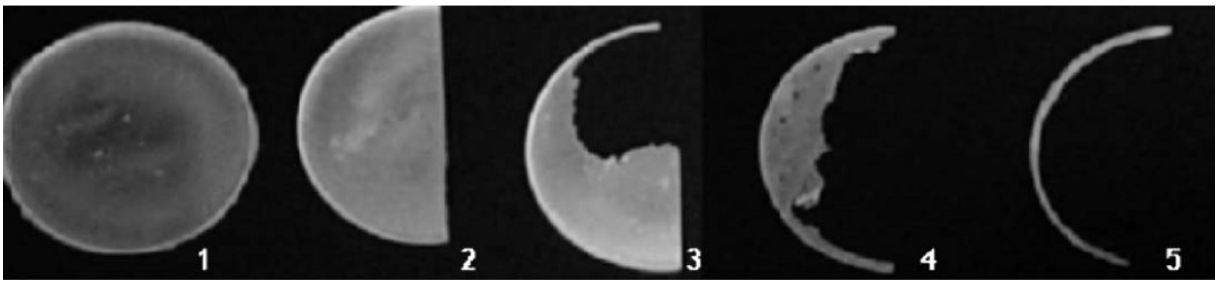


Figure 1.6: Degradation of P(HB-*co*-12mol%HHx) in activated sludge at start (1), after: 1 week (2), 2 weeks (3), 3 weeks (4) and 4 weeks (5) (Wang et al. 2004).

Global production of biopolymers

Whereas the worldwide production of bioplastic was annually stagnated between 2011 and 2013 at 1.–1.6 x 10^6 t, there is now the prediction of a rapidly increasing annual production to almost 7 x 10^6 t (Endres and Siebert-Raths 2011; Figure 1.7; Figure 1.8). Mostly this is due to a massive increase of the non-biodegradable bio-PET30, which will double its share from all produced biopolymers from 37–74%. Whereas, the share of the biodegradable polymers will be more than cut in half by 2018 (38% → 17%). The predicted ratio between PLA/PHA will stay constant with 6:1 with a share loss of 50% on the bioplastic market. However, the total annual PLA/PHA production will increase more than twofold in the next couple of years, whereas it stagnated in in last couple of years.

The reason in the low market share of PHA can be found mostly in its high price between 6–15 € per kg, depending in the production process, but mostly influenced by the used carbon feedstock. However, the bioplastic market is fast-moving and with a cheaper PHA production processes to be found, a rapid increase is imaginable (*e. g.,* in 2011, the share of bio-PET was less than 0.1%) (Endres and Siebert-Raths 2011).

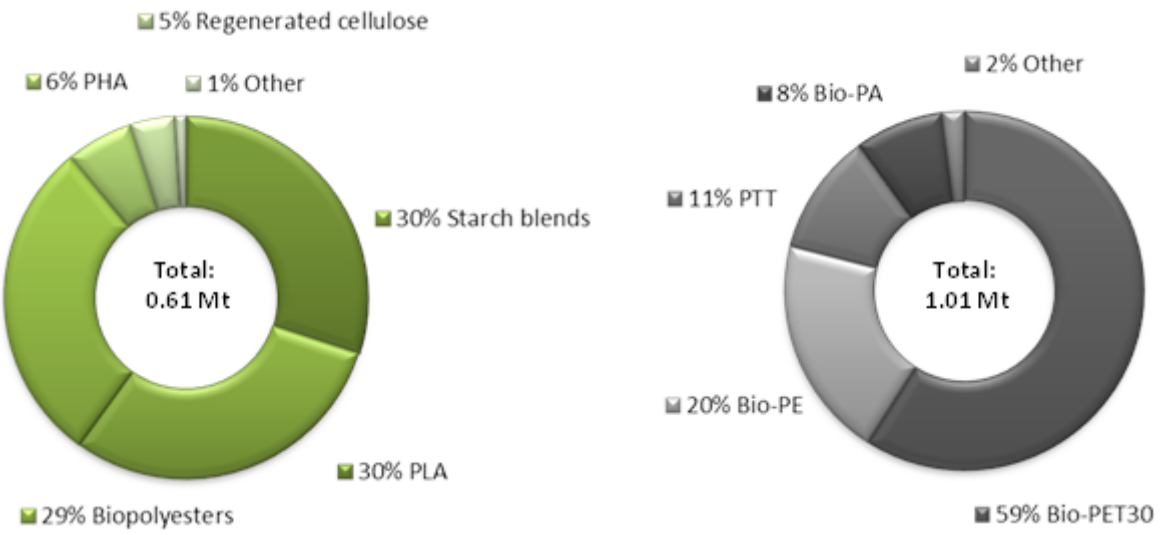


Figure 1.7: Global production capacities of bioplastics 2013 by material type. Green = biodegradable, grey = biobased/ non-biodegradable, PHA = polyhydroxyalkanoate, PLA = polylactic acid, Bio-PA = bio-polyamide, PTT = polytrimethylene terephthalate, Bio-PE = bio-polyethylene, Bio-PET30 = bio-polyethylene terephthalate 30 (raw data from Aeschelmann et al. 2015).

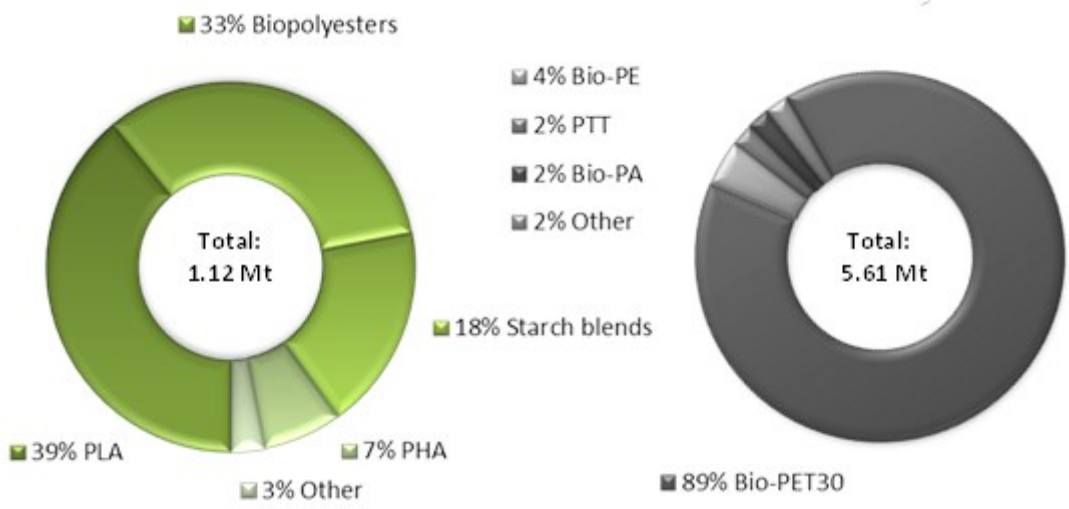


Figure 1.8: Global production capacities of bioplastics 2018 by material type. Green = biodegradable, grey = biobased/ non-biodegradable, PHA = polyhydroxyalkanoate, PLA = polylactic acid, Bio-PA = bio-polyamide, PTT = polytrimethylene terephthalate, Bio-PE = bio-polyethylene, Bio-PET30 = bio-polyethylene terephthalate 30 (raw data from Aeschelmann et al. 2015).

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CHAPTER II

Growth conditions for hydrophobic carbon sources

This chapter was modified from a previously published article in Applied Microbiology and Biotechnology, 2011. 89: 1611-1619 'Growth and polyhydroxybutyrate production by *Ralstonia eutropha* in emulsified plant oil medium' Charles F. Budde, Sebastian L. Riedel, Florian Hübner, Stefan Risch, Milan K. Popović, ChoKyun Rha, and Anthony J. Sinskey © Springer-Verlag.

Goal of Chapter II

The Chapter establishes general methods used in this study. A method for growing *R. eutropha* in plant oil medium with an emulsifying agent is described. Furthermore this chapter focuses on the analytics used during fermentations with lipids as a carbon source. An extraction method was established, which allows the monitoring of oil consumption during the fermentation and changes of the lipid profile of the residual oil. The developed culture method was successfully applied during six fold fermentations with the abovementioned analytics.

Introduction

Palm oil was chosen as carbon feedstock for the cultivation of *R. eutropha* to produce PHA. At the beginning, a basic cultivation method was developed to study the growth of *R. eutropha* with palm oil. The goal was to monitor cell growth, PHA accumulation and oil consumption over the course of the experiment. Through the hydrophobic properties of the palm oil it forms a top layer on the aqueous media, which leads to heterogeneous conditions at the beginning of the cultivation. *R. eutropha* will finally establish homogenous conditions through emulsification of the palm oil due to excretion of an external lipase (Lu et al., 2013). However, until the emulsion is built, no monitoring of oil consumption is possible. Also, the time needed for the oil to become emulsified served to extend the lag phase of the culture, since the oil is not significantly bioavailable for cell growth at this time.

In the first part of Budde et al. (2011), gum arabic, a natural glycoprotein synthesized by the acacia tree (Qi et al. 1991; Goodrum et al. 2000), was identified as a suitable emulsifying agent for palm oil cultivations with *R. eutropha.* Gum arabic is already used in the food industry as stabilizer, thickener and emulsifier (FDA, U.S. Code of Federal Regulations, 21CFR184.1330). Gum arabic could not be utilized as either a carbon or nitrogen source during cultivations with *R. eutropha*. Also, it did not significantly influence cell growth or PHA accumulation. Alternative potential emulsifying agents, such as the surfactants SDS,

Tween 80 or Triton X, were able to be used as carbon source from *R. eutropha* and/or inhibited cell growth. (Budde et al. 2011).

In the second part of Budde et al. (2011), this Chapter, the developed shaking flask cultivation conditions were transferred to batch fermentation conditions. Also a method to monitor and quantify the oil consumption, including oil degradation products, was developed.

Materials and methods

Bacterial strain and cultivation conditions

Experiments were performed with *R. eutropha* H16 (ATCC 17699). Tryptic soy broth (TSB) medium, without dextrose, (Becton Dickinson, Sparks, MD) was used as rich medium for precultures. As main medium a phosphate buffered minimal medium (pH 6.8), which was described previously (Budde et al. 2010), was used for the fermentations. To trigger PHA production, 0.1% NH_4Cl was used as nitrogen source. All media contained 10 µg/mL gentamicin sulfate. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Preparation of emulsified oil medium

A 2% palm oil medium was emulsified with 0.5% gum arabic as followed: At first a 10× solution of gum arabic was prepared in water. The gum arabic solution was then centrifuged (10,500×*g*) to separate out insoluble particles. The sodium phosphate, K_2SO_4, clarified gum arabic, and palm oil were combined, along with an appropriate amount of water, and emulsified by homogenizing with a Sorvall Omni-Mixer for one minute. After emulsifying the oil, the medium was autoclaved, cooled, and $MgSO_4$, $CaCl_2$, trace elements, and gentamicin were all added from sterile stocks.

Pre-culture conditions

R. eutropha was grown overnight in 4 mL TSB culture tubes, inoculated from a single colony. 0.5 mL of the overnight culture was used to inoculate 50 mL minimal medium flask precultures containing 2% fructose and 0.1% NH_4Cl.

Fermentation Conditions

Emulsified palm oil fermentations were carried out using an Infors Sixfors multiple fermenter system (Bottmingen, Switzerland). Cells from the minimal medium precultures were used to inoculate each fermenter to an initial OD600 of 0.1. Each vessel contained 400 mL of emulsified palm oil medium with 2% palm oil, 0.5% gum arabic, and 0.1% NH4Cl. The

temperature of each fermenter was kept constant at 30°C. The pH of each culture was maintained at 6.8 ± 0.1 through controlled addition of 2 M NaOH. Stirring was provided by two six-blade Rushton impellers at speeds of 500–900 rpm. Air was supplied at 1 vvm and the dissolved oxygen concentration was maintained above 40% through controlled addition of pure oxygen.

Analytical methods

Determination of cell dry weight, residual cell dry weight, PHB accumulation and NH_4Cl concentration

The CDW and PHB content of samples from fructose cultures were measured as described previously (Budde et al. 2010). rCDW was calculated as the total CDW minus the mass of PHB. CDW of samples from oil fermentations was measured by taking 10 mL samples in 15-mL pre-weighed plastic tubes, centrifuging, washing with 5 mL cold water plus 2 mL cold hexane, centrifuging, resuspending the cells in 2 mL cold water, freezing at -80°C, and lyophilizing. The hexane was included in the washing step to remove unused oil. The PHB content of cells was measured by transferring freeze dried biomass to screw top glass test tubes, and reacting the samples with methanol and sulfuric acid in the presence of chloroform to convert the PHB monomers into methyl 3-hydroxybutyrate (Brandl et al. 1988). The amount of methyl 3-hydroxybutyrate was quantified using an Agilent 6850 gas chromatograph (Santa Clara, CA) equipped with a flame ionization detector and a DB-Wax column (Agilent, 30 m × 0.32 mm × 0.5 µm). The carrier gas was hydrogen (3.0 mL/min) and the temperature program was 80°C for 5 min, ramp to 220°C at 20°C/min, and hold at 220°C for 5 min. A calibration curve for the instrument was generated by treating known amounts of pure PHB as described above and measuring the resulting peak areas on the chromatograms. The ammonium concentration in fermentation samples was determined from cell free supernatants using an enzymatic ammonium assay kit from Sigma Aldrich (Cat. No. AA0100). The assay was carried out following the manufacturer's instructions.

Quantification of palm oil during cultivation

A previous report described a method for measuring plant oil concentrations in which oil was extracted from 2 mL of medium using hexane (Kahar et al. 2004). This method was tested with emulsified palm oil medium and could not achieve quantitative oil recovery. Therefore a new method for measuring the concentration of oil and other lipids in emulsified oil medium was developed. For each sample, 10 mL medium was taken in a 15-mL plastic test tube and

centrifuged 10 min using a swinging bucket rotor. (In this method, all centrifugations were performed at room temperature (RT) and 7,200×g.) The supernatant was transferred to a 50-mL plastic test tube, and the pellet was washed with 5 mL water and centrifuged again to recover oil that had been associated with the cell pellet. This supernatant was then combined with the supernatant from the previous centrifugation (15 mL total). 20 mL of a 2:1 (v/v) mixture of chloroform and methanol were added to the tube, and the sample was vortexed for one minute. The sample was then centrifuged and 5 mL of the organic phase (the bottom phase) was transferred to a pre-weighed glass test tube. Solvent was removed by incubating the test tube in a heat block at 40°C, then transferring the test tube to a vacuum oven at 80°C. After drying, samples were weighed to determine the mass of recovered lipid. The lipid concentration in the medium was calculated:

$$\text{Lipid concentration} = \text{Mass recovered} \left(\frac{V_{org.}}{V_{trans.}}\right)\left(\frac{1}{V_{sampel}}\right) \quad [1]$$

In this equation, $V_{organic}$ is the volume of the organic phase after the extraction. Because the solvent mixture includes methanol, not all of the solvent remains in the organic phase after contact with the aqueous medium. $V_{organic}$ was measured to be 13.33 mL when using chloroform/methanol. $V_{transferred}$ was 5 mL and V_{sample} was 10 mL (the volume of medium taken from the culture).

The lipid species extracted from fermentation samples were examined qualitatively by thin layer chromatography (TLC). Lipid samples were dissolved in chloroform and 30 µg were spotted onto a silica gel TLC plate (250 µm thickness). The standard mixture spotted on the plate contained a TAG (1,2-distearoyl-3-oleoyl-rac-glycerol; 10 µg), DAG (1,2-dipalmitoyl-rac-glycerol; 20 µg), MAG (1-palmitoyl-rac-glycerol; 20 µg), and FFA (palmitic acid; 10 µg). The plate was first developed with chloroform/methanol/water (60:35:5, by volume) to 5 cm from the origin. It was then developed with hexane/diethyl ether/acetic acid (70:30:1, by volume) to the top of the plate. Finally analytes were made visible by spraying the plate with 3% cupric acetate in an 8% phosphoric acid solution and incubating the plate at 200°C.

Results

Quantification of oil utilization

As bacteria grow and oil is broken down, other lipids will appear in the culture medium, including MAGs, DAGs, and FFAs. To accurately measure the total amount of lipid present in a medium, an extraction procedure must also recover these polar lipids. To test the extraction of polar lipids, recovery of oleic acid and a 1:1 mixture of oleic acid and palm oil

from gum arabic emulsions was measured. Results of these experiments are shown in Table 2.1.

Table 2.1: Palm oil, oleic acid (OA), and a 1:1 mixture of the two were extracted from minimal medium standards in which the lipids were emulsified with gum arabic. 10 mL samples were taken from each standard and 20 mL of each solvent was used for extraction. All extractions were performed four times, and the reported values represent the means ± SD.

Solvent	Mass of lipid recovered (%)					
	1% Lipid + 0.3% gum arabic			2% Lipid + 0.5% gum arabic		
	Palm oil	OA	PO/OA	Palm oil	OA	Palm oil/OA
Hexane	31.4 ± 1.1	62.1 ± 4.5	50.9 ± 2.7	23.5 ± 0.8	47.6 ± 1.8	42.0 ± 1.7
Chloroform	100.5 ± 1.7	99.8 ± 1.2	101.6 ± 1.6	97.2 ± 4.7	90.6 ± 3.5	96.0 ± 2.1
Chloroform/ methanol	102.2 ± 1.2	103.5 ± 2.1	100.2 ± 1.7	98.7 ± 2.7	98.1 ± 0.6	98.0 ± 2.7

Both chloroform and chloroform/methanol recovered greater than 90% of the lipids from each standard. Chloroform/methanol was chosen for measuring lipid concentrations in experimental samples, as it yielded slightly better results with the 2% lipid emulsions. Hexane could not be used to quantitatively recover palm oil or oleic acid. It was found that hexane could quantitatively extract emulsified palm oil if sodium hydroxide was added to the samples to break the emulsions (data not shown). Even with this sodium hydroxide treatment, however, oleic acid could not be recovered efficiently. Control standards were tested that contained either 0.3% or 0.5% gum arabic and no lipid. The mass recovered from these samples was negligible, indicating that gum arabic is not extracted by the solvents used in this study (data not shown).

When cells are present in a sample, some of the lipids in the medium are associated with the cell pellet. To estimate how much lipid was associated with the cells, the pellet was washed with hexane after the water washing steps. (Chloroform/methanol could not be used for this step because the chloroform extracts PHB from biomass.) It was determined that if the hexane wash is included in the lipid recovery procedure, the observed lipid concentration increases by 1–4% (data not shown). Given the small impact this has on the measurement, it was determined that this step does not need to be included in the standard method, but could be added if higher precision is required.

It was also discovered that the use of chemical antifoams in fermentations interferes with the lipid recovery method and should be avoided. Both polypropylene glycol and silicone oil are extracted by the chloroform/methanol method, which will artificially increase the measured

lipid concentration in the medium. In this experience, foam formation in emulsified oil cultures was not a significant issue at the cell densities used in this study.

Emulsified oil fermentations

Fermentations were carried out in medium containing 20 g/L palm oil emulsified with 0.5% gum arabic, and cell growth and oil utilization were measured (Figure 2.1). A measureable increase in CDW by the 9 h time point was observed. This demonstrates that the method leads to cultures with short lag phases, and that it was able to take accurate measurements early in the experiment. By 12 h all the ammonium in the medium was depleted (data not shown), and by 16 h the rCDW reached its maximum value. After this point cell division stopped and further increases in CDW were due to storage of PHB. By 72 h, 79% of CDW consisted of PHB. Six fermentations were carried out in this experiment, and the small error bars in Figure 2.1 show that this method allows for reproducible growth of *R. eutropha*.

The initial measured concentration of oil in the medium was 17 g/L, which is lower than the 20 g/L added to each vessel. This discrepancy is attributed to the fact that some oil collected on the walls of the vessels and was therefore removed from the medium. Significant oil consumption by the cells was first observed between the 9 and 12 h time points. Oil consumption continued throughout the experiment, although the rate of consumption decreased over time. Measurement of lipid utilization allows for calculation of product yields. It was found that over the course of the entire fermentation, polymer was formed at a yield of 0.61 g PHB/g palm oil. If only the PHB production phase of the culture is considered, the PHB yield is 0.84 g/g palm oil. These values are similar to other yields from plant oils reported in the literature (Kahar et al. 2004).

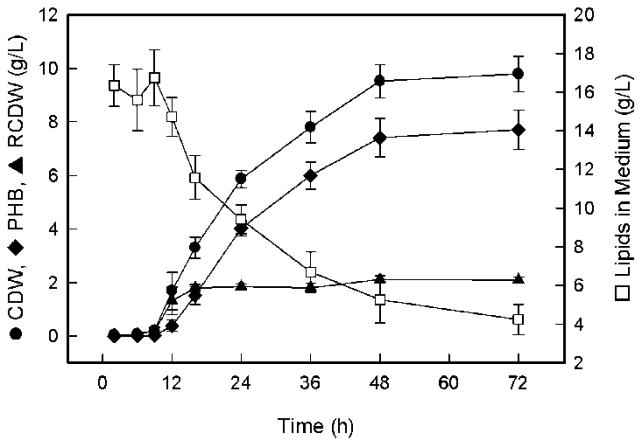

Figure 2.1: *R. eutropha* was grown in emulsified palm oil fermentations. Cell growth, PHB production, and oil consumption were measured over time. Values reported are means from six fermentations and error bars represent standard deviations.

TLC analysis revealed that over time TAGs in the medium were broken down and the concentrations of FFAs, MAGs, and DAGs increased (Figure 2.2).

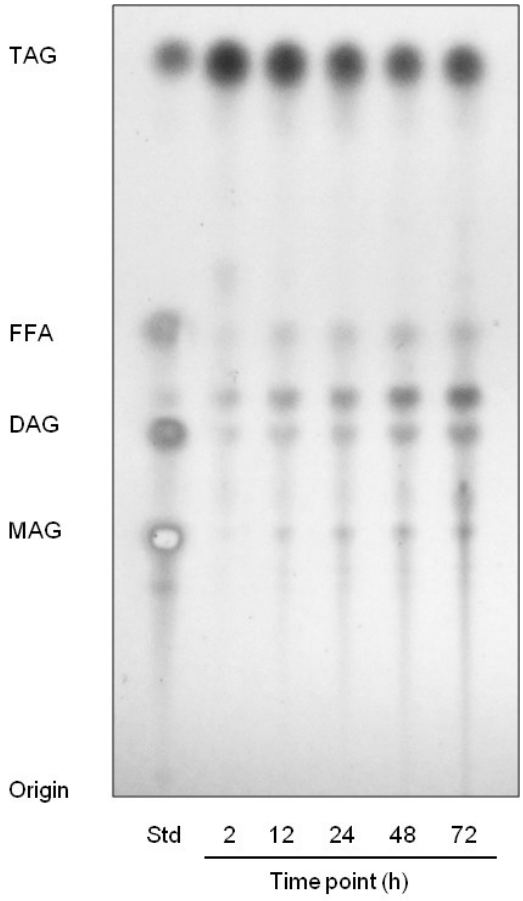

Figure 2.2: The types of lipids present in the medium of an emulsified palm oil fermentation were analyzed with TLC. Samples were taken at various times during the experiment and lipids were extracted. 30 µg of lipid were loaded for each time point. As the cells grew MAGs, DAGs, and FFAs were released from the palm oil.

Discussion

While *R. eutropha* is able to grow on non-emulsified palm oil, these cultures exhibit variable lag times and representative samples cannot be taken early in experiments. Oil will eventually become emulsified as the bacteria grow, but the mechanism by which this emulsification occurs is unknown. Some bacteria excrete surfactants (Rosenberg and Ron 1999), but there is no evidence that *R. eutropha* synthesizes these compounds. It is possible that polar lipids released during the breakdown of TAGs (*i.e.* MAGs, DAGs, and FFAs) could themselves emulsify the oil. Our group recently showed that the putative lipase encoded by gene H16_A1322 (GeneID: 4249488) is essential for robust growth of *R. eutropha* on non-emulsified plant oil (Brigham et al. 2010, Lu et al. 2013).

In order to conduct quantitative, reproducible experiments with plant oil as the carbon source, an emulsified oil culture method for *R. eutropha* was developed. *R. eutropha* fermentations with emulsified palm oil demonstrated the effectiveness of this method. Cultures had short lag phases and were highly reproducible (Figure 2.1). The emulsified oil medium can be used in both fermenters and shake flasks. The cells accumulated high levels of bioplastic, with PHB content of 79% of CDW reached after 72 h. However, while this method is a useful experimental tool, it is unlikely to have industrial applications due to the cost of gum arabic. A lipid extraction method was also developed, which allowed us to monitor oil consumption by the cells and the breakdown of oil in the medium (Figure 2.2).

After this chapter established several basic methods for analyzing growth of *R. eutropha* on palm oil during PHB accumulation with the wild type, the next chapter focuses on the metabolic engineering of *R. eutropha* to accumulate P(HB-*co*-HHx).

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CHAPTER III

Selection of P(HB-*co*-HHx) production strain

This chapter was modified from a previously published article in Applied and Environmental Microbiology, 2011. 77(9): 2847-2854 'Production of Poly (3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) from Plant Oil by Engineered *Ralstonia eutropha* Strains' Charles F. Budde, Sebastian L. Riedel, Laura B. Willis, ChoKyun Rha, and Anthony J. Sinskey © American Society for Microbiology.

Goal of Chapter III

The goal of this Chapter was the generation and selection of a PHA production strain, which produces the novel copolymer P(HB-*co*-HHx), with a high (>10 mol%) HHx content, from plant oils. Besides the requirement of (i) the high HHx content per polymer, the strain needs to meet two more criteria: (ii) a high accumulation of PHA per CDW (>65%) and (iii) a stable growth to high cell densities.

Palm oil batch fermentation conditions with eight times higher nitrogen content compared to shaking flask experiments, without the usage of an emulsifying agent, for *R. eutropha* were established during this study.

Introduction

In the first part of Budde et al. 2011, the metabolic engineering of *R. eutropha* is described to produce P(HB-*co*-HHx). The wildtype strain of *R. eutropha,* H16, only synthesizes *scl*-PHA, because it contains a class 1 PHA-Synthase, which cannot incorporate *mcl*-HA monomers into the polymer chain. The first organism identified that naturally accumulates P(HB-*co*-HHx) was *Aeromonas caviae* (Shimamura et al. 1994). Two genes were therefore critical: First, its gene for a class II PhaC ($phaC_{Ac}$) for the *mcl*-HA incorporation and the gene $phaJ_{Ac}$, encoding an enoyl-CoA hydratase, which converts fatty acid β-oxidation intermediate to PHA precursors (Fukui and Doi 1997; Fukui et al. 1998). Following, several groups studied P(HB-*co*-HHx) accumulation, with *Aeromonas* strains or recombinant *R. eutropha* strains with *Aeromonas* genes. However, nobody was able to engineer a strain that accumulates P(HB-*co*-HHx) with a high HHx concentration (>10 mol%) using plant oils as the sole carbon sources. Only the use of shorter chain length fatty acids as a feedstock leads to a high HHx accumulation in the polymer (Fukui and Doi 1998; Chen et al. 2001; Kahar et al. 2004; Loo et al. 2005; Mifune et al. 2008; Mifune et al. 2010). The use of shorter chain fatty acids for PHA production is not preferable, since production of these feedstocks or separation from raw material is costly.

Budde et al. (2010) discussed, that the concentration of HB-CoA molecules influences the HHx incorporation during a P(HB-*co*-HHx) synthesis. Based on this theory, they engineered *R. eutropha* with deletions of the acetoacetyl-CoA reductases (*phaB*$_{1\text{-}3}$). This strain therefore could not synthesize PHB through the classic pathway, starting with acetyl-CoA. HB-CoA precursor molecules could only be generated through β-oxidation intermediates when grown on lipids. As a result, the strain made significantly less PHB than the wild type (Budde et al. 2010). Based on these findings, the recombinant strains for this chapter were engineered with different levels of acetoacetyl-CoA reductase activities. PHA synthase genes from *Rhodococcus aetherivorans* I24 for HHx incorporation (Buckland et al. 1999; Williams et al. 1994), and *phaJ* genes from *A. caviae* (Fukui and Doi 1997) and *Pseudomonas aeruginosa* (Davis et al. 2008; Sato et al. 2011) for increasing the HHx-CoA concentration from β-oxidation intermediates were used to engineer the strains. Also, the expression levels of the genes where modified among the strains through using an overexpression plasmid. Plasmid stability was generated through the creation of a proline auxotrophy. The gene *proC*, involved in the proline synthesis, was deleted and added to the plasmid to generate an addiction system.

The recombinant strains were screened in shaking flask experiments with palm oil as the sole carbon source for both high accumulation of HHx / PHA and high PHA accumulation per CDW. The two best performing strains were used to choose the PHA production strain for palm oil batch fermentations.

Materials and methods

Bacterial strains and cultivation conditions

All PHA production experiments in this study were conducted with *R. eutropha* H16 and mutants derived from this strain (Table 3.1). The rich medium used for growth of *R. eutropha* was dextrose-free TSB medium (Becton Dickinson, Sparks, MD). The salt concentrations in the *R. eutropha* minimal medium have been reported previously (Budde et al. 2010). Carbon and nitrogen sources were added to the minimal medium as described in the text. All media contained 10 µg/mL gentamicin sulfate. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless noted otherwise. *R. eutropha* strains were always grown aerobically at 30°C. In shake flask experiments, 50 mL media was used in 250-mL flasks. The shaker was set to 200 rpm.

Fermentation conditions

Strains Re2058/pCB113 and Re2160/pCB113 were grown to higher densities than is possible in shake flasks using an Infors Sixfors multiple fermenter system (Bottmingen, Switzerland). Cultures were prepared by first growing the strains overnight in TSB containing 200 µg/mL kanamycin. These cultures were used to inoculate 50 mL minimal medium flask pre-cultures containing 2% fructose and 0.1% NH_4Cl. The minimal medium pre-cultures were used to inoculate the fermenters so that the initial OD_{600} of each 400 mL culture was 0.1. Each fermenter contained 4% (Re2160/pCB113) or 4.5% (Re2058/pCB113) PO and 0.4% NH_4Cl. Neither the fructose nor palm oil minimal medium cultures contained kanamycin. The temperature of each fermenter was kept constant at 30°C. The pH of each culture was maintained at 6.8 ± 0.1 through controlled addition of 2 M sodium hydroxide. Stirring was provided by two six-blade Rushton impellers at speeds of 500–1,000 rpm. Air was supplied at 0.5–1 vvm and the dissolved oxygen concentration was maintained above 40% through controlled addition of pure oxygen. Silicone oil AR200 was used as antifoam in these experiments and was added to cultures by hand as necessary.

Analytical methods

The CDW of cultures were measured by taking 8–14 mL samples in pre-weighed plastic test tubes. The samples were centrifuged and the pellets were washed with 5 mL cold water. For experiments using palm oil as the carbon source, 2 mL cold hexane was also included during the wash step to remove unused oil from the samples. Samples were then centrifuged again, resuspended in 1 mL cold water, frozen at -80°C, and lyophilized. The dried samples were weighed and CDWs were determined. rCDW values were calculated for each sample, which are defined as the total CDW minus the mass of PHA. Ammonium concentrations in clarified culture supernatants were measured with an ammonium assay kit (Sigma-Aldrich, Cat. No. AA0100) following the manufacturer's instructions.

The PHA contents and compositions of dried samples were determined using a methanolysis protocol adapted from (Brand et al. 1988). Dried cells were weighed into screw top glass test tubes and reacted with methanol and sulfuric acid in the presence of chloroform for 2.5 h at 100°C. This reaction converts PHA monomers into their related methyl esters. The concentrations of methyl esters were determined via gas chromatography with an Agilent 6850 GC (Santa Clara, CA) equipped with a DB-Wax column (Agilent, 30 m x 0.32 mm x 0.5 µm) and a flame ionization detector. 2 µL of each sample were injected into the GC with a split ratio of 30:1. Hydrogen was used as the carrier gas at a flow rate of 3 mL/min. The oven

was held at 80°C for 5 min, heated to 220°C at 20°C/min, and held at 220°C for 5 min. Pure standards of methyl 3-hydroxybutyrate and methyl 3-hydroxyhexanoate were used to generate calibration curves for the methanolysis assay.

Table 3.1: Strains used in this study

Strains	Description[a]	Reference or source
R. eutropha		
H16	Wild type strain, Gm resistant	ATCC 17699
Re1034	H16 Δ*phaC1*	York et al. 2001
Re2000	Re1034::$phaC1_{Ra}$,	This study
Re2001	Re1034::$phaC2_{Ra}$,	This study
Re2058	Re1034 Δ*proC*,	This study
Re2115	H16 Δ*phaB1* Δ*phaB2* Δ*phaB3*	Budde et al. 2010
Re2133	Re2115 Δ*phaC1*,	This study
Re2135	Re2133:: $phaC2_{Ra}$,	This study
Re2136	Re2133:: $phaC_{Ac}$,	This study
Re2151	Re2135::*phaB2*,	This study
Re2152	Re2135::$phaJ1_{Pa}$,	This study
Re2153	Re2135::$phaJ_{Ac}$,	This study
Re2154	Re2136::*phaB2*,	This study
Re2155	Re2136::$phaJ1_{Pa}$,	This study
Re2156	Re2136::$phaJ_{Ac}$,	This study
Re2160	Re2133 Δ*proC*, m	This study
E. coli strains		
S17-1	Strain for conjugative transfer of plasmids to *R. eutropha*	Simon et al. 1983
Other strains		
R. aetherivorans I24	Source of $phaC1_{Ra}$ and $phaC2_{Ra}$	Buckland et al. 1999
P. aeruginosa PA01	Source of $phaJ1_{Pa}$	Stover et al. 2000

[a]Abbreviations: Gm, gentamicin; Km, kanamycin

Results

Re2000 and Re2001 containing $phaC1_{Ra}$ were able to accumulate P(HB-*co*-HHx), but only with very low levels of <2 mol% HHx when grown on palm oil (Table 3.2). It is hypothesized that high intracellular concentrations of HB-CoA may limit HHx incorporation into the PHA made by the recombinant strains. Our group previously constructed a strain with low acetoacetyl-CoA reductase activity that accumulates significantly less PHB than H16 (Re2115). The *phaC1* gene from the genome of Re2115 was deleted, and $phaC2_{Ra}$ (Re2135) or $phaC_{Ac}$ (Re2136) was inserted in its place. Both Re2135 and Re2136 made PHA with high

HHx content from PO, but these strains did not accumulate significant polymer (~25% of CDW after 72 h).

Additional genes were therefore inserted into the genomes of these strains at the *phaB1* locus, with the goal of increasing total polymer accumulation. One of these genes was *phaB2*, which encodes a low activity acetoacetyl-CoA reductase (Budde et al. 2010). It was hypothesized that expression of this gene would increase HB-CoA production, but not to the level of H16. Also *phaJ* genes from *P. aeruginosa* and *A. caviae* were inserted, which would allow the strains to convert intermediates of fatty acid β-oxidation into HA-CoA molecules. All of these strains exhibited greater PHA production than Re2135 and Re2136 when grown on palm oil (Table 3.2). The strains containing $phaC_{Ac}$ (Re2154-Re2156) made the most polymer, but the HHx content of the PHA was reduced to 4–5 mol%. The strains containing $phaC2_{Ra}$ (Re2154, Re2155 and Re 2156) made the most polymer, but the HHx content of the PHA was reduced to 4 to 5 mol% at 72 h. The strains containing $phaC2_{Ra}$ (Re2151, Re2152, and Re2153) made more PHA than Re2135, and the polymer still contained significant HHx. Of these strains Re2152 was the most promising, as it accumulated 40% P(HB-*co*-HHx) with 22 mol% HHx.

Analysis of engineered *R. eutropha* strains harboring plasmids

It was hypothesized that polymer accumulation could be increased in the engineered *R. eutropha* strains by increasing expression of the PHA biosynthetic genes. To accomplish this, the engineered PHA operon from Re2152 ($phaC2_{Ra}$-*phaA*-$phaJ1_{Pa}$) was amplified and cloned into pBBR1MCS-2. The cloned region included 460 bp from the genome upstream of the start codon of $phaC2_{Ra}$, so that the operon in the plasmid would be expressed from the native *R. eutropha* promoter. The resulting plasmid (pCB81) was transformed into Re1034 and Re2133, to determine how the different acetoacetyl-CoA reductase activity levels of the two strains would influence PHA synthesis. When these strains were grown in palm oil minimal medium containing kanamycin, both accumulated >65% P(HB-*co*-HHx) (Table 3.2) at 72 h. At this time point, the PHA from Re1034/pCB81 contained 12 mol% HHx, while the PHA from Re2133/pCB81 contained 23 mol% HHx.

While both strains harboring pCB81 accumulated significant P(HB-*co*-HHx) with high HHx content, these strains were not suitable for industrial PHA production from palm oil. The use of plasmid pCB81 would require the addition of expensive antibiotics to fermentations, which would add excessive cost at the industrial scale.

Table 3.2 Cell dry weights and levels of PHA and HHx[a]

Strain	48 h			72 h		
	CDW (g/L)	PHA (%/CDW)	HHx (mol%)	CDW (g/L)	PHA (%/CDW)	HHx (mol%)
H16	5.3 ± 0.4	71 ± 1	0	6.0 ± 0.2	79.2 ± 0.9	0
Re2000	6.1 ± 0.1	75.3 ± 0.3	1.5 ± 0.1	7.3 ± 0.1	82 ± 4	1.1 ± 0.3
Re2001	1.89 ± 0.04	49 ± 2	1.6 ± 0.2	2.19 ± 0.09	50 ± 3	1.5 ± 0.2
Re2115	0.78 ± 0.04	16.9 ± 0.2	1.68 ± 0.01	1.13 ± 0.06	22 ± 3	1.7 ± 0.3
Re2135	1.0 ± 0.1	22.3 ± 0.2	31.4 ± 0.2	1.22 ± 0.08	26 ± 2	31.4 ± 0.8
Re2136	0.72 ± 0.04	21.3 ± 0.2	15.01 ± 0.02	1.05 ± 0.01	25.5 ± 0.7	13.9 ± 0.5
Re2151	0.83 ± 0.01	28.63 ± 0.01	15.04 ± 0.01	1.01 ± 0.07	33 ± 3	12 ± 1
Re2152	1.15 ± 0.07	35.27 ± 0.07	23.29 ± 0.02	1.40 ± 0.02	40.4 ± 0.4	22.44 ± 0.08
Re2153	1.0 ± 0.1	31.5 ± 0.8	22.29 ± 0.01	1.32 ± 0.09	37 ± 2	22.29 ± 0.07
Re2154	1.26 ± 0.08	45.8 ± 0.8	5.8 ± 0.2	1.92 ± 0.04	53 ± 3	4.83 ± 0.01
Re2155	1.87 ± 0.01	54.9 ± 0.5	3.85 ± 0.07	2.55 ± 0.06	63 ± 3	4.00 ± 0.04
Re2156	2.2 ± 0.2	53 ± 3	3.8 ± 0.3	2.45 ± 0.09	57 ± 2	2.80 ± 0.4
Re1034/pCB81	3.3 ± 0.2	68.8 ± 0.8	13.6 ± 0.2	4.0 ± 0.2	73.0 ± 0.9	11.6 ± 0.2
Re2058/pCB113	3.24 ± 0.03	68 ± 2	15.3 ± 0.4	3.6 ± 0.3	73.1 ± 0.2	12.7 ± 0.3
Re2133/pCB81	2.3 ± 0.1	60 ± 4	24.3 ± 0.8	2.9 ± 0.1	67.0 ± 0.3	23.3 ± 0.2
Re2160/pCB113	2.00 ± 0.01	56 ± 0.5	25.32 ± 0.09	2.74 ± 0.06	63.99 ± 0.03	24.13 ± 0.02

[a] *R. eutropha* strains were grown in minimal medium with 1% palm oil and 0.05% NH_4Cl. Samples were harvested after 48 and 72 h of growth to analyze CDW and P(HB-*co*-HHx) content. Re1034/pCB81 and Re2133/pCB81 cultures contained kanamycin. All values represent means from duplicate or triplicate cultures, with the uncertainties indicating the range of observed values.

A common strategy for maintaining plasmid stability without the use of antibiotics is to create an auxotrophic mutant through a genome mutation, and then to complement the mutation with a plasmid containing the deleted gene (Kroll et al. 2010). The *proC* gene from Re1034 and Re2133 was therefore deleted. These strains were unable to grow in minimal medium that did not contain proline (data not shown). Plasmid pCB113 was created by cloning the *proC* region of the *R. eutropha* genome into pCB81. When pCB113 was transformed into Re2058 and Re2160, the ability of these strains to grow in minimal medium without proline was restored. PHA production from palm oil in kanamycin-free medium by Re2058/pCB113 and Re2160/pCB113 closely matched the results observed for Re1034/pCB81 and Re2133/pCB81 (Table 3.2). It was also found that these strains made the desired PHA copolymers only when oil or fatty acids were provided as carbon sources. When Re2058/pCB113 and Re2160/pCB113 were grown in fructose minimal medium, these strains accumulated only 40% and 17% PHA, respectively, and no HHx was detectable in the polymer (data not

shown). The PHA production pathway from TAGs and respective FA is shown in Figure 3.1 for Re2058/pCB113 and in Figure 3.2 for Re2160/pCB113.

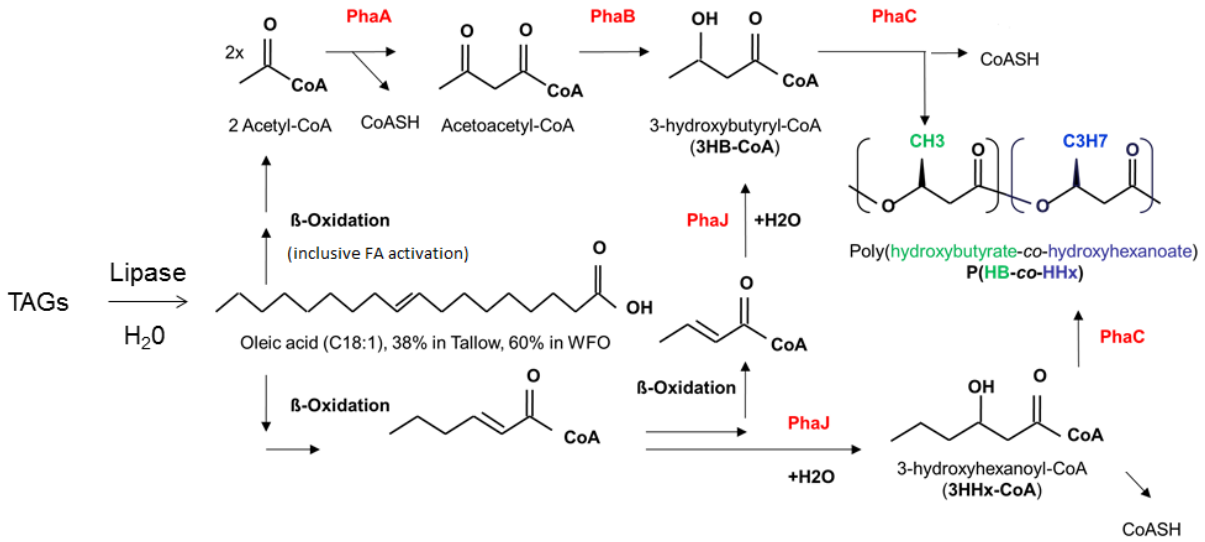

Figure 3.1: PHA production pathway in Re2058/pCB113.

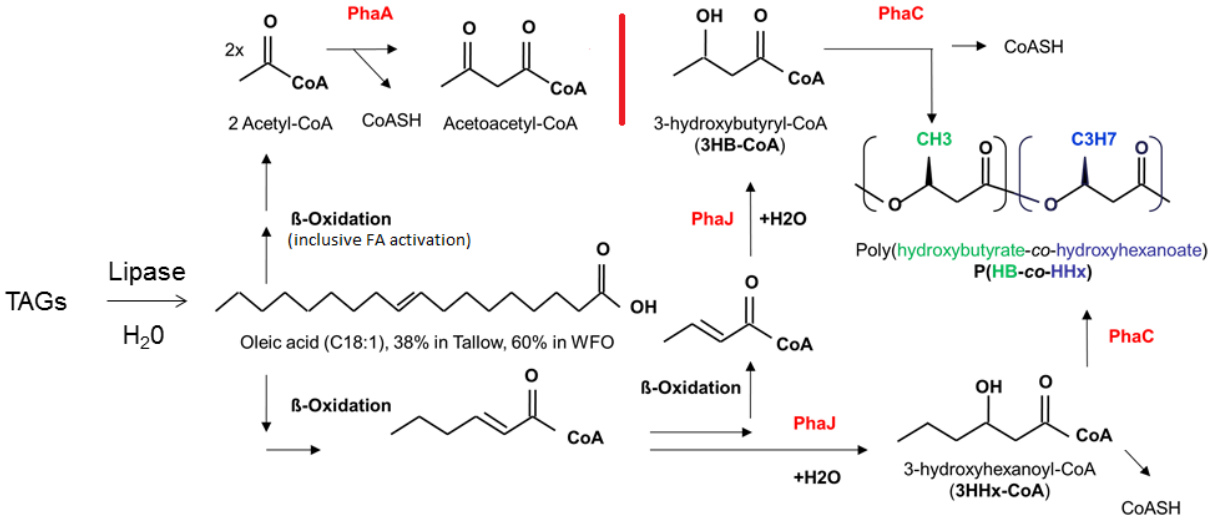

Figure 3.2: PHA production pathway in Re2160/pCB113.

The performance of Re2058/pCB113 and Re2160/pCB113 in higher-density palm oil cultures was evaluated by growing these strains in fermenters, using medium with an NH_4Cl concentration eight times that of the medium in the flask cultures (Figure 3.3). No kanamycin was added to the fermentation medium or the minimal medium pre-cultures. Both strains grew in the high-nitrogen medium, although Re2160/pCB113 exhibited a lag phase of 24 h. By the ends of the fermentations, Re2058/pCB113 accumulated 71% PHA with 17 mol% HHx, while Re2160/pCB113 accumulated 66% PHA with 30 mol% HHx. The PHA contents of the cells in both fermentations closely matched the values measured in the low density flask cultures, suggesting that plasmid loss did not occur at the higher cell densities. When samples taken

from similar fermentations were diluted and plated onto solid TSB without kanamycin, equal numbers of colonies were observed (data not shown), further indicating that plasmid loss does not occur with theses strains.

Several interesting observations were made when analyzing the PHA made in these experiments. In both fermentations the HHx content of the polymer was extremely high (>40 mol%) early in the cultures. Over time the HHx content decreased and then remained stable over the final 48 h of each experiment. The final HHx content in the PHA was higher in the fermenter cultures than the low-density flask cultures (Table 3.2). When analyzing the gas chromatograms of the methanolysis samples from both fermentations, small peaks with the same retention time as methyl 3-hydroxyoctanoate were observed (data not shown). These peaks were also present when polymer purified from dried cells of both strains was subjected to the methanolysis assay. This suggests that the PHA produced in these fermentations contained trace amounts of 3-hydroxyoctanoate in addition to HB and HHx.

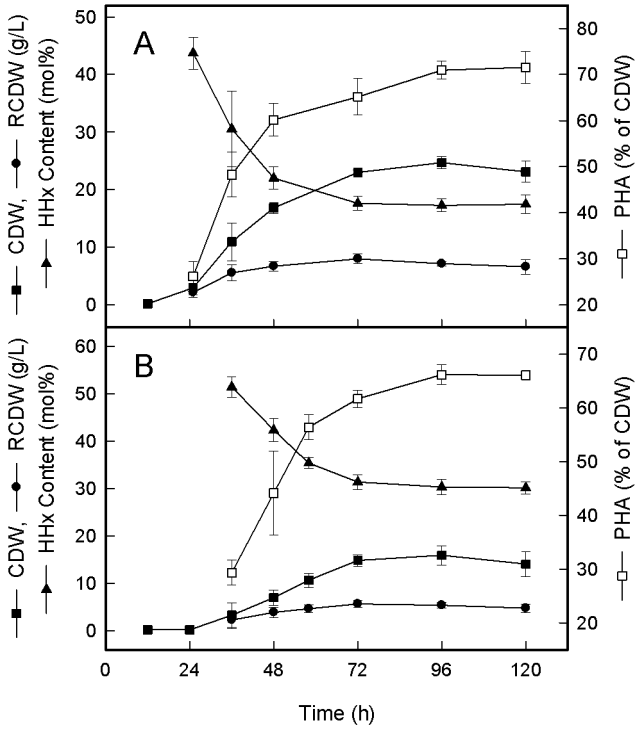

Figure 3.3: Re2058/pCB113 (A) and Re2160/pCB113 (B) fermentations were carried out using palm oil as the sole carbon source. Plasmid pCB113 was retained by the cells without the use of kanamycin. Both strains accumulated P(HB-*co*-HHx) with higher HHx than was achieved in flask cultures. Data points are means from triplicate fermentations and error bars indicate SD.

Discussion

Two novel PHA synthases from the bacterium *R. aetherivorans* I24 were used for *mcl*-PHA accumulation with *R. eutropha*. These strains accumulated P(HB-*co*-HHx) when grown on

palm oil, but the HHx content of the PHA was low. The PHA from Re2001 contained only 1.5 mol% HHx when the strain was grown on palm oil. It has previously been observed that HHx content in PHA decreases as the lengths of the fatty acids fed to recombinant *R. eutropha* increase (Mifune et al. 2008). As the most abundant fatty acids in palm oil are palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) (Sambanthamurthi et al. 2000).

In order to increase the HHx content of the PHA, *R. eutropha* strains that expressed recombinant PHA synthases and had low acetoacetyl-CoA reductase activity were constructed. It was previously discovered that *R. eutropha* strains in which the acetoacetyl-CoA reductase (*phaB*) genes had been deleted made significantly less PHB than the wild type, presumably because the HB-CoA synthesis pathway had been disrupted (Budde et al. 2010). The PHA made by the *phaB* deletion strains with recombinant synthases had high HHx content, but the strains stored little polymer (Table 3.2). Notably, the strain containing *phaC2*$_{Ra}$ (Re2135) made PHA with a HHx content much higher than that of the analogous strain containing *phaC*$_{Ac}$ (Re2136). The PhaC$_{Ac}$ synthase has been the most widely studied enzyme for synthesis of P(HB-*co*-HHx) (Fukui and Doi 1997, Fukui and Doi 1998, Loo et al. 2005, Mifune et al. 2008, Mifune et al. 2010).

In order to increase synthesis of HB-CoA and HHx-CoA from fatty acid β-oxidation intermediates, *phaJ* genes were inserted into the genomes of the recombinant strains. PhaJ enzymes from *A. cavie* and *P. aeruginosa* have been shown to hydrate crotonyl-CoA and 2-hexenoyl-CoA at similar rates (Fukui et al. 1998, Tsuge et al. 2000). It was found that insertion of either *phaJ*$_{Ac}$ or *phaJ1*$_{Pa}$ into our recombinant strains led to increased PHA accumulation, with the strains expressing *phaJ1*$_{Pa}$ generating polymer with slightly higher HHx content (Table 3.2).

Expression of the PHA biosynthesis genes was increased using a plasmid-based system. Plasmid stability issues have been reported in high density *R. eutropha* cultures, even in the presence of antibiotics (Srinivasan et al 2003). In order to ensure that our strains would produce PHA in high density cultures without the need for kanamycin, a plasmid stability system was adapted that has been used successfully with other species of bacteria (Schneider et al. 2005). The *proC* gene was deleted from the genomes of *R. eutropha* strains and expressed from plasmid pCB113. One scenario that could lead to plasmid loss in this system is if some cells produce excess proline and excrete it into the medium, which would allow other cells to grow and replicate without pCB113. Plasmid loss was not observed in

Re2058/pCB113 or Re2160/pCB113 cultures, suggesting that proline excretion does not occur under the conditions tested.

The data presented in Figure 3.3 show that the polymer produced by Re2058/pCB113 and Re2160/pCB113 varied over time in the fermentations. The HHx content in the PHA is very high early in the cultures, then decreases, and eventually it stabilizes. This means that late in the cultures, newly synthesized polymer has lower HHx content than the overall average. For example, Re2058/pCB113 produced 10.1 g/L PHA with 22.0 mol% HHx by the 48 h time point. By the 96 h time point, this strain had produced 17.5 g/L PHA with 17.3 mol% HHx. Therefore, from 48 to 96 h, 7.4 g/L PHA was accumulated with an average HHx content of 10.9 mol%. The reason for higher HHx content in the PHA early in cultures is not completely understood. Some of the HB-CoA made by the strains is produced from acetyl-CoA through the actions of a β-ketothiolase (PhaA) and an acetoacetyl-CoA reductase (PhaB1 in Re2058/pCB113, unknown reductases in Re2160/pCB113). It has been shown that during the *R. eutropha* growth phase, the high intracellular concentration of free CoA inhibits PhaA, slowing the rate of HB-CoA synthesis (Oeding et al. 1973). This suggests that early in the cultures the ratio of HHx-CoA to HB-CoA is high, causing more HHx to be incorporated into the PHA. This could also explain the higher HHx content observed in fermenter cultures relative to flask cultures. The fermentation medium contained more NH_4Cl than the flask medium, leading to a longer growth phase in which more HHx is included in the PHA.

Both, Re2058/pCB113 and Re2160/pCB113 met the requirements (i – high HHx (>10 mol%) content per polymer) and (ii – high PHA accumulation per CDW (>65%)) set for the desired P(HB-*co*-HHx) production strain (see goal of Chapter III). However, Re2058/pCB113 was chosen as the PHA production strain for further process development, since Re2058/pCB113 accumulated significantly more P(HB-*co*-HHx) per CDW as Re2160/pCB113 (Figure 3.3).

After (in this chapter) the PHA production strain was selected, the next chapter focuses on increasing the total PHA production over the development of high cell density fermentations.

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CHAPTER IV

Development of a high cell density fermentation for PHA production from palm oil with *Ralstonia eutropha*

This chapter was modified from a previously published article in Biotechnology and Bioengineering, 2012. 109(1): 74-83 'Production of Poly (3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) by *Ralstonia eutropha* in High Cell Density Palm Oil Fermentations' Sebastian L. Riedel, Johannes Bader, Christopher J. Brigham, Charles F. Budde, Zainal Abidin Mohd Yusof, ChoKyun Rha and Anthony J. Sinskey © Wiley Periodicals, Inc.

Goal of Chapter IV

In Chapter III *R. eutropha* Re2058/pCB113 was identified as the best producer of the desired copolymer P(HB-*co*-HHx), in terms of a high PHA accumulation per CDW and a high HHx concentration in the polymer. This chapter focuses on the development of a high cell density fermentation process with palm oil as the sole carbon source, which should have the potential to being up scaled for industrial PHA production. Analytical methods, which were developed in Chapter II where used to analyze the oil consumption behavior of *R. eutropha* during fed-batch fermentations. Also the change of the molecular weight, of the polymer, was monitored over the time of experiment.

Introduction

A summary of 24 PHA-producing companies offered by (Chen 2009) demonstrates the global interest in production of these polymers. It is expected that lower production costs will help accelerate further commercialization of PHA. For low-cost industrial PHA production, high space-time yields are required. Optimal fermentation processes would be based on either renewable carbon substrates with a high yield per hectare or inexpensive secondary products. Tian *et al.* (2009), review the utilization of waste water, whey, molasses and various plant oils as carbon substrates for PHA production. The advantage of utilizing plant oils is their high carbon content, as well as high conversion rate to PHA (Akiyama et al. 2003; Fukui and Doi 1998; Kahar et al. 2004; Loo et al. 2005; Ng et al. 2010). Because of their high carbon content, low-flow rate feed streams can be applied, reducing the dilution of the fermentation broth and optimizing product concentration. Compared with all other oilseed plants, the production of palm oil offers the highest yield per area with an average oil production of four metric tons per hectare (Basiron 2007).

In Chapter III, the recombinant *R. eutropha* strain Re2058/pCB113 was described, which was engineered from *R. eutropha* H16 (ATCC 17699) and which produces P(HB-*co*-HHx) when grown on palm oil as the sole carbon source (Budde et al. 2011b). In this chapter, the

optimization of the fermentation conditions of Re2058/pCB113 with palm oil as the sole carbon substrate is discussed.

Material and methods

Bacterial strain

Experiments were performed with the recombinant *R. eutropha* strain Re2058/pCB113, which was engineered from strain H16 (ATCC 17699) and produces P(HB-*co*-HHx) when grown on plant oils (Budde et al. 2011b).

Growth media and pre-culture cultivation conditions

Dextrose-free TSB medium (Becton Dickinson, Sparks, MD) was used for overnight cultivations. To ensure maintenance of the plasmid pCB113 in rich media, TSB medium was always supplemented with 200 µg/mL kanamycin sulfate. The addition of kanamycin for plasmid maintenance in minimal media cultures was unnecessary, due to the presence of an addiction system based on proline auxotroph (Budde et al. 2011b). All growth media contained 10 µg/mL gentamicin sulfate. Phosphate buffered minimal medium used for precultures and fermentations was described previously (Budde et al. 2010). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

The carbon sources fructose or palm oil (Wilderness Family Naturals, Silver Bay, MN) and nitrogen sources (NH_4Cl, NH_4OH or urea) were all used as described in the text. Cultures were always grown aerobically at 30°C, and nitrogen limitation was used to trigger maximum PHA production.

Re2058/pCB113 was initially grown overnight in 4 mL TSB from a single colony. Cells were centrifuged at 16,100 x *g* and the pellet was resuspended in 0.85% saline and used to inoculate 50 or 100 mL minimal medium flask cultures containing 2% fructose and 0.1% NH_4Cl or 0.06% urea to an initial OD_{600} of 0.05. After approximately 24 h of incubation, cells were centrifuged at 6,500 x *g* and the pellet was resuspended in 0.85% saline for inoculation of the fermenter culture to an initial OD_{600} of 0.1.

General fermentation conditions

A Bioengineering multiple fermenter system (R'ALF PLUS TRIO) consisting of 2-L double jacketed glass vessels with a working volume of 1.2 L (Wald ZH/Switzerland) was used for fermentation studies. The temperature of the cultures was kept constant at 30°C, and the pH was maintained at 6.8 ± 0.1, through controlled addition of 2 M NaOH (NH_4OH was used for

pH controlled nitrogen feeding) and 0.67 M H_3PO_4. Cultures were stirred using two six-blade Rushton impellers at speeds ranging from 300–1,500 rpm. Air was supplied through a ring sparger at 0.5 vvm unless noted otherwise. The dissolved oxygen concentration was maintained at levels above 40% by addition of pure oxygen and kept at a constant flow rate by a mass flow controlled pO_2 cascade. Foam in the cultures was broken mechanically with pairs of cable ties attached to the shaft of the impellor.

Extended-batch fermentation

Cultures consisting of 1 L minimal medium with 40 g/L palm oil and 4.5 g/L urea (150 mM nitrogen) were inoculated from precultures as described above. A bolus of 20 g/L palm oil, based on initial culture volume, was fed to the fermenter after 32 h of cultivation. Air was supplied at 0.2–1 vvm during fermentation.

Fed-batch fermentation (NH_4OH), with pH controlled nitrogen feeding

Fermenter cultures containing 1 L of minimal medium with 20 g/L palm oil and 4 g/L NH_4Cl (75 mM nitrogen) were inoculated with precultures, as described above. During cultivation, palm oil was fed between 24–84 h in approximately 5 g/L steps, pulse-wise with a pump in 2 h intervals, to a total concentration of approximately 170 g/L, based on initial volume. For initial pH control and nitrogen feeding, a calculated volume of a stock solution of 14% NH_4OH was provided to the culture, from the base reservoir of the fermenter, to reach a total nitrogen concentration of 480 mM nitrogen, based on initial volume. After the NH_4OH solution was depleted, the pH control was switched to 2 M NaOH in order to initiate nitrogen limitation. After 48 h cultivation, a solution consisting of trace metal salts (Budde et al. 2010), magnesium sulfate, calcium chloride, and potassium sulfate was added in initial concentrations to prevent undesired nutrient limitation.

Fed-batch fermentation (urea)

Cultures consisting of 1 L minimal medium with initial concentrations of 20 g/L palm oil and 2.2 g/L urea (75 mM nitrogen) were inoculated with precultures as described above. Palm oil was fed to the fermenter culture as described above. Urea was fed in approximately 0.2 g/L steps (7 mM nitrogen) in 30 min intervals, starting at 18 h, until a total concentration of 14.4 g/L urea (480 mM nitrogen) was reached based on initial volume. After 48 h cultivation, a solution consisting of trace metal salts (Budde et al. 2010), magnesium sulfate, calcium chloride and potassium sulfate was added in initial concentrations to prevent undesired nutrient limitation.

Analytical methods

Aliquots of 3–14 mL from fermenter cultures were sampled in pre-weighed polypropylene test tubes. The samples were centrifuged for 10–15 min at 6,500 x *g* and 1 mL of the supernatant was frozen at -20°C. The pellets were washed with a mixture of 5 mL cold water and 2 mL cold hexane to remove residual oil. The washed cell pellet was then resuspended in 2 mL cold water, frozen at -80°C, lyophilized and then the CDW was determined. The content and composition of PHA from dried cells were determined using a methanolysis protocol described previously (Budde et al. 2011b). In this procedure, pure standards of methyl 3-hydroxybutyrate and methyl 3-hydroxyhexanoate were used to generate calibration curves. rCDW was defined as CDW minus the mass of the PHA.

For molecular weight determinations of P(HB-*co*-HHx), the polymer was extracted from freeze-dried cells with chloroform. Equal masses of freeze-dried cells were weighed in screw capped glass tubes to form a 3 mg/mL PHA solution with a total volume of 2 mL. Samples were kept at 50°C for 4 h and briefly shaken by hand every 30 min. After PHA extraction, the samples were filtered through a 0.2 µm polytetrafluoroethylene (PTFE) membrane and the molecular weight was measured via gel permeation chromatography (GPC) relative to polystyrene standards as described previously (Budde et al. 2010).

For testing the nitrogen content of cell supernatants, frozen culture supernatants were thawed at RT, centrifuged for 5 min at 16,100 x *g,* and filtered through a 0.2 µm PTFE membrane (if necessary). Ammonium and urea concentrations were measured from clarified supernatants with an ammonium assay kit (Sigma-Aldrich, Cat. No. AA0100) or a urea assay kit (BioVision, Cat. No. #K375-100) respectively, as defined in the text.

Lipids were extracted from the culture by using a mixture of chloroform/methanol (2:1, v/v) as previously described (Budde et al. 2011a). Samples for lipid recovery were taken prior to any palm oil feeding steps at a given time point. The distribution of fatty acids in the recovered lipids was determined by the same methanolysis assay described above, which leads to formation of fatty acid methyl esters. The species and proportion of fatty acids in the lipids recovered from the chloroform/methanol extraction were identified by TLC. The extracted lipids were dissolved in chloroform to a final concentration of 3 mg/mL. Aliquots of 10 µL (30 µg lipids) were spotted on a silica gel TLC plate (250 µm thickness; EMD Chemicals, Gibbstown, NJ). A mixture of defined standards was also spotted: TAG (1,2-distearoyl-3-oleoyl-rac-glycerol; 10 µg), DAG (1,2-dipalmitoyl-rac-glycerol; 20 µg), MAG (1-palmitoyl-rac-glycerol; 20 µg) (Nu-check Prep, Inc., Elysian MN) and FFA.

Results

The polymer P(HB-*co*-HHx) has been shown to exhibit thermal and mechanical properties that rival those of petroleum-based polymers (Doi et al. 1995; Noda et al. 2005). Our group is interested in the production of high concentrations of P(HB-*co*-HHx) in fermentation cultures using plant oils as the sole carbon source, with high yields of PHA from oil. The production of P(HB-*co*-HHx) containing a high concentration (≥15 mol%) of HHx monomers is a novel undertaking, as such polymers have not been produced in high quantities thus far by microorganisms grown on raw carbon sources. We have recently described the engineered *R. eutropha* strain Re2058/pCB113, which accumulates high amounts of PHA per CDW with a high HHx level when grown on plant oils. In that work, we demonstrated the strain's performance in a batch fermentation in which it produced 25 g/L CDW with a PHA content of 71% with 17 mol% HHx after 96 h of cultivation in minimal medium containing 40 g/L palm oil as the carbon source and 4 g/L NH_4Cl (75 mM nitrogen) as the nitrogen source (Budde et al. 2011b).

In this study, to further enhance PHA production, we evaluated different fermentation strategies with Re2058/pCB113 to increase biomass concentrations, which allows for higher PHA titers.

Extended-batch fermentation

To begin to optimize the yield of PHA produced by *R. eutropha* Re2058/pCB113, an extended-batch fermentation was performed with a two fold increase in the culture's total nitrogen concentration (2.1 g/L urea, 150 mM nitrogen) compared to the batch fermentation described previously (Budde et al. 2011b). Urea was used as nitrogen source instead of NH_4Cl, as urea is a less costly nutrient and allows for better growth of *R. eutropha* (Khanna and Srivastava 2004; Ng et al. 2010). The total palm oil concentration of the culture was increased by 1.5 fold to 60 g/L. In the extended-batch fermentation, it was observed that the PHA content was already 45% of CDW at 24 h (Figure 4.1), before the culture had reached nitrogen limitation. After 48 h, nitrogen became limiting, resulting in a constant residual biomass and allowing for maximum PHA production. After 96 h, cultures had produced 32.5 g/L PHA (72% of CDW) with an HHx level of 17 mol% (Figure 4.1). Over the entire fermentation, polymer was formed at a yield of 0.52 g PHA/g palm oil. During 48–96 h the PHA yield increased to 0.77 g PHA/g PO.

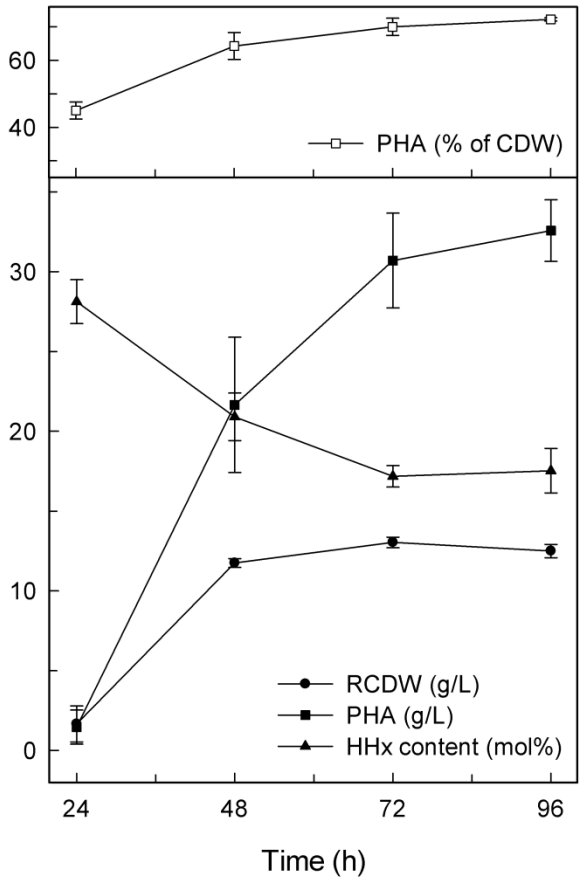

Figure 4.1: Extended-batch fermentations for P(HB-*co*-HHx) production by *R. eutropha* Re2058/pCB113 using palm oil as the sole carbon source. Palm oil (initial concentration = 40 g/L) and urea (initial concentration = 4.5 g/L; 150 mM nitrogen) were added as carbon and nitrogen sources, respectively to 1 L minimal medium. A bolus of 20 g/L Palm oil was added to the culture after 32 h based on initial volume. PHA content of cell dry weight (%, top), HHx content of PHA (mol%, bottom, filled triangles), residual cell dry weight (g/L, bottom, filled circles) and PHA produced (g/L, filled boxes) are shown. Data points are mean values from duplicate fermentations. Error bars indicate maximum and minimum values.

Fed-batch fermentation (NH_4OH), with pH controlled nitrogen feeding

While the results of the extended-batch fermentation represented an improvement over the initial batch fermentation, we continued to improve fermentation performance using fed-batch strategies. Thus, we implemented two fed-batch strategies with two different kinds of nitrogen feeding. In both strategies, the initial nitrogen concentration of the culture was 75 mM, and nitrogen was fed to a final concentration of 480 mM as described in Materials and Methods.

In the first fed-batch cultivation (Figure 4.2), NH_4Cl was the initial nitrogen source and NH_4OH was fed to the culture for pH control. During the nitrogen feeding stage of the culture, the nitrogen level remained constant, equivalent to the initial level of 4 g/L NH_4Cl (75 mM nitrogen). As the initial NH_4^+ from NH_4Cl was consumed, the decrease in culture pH resulted in the addition of NH_4OH by the pH controller, resulting in nitrogen levels returning to their initial concentration during NH_4OH feeding causing the nitrogen concentration to remain

approximately constant during NH_4OH feeding. It was observed, during NH_4OH feeding (between 12–48 h), that the PHA content of CDW increased from 28% to 59%, even though nitrogen was still present in the culture. After 60 h of cultivation, nitrogen limitation was observed and by the end of the fermentation the culture reached 98 g/L CDW with a PHA content of 70% PHA containing 24 mol% HHx.

Fed-batch fermentation (urea)

In the second fed-batch culture (Figure 4.3), urea was used as the sole nitrogen source with an initial concentration of 2.2 g/L (75 mM nitrogen). After 15 h, the PHA content of CDW was already at 43%. The PHA content increased to 61% after 48 h, and nitrogen limitation was observed after 63 h of cultivation. At the end of fermentation, 102 g/L PHA (73% of CDW) was produced containing 19 mol% HHx. Residual lipids from culture supernatants were isolated to follow the utilization of palm oil during the course of the experiment and also to calculate the PHA yield from palm oil (g PHA/g palm oil). The concentration of recovered lipids stayed almost constant around 5 g/L during the entire fermentation, which indicated a balanced palm oil feed (Figure 4.3). Over the course of the entire cultivation, polymer was accumulated at a yield of 0.63 g PHA/g palm oil. During 63–96 h, the PHA yield was 0.78 g/g palm oil.

Recovery and analysis of residual lipids from culture supernatants

Recovery of residual lipids in fermenter cultures is useful for determination of many different process parameters, such as PHA production yield (g PHA/g oil), fatty acid composition of residual lipids, and lipid consumption profiles. Methanolysis was used to identify the residual fatty acids and quantify their distribution in the lipids extracted from culture supernatants from the fed-batch fermentation in which urea was the nitrogen source. One of the key observations that can be seen in Figure 4.4 is that the proportion of residual fatty acids stayed almost constant during palm oil feeding (24–84 h). Approximately 12 h after the last palm oil feeding step, at 96 h, the proportion of stearic acid was over 5 times greater than it had been at 48 h. Linoleic acid had decreased almost 4 fold by that time, and the proportion of oleic acid decreased from 51% to 39%. The proportion of palmitic acid stayed constant over the 96 h course of the fermentation, however the standard deviation (SD) for these values was large at 96 h (over 9%). Myristic acid was present throughout the fermentation in a proportion of less than 1% of the total fatty acids. An increase of certain fatty acids in the supernatant over the course of the fermentation could be due to the cells preferring some fatty acids as carbon sources, thus certain fatty acids are not readily utilized and build up in the culture media. On

the other hand, fatty acids that decrease in concentration might be more readily consumed by the cells.

A biohydrogenation of the unsaturated fatty acids linoleic acid ($C_{18:2}$) and oleic acid ($C_{18:1}$) to produce stearic acid ($C_{18:0}$), has been shown to occur using a consortium of rumen bacteria (Jenkins et al. 2008). This phenomenon could potentially occur in palm oil cultures, and could account for a decrease in linoleic and oleic acids and a concomitant increase of stearic acid. However, homologs of genes and enzymes responsible for this process have not yet been identified in *R. eutropha*.

TLC analysis of the same extracted lipids (Figure 4.5) showed that the concentration of TAGs in the lipid extracts decreased continuously from 24 h until all TAGs had been broken down by the end of the fermentation (96 h). DAGs also decreased proportionally until the end of fermentation. At the same time, the concentration of FFAs in the extracted lipids continuously increased up until 91 h. The concentration of MAGs fluctuated over the course of fermentation. The palm oil feeding between 24 h to 84 h did not result in an increase in the proportion of TAGs, indicating balanced palm feeding throughout the experiment. At 48 h, a new species was observed below the spot corresponding to MAG migration. This new species appears in the TLC area thought to be for polar lipid separation (King et al. 1977). We hypothesize that this spot represents a polar lipid (or polar lipids) extracted from culture supernatants. From 48–91 h, the intensity of the spot representing the unknown species decreased.

Comparison of PHA production from the different fermentations

The amount of biomass produced in Re2058/pCB113 fermentations was increased using different strategies, as described above. The amount of total PHA produced increased due to the increase in the total nitrogen supplied to the cultures from either urea or NH_4^+ (Table 4.1), along with the adjustment of palm oil concentration. These increases in the concentrations of growth substrates allowed for the production of larger quantities of biomass, resulting in larger amounts of PHA per culture. The key observation of these experiments was the comparison of the PHA production of the batch culture and of the fed-batch culture using urea as the nitrogen source. In this case, the fold increase of the total nitrogen (6.4 fold, Table 4.1) added to the culture correlates with the increase in PHA produced by the cultures (5.8 fold, Table 4.1).

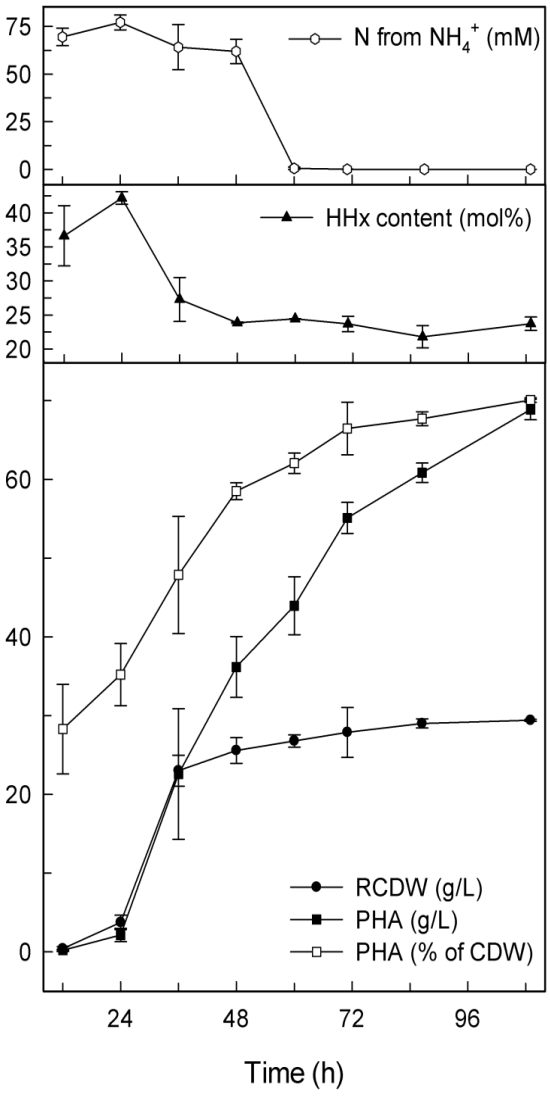

Figure 4.2 Fed-batch fermentations for P(HB-*co*-HHx) production by Re2058/pCB113 from palm oil, using NH_4^+ as nitrogen source. Initial concentrations of 20 g/L PO and 4 g/L NH_4Cl (75 mM nitrogen) were used in 1 L media. PO was fed between 24–84 h to a total concentration of 170 g/L based on initial volume. Nitrogen was fed over pH control (pH 6.8 ± 0.1) using a 14% NH_4OH stock solution to a total nitrogen concentration of 480 mM nitrogen. Concentration of nitrogen from ammonium (g/L, top), HHx content of PHA (mol%, middle), residual cell dry weight (g/L, bottom, filled circles), PHA produced (g/L, bottom, filled boxes) and PHA content of CDW (%, bottom, open boxes) are shown. Data points are mean values from duplicate fermentations. Error bars indicate maximum and minimum values.

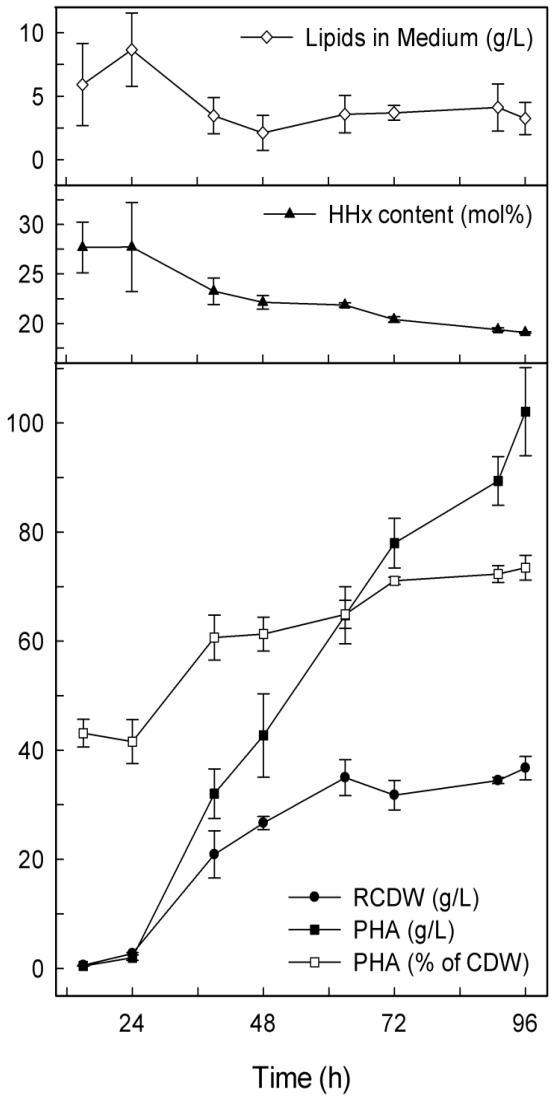

Figure 4.3 Fed-batch fermentations for P(HB-*co*-HHx) production by Re2058/pCB113 using palm oil as carbon source and urea as nitrogen source. Initial concentrations of 20 g/L PO and 2.2 g/L urea (75 mM nitrogen) were added to 1 L media. PO and urea were fed between 24–84 h and 18–48 h, respectively, to total concentrations of 170 g/L PO and 480 mM nitrogen, based on initial volume. Concentration of lipids in the medium (g/L, top), HHx content of PHA (mol%, middle), residual cell dry weight (g/L, bottom, filled circles), PHA produced (g/L, bottom, filled boxes) and PHA content of cell dry weight (%, open boxes) are shown. Data points are means from triplicate fermentations and error bars indicate ± SD. Values for 15 h and 91 h data points are means of duplicate samples.

These results suggest that the fed-batch strategy with urea as the nitrogen source is the superior PHA production strategy.

For efficient industrial scale PHA production, it is important for a production strain to produce large amounts of PHA in a relatively short time. Thus, the space time yield (STY) for all fermentations was calculated. Figure 4.6 shows that we were able to increase the STY of PHA production from 0.2 g/L/h from the initially described batch fermentation (Budde et al. 2011b) to 1.1 g/L/h from the fed-batch fermentation (urea) with a total PHA production of 102 g/L.

In all fermentations discussed here, the HHx content of PHA was observed to decrease over time (Table 4.2, Figures 4.1–4.3). At early time points, a high level of HHx monomer was seen in cultures, reaching above 40 mol% in some cases. Over the course of the fermentation, the HHx monomer percentage decreased and eventually leveled off. This phenomenon was also observed in the batch fermentation published previously (Budde et al. 2011b).

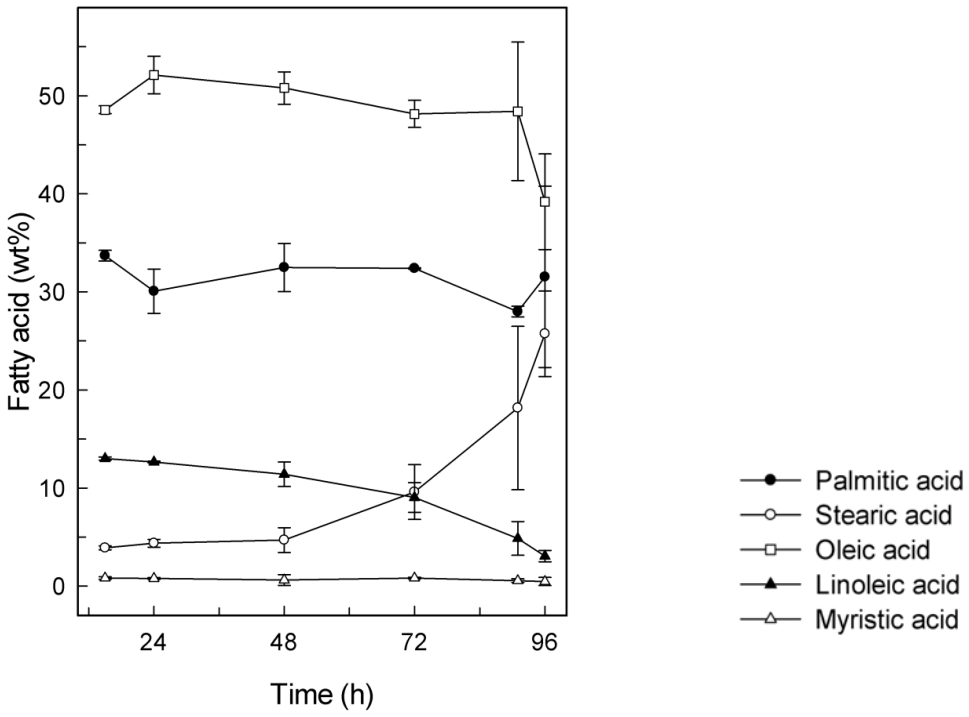

Figure 4.4: Fatty acid distributions in lipid samples extracted from medium over the course of the fed-batch fermentations (urea), were determined. Data points are means from triplicate fermentations (with the exception of the 91 h data point, which represents the mean of duplicate) and error bars indicate ± SD. Fatty acid content was determined by quantification of fatty acid methyl esters using known quantities of standard compounds.

Table 4.1: Improvement of PHA Production from palm oil by Re2058/pCB113 [a] Results obtained from triplicate cultures with error bars indicating ± SD [b] Results obtained from duplicate cultures with error bars indicating max. and min. values. [c] From Budde et al. 2011b

Fermentation	Total N (mM)	N Source	PHA (% of CDW)	PHA (g/L)
Batch[a,c]	75	NH_4Cl	70.9 ± 1.9	17.5 ± 1.1
Extended-batch[b]	150	Urea	72.3 ± 0.5	32.6 ± 1.9
Fed-batch (NH_4OH)[b]	480	NH_4Cl/NH_4OH	70.1 ± 0.3	68.9 ± 1.3
Fed-batch (urea)[a]	480	Urea	73.5 ± 2.3	102.1 ± 8.1

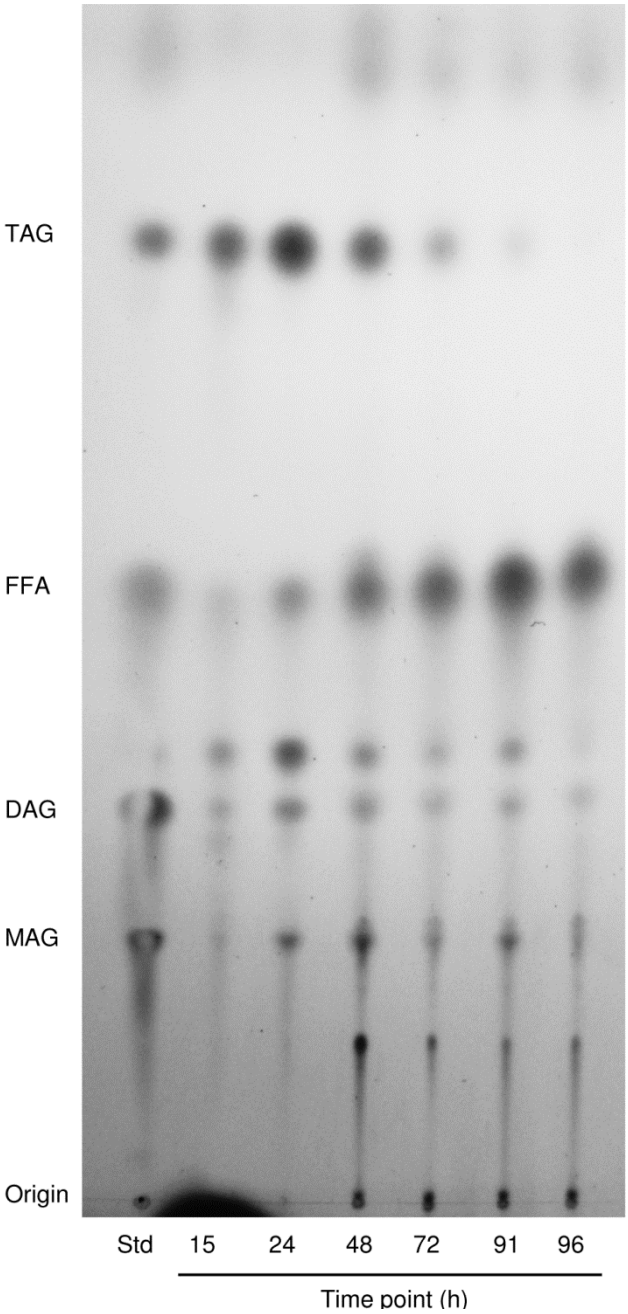

Figure 4.5: Thin layer chromatography indicating the time course of residual lipids and fatty acid present in the medium of fed-batch fermentations (urea) with palm oil as the sole carbon source. Proportions of triacylglycerol's (TAG), diacylglycerols (DAG), monoacylglycerols (MAG) and free fatty acids (FFA) are shown. Std = lipid standards as described in Materials and methods.

Molecular weight analysis of PHA

Molecular weight of PHA polymer chains plays an important role in determining whether the polymer can be processed (Sim et al. 1997; Tsuge et al. 2007). Using GPC analysis, the weight average (M_w) and number average (M_n) molecular weights were determined of the PHA polymer produced over the course of the fed-batch fermentation (urea) (Figure 4.7). The average molecular weight of the PHA decreased from 500,000 Da after 39 h to 300,000 Da after 96 h. A similar decrease over time in molecular weight of PHA was also seen in our previous study (Budde et al. 2011b) where the molecular weight decreased from 400,000 Da after 48 h to 300,000 Da after 96 h. The polydispersity index of PHA from the fed-batch culture increased from 1.9 to 2.1 during 39–96 h, which indicates narrow molecular weight distributions.

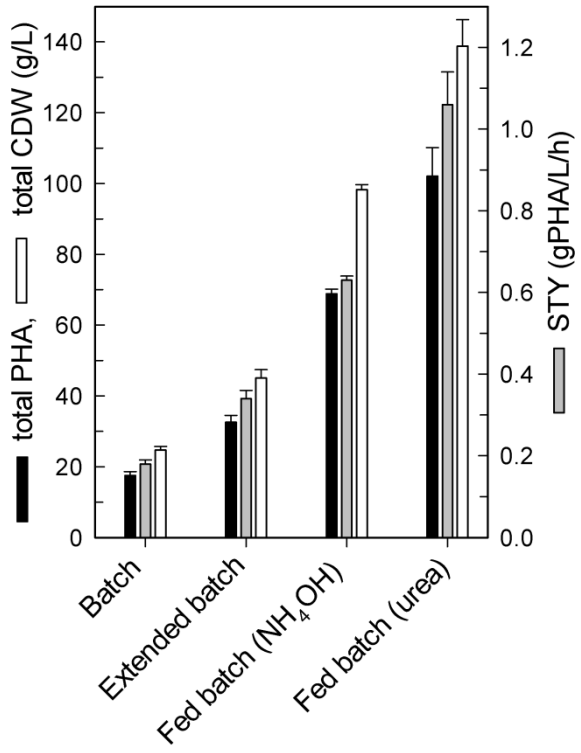

Figure 4.6: Improvements to PHA production (g/L), the space time yield of PHA production (g/L/h), and cell dry weight production (g/L) comparing batch culture (Data points are means from triplicate and error bars indicate ± SD, Budde et al., 2011b), extended-batch culture (Data points are means from duplicate with error bars indicating maximum and minimum values), and fed-batch cultures (fed-batch (urea) in triplicate with error bars indicating SD. Fed-batch (NH_4OH) in duplicate with error bars indicating maximum and minimum value.

Discussion

Production of P(HB-*co*-HHx) was increased by using different fermentation strategies to attain a maximum concentration of 102 g/L PHA (Figure 4.6). It was shown that the addiction

system present in Re2058/pCB113 for plasmid maintenance (Budde et al. 2011b) was robust in high cell density fermentations of up to 140 g/L CDW. The PHA content of CDW was always over 70% with high HHx level (>17 mol%) at the conclusion of all fermentations (Table 4.2), indicating that the plasmid-borne PHA production genes were still present at the conclusion of fermentation. Therefore, this system overcomes previously reported issues of plasmid instability in high cell density fermentations (~100 g/L CDW) of *R. eutropha* (Srinivasan et al. 2003).

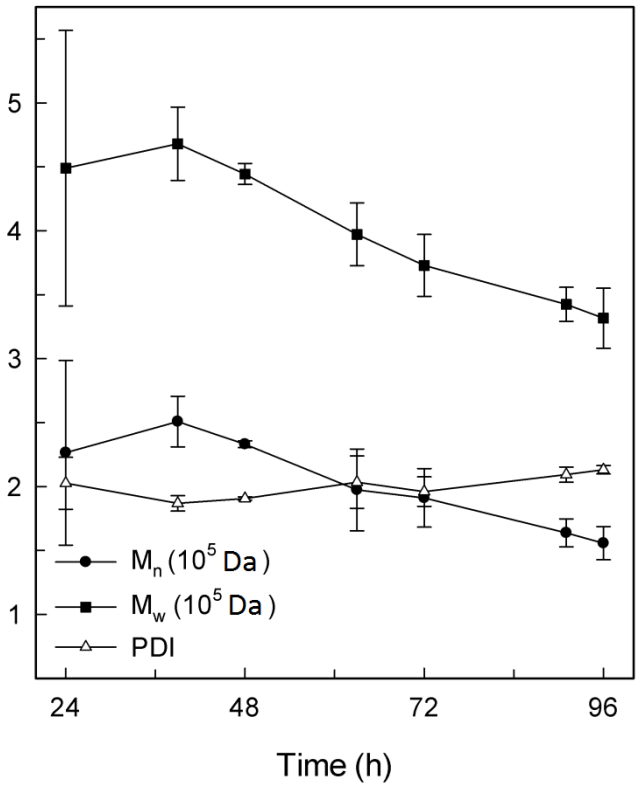

Figure 4.7: GPC analyses of P(HB-*co*-HHx) during the fed-batch fermentations (urea). PHA was extracted from freeze-dried cells with chloroform and molecular weights determined relative to polystyrene standards. The number average molecular weight (M_n, 10^5 Da, filled circles), weight average molecular weight (M_w, 10^5 Da, filled boxes) and polydispersity index (PDI, open triangles) are shown. Data points are means from triplicate fermentations (with the exception of the 91 h data point, which represents the mean of duplicate) and error bars indicate ± SD.

Plant oils such as palm oil are favorable feedstocks because of their lower price per mass and higher carbon content compared to sugars. Furthermore, plant oils are shown to be an excellent carbon source for PHA production in *R. eutropha* cultures (Akiyama et al. 2003; Fukui and Doi 1998). They can also be utilized from pure stocks, which minimize added volume during carbon substrate feeding. Brigham *et al*. (2010) showed that *R. eutropha* expresses lipases which are essential for growth on unemulsified plant oils. In a previous study, we developed an emulsification process to allow for immediate availability of palm oil

in growth media, thus shortening the lag phase in growth (Budde et al. 2011a). We did not use this method in the current study in order to avoid the costs of external emulsification agents. After 24 h in fed-batch fermentations, the palm oil added initially appeared to be emulsified, thus feeding was initiated after that time. Additional palm oil was emulsified shortly after addition to the cultures. An excess of palm oil during cultivation (*e.g.* during feeding) resulted in excessive foaming and thickening of the culture broth (data not shown), which likely inhibited the oxygen transfer, and thus resulted in inefficient production of PHA. It is largely for this reason that proper dosage of palm oil during feeding is crucial for maximum PHA productivity. During cell growth, MAGs, DAGs, and FFAs were produced from the breakdown of TAGs (Figure 4.5). These lipids may act as emulsifiers when interacting with unemulsified palm oil. A 2% initial palm oil concentration and a linear feeding strategy after 24 h in small steps every 2 h was identified as an efficient process for oil addition. PHA yield in the storage phase of the fed-batch fermentation urea was 0.78 g/g PO, which is similar to PHB production yields described in previous studies (Budde et al. 2011b; Kahar et al. 2004; Ng et al. 2010) in which plant oils were used as the sole carbon source.

Table 4.2: HHx monomer content of PHA produced by Re2058/pCB113 at early and late stages in fermentations using palm oil as the sole carbon source. All values represent means from duplicate or triplicate cultivations.

Fermentation	Early Time Point				End of Cultivation			
	t (h)	PHA (%/CDW)	HHx (mol%)	CDW (g/L)	t (h)	PHA (%/CDW)	HHx (mol%)	CDW (g/L)
Batch[a,c]	25	26.2 ± 3.3	43.7 ± 2.8	3.0 ± 1.2	96	70.9 ± 1.9	17.3 ± 1.1	24.7 ± 01.0
Extended-batch[b]	24	45.0 ± 2.5	28.1 ± 1.4	3.1 ± 2.2	96	72.2 ± 0.5	17.5 ± 1.4	45.1 ± 2.4
Fed-batch (NH_4OH)[b]	24	35.2 ± 3.9	42.2 ± 0.9	5.9 ± 1.8	109	70.1± 0.3	23.7 ± 1.0	98.3 ± 1.4
Fed-batch (urea)[a]	24	41.6 ± 4.0	27.7 ± 4.5	4.7 ± 0.7	96	73.5 ± 2.3	19.1 ± 0.1	138.0 ± 7.5

[a] Results obtained from triplicate cultures with error bars indicating ± SD; [b] Results obtained from duplicate cultures with error bars indicating maximum and minimum values; [c] From Budde et al., 2011b

The maximum cell growth, and consequently maximum PHA production, was highly dependent on the nitrogen source used. In the fed-batch fermentation with urea, we could produce 48% more PHA compared to the fed-batch fermentation with NH_4OH feeding and NH_4Cl as the initial nitrogen source. Previous studies have described urea as the best nitrogen source for PHA production by *R. eutropha* (Khanna and Srivastava 2004; Ng et al. 2010). CO_2 remains after the consumption of nitrogen from urea (data not shown), which could potentially have a positive effect on cell growth (*i.e.* as a secondary carbon source). With a STY of 1.1 g/L/h PHA in fed-batch fermentation with urea as the nitrogen source, we

establish a high productivity process. However, a further increase of the STY would result in an increase of the total amount of PHA produced and also shorten the fermentation process. A higher biomass could be reached with higher total carbon and nitrogen concentrations along with an adjusted feeding strategy. The lag phase could potentially be shortened by first using a soluble carbon source (*e.g.* sodium butyrate) in the growth media, so that the carbon would be immediately available for consumption by the cells. Such a procedure would allow for faster growth. A second feeding of oil could then follow, which would be quickly emulsified due to a high cell concentration generating more lipase activity. Another potential method for shortening the lag phase in growth would be to emulsify the initial palm oil added to the culture while also decreasing the initial palm oil concentration (decreasing the amount of emulsifying agent needed), and then feeding unemulsified palm oil.

PHA production is normally triggered through a nutrient limitation (Anderson and Dawes 1990), which in our fermentations was nitrogen. However, it was observed at early time points in the fermentations (where no nutrient limitation yet occurred) a surprisingly high PHA accumulation of over 40% CDW (Figures 4.1–4.3). This early high PHA production is possibly due to the PHA production genes being located on a plasmid that results in higher gene dosage and consequently higher gene expression.

Moving forward, the results of high density PHA production on different oil palm products will be examined and their effects on copolymer content (*e.g.* HHx) and on molecular weight will be determined. The presented fed-batch fermentation strategy using urea as the nitrogen source offers the possibility for production of P(HB-*co*-HHx) with a high HHx concentration (19 mol%), which will allow high cell density fermentation processes with a yield of 102 g/L PHA. A scale-up of the developed process to industrial scale is being planned. The design and implementation of an integrated biorefinery for PHA production is imaginable. Palm oil mills usually produce excess heat and electricity by burning waste material (*e.g.* fruit fibre) (Basiron 2007), which could be also used to power a PHA plant. Hence the results presented in this study may contribute to commercialization of biodegradable bioplastics made from palm oil products, and reduce the dependency of the plastics industry on fossil fuels.

After (in this chapter) the development of a high cell density fermentation process for high yield PHA production from palm oil was shown, the next chapter focuses on the use of alternative, biogenic waste-based, carbon feeds to palm oil for PHA production.

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CHAPTER V

Polyhydroxyalkanoates production with *Ralstonia eutropha* from low quality waste animal fats

This chapter was modified from a paper published in the Journal of Biotechnology, 2015, 214:119-127. 'Polyhydroxyalkanoates production with *Ralstonia eutropha* from low quality waste animal fats' Sebastian L. Riedel, Stefan Jahns, Steven Koenig, Martina C. E. Bock, Christopher J. Brigham, Johannes Bader, Ulf Stahl.

Goal of Chapter V

In Chapter IV a high density fermentation process for the production of P(HB-*co*-HHx) with recombinant *R. eutropha* from palm oil was described. This chapter focusses on the usage of alternative, TAG based, carbon feedstocks for the described process. Waste animal fats were chosen because they are biogenic waste, they have no role or value to the food industry. Also, it was goal to use waste animal fats with a very low quality to avoid competition to other industries as the biodiesel production. The challenge of using initially unmanageable waste fats as carbon source for profitable PHA production is described here.

Introduction

So far, high production costs together with the low price of conventional plastic prevent the use of PHAs as bulk material. To produce low-cost PHAs at industrial scale, high productivities along with a high total production from inexpensive, widely-available carbon feedstocks, is necessary. Since current cost of carbon feedstocks is one of the most significant price-enhancing factors (up to 50% of process costs), PHA production from waste materials is becoming a promising approach to meet the aforementioned parameters for increasing industrial PHA production (Koller et al. 2010). PHA production from various waste raw materials has been reviewed by Tian et al. (2009) and Koller et al. (2010). Waste lipids, *e.g.* used cooking oils, are favourable feedstocks, since they are readily available and have high carbon contents. Efficient PHA production in high cell density cultures from (waste) plant oils, (raw) glycerol and short-chain fatty acids have been summarized (Riedel et al. 2014). Waste animal lipids from, inter alia, the food processing and slaughtering industries have a huge potential as carbon feedstock for PHA production. In 2010, the United States produced the largest amount of 2.7×10^6 t inedible tallow and grease (U.S. Census Bureau 2011). Previously, PHA accumulation from tallow has been shown (Cromwick et al. 1996; Taniguchi et al. 2003) in shaking flask cultures.

However, no upscale to higher cell density fermentation cultures was performed following these initial works. One potential major issue is the high T_m of these fats, which makes them difficult to consume by microorganisms. The saturated fraction of fatty acid esters (FAE) from fats were used as carbon feedstocks for PHA production. The origin of this fraction was the European project ANIMPOL focused on the usage of byproducts of the biodiesel process starting from waste animal fats (Kettl et al. 2011; Titz et al. 2012). Using waste animal fat-based raw material also has the potential to lower the carbon footprint (Kettl et al. 2012; Shahzad et al. 2013).

In this study, we evaluated the direct use of waste animal fats as carbon feedstocks for PHA production with *R. eutropha* as the biocatalyst. Various industrially rendered fats of different qualities of category 2 and 3 with respect to the European Union regulation of animal byproducts (No 1069/2009) and tallow were used. Besides their hydrophobicity, these fats have high T_m, up to 55°C, making them poorly or non-consumable for *R. eutropha* in the absence of necessary process alterations. Bioavailability for growth was initially achieved with an emulsifying agent and finally with a developed emulsification strategy, which did not require any mechanical or chemical treatments.

PHA accumulation was first tested in shaking flask experiments, followed by PHA production studies at lab fermenter scale with the lowest quality waste fat available, in order to achieve maximum cost reduction and prevention of a competition for the raw material with other industries (*e.g.*, biodiesel production).

Material and methods

Bacterial strain

The wild type *R. eutropha* strain, H16 (DSM428), was used for PHB production. The recombinant *R. eutropha* strain Re2058/pCB113, which was engineered from strain H16 previously (Budde et al. 2011b), was used for the production of P(HB-*co*-HHx). During the metabolic engineering process of strain Re2058, the *R. eutropha* H16 *phaC*, which encodes for a *scl*-PHA synthase, was removed by in-frame deletion. Also, the strain Re2058 harbors a *proC* gene, which encodes for a pyrroline-5-carboxylate enzyme that is necessary for proline biosynthesis. Thus, Re2058 is unable to grow on minimal medium without the addition of proline. The PHA production plasmid pCB113 contains the *proC* gene, to ensure plasmid stability in minimal medium. Also, pCB113 expresses a *scl*/*mcl*-PHA-synthase from *Rhodococcus aetherivorans*, which is able to facilitate synthesis of P(HB-*co*-HHx). Additionally, *phaJ*, an (enoyl-CoA) hydratase gene from *Pseudomonas aeruginosa*, was

added to pCB113 to boost synthesis of HHx precursor molecules from β-oxidation intermediates (Budde et al. 2011b).

Plant waste oil

Locally obtained waste plant oil

Waste frying oil was obtained from "Cookinchen", a local snack bar, in Berlin. The Waste frying oil was filtered at RT through a standard cellulose coffee filter (Contal, size 4) to remove impurities. The major fatty acid species present in waste frying oil where $C_{16:0}$ and $C_{18:1}$ (Table 6.1).

Waste animal fats

The waste animal fats were obtained from ANiMOX GmbH (ANiFATs), except for tallow (see below), and were byproducts of a high pressure thermolysis process of animal waste streams of different quality and origin. Poultry, swine, cattle, sheep and wild animals could be potential sources. The major fatty acids present in waste animal fats where $C_{16:0}$, $C_{18:0}$ and $C_{18:2}$ (Table 5.1).

Locally obtained waste animal fat

Tallow was processed during this study as follows: suet, as obtained from a local butcher, contained considerable amounts of impurities (*e.g.*, blood, fibres and tissues). From 5 kg suet, 3.5 kg tallow was recovered. Removal of impurities was achieved by heating in a microwave oven for 2 min at 500 W followed by mechanical homogenization (Braun Multiquick MR 6500 M) and heating to 100°C. The proteinaceous phase was removed by filtering through a standard cellulose coffee filter (Contal, size 4) overnight in an incubator at 80°C. The resulting liquid was clear and did not contain visible particles. When cooled to RT, the clarified fat became a white solid.

Table 5.1: Fatty acid distribution in per cent of waste animal fats (ANiFATs, tallow) and waste frying oil (WFO). ± are indicating minimum and maximum values of a repeated determination.

Fat/Oil	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
Animal						
ANiFAT_P	1.32 ± 0.11	24.39 ± 1.28	2.33 ± 0.05	9.66 ± 0.38	52.83 ± 1.98	9.48 ± 0.26
ANiFAT_C	0.81 ± 0.10	27.00 ± 0.02	5.62 ± 0.11	5.89 ± 0.00	42.31 ± 0.71	18.36 ± 0.53
ANiFAT_RP	1.57 ± 0.08	28.90 ± 0.21	1.80 ± 0.04	14.55 ± 0.24	42.89 ± 0.32	10.29 ± 0.15
ANiFAT_R3	1.44 ± 0.02	28.55 ± 0.12	1.36 ± 0.02	16.69 ± 0.08	42.33 ± 0.47	9.63 ± 0.14
ANiFAT_R2		31.37 ± 2.27		12.85 ± 0.18	47.68 ± 0.97	8.10 ± 0.51
Tallow		28.95 ± 2.86		39.48 ± 3.95	29.30 ± 0.86	2.27 ± 0.14
Plant						
WFO		44.71 ± 0.94		2.12 ± 0.02	46.17 ± 0.39	7.00 ± 0.08

Industrially obtained animal waste fats

The company ANiMOX GmbH (Berlin, Germany) produces protein hydrolysates from animal waste streams for a wide range of applications. The peptides and amino acids produced are used for the production of: binders, foams, and coatings, nitrogen sources for the growth of industrial microorganisms, fish food, and more (www.animox.de). As a byproduct from the protein hydrolysates production (pressure thermolysis), fat of different qualities (regarding total lipid content and FFA ratio) is obtained. The European Union regulation of animal byproducts (No. 1069/2009) classifies animal waste streams into three categories:

Category 1: Material with high risk *e.g.*, from animals that succumbed to infectious diseases. This material must be destroyed.

Category 2: Material that is not infectious *e.g.*, from killed (not slaughtered) animals.

Category 3: Waste material or edible byproducts from slaughterhouse, animal products which are no longer edible (sell by date expired) or canteen garbage.

In this study, different waste animal fats produced from category 2 or 3 material by ANiMOX were tested of their usability as carbon feedstocks for PHA production with *R. eutropha* (Table 5.2).

Table 5.2: Classification, physical and chemical data of waste animal fats (ANiFATs, tallow) and waste frying oil (WFO), FFA = Free fatty acids, AN = Acid number, RM = Residual moisture, T_m = melting temperature, Ca = Category, n.a. = not available.

Fat/oil	Origin	Ca[a]	Total Lipids (%)	FFA (%)	AN	Total N (%)	Total Protein (%)	RM (%)	Ash (%)	T_m (°C)
Animal										
ANiFAT_P	swine	3	99.8	1.93	3.93	0.02	0.13	0.2	<0.01	45
ANiFAT_C	poultry	3	99.8	3.68	7.48	0.03	0.19	0.2	<0.01	34
ANiFAT _RP	swine/cattle	3	99.5	2.53	5.14	0.08	0.51	0.5	<0.01	45
ANiFAT_R3	mix	3	99.8	5.59	11.4	0.02	0.13	0.2	<0.01	45
ANiFAT_R2	mix	2	98.3	51.97	90.7	0.04	0.26	1.7	0.23	47
Tallow	cattle	3	99.0	0.43	0.88	0.05	0.31	0.7	<0.01	55
Plant										
WFO	n.a	-	99.4	0.46	0.93	0.09	0.56	<0.01	<0.01	RT[b]

[a] After European Union Regulation of animal byproducts (No. 1069/2009), [b] Room temperature, was the lowest temperature tested

Analyses of carbon feedstocks

The T_m values of the different fats were measured prior to their use as a carbon source for *R. eutropha* cultures. To this end, 1 g of each sample was transferred to glass tubes and heated until the fat was liquefied completely. The distribution of fatty acids (FA) in the fat samples was determined using a methanolysis protocol described previously (Budde et al. 2011b). Methods that were modelled after DIN norms (German Institute for Standardization) were used for the determination of: FFA content and acid number (DIN EN 14104), residual moisture and ash content (DIN 38409-H1) and total lipid content (LMBG L40.00-70) in the fat samples. Total nitrogen content was determined using the Dumas method (Herrmann, 1955). The total protein concentration was estimated by multiplying the total nitrogen content with the factor 6.25 (Kjeldahl, 1883).

Overnight cultivations

Initially, *R. eutropha* was grown in 4 mL TSB from single colony to an optical density (OD_{600}) of not higher than 5.0.

Shaking flask experiments

Overnight cultures were pelletized and resuspended in 0.85% saline before inoculation of shaking flask cultures to an initial OD_{600} of 0.05. Baffled flasks (250-mL), containing 50 mL minimal media, were shaken at 200 rpm for 72 h. In flask cultures, 0.05% NH_4Cl was used as nitrogen source and 1% waste animal or plant fat/oil was used as carbon source. The different

fats were pre-emulsified in the media with 0.5% gum arabic as an emulsifying agent (adapted from Budde et al. 2011a) as follows: all media components (prior to autoclavation) were heated to 60°C prior to emulsification with a homogenizer at 24,000 rpm for 1 min. Only pre-heated lab equipment was used during the homogenization process. For the emulsification of tallow, the temperature was increased to 80°C. Waste frying oil was used in cultures directly, non-emulsified, as the carbon source. Prior to addition of fat to the media, the shaking flasks were pre-heated at 30°C.

Fermentation culture conditions

BIOSTAT Aplus laboratory scale fermenter (Sartorius Stedim Biotech S.A., Aubagne Cedex, France) with a maximum working volume of 5-L was used for fermentation studies. Temperature was controlled constantly at 30°C. The culture pH was maintained at 6.8 ± 0.1, through controlled addition of 2 M NaOH or 1 M H_3PO_4. The gas flow of air was set to 0.5 vvm for aeration. The dissolved oxygen concentration was kept over 40% through a stirrer cascade with two six-blade Rushton impellers at speeds ranging from 300–1,200 rpm. Foam was broken mechanically with pairs of cable ties attached to the shaft of the impellor.

Pre-culture conditions

Fermentations were performed with or without pre-emulsification of fat, as discussed above. Pre-cultures for fermentations with pre-emulsified fat were grown for approximately 20 h in 250-mL shaking flasks with minimal media containing 0.1% NH_4Cl and 2.0% fructose. Cells were pelletized and resuspended in 0.85% saline before inoculation of the fermenter to an OD_{600} of 0.1. Pre-cultures for fermentations with non-emulsified fat were grown for approximately 24 h in 1-L shaking flasks with the initial fermenter minimal media.

Non-emulsified waste frying oil (1.0%) or non-emulsified ANiFAT_C (1.0%) and 0.4% NH_4Cl or 0.45% urea were used as described in the text. The pre-culture was used directly for inoculation of the fermenter culture. For OD_{600} determination from pre-cultures grown on fat, the cells were washed twice in 0.85% saline.

Fermentation with tallow

Minimal medium (2.5 L) with 30 g/L pre-emulsified tallow (with 0.8% gum arabic) and 0.4% NH_4Cl (75 mM nitrogen) was used in batch fermentations. In extended-batch fermentations, 0.45% urea (150 mM nitrogen) with an initial amount of 15 g/L pre-emulsified tallow was used. Based on initial culture volume, 15 g/L non-emulsified tallow was fed pulse-

wise after 38 h, 46 h and 70 h of fermentation to a total, final tallow concentration of 60 g/L. Before feeding, tallow was liquefied at 80°C.

Fermentations with ANiFAT_R2 as main carbon source

Minimal medium (2.5 L) with 10 g/L non-emulsified ANiFAT_C or 10 g/L non-emulsified waste frying oil and 0.4% NH_4Cl or 0.45% urea were inoculated with precultures, as described above. After the initial carbon source was emulsified naturally by *R. eutropha* cells (Lu et al. 2013), non-emulsified ANiFAT_R2 was fed as described in the text. Before feeding, the waste animal fat was liquefied at 60°C.

Analytical methods

For CDW determination, culture aliquots of 5–14 mL were sampled in pre-weighed polypropylene test tubes. The cells were pelletized at 4020 × *g* for 10–20 min. Supernatant was frozen at -20°C. The pellets were washed with a mixture of 5 mL cold water and 2 mL cold hexane to remove residual fat. The washed cell pellet was then resuspended in 1–2 mL cold water, frozen at -80°C, and lyophilized. Alternatively, the washed cell pellet was dried in an oven at 80°C to constant mass.

PHB content from dried *R. eutropha* H16 cells was determined using the crotonate assay protocol described previously (Karr et al. 1983). Samples were analyzed by HPLC with 20 µL injection volume at 30°C for 60 min at 0.4 mL/min on a Macherey-Nagel column (NUCLEOGEL ION 300 OA) with 5 mM sulfuric acid as the mobile phase. PHA content per CDW and HHx mole fraction of P(HB-*co*-HHx) from Re2058/pCB113 dried cells were determined using a methanolysis protocol described previously (Budde et al. 2011b). For testing the nitrogen content of cell supernatants, frozen culture supernatants were thawed at RT, centrifuged for 5 min at 13000 x *g,* and filtered through a 0.2 µm cellulose acetate membrane (if necessary). Ammonium concentrations were measured from clarified supernatants with an ammonium assay kit (Spectroquant, Merck KGaA) according to the instructions provided by the manufacturer.

Results

In this study, waste fats of different origin were evaluated for their direct use as carbon feedstocks for microbial PHA production using *R. eutropha* as the biocatalyst. The final goal was the direct use of low quality waste animal fats as carbon sources, due to their inexpensive world market price (100–200 $USD/t) and non-competition to other lipid-consuming industries, such as biodiesel manufacture. Utilization of these substances as feedstocks for

biodegradable plastic production is favourable as these inexpensive carbon sources can lower process costs, and their use does not result in a "food vs. commodity chemical" controversy. Our group is mainly interested in the production of the PHA copolymer P(HB-*co*-HHx), which has thermal as well as mechanical properties that are comparable to petroleum-based plastic materials and which are largely affected by the HHx content of the polymer (Doi et al. 1995; Noda et al. 2005). With the engineered *R. eutropha* strain Re2058/pCB113 (Budde et al. 2011b), we recently demonstrated robust production of P(HB-*co*-HHx) in high cell density fed-batch fermentations with palm oil as the sole carbon source. The fermentation exhibiting the highest productivity resulted in a total PHA titer of 102 g/L (139 g/L CDW with 74% PHA) containing 19 mol% HHx (Riedel et al. 2012), which is among the highest reported yield data on the production of this type of polymer.

In the present work, we initially analyzed the composition of the different fatty waste feedstocks, since it has been demonstrated that the fatty acid composition of the carbon source may affect the HHx content of the produced polymer (Riedel et al. 2012; Wong et al. 2012). Following analysis of carbon source, initial growth experiments were performed in shaking flasks with wild type *R. eutropha* and the P(HB-*co*-HHx) production strain Re2058/pCB113. Then, the PHA production was scaled up to 5-L laboratory fermenter scale using different lipid waste fats. There were important handling issues to overcome by using the waste animal fats directly as feedstocks in fermentation process, as discussed below. An emulsifying strategy for fats, which were not easily consumable for *R. eutropha* cells, was developed. Finally, we demonstrated a fed-batch fermentation using the lowest quality waste fat available directly for a high-yield production of P(HB-*co*-HHx) with Re2058/pCB113 as the biocatalyst.

Feedstocks characterization

Waste animal fats

Before cultivation of bacteria, various chemical properties of these fats were determined, such as total lipid, FFA and saturated fatty acid contents (Table 5.2, Figure 5.1). Also, the fatty acid composition of the fats was analyzed (Table 5.1).

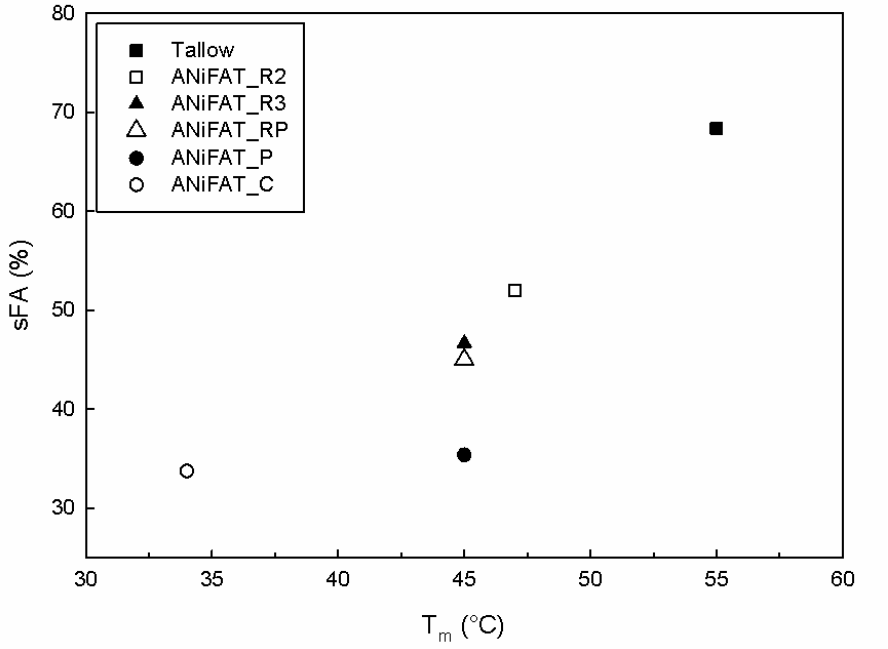

Figure 5.1: Melting temperatures (T_m) of waste animal fats dependant on their saturated fatty acid (sFA) contents. The average chain length of all animal waste fats was between $C_{17.3}$ and $C_{17.4}$

All fats had a very high purity, >95%, and residual protein and nitrogen content of the fats were negligible. The FFA content was between 0.4–7.4%, with the exception of ANiFAT_R2, which had a very high FFA content of 52%. ANiFAT_R2, which also had the lowest purity among all fats, was among the fats with the lowest quality used in this study. The T_m for the waste fats was between 30°C and 55°C, thus solid at RT. The fatty acid compositions of all feedstocks were similar to each other, chain lengths were between C_{14} and C_{18}, inclusive. Among the animal fats, ANiFAT_C exhibited the lowest T_m of 30°C, and had both the lowest amounts of sFA (34%) and C18 FA (67%). Tallow, which had the highest T_m of 55°C, had both the highest amounts of sFA (67%) and C18 FA (82%). Figure 5.1 shows that the different T_m of the animal fat samples depends mainly on the amount of sFA. Effects due to the average FA chain length are negligible, since all samples had an average chain length of between $C_{17.1}$-$C_{17.6}$. Also the amount of FFA had no effect on the T_m of the fats, according to the data in Table 5.2.

Waste plant oil

The waste frying oil used had a low FFA content (0.46%) and low residual moisture (<0.01%, Table 6.2). In contrast to the animal based feedstocks waste frying oil, which mainly consists of palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) (Table 6.1), was liquid at RT.

Evaluation of growth and PHA production in shaking flask experiments

Shaking flask experiments were carried out for an initial evaluation of growth and PHA production from the animal and plant waste feedstocks with *R. eutropha*. All fats used in this study were emulsified, as described in Materials and Methods, prior to inoculation, to increase the bioavailability of the fats and to shorten the lag phase of growth (Budde et al. 2010; Lu et al. 2013), whereas waste frying oil was used directly as carbon source.

PHB from waste animal fats

Experiments with *R. eutropha* H16 demonstrated that the cells grew on all ANiFATs, with the exception of ANiFAT_R2, to a CDW of 4–4.5 g/L and accumulated 72–75% PHB/CDW (Table 5.3). The CDW accumulated with ANiFAT_R2 and tallow as carbon sources was lower, at 3.1 g/L and 2.5 g/L, respectively. Also, the PHB content of the cells was lower, at 56% and 61% PHB/CDW, respectively (Table 5.3).

P(HB-co-HHx) from waste animal fats

The final CDWs (1.5–3.4 g/L) of the recombinant strain Re2058/pCB113 obtained from all waste animal fats with the exception of ANiFAT_C, were always below those of the wild type strain. PHA accumulation per CDW was >70% from ANiFAT_C and -P, and from all others 49–65%. Nevertheless, the P(HB-*co*-HHx) synthesized from all animal fats had a high HHx content of 16–27 mol%.

It is notable that, in all shaking flask experiments using these fats as carbon sources, the largest standard deviations in the data during these triplicate experiments were found in the cultivations with the fats having the highest T_m (ANiFAT_R2: 47°C; tallow: 55°C). During these experiments, non-uniform fat agglomeration in the media occurred, as well as fat deposits sticking to the wall of the shaking flasks, both of which reduced the bioavailability of the fat and likely led to the large deviation.

PHB and P(HB-co-HHx) from waste frying oil

In shaking flask experiments using waste frying oil as a carbon source, both strains of *R. eutropha* used in this work grew to over 3 g/L of CDW with a high PHA content per CDW (>78%). The P(HB-*co*-HHx) from Re2058/pCB113 had an HHx content of 14 mol%. The PHA accumulation per CDW was higher, but the HHx content was lower compared to that of any other waste animal fat (Table 5.3).

Table 5.3: Evaluation of PHB production with *R. eutropha* H16 and P(HB-*co*-HHx) production with Re2058/pCB113 from various waste animal fats (ANiFATs, tallow) and waste frying oil (WFO) in shaking flask experiments. Bacteria were grown for 72 h in minimal medium with 0.05% NH_4Cl as nitrogen source and 1.0% fat or oil as carbon source. Gum arabic (0.5%) was used to emulsify only the waste animal fats prior to inoculation. Error bars indicate standard deviation of the arithmetic mean from triplicate cultivations.

Fat/oil	*R. eutropha* H16		Re2058/pCB113		
Animal	CDW (g/L)	PHB/CDW (%)	CDW (g/L)	PHA/CDW (%)	HHx/PHA (mol%)
ANiFAT_C	4.3 ± 0.1	72.2 ± 0.5	4.6 ± 0.4	71.9 ± 4.7	17.2 ± 1.1
ANiFAT_P	4.0 ± 0.2	73.1 ± 3.2	3.4 ± 0.3	70.9 ± 3.8	15.9 ± 0.3
ANiFAT_R2	3.1 ± 1.8	56.2 ± 5.2	1.5 ± 1.3	48.8 ± 10.0	27.4 ± 6.1
ANiFAT_R3	4.0 ± 0.3	75.3 ± 4.1	2.2 ± 0.6	58.4 ± 3.2	21.8 ± 1.5
ANiFAT_RP	4.5 ± 0.3	72.0 ± 0.4	3.0 ± 0.6	64.1 ± 5.2	16.6 ± 1.1
Tallow	2.5 ± 1.0	61.3 ± 12	n.a.	n.a.	n.a.
Plant					
WFO	4.2 ± 0.4	79.2 ± 2.1	3.1 ± 0.1	78.1 ± 1.5	14.0 ± 0.3

PHB production from tallow and waste frying oil in batch fermentation

R. eutropha H16 was grown with 0.4% NH_4Cl (75 mM total N) as nitrogen source and 3% of waste frying oil or tallow, as reported previously under batch fermentation conditions (Budde et al. 2011b). It is interesting to note that the first fermentation with tallow (high T_m) failed because *R. eutropha* was not able to emulsify the carbon source for growth (data not shown). The fermentation was repeated with pre-emulsified tallow (mechanical mixing with the addition of 0.8% gum arabic before sterilization in the fermenter). In both pre-emulsified tallow and waste frying oil cultures, *R. eutropha* H16 accumulated >70% PHB per CDW. However, the total PHA yield from waste frying oil was higher (18 g/L PHB) as compared to tallow (14 g/L). Even though the tallow was pre-emulsified in the culture, *R. eutropha* grew faster on non-emulsified waste frying oil (Table 5.4).

PHB production from tallow in extended-batch fermentation

To optimize the yield of PHB produced by *R. eutropha* H16 from tallow, the initial nitrogen concentration was doubled to 150 mM using urea instead of NH_4Cl, as compared to the batch fermentation conditions, and also the total overall tallow concentration increased to 6%. Urea was used as nitrogen source instead of NH_4Cl, as urea is a less costly nutrient and previously allowed for more robust cell growth, with *R. eutropha* strains grown in high cell density palm oil and other fermentations, (Chapter IV).

The initial tallow concentration was cut in half and also pre-emulsified with the half amount of gum arabic (0.4%) as used under batch conditions. Use of an emulsifying agent during a fermentation process is not ideal, since it increases the production costs (increased effort before inoculation and material costs of the emulsifying agent). However, in this approach the amount of the emulsifying agent was kept to a minimum, and it was investigated if it was possible to feed non-emulsified tallow during the fermentation process.

At the beginning of the fermentation, the artificially generated tallow emulsion broke mostly into tallow chunks suspended in a thin emulsion, resulting in a very low cell density of 1.5 g/L after 23 h. However, *R. eutropha* was finally able to emulsify all tallow during the course of the fermentation. Moreover, the liquefied, non-emulsified tallow (at 80°C) added was easily emulsified by action of the cells in culture. At the end of fermentation, *R. eutropha* produced almost double the amount of CDW as under batch conditions (39 vs. 20 g/L). However, the final polymer accumulation of 63% PHB per CDW was ~10% lower compared to batch conditions.

Table 5.4: PHB production with *Ralstonia eutropha* H16 with waste frying oil (WFO) or tallow as carbon source in batch and extended-batch fermentations. As nitrogen sources, 75 mM N (NH_4Cl) was used in batch fermentations and 150 mM N (urea) was used in extended-batch fermentations. Tallow was pre-emulsified with gum arabic before inoculation. In the extended-batch fermentation liquefied tallow (at 80°C) was fed directly in 1.5% steps after 38, 46 and 70 h based on initial volume. Error bars indicate maximum and minimum values of duplicate fermentations.

Carbon	Batch				Extended-batch	
Initial	3% WFO		3% Tallow		1.5% Tallow	
Total	3% WFO		3% Tallow		6.0% Tallow	
Time (h)	CDW (g/L)	PHB/CDW (%)	CDW (g/L)	PHB/CDW (%)	CDW (g/L)	PHB/CDW (%)
23	07.6 ± 0.3	27.5 ± 2.1	07.9	40.3	01.5	18.4
39	25.1 ± 1.3	71.2 ± 1.6	15.3	57.4	10.5	10.9
47			20.1	70.2	18.1	20.7
63					34.4	46.3
71					38.6	63.3

P(HB-*co*-HHx) production from tallow and industrially produced waste animal fats

Tallow as carbon source with emulsifying agent

Re2058/pCB113 was grown with 3% pre-emulsified tallow under similar conditions as the PHB production batch fermentations discussed above. Similar problems occurred with the artificially created emulsion during these fermentations as in the tallow fermentations with the

wild type strain, resulting in a long initial lag phase: after 23 h, the accumulated CDW was >1 g/L. After 39 h, the RCDW reached 5 g/L and was constant at 5.9 g/L from 63 until 90 h of culture time. The PHA content per CDW increased from 27 to 75% whereas the HHx concentration of the polymer decreased from 48 to 19 mol% between 23 to 90 h of fermentation. The final PHA production was 18 g/L (Table 5.5).

Table 5.5: P(HB-*co*-HHx) production with Re2058/pCB113 with animal waste fats as carbon source. Tallow was pre-emulsified with gum arabic before inoculation. ANiFAT_C (AF_C) and ANiFAT_R2 (AF_R2) were used directly. Error bars are indicating standard deviation of the arithmetic mean of triplicate fermentations.

Initial Carbon	3% Tallow[a]				1% AF_C[b]			
Total Carbon	3% Tallow				3% (1% AF_C + 2 % AF_R2)			
Time (h)	CDW (g/L)	rCDW (g/L)	PHA/ CDW (%)	HHx (mol%)	CDW (g/L)	rCDW (g/L)	PHA/ CDW (%)	HHx (mol%)
21					01.1 ± 0.4	0.6 ± 0.3	47.2 ± 8.6	46.6 ± 0.1
23	0.9	0.6	27.2	47.5	02.6 ± 1.5	1.3 ± 0.8	51.3 ± 2.5	32.8 ± 7.8
45[b]/48[a]	12.9	5.2	59.5	25.9	11.3 ± 1.4	3.7 ± 0.4	67.2 ± 0.5	29.9 ± 1.8
68[b]/72[a]	16.9	5.8	65.5	21.0	19.6 ± 2.5	5.8 ± 0.1	70.1 ± 3.5	24.8 ± 5.9
90	23.7	5.9	74.9	18.6				

Use of waste fats in the absence of emulsifying agent as carbon source

ANiFAT_R2 was chosen from the industrially available waste animal fats since it had the lowest quality (Table 5.2) and is therefore considered to be the least costly feedstock among the other waste animal fats. ANiFAT_R2 has a high T_m of 47°C and thus is solid at RT, as is tallow (T_m = 55°C). This time, no gum arabic was used for pre-emulsification of the fat, since it was not effective for tallow and also would increase production costs at larger scale (see above). In order to increase the bioavailability of the solid waste fat, another strategy was chosen. The fermentation was initiated with non-emulsified 1% ANiFAT_C as the carbon source, which had the lowest T_m among the tested fats at 34°C. *R. eutropha* was able to naturally emulsify ANiFAT_C under culture conditions described in Materials and Methods. Afterwards, solid ANiFAT_R2 was liquefied at 60°C and fed pulse-wise to the culture. The ANiFAT_R2 was readily emulsified by the metabolically active cells in the culture. After 24 h, the cell density was three times higher as compared to the tallow fermentation. At the end of the fermentation, 20 g/L CDW of Re2058/pCB113 cells with a PHA content of 70% were harvested. The rCDW (5.8 g/L) is equivalent to the rCDW achieved from the tallow fermentation. The HHx content of the polymer decreased from 47–25 mol% over the course of experiment (Table 5.5).

To further increase the P(HB-*co*-HHx) production from ANiFAT_R2, another set of fermentations were performed with the goal of doubling the PHA yield. Therefore, the initial amount of nitrogen was doubled. This time, waste frying oil was used as the initial carbon source instead of ANiFAT_C. Since waste frying oil is liquid at RT, it should therefore be emulsified more readily by *R. eutropha* at the culture conditions of 30°C as compared to ANiFAT_C (T_m = 34°C).

After 24 h of cultivation, the rCDW was 3 g/L, the highest for the Re2058/pCB113 fermentations at this time. All added liquefied ANiFAT_R2 was emulsified through the existing emulsion. The PHA content plateaued after 56 h, whereas cell growth and therefor PHA production continued until the end of the fermentation. The final CDW after 72 h was 45 g/L. Although the final PHA accumulation per CDW was less than 60%, the total PHA production doubled to 26 g/L, as compared to the previous ANIFAT_C/ ANiFAT_R2 fermentation (Figure 5.2).

Discussion

Previously, plant oils have been shown to be highly favorable carbon feedstocks for PHA production using *R. eutropha,* due to their high carbon content, low price per mole of carbon, high obtained PHA yield (0.78 PHA/g plant oil) and high PHA production (>100 g PHA/L) (Akiyama et al. 2003; Fukui and Doi 1998; Kahar et al. 2004; Riedel et al. 2012; Sato et al. 2013). Crude palm oil is still the most available and inexpensive plant oil on the market, even though its price has increased from $US 424/ton to $US 865/ton from 2006 to 2014 (MPOB, 2015). A main reason for the price increase can be found in the increasing demand of biodiesel from palm oil (de Vries et al. 2010; Manik and Halog 2013; Mekhilef et al. 2011; Stichnothe et al. 2014).

In order to emulsify oils/fats for consumption, *R. eutropha* secretes a lipase into the culture broth (Lu et al. 2013). However, *R. eutropha* was not able to emulsify waste animal fats with T_m >30°C in fermentation cultures. These fats agglomerated after addition to the aqueous media (at the cultivation temperature of 30°C), and no emulsification or cell growth occurred. In this study, we used an emulsifying agent for initial experiments. However, this technique is not optimal for an industrial scale up, due to the potentially high production costs. Also, poor stability of the artificial emulsion was observed following sterilization of the media. During this study, a more suitable alternative was found in using the waste animal fats (T_m >34°C) in the media directly as a carbon source. As an initial carbon source, 1% of an oil/fat (T_m ≤34°C) was used, which *R. eutropha* itself readily emulsified. After the initial emulsion was built, the

animal fat (T_m >34°C) was repeatedly manually fed directly to the culture. The fat was emulsified smoothly in the media during cell growth. With this emulsification strategy, we were able to produce 45 g/L CDW with a P(HB-*co*-HHx) content of 60% per CDW and a final HHx concentration of 19 mol% using Re2058/pCB113 cultured on 1% initial waste frying oil and 5% low quality ANiFAT_R2.

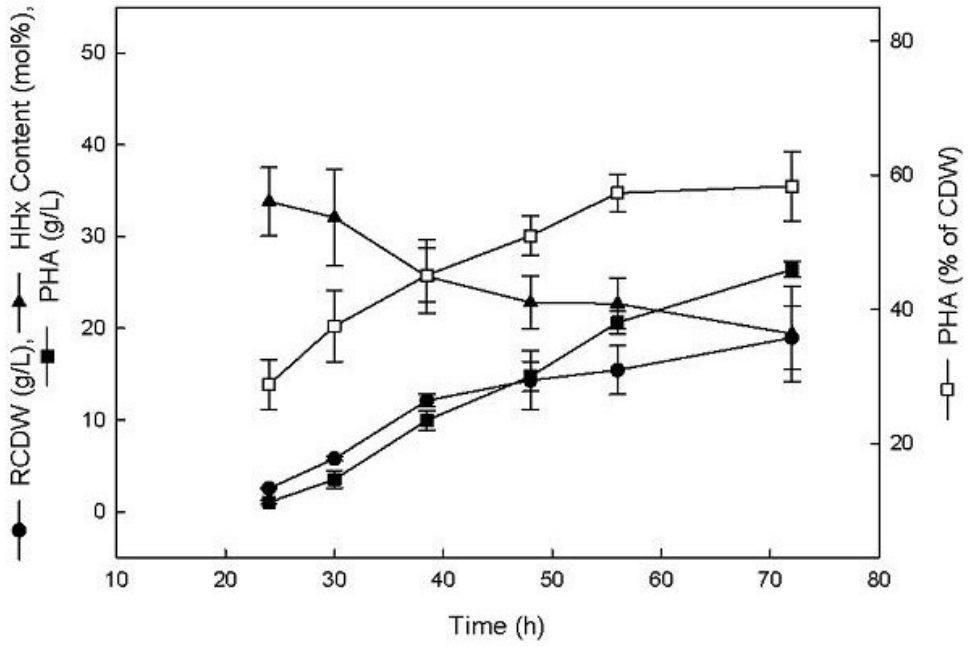

Figure 5.2: Fed-batch fermentations for P(HB-*co*-HHx) production by *R. eutropha* Re2058/pCB113 from waste frying oil and animal waste fat ANiFAT_R2 as carbon source and urea as nitrogen source. Initial concentrations of 10 g/L waste frying oil and 4.5 g/L urea (150 mM N) were added to 2.5 L minimal media. After the initial waste frying oil was emulsified during growth of Re2058/pCB113, liquefied (at 60°C) ANiFAT_R2 was fed in 10 g/L steps after 6, 9, 24, 26 and 30 h to a total carbon concentration of 60 g/L, based on initial volume. PHA content per cell dry weight (CDW), PHA production in g/L, residual cell dry weight (RCDW) in g/L and the HHx content (mol%) of the copolymer are given over the course of triplicate fermentations. Error bars are indicating the standard deviation of the arithmetic mean.

The HHx content of the polymer decreased continuously over the cultivation time, which is a phenomenon that has been discussed previously (Budde et al. 2011b; Riedel et al. 2012). It is notable that the PHA concentration per CDW leveled at the end of the fermentation, whereas the PHA mass concentration still increased until the end of the fermentation (Figure 5.2). This phenomenon has been previously observed during nitrogen-limited PHA fermentation independently from the bacterium, carbon or nitrogen source used (Riedel et al. 2012; Obruca et al. 2010; Chen et al. 2001). However, it should be noted that this phenomenon did not occur during fermentations with phosphate limitation (Kahar et al. 2004; Chen et al. 2001) and continuously low nitrogen (Tsuge et al. 2001). The P(HB-*co*-HHx) production data from this

study are very similar to the production data published previously from 6% palm oil, where the oil was also fed in 1% additions (Riedel et al. 2012). This previous study also showed an increase in PHA production up to 100 g/L during high cell density fed-batch fermentations (Riedel et al. 2012). We are convinced that these high production values are also achievable with ANiFAT_R2 as the carbon source by using a similar automated feeding strategy.

We faced many operational challenges in the development of an automated fed-batch strategy, due to the high T_m of ANiFAT_R2. Even if the fat and feeding lines were heated, solidification through the low pumping rates occasionally occurred. Technical challenges typically increase during a scale-up from laboratory to industrial (pilot) plant scale. However, in this case we foresee alleviation of the described problems. Faster pumping rates will successfully prevent a solidification of the fat in the feeding lines. Also, massive foaming, another issue which often occurs during fermentations with oil/fats, will be easier to address in large scale through more head space in the fermenter and the potential use of foam centrifuges, which were not available for the laboratory scale fermenters. Additionally, to inhibit foaming during the process, the aeration was set at 0.5 vvm. To ensure the DO set point was at 40% with the applied stirrer cascade, only half of the working volume of the fermenter was used. ANiFAT_R2 consisted of over 50% FFA, which caused an even greater increase of the strong foaming compared to the other used fats, especially after the pulse-wise additions. Foaming was also increasing with an increasing cell density of the culture. The use of chemical antifoam agents was not possible: Polyethylene glycol can be utilized by *R. eutropha* as a carbon source and also reduces the molecular weight of the accumulated PHA in the cell (Shi et al. 1996), and silicon oil also gets emulsified during cultivation (Sinskey Laboratory, unpublished), therefore large amounts would be needed in order to reduce foam continuously. Large amounts of silicon oil will increase the viscosity of the culture broth, which inhibits the oxygen transfer and can also have negative (unknown) effects on the cell physiology (Routledge 2012). Additionally, the silicon oil would need to be removed during the downstream processing.

However, even though the approach of developing an automated fed-batch fermentation strategy to achieve high-cell densities (≥140 g/L) could not be completed during this time of study, the outcome of the presented work is very promising for a potential PHA production on an industrial scale. The operational problems encountered are less critical in industrial scale (see above). The achieved yield (45 g/L CDW, 26 g/L PHA) in the manually controlled fed-batch fermentations of 0.4 g PHA/g fat and 0.36 g PHA/(L*h) was still very favorable, especially considering the low costs of the feedstock. In general, the cost of the feedstock for

the commercial production of a biotechnological product should not exceed 15% of the final product price. In projection of the PHA production from low quality animal waste fats with our shown production data and prices for the feedstock, a potential market price in the range of US$1.6–3.3 / kg PHA is possible.

To the best of our knowledge, this is the first known demonstration of PHA production directly from (low quality) waste animal fats and tallow as carbon feedstocks in fermentation scale. In the European project ANIMPOL, PHA production is described from waste animal fat-based feedstocks. In this project, the animal waste fats were first turned into FAEs, which then were split into high-quality fractions for biodiesel production and low quality fractions (saturated fractions), which were used as feedstock for PHA production (Kettl et al. 2011; Titz et al. 2012). The same group reported from these FAEs fractions a *mcl*-PHA production of maximum 0.14 g *mcl*-PHA/(L*h) from *Pseudomonas*. The PHA accumulation was a maximum of 29% per CDW, which is comparatively very low (Muhr et al. 2013a; Muhr et al. 2013b).

After this and the last chapter, the high yield PHA production from waste animal fats, respectively palm oil was described, the next chapter focuses on the PHA recovery.

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CHAPTER VI

PHA Recovery

This chapter was modified from a previously published article in Biotechnology and Bioengineering, 2013. 110(2): 74-83 'Recovery of Poly (3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) From *Ralstonia eutropha* Cultures with Nom-Halogenated Solvents' Sebastian L. Riedel, Christopher J. Brigham, Charles F. Budde, Johannes Bader, ChoKyun Rha, Ulf Stahl and Anthony J. Sinskey © Wiley Periodicals, Inc.

Goal of Chapter VI

In Chapter IV and Chapter V high cell density fermentation processes for the production of novel P(HB-*co*-HHx) from palm oil, respectively waste animal are shown. This chapter focuses on the recovery of the produced polymer from Chapter IV and Chapter V with non-halogenated solvents.

Introduction

In this work, we examine the recovery of P(HB-*co*-HHx) polymer from wet and dry biomass using non-halogenated solvents. All of the PHA recovered was produced using high cell density palm oil fermentations, similar to the process described in a recent publication by our group (Riedel et al. 2012). In this previous work, a fed-batch fermentation process resulted in >139 g/L of biomass with a PHA content of 74% and a STY of better than 1 g PHA/L/h. The P(HB-*co*-HHx) produced had high HHx monomer content (19 mol%), indicating that it is a unique bio-based PHA polymer. The goal of the present study was to develop an effective way to isolate this PHA from other components in the fermentation broth, including residual triacylglycerol's and fatty acids from the palm oil. Non-halogenated solvents were evaluated for their ability to produce high purity PHA at a high yield. Interestingly, some solvents used in this work were found to promote separation of different polymers based on HHx monomer content. These observations suggest the development of a unique polymer purification and separation procedure.

Materials and methods

Production of cell material for P(HB-*co*-HHx) recovery

R. eutropha Re2058/pCB113, a strain engineered from the *R. eutropha* wild-type strain H16 (ATCC 17699) (Budde et al. 2011), was grown using fermentation conditions described previously (Riedel et al. 2012), with triacylglycerol's and fatty acids from different plant oils as sole carbon sources, to produce biomass containing P(HB-*co*-HHx)

with high levels of HHx (>15 mol%). Cells from fermentation broths were harvested, frozen at -80˚C, and processed as described below.

Recovery of PHA with chloroform/methanol

For every 50 g dried cells, polymer extraction was performed with 500 mL chloroform (Mallinckrodt Chemicals, Phillipsburg, NJ) for 4 h in a 1-L round bottom flask. A reflux condenser was used to prevent evaporation of chloroform. After extraction of PHA, the chloroform was filtered once with a dense strainer followed by a glass microfiber filter (pore size = 55 mm) into a 4 L glass flask. The PHA was precipitated by adding 2.5 L cold methanol (-20°C) to the filtrate and allowing the mixture to incubate at -20°C for 4 h. The solid PHA was separated from the liquid by centrifuging for 10 min at 5,800 × *g* and 4°C. The pellet was then removed and transferred to a 50-mL polypropylene tube. The tube was incubated in a 45°C heat block for 24 h. The remainder of the solvent was evaporated under vacuum overnight. The quality of the recovered plastic was determined via methanolysis analysis (see below).

Test of solvents for PHA recovery

To test solvents, the PHA recovered with chloroform / methanol (see above) was used as starting material. Aliquots of 2.5 mL of the non-halogenated solvents methyl isobutyl ketone, methyl ethyl ketone, butyl acetate (MIBK, MEK, BA, Sigma-Aldrich, St. Louis, MO) , ethyl acetate (EA, Muskegon, MI) and isoamyl alcohol (IA, Mallinckrodt Chemicals, Phillipsburg, NJ), were added to 0.25 g PHA in screw top test tubes (10% solutions). The PHA was dissolved by heating at 50°C for 2 h in a heat block and stirring with a magnetic stirrer. After incubation, each solution was filtered through a 0.2 µm PTFE membrane filter, and 1.5 mL of each filtrate were transferred into a pre-weighed borosilicate glass test tube. The glass tubes were incubated at temperatures 10°C below the boiling point of each solvent until dry. The samples were further dried under vacuum until they reached a constant weight.

Test of precipitants for recovery of PHA

To test precipitants, 5% stock solutions of PHA in MIBK and BA were prepared in sealed vessels. For each precipitant tested, 1 mL of the 5% PHA solution was transferred into pre-weighed borosilicate glass test tubes. PHA was then precipitated by addition of 0.5–5 volumes of precipitant (*n*-hexane or *n*-heptane) at RT. The tubes were briefly vortexed and incubated at RT for 1 h. Following mixing, the tubes were centrifuged for 10 min at 2,500 × *g* and 20ºC. The supernatant was discarded and the PHA pellet was initially dried in a heating block at 50ºC and finally in a vacuum oven at 80ºC until dry.

Test of lipid and fatty acid precipitation

Since PHA investigated in this work was produced by cultures grown on plant oils, it was necessary to determine if residual lipids from the palm oil culture broth could be precipitated by methods used for polymer precipitation. Solutions (5%) of palm oil, oleic acid ($C_{18:1}$), palmitic acid ($C_{16:0}$), and lauric acid ($C_{12:0}$) were prepared using BA or MIBK as the solvents in screw-capped tubes. Three volumes of *n*-hexane were added, and the solutions were observed for precipitation of triacylglycerol's or fatty acids during overnight incubation at RT or 4ºC.

Recovery of PHA from dry cells in 2 mL scale

Sealed bottles containing 600 mL volumes of fermentation broth were thawed in warm water, and then centrifuged for 20 min at 7,200 × *g*. The cell pellet was washed with a mixture of 500 mL water and 100 mL *n*-hexane (Mallinckrodt Chemicals, Phillipsburg, NJ) to remove any residual oil. The wet cell pellet was homogenized by mixing with a spatula, frozen at -80˚C and then freeze-dried. The PHA content of the freeze-dried cells was determined as described below. Equivalent masses of freeze-dried cells, containing 40 mg of PHA, were weighed in screw capped sealed glass tubes. In each tube, 2 mL solvent was added to form 2% PHA/solvent mixtures. Chloroform, MIBK, MEK, BA, or EA were used as solvents for polymer recovery. PHA was extracted by incubating samples at 50˚C, 75˚C or 100˚C for 4 h and were mixed by briefly shaking tubes by hand every 30 min. Samples were cooled to RT and centrifuged at 2,000 × *g* for 10 min at RT. In some cases, the formation of a gel-like phase between the residual cells and organic phase was observed. This gel-like phase will thus be referred to as PHA/solvent-gel, in contrast to the PHA/solvent-solution (*e.g*. PHA/MIBK-gel or PHA/BA-solution). A typical PHA/solvent-gel that formed during a MIBK extraction is shown in Figure 6.1. PHA/solvent-solutions and PHA/solvent-gel, if present, were each transferred to individual, pre-weighed borosilicate glass tubes. PHA was then precipitated with 3 volumes of *n*-hexane, briefly vortexed at RT, centrifuged at 2,000 × *g* and then washed twice with *n*-hexane. The washed polymer was dried overnight at 50˚C. Monomer composition of the P(HB-*co*-HHx) copolymer was determined by methanolysis, as described below.

Recovery of PHA from dry cells in 40 mL scale

Samples of freeze-dried biomass, containing 0.8 g PHA, were each extracted with 40 mL of non-halogenated solvents (EA, MIBK, MEK or BA) to form 2% PHA/solvent mixtures. Extraction occurred at 100˚C in 125-mL flat-bottom flasks under reflux cooling conditions for

4 h. The samples were cooled to RT and centrifuged at 6,000 × *g* for 10 min in 50 mL polypropylene tubes. The flat-bottom flask was rinsed twice with 2.5 mL solvent and used to wash residual cell material. The PHA was precipitated with three volumes of *n*-hexane at RT and washed three times with *n*-hexane. The washed polymer was dried at 50˚C overnight. Both the monomer compositions of PHA polymer and residual cell material were determined as described below.

Larger scale recovery from dry cells with ethyl acetate (EA)

A 2% PHA solvent mixture was created by adding 1.78 L of EA to freeze-dried cells containing 35.5 g PHA. PHA was extracted in a 5-L round bottom flask for 4 h at 80–90˚C. The sample was centrifuged at 2,200 × *g* for 20 min at RT. Aliquots of 1 L PHA/EA-solution were precipitated with 3 L *n*-hexane at RT in 4-L Erlenmeyer flasks with stirring. Supernatant was removed through decantation and the precipitated PHA was washed twice with *n*-hexane, manually crushed into smaller particles with a spatula, placed in a glass bowl, and then dried at 50˚C overnight.

Larger scale recovery from dry cells with methyl isobutyl ketone (MIBK)

A total volume of 1.35 L MIBK was added to freeze-dried cells containing 27 g PHA, to form a 2% PHA solvent mixture. The PHA mixture was then transferred to a 5-L round bottom flask. Polymer was extracted at 100˚C with stirring under reflux conditions for 4 h. The sample was cooled to RT overnight and centrifuged in glass centrifuge bottles at 2,200 × *g* for 10 min at RT. Aliquots of 1 L PHA/solvent-solution were precipitated with 3 L *n*-hexane at RT in 4-L Erlenmeyer flasks with stirring. Supernatant was removed through decantation and the precipitated PHA was washed twice with *n*-hexane and then dried at 50˚C overnight. Before drying, the washed polymer pellet was manually crushed into smaller particles with a spatula and transferred into a flat-bottom glass bowl.

Larger scale recovery from wet cells

Equal volumes (400–750 mL) of fermentation broth (containing cells and PHA) were thawed in warm water and centrifuged for 20 min at 7,200 × *g* at RT. The wet cell pellet was transferred into a 5-L round-bottom flask, and solvent was added to form a 2% PHA solvent mixtures (*e.g.* wet cells containing 60 g PHA in 3 L solvent). PHA was extracted at 100˚C with stirring under reflux conditions for 4 h. At the beginning of the extraction, 0.33 L of water per 1 L solvent was added to enhance mixing of the wet cell pellet with the solvent. After extraction, the sample was cooled to RT overnight and centrifuged in glass centrifuge bottles at 2,200 × *g* for 10 min at RT. Aliquots of 1 L PHA/solvent-solution were precipitated

with 3 L *n*-hexane at RT in 4-L Erlenmeyer flasks under stirring. Supernatant was removed through decantation and the precipitated PHA was washed 3 times with *n*-hexane and dried at 50˚C overnight. Before drying, the washed polymer pellet was manually crushed into smaller particles using a spatula and transferred into a flat-bottom glass bowl.

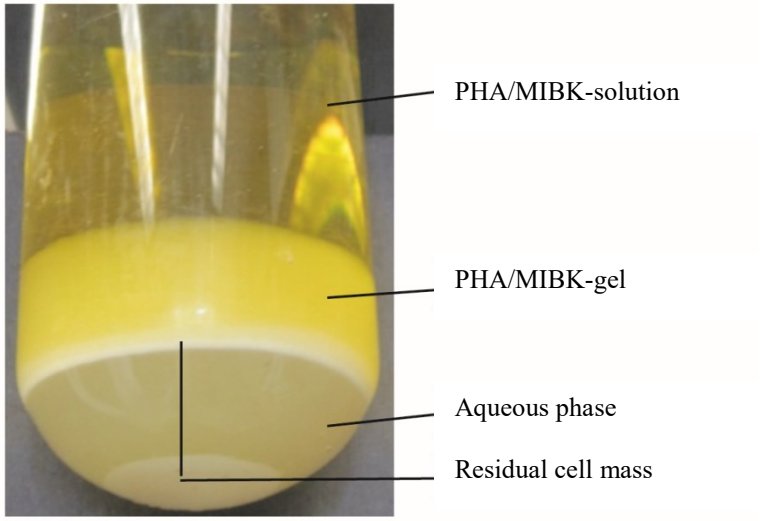

Figure 6.1 Separation of PHA/MIBK-solution, PHA/MIBK-gel and residual cell mass, following PHA extraction from wet cells with MIBK. Polymer in solution was extracted for 4 h at 100°C under reflux conditions. The sample was cooled to room temperature and centrifuged for 10 min at 2,200 × *g*. Multiple phases are observed, as discussed in the text.

After polymer extraction from wet cells using EA, residual cell material was further separated from residual PHA/solvent-solution by centrifugation at 6,700 × *g* in polypropylene tubes. During centrifugation, residual cell material separated into two different fractions. Part of the residual cell material collected at the solvent/water interface, while the rest formed a pellet at the bottom of the tube. The interface between the organic and aqueous phases had a yellow colored top portion and a white bottom portion Three separate sections of the centrifuged material (residual cells/interface-top, residual cells/interface-bottom and residual cell pellet) from one polypropylene tube were transferred into different polypropylene tubes, washed three times with water, freeze-dried and analyzed to determine PHA content by methanolysis.

Analytical methods

PHA concentration per CDW, purity of the recovered PHA, and HHx content of the copolymers were determined using a methanolysis protocol described previously (Budde et al. 2011).

In this procedure, pure standards of poly-3-hydroxybutyrate and methyl 3-hydroxyhexanoate (Sigma-Aldrich, St. Louis, MO) were used to generate calibration curves. Recovery yield (RY) was defined as:

$$\text{Recovery yield (RY,\%)} = \frac{\text{mass PHA recovered (g) * purity (\%)}}{\text{mass PHA in cells used in recovery batch (g)}} \qquad [2]$$

Results

Many non-halogenated solvents that can serve as alternatives to chloroform for PHA recovery have been identified in the academic and patent literature (Kinoshida et al. 2006; Noda 1998; Noda et al. 2005; VanWalsem et al. 2007). We chose to investigate MIBK, MEK, BA, EA, and IA as potential solvents for P(HB-*co*-HHx) produced from palm oil (Riedel et al. 2012). Physical properties and safety characteristics of the chemicals used in this study are compiled in Table 6.1. These properties would determine how effective a solvent would be in an industrial recovery process. Isolation of PHA from bacterial cells requires extraction, separation, and washing steps. All non-halogenated solvents used in this study have lower densities than water, which allowed for simple decantation of PHA solutions after extraction and centrifugation. Also, the residual biomass remained in the aqueous phase, separated from the polymer solution. This phenomenon is a process advantage over chloroform, which has higher density than water. Thus, PHA-chloroform solutions will form the bottom phase along with the residual cell material. A general flow diagram of the recovery studies performed in this work is presented in Figure 6.2.

Testing PHA solubility in chosen solvents

To evaluate which solvents were capable of dissolving our PHA copolymer, previously-extracted P(HB-*co*-33mol%HHx), with a purity of 86% was used as the starting material. With the exception of IA, all solvents tested were able to dissolve the copolymer (Figure 6.3). Recovery yields of up to 95% were achieved from the 10% PHA solutions. However, after the incubation with IA, it was not possible to filter the solution as it was too viscous and cloudy, indicating that the PHA did not dissolve.

Test of precipitants for recovery of PHA

The precipitants *n*-hexane and *n*-heptane were tested for PHA precipitation. PHA was precipitated from 5% solutions at RT**.** The various combinations of solvents and precipitants gave similar results. A threefold volume of either precipitant (per volume of PHA solution) was sufficient to recover almost 100% of the PHA (Figure 6.4).

Test of lipid precipitation by *n*-hexane

In order to determine if residual lipids from the palm oil culture broth would also come out of solution upon addition of *n*-hexane, attempts were made to precipitate oil or fatty acids from different 5% lipid solutions in solvent (MIBK, BA). These test solutions contained a significantly higher concentration of lipids than one would expect to co-extract with PHA. All lipids went into solution in MIBK or BA at RT, although with palmitic acid, the fatty acids precipitated when incubated at 4ºC. After addition of *n*-hexane, no lipid precipitation was observed at RT, but when solutions were incubated at 4ºC, lipids in the palmitic acid solution once again came out of solution. These findings indicate that substantial co-precipitation of oil and fatty acids, during PHA precipitation, is unlikely using the methods described in this work.

However, precipitated PHA must still be washed with additional volumes of precipitant in order to remove the residual solvent, which can contain residual lipids, to ensure that lipid contamination of the polymer does not persist upon drying.

Copolymer recovery from *R. eutropha* cells at 2 mL scale

To further evaluate solvents for PHA recovery from biomass, P(HB-*co*-HHx) was extracted from dry cells containing 76% PHA of CDW with an HHx concentration of 15 mol%. Extractions were performed at various temperatures (50˚C, 75˚C, and 100˚C) with a PHA to solvent ratio targeting 2% PHA solutions at the 2 mL scale (Table 6.2). Chloroform was used as a control solvent and was able to recover almost all PHA present in cells (≥ 98%) at 75˚C or 100˚C. At 50˚C, the recovery yield from chloroform solutions was slightly lower at 95%, and the HHx content of the recovered polymer also increased slightly as compared to samples incubated at higher temperatures.

Along with the typical PHA/solvent-solutions, PHA/solvent-gel formation was observed at the bottom of the organic phase during polymer extraction with MIBK or BA (Figure 6.1). The PHA/solvent-gel formation was observed with these solvents only at temperatures of 100˚C and 75˚C. The final yield of polymer from PHA/MIBK-solution or PHA/BA-solution decreased with a decrease in recovery temperature, whereas the amount of polymer in the PHA/MIBK-gel was higher at the lower temperature. The recovery yield from PHA/BA-gel did not change as temperature decreased. The total recovery yield, taking into account PHA in solution and in the gel phase, decreased for both MIBK (79% to 72%) and BA (74% to 60%) extractions at 75°C compared to 100°C.

Table 6.1: Property data for chemicals that could potentially be used in a PHA recovery process. The top group of compounds consists of potential PHA solvents, with water included as a reference. The bottom three compounds (n-hexane, n-heptane, and n-octane) are used as PHA precipitants.

Compound	Boiling Point (°C)[a]	Density (g/cm^3) [a]	Viscosity (cP) [a]	Heat capacity ($J\ mol^{-1}\ K^{-1}$) [a]	Solubility in water (ppmw) [a]	PEL (ppm)[b]	FDA class[c]
Water	100	1.03	0.91	76	N.D.	N.D.	Safe
Chloroform	61	1.48	0.54	112	7.50×10^3	50	2
Ethyl acetate	77	0.89	0.42	171	7.37×10^4	400	3
Butyl acetate	126	0.88	0.68	228	6.80×10^3	150	3
Methyl isobutyl ketone	117	0.80	0.60	212	1.90×10^4	100	3
Methyl ethyl ketone	80	0.80	0.40	160	2.48×10^5	200	3
Isoamyl alcohol	131	0.81	3.69	165	2.70×10^4	100	3
n-hexane	69	0.66	0.30	193	1.33×10^1	500	2
n-heptane	98	0.68	0.39	230	2.24	500	3
n-octane	126	0.70	0.51	255	0.43	500	N.D.

[a] Physical property data is from (Yaws 1999), measured at 25°C and 1 atm; [b] PEL is the Permissible Exposure Limit established by the United States Occupational Safety and Health Administration (OSHA, standard number: 1910.1000 TABLE Z-1); [c] The FDA rates chemicals for use in manufacturing of biomedical products, where 1 is most toxic and 3 is least toxic, Q3C Feb 12 (www.fda.gov/RegulatoryInformation/Guidances/ucm128290.htm) N.D. indicates no data was available

Interestingly, in the recovery processes where a gel phase was observed, the monomer compositions of polymer recovered from PHA/solvent-solution and PHA/solvent-gel were different from each other. Polymer recovered from PHA/solvent-solution had a notably higher HHx level (17 mol%) compared to the polymer recovered from the PHA/solvent-gel (14–15 mol%) or compared to the total polymer recovered using chloroform as the solvent (15 mol%). The polymer recovered from the PHA/MIBK-solution or PHA/BA-solution at an extraction temperature of 50˚C had an even higher HHx concentration at 21 mol% or 19 mol%, respectively. PHA recovery using MEK or EA exhibited a high recovery yield (≥ 95%) at temperatures of 75˚C and 100˚C. However, at 50˚C, the recovery yield decreased to 87% or 76%, respectively, concomitant with a slight increase in HHx content to ~17 mol%. No PHA/solvent-gel formation was observed during recovery with MEK and EA.

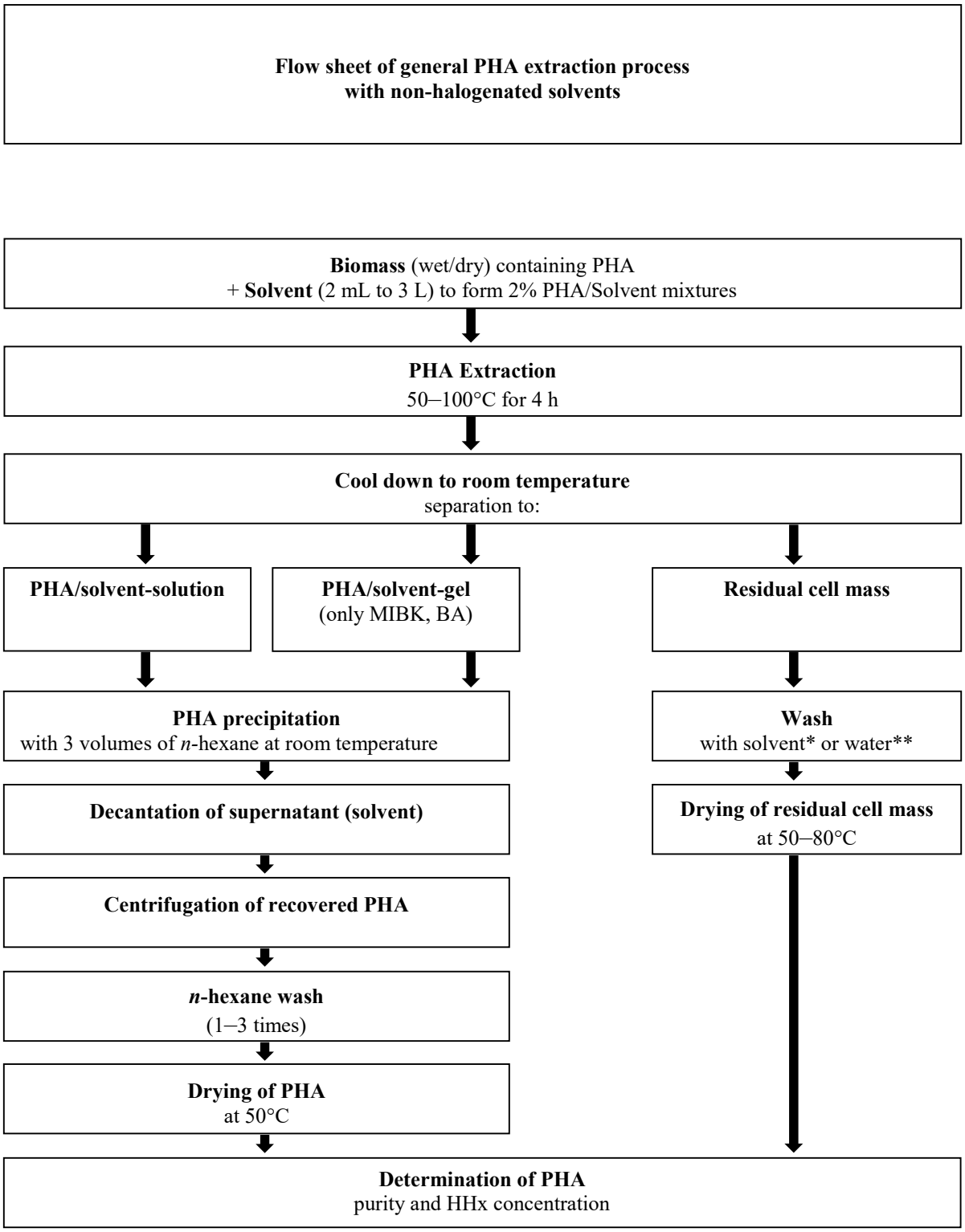

Figure 6.2: Flow sheet of a general PHA extraction process. P(HB-*co*-HHx) was extracted from wet or dry biomass using the non-halogenated solvents: EA, MEK, BA or MIBK. (*) washed twice with 2.5 mL solvent during the 40 mL extraction process from dry cells (**), or three times with water after the 1.5 L EA extraction from wet cells.

Copolymer recovery from dried *R. eutropha* cells at 40 mL scale

At the 2 mL scale, the extractions at 100˚C yielded the best results (see recovery yield and purity, Table 6.2). Larger volume (40 mL) extractions were performed from dry cells, containing 62% PHA with 22 mol% HHx. The purities of all recovered polymer samples from PHA/solvent-solutions were ≥95 %.

The data from 40 mL scale recovery experiments are shown in Table 5.3. A PHA/solvent-gel was observed using the solvents MIBK and BA, similar to results seen during the 2 mL extractions. The purities of the recovered polymer samples from the PHA/solvent-gel were lower relative to polymer from solution, with purities of 73% or 62%, using MIBK or BA, respectively. The HHx concentrations of polymer recovered from either PHA/MIBK-solution or PHA/BA-solution were, at 24 mol%, slightly higher compared to the polymer present in cells (22 mol% HHx). Furthermore, the HHx content of the polymer recovered from the PHA/MIBK-gel or the PHA/BA-gel was much lower, at 12 or 11 mol%, respectively. Extraction with either EA or MEK gave a high polymer recovery yield of ≥94%. The HHx content of polymer from the PHA/EA-solution or PHA/MEK-solution was ~21 mol%, which was similar to the HHx content measured in whole cells. Analysis of the residual cell mass from extractions using MEK or EA showed that only minor amounts of unrecovered PHA (2% of cell mass) were present. However, the PHA content of residual cell mass from cells treated with either MIBK or BA was much higher, at 13% or 24%, respectively. The HHx monomer content of PHA not extracted from the cells with these solvents was 11–12 mol%. The total recovery yield from MIBK extraction was 84% (71% from PHA/MIBK-solution and 13% from PHA/MIBK-gel). The total recovery yield was lowest with BA, reaching only 76% (68% from PHA/BA-solution and 8% from PHA/BA-gel).

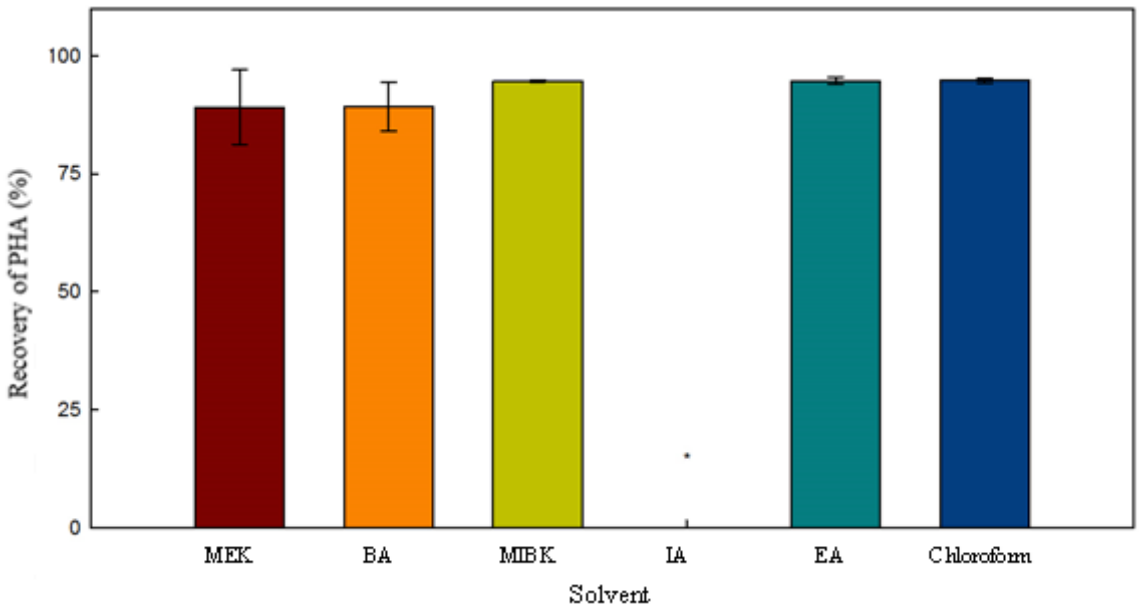

Figure 6.3 Solubility of P(HB-*co*-HHx). A 10% PHA solution was made using the MEK, BA, MIBK, IA, EA or chloroform. Data indicating recovery of the PHA polymer (% input) from polymer solutions are presented. Maximum and minimum values of two replicates are shown. Asterisk (*) indicates that, after incubation with IA, it was not possible to filter the solution and determine a recovery value.

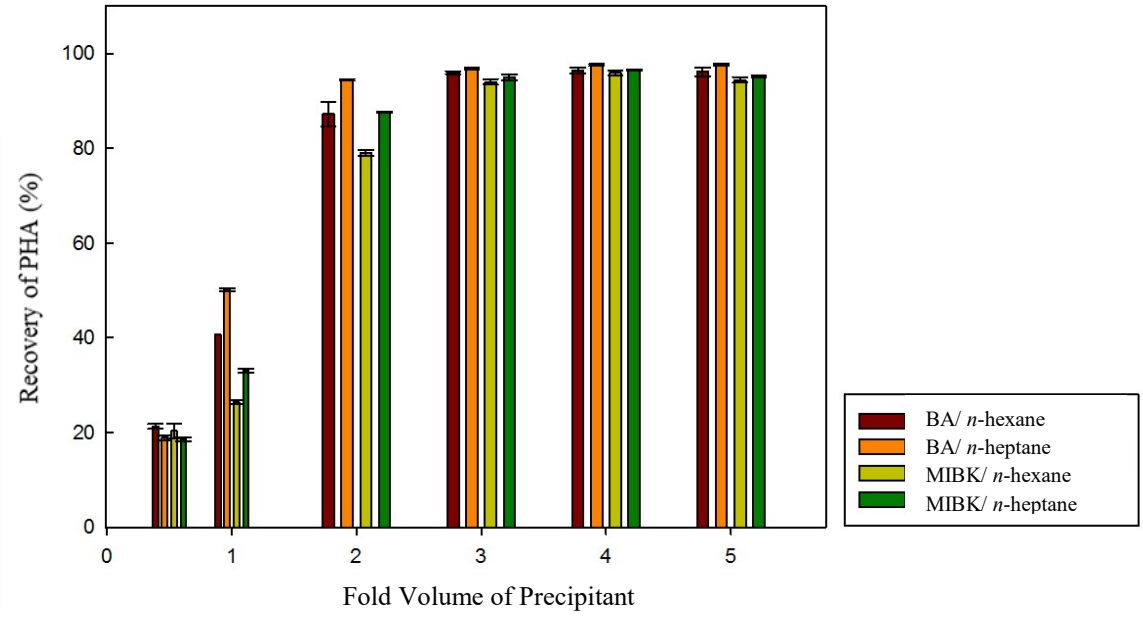

Figure 6.4 Examination of precipitants for P(HB-*co*-HHx) recovery. Using MIBK or BA as PHA solvents, 5% PHA solutions were made. The polymer was precipitated by addition of *n*-hexane or *n*-heptane to the solution at room temperature. Averages of two replicates are shown.

Table 6.2: P(HB-*co*-HHx) recovery from dry *R. eutropha* cells on a 2 mL scale. PHA was extracted for 4 h at 100˚C, 75˚C or 50˚C with the non-halogenated solvents (52.6 mg cells, 76% CDW of PHA, 2 mL solvent). Chloroform extractions were used as controls. In all cases, the extracted polymer was precipitated with 3 volumes of *n*-hexane at room temperature from PHA/solvent-solution (S) or PHA/solvent-gel (G) and dried at 50˚C. All values represent means from triplicate extractions with error bars indicating ± S.D.

Solvent (PHA/solvent-solution or gel)	Temperaure (˚C)	Recovery Yield (%)	Purity (%)	HHx (mol%)
Chlorofrom[c] (S[a])	100	99 ± 1	100 ± 1	15 ± 1
	75	98 ± 0	100 ± 0	15 ± 0
	50	95 ± 1	100 ± 1	16 ± 0
MIBK[b] (S)	100	55 ± 2	99 ± 1	17 ± 0
	75	37 ± 2	99 ± 1	17 ± 0
	50	37 ± 2	100 ± 0	21 ± 0
MIBK (G[a])	100	24 ± 4	96 ± 2	14 ± 0
	75	35 ± 5	98 ± 2	15 ± 0
MEK[b,c] (S)	100	95 ± 1	100 ± 1	16 ± 0
	75	95 ± 1	99 ± 0	15 ± 0
	50	87 ± 3	100 ± 0	17 ± 0
BA[b] (S)	100	42 ± 0	100 ± 1	17 ± 1
	75	27 ± 1	100 ± 1	17 ± 0
	50	41 ± 1	99 ± 2	19 ± 0
BA (G)	100	33 ± 5	100 ± 1	15 ± 0
	75	33 ± 3	95 ± 3	15 ± 0
EA[b,c] (S)	100	99 ± 0	100 ± 0	16 ± 0
	75	95 ± 0	97 ± 0	15 ± 0
	50	76 ± 0	100 ± 1	17 ± 1

[a] (S) indicates PHA/solvent-solution, (G) indicates PHA/solvent-gel; [b] MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; BA = butyl acetate; EA = ethyl acetate; [c] No PHA/solvent-gel formation was observed using MEK, EA or chloroform

Larger scale PHA recovery

To demonstrate the scalability of a PHA recovery process, polymer was recovered from cell biomass using up to 3 L volumes of solvent (Table 6.4). Wet cells were used, instead of dry cells, to avoid an energy and time consuming drying step for these large quantities of cell material. For comparison, representative gel forming and non-gel forming solvents were used in this laboratory scale up process. MIBK was chosen over BA for the PHA/solvent-gel forming solvent, due to better yields in the previous experiments (Table 6.3). EA was chosen over MEK, because of its lower solubility in water, which would enhance separation of wastewater and solvent following the PHA extraction step of a potential industrial process. In recovery with MIBK at the 3 L scale from wet cells, PHA separation based on the HHx

content was observed, with polymer from PHA/MIBK-solution at ~20 mol% HHx and polymer from PHA/MIBK-gel at ~15 mol% HHx (average HHx content of total PHA before recovery was 20 mol%). The purity of the polymer from PHA/MIBK-solution was observed to increase to >99% with efficient washing of the recovered polymer with *n*-hexane. The overall recovery yield from both PHA/MIBK-solution and PHA/MIBK-gel was 84%.

PHA recovery with EA from wet cells in a 1.5 L scale showed no PHA/EA-gel formation, as expected. The recovered polymer had a purity of 98% with an HHx content of 21 mol%. During centrifugation of the extraction mixture, a separation of the residual cell material into three distinct phases was observed, as described in Materials and Methods. The PHA content from the residual cells/interface-top (the upper phase) had a PHA content of 58%, the residual cells/interface-bottom (the middle phase) had a PHA content of 31% and the residual cell pellet (bottom phase) a PHA content of 27%. We hypothesize that, during recovery of PHA from wet cells using EA as the solvent, a mixture of EA, water, and residual cell debris formed, resulting in a significant portion of the polymer remaining with the wet cell mass. All three phases had an average HHx content of 22 mol%. The recovery yield from the PHA/EA-solution from wet cells was only 71%, whereas PHA recovery from dry cells in a 1.8 L extraction gave a recovery yield of 93% with a purity of 95%.

Table 6.3: P(HB-*co*-HHx) recovery from dry *R. eutropha* cells (PHA content of 62% with HHx concentration of 22 mol%) at the 40 mL scale. PHA was extracted for 4 h at 100˚C using non-halogenated solvents. The extracted polymer was precipitated with 3 volumes of *n*-hexane at room temperature from PHA/solvent-solution or PHA/solvent-gel. PHA and the residual cell mass were dried at 50˚C. All values represent minimum and maximum data from duplicate extractions.

Solvent	PHA/solvent-solution			PHA/solvent-gel			Rcells		Total
n=2	Purity (%)	HHx (mol%)	RY[a] (%)	Purity (%)	HHx (mol%)	RY[a] (%)	PHA (%)	HHx (mol%)	RY (%)
MIBK[c]	96 ± 1	24 ± 0	71 ± 0	73 ± 2	12 ± 0	13 ± 0	13 ± 4	11 ± 0	84 ± 0
BA[c]	95 ± 2	24 ± 0	68 ± 0	62 ± 2	11 ± 0	8 ± 0	24 ± 1	12 ± 0	76 ± 0
EA[c, d]	97 ± 1	21 ± 0	94 ± 0				2 ± 0	20 ± 0	94 ± 0
MEK[c,d]	97 ± 2	21 ± 0	95 ± 0				2 ± 1	20 ± 0	95 ± 0

[a] PHA recovery yield (RY), total RY combines RY from PHA/solvent-solution and PHA/solvent-gel; [b] Residual cell mass; [c] MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; BA = butyl acetate; EA = ethyl acetate; [d] No PHA/solvent-gel formation was observed

Table 6.4: Larger scale recovery of P(HB-*co*-HHx) from dry and wet cells. PHA was extracted for 4 h at 100˚C, with the non-halogenated solvents to form a 2% PHA mixture. The extracted polymer was precipitated with 3 volumes of *n*-hexane at room temperature and dried at 50˚C.

Solvent, Vol.	Biomass (mol% HHx/PHA)	PHA recovered (g)		Purity (%)		HHx (mol%)		Recovery Yield (%)		
		S[a]	G[a]	S	G	S	G	S	G	S + G
MIBK[b], 3 L	wet (20)	45	10	92	90	20	15	69	15	84
MIBK, 3 L	wet (20)	37	2	>99	80–99	21	14	61	3	64
MIBK, 1.35 L	dry (29)	20	2	>99	78–99	30	12–18	74	5	79
EA[b], 1.5 L	wet (22)	21	nd[c]	95	nd[c]	21	nd[c]	71	nd[c]	71
EA, 1.78 L	dry (18)	33	nd	>95	nd	17	nd	93		93

[a] S = PHA/solvent solution; G = PHA/solvent gel; [b] MIBK = methyl isobutyl ketone; EA = ethyl acetate; [c] No PHA/solvent gel was detected in EA-based recovery of PHA.

Discussion

There are several requirements that must be met for a PHA production process to be sustainable and economically viable. High yield PHA production must be reached from a readily available carbon source [*e.g.* palm oil; (Riedel et al. 2012)]. Also, there must be an efficient recovery process that allows for consistent isolation of high purity polymer (Jacquel et al. 2008). The use of chlorinated solvents such as chloroform, methylene chloride or 1,2-dichlorethane has been shown to lead to high purity levels during PHB recovery (Ramsay et al. 1994). Use of non-halogenated solvents will reduce the hazards for the operators and for the environment. In this study, we designed a process for the recovery of P(HB-*co*->15mol%HHx) from bacterial biomass. Based on their physical properties and safety characteristics (Table 5.1), which are important for industrial scale-up process (*e.g.* energy to pump, energy to heat or cool, solvent separation from wastewater) and use of recovered bioplastics for different applications (*e.g.* food service, household, and medical products), respectively, the following solvents were chosen for evaluation of PHA recovery: MIBK, BA, EA and IA. All solvents, with the exception of IA, were able to effectively dissolve this polymer. We demonstrated PHA extraction from dry and wet cells at different scales, from 2 mL up to 3 L. We decided to focus on BA and MIBK, due to their lower miscibility's with water as compared to EA and MEK (Table 6.1), resulting in a better separation of the organic phase from the aqueous phase during PHA recovery from wet cells. Recovery from wet cells eliminates a biomass drying step from the downstream process, saving time and cost. With MIBK, we were able to recover PHA from wet cells with the same efficiency (recovery yield 84%) as from dry cells with purities reaching 99% (Table 6.3;

Table 6.4). With EA, the recovery yield with wet cells was 71% (Table 6.4), which was significantly lower compared to the recovery yield from dry cells (93–99%) (Table 6.2–6.4).

During PHA recovery with BA and MIBK, a separation of the copolymer occurred based on HHx content. PHA with a higher fraction of HHx monomer (17–30 mol%) was observed in the PHA/solvent-solution, whereas polymer with lower HHx fraction formed a PHA/solvent-gel (11–16 mol%) located between residual cell material and the PHA/solvent-solution. Also, small amounts of PHA containing low levels of HHx (11–12 mol%) remained in the residual cell material. This indicates that higher HHx content makes the polymer more soluble, as has been observed previously (Noda et al. 2005). It is unclear whether the gel was present throughout the extraction, or only appeared as the solution cooled during centrifugation. The fractionation of PHA during recovery confirms our previous finding that the strain used in this study makes PHA with varying HHx content during fermentation on palm oil (Budde et al. 2011).

The recovery yield from the MIBK and BA PHA/solvent-solutions were observed to be much lower in 2 mL extractions compared to the other extractions (Table 6.2–6.4). These results can be explained through a better separation of the PHA/solvent-solution from the PHA/solvent-gel due to greater force (higher rpm) during the centrifugation step of the larger scale extractions, as compared to the 2 mL extractions. Overall, MIBK had the capacity to recover more PHA from cells than BA in our studies (Table 6.2; Table 6.3). With non-gel forming solvents (MEK and EA) high recovery yields from 93 to 99% could be reached using dry cells (Table 6.2–6.4).

Chen et al. (2001) demonstrated the recovery of P(HB-*co*-11mol%HHx) from dry cells at an industrial scale using EA. In the aforementioned study, 5,000 L of EA was added to 200–500 kg dry cells, with a PHA content of 50%, to form 2–5% PHA solvent mixtures. Polymer was then precipitated with 3 volumes of *n*-hexane or *n*-heptane. Recovery yield or purity data from these extractions are not available. However, direct recovery from wet biomass would eliminate a drying step of the cells, potentially saving time and cost. In our study, the recovery yield from the EA extraction using wet cells as starting material in 1.5 L scale was 71%, much lower than the 93% recovery yield observed from the 1.8 L extraction using dry cells as starting material. The solubility of EA in water is 4 fold higher than that of MIBK (Table 6.1). The intermixture of the PHA/EA-solution with water and the wet residual cell material may explain the lower recovery yield from PHA/EA-solution using wet cells compared to dry cells. The residual cell mass from the EA extraction using wet cells showed a high PHA

concentration, which may have resulted from PHA solution becoming trapped in the biomass, whereas the PHA content of the residual cell mass from the dry 40 mL extraction was negligible (Table 6.3). Another possibility is that the presence of water simply reduced the solvating power of the EA, leaving some polymer unextracted. The 3 L scale up with MIBK using wet biomass exhibited a recovery yield up to 84%, which is the same recovery yield observed using dry cells in 40 mL extractions.

The purity of polymer from the 3 L MIBK extractions from wet cells improved to 99% by extra washing with *n*-hexane. The purity of the PHA recovered from wet cells with EA was slightly lower at 95%, although the same *n*-hexane wash was performed. The higher PHA purity reached with MIBK could be explained by the PHA/MIBK-gel formation, which covers the residual cell mass, separating it from the PHA/MIBK-solution. We did not filter PHA/solvent-solutions before the polymer precipitation in extractions of greater than 2 mL volumes. Therefore, the slight contamination seen in the EA extraction probably comes from residual cell material during the separation of the residual biomass from the PHA/EA-solvent-solution prior to PHA precipitation. All PHA extractions in our studies were performed using a PHA solvent ratio of 2%. All solvents used were shown to be capable of dissolving P(HB-*co*-33mol%HHx) to concentrations of 10%. Higher PHA concentrations would reduce the amount of solvent used, but would also result in more viscous PHA solutions (Van Walsem et al. 2007), which are more difficult to pump, centrifuge, or filter during downstream processing. The viscosity of polymer solutions is dependent on polymer structure, polymer molecular weight, concentration, solvent type and temperature (Flory 1953).

To recover dissolved polymer we chose to precipitate the polymer with alkanes, instead of evaporating the solvent. Evaporation can be problematic in batch operations because the polymer tends to coat the vessel after the solvent is removed. Additionally, any contaminants that are also present in the solvent (*e.g.* residual lipids from plant oil fermentations) will co-purify with the PHA. We determined that adding a threefold volume of precipitant to PHA/solvent solution at RT precipitated the polymer sufficiently (Figure 6.4; Table 6.2). A smaller ratio may be possible at a lower precipitation temperature. The boiling point of *n*-hexane is lower than that of *n*-heptane. This suggests that *n*-hexane should be easier to separate from both BA and MIBK, making it a more promising precipitant for these solvents, due to lower cost during solvent recycling. However, *n*-heptane is rated as a class 3 chemical by the FDA, while *n*-hexane is class 2, and is therefore considered as less safe than *n*-heptane (www.fda.gov). If PHA is destined for biomedical applications, then *n*-heptane may be the

preferred precipitant. If EA or MEK was chosen as the solvent, *n*-heptane or *n*-octane could be used as a precipitant due to the greater differences in their boiling points, as compared to *n*-hexane with the solvents.

It is possible that some residual palm oil and fatty acids may be associated with the biomass at the end of a high density fermentation. It was shown that these lipids dissolve in the solvents used in this work, but were not precipitated during the recovery process. However, after precipitation, residual solvent can be removed from the polymer by washing with precipitant, to avoid contamination of PHA with residual lipids.

For a recovery process using wet cells as starting material, we recommend the solvent/precipitant pair of MIBK/*n*-hexane, based on the polymer recovery results obtained in this work, as well as the large differences in boiling points, which predicts effective recycling of solvent through distillation. BA could be used alternatively to MIBK because it is less miscible with water, has a higher boiling point, is less flammable, and has a higher permissible exposure limit. However, the performed recovery studies showed higher recovery yields using MIBK. One potential issue with BA is that it can degrade by hydrolysis in the presence of water (Sakamuri 2005), which is clearly a concern given that in a sustainable process, solvent would be continuously recycled.

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CHAPTER 7

Conclusions and future work

Summary of goals and achievements

The general goal was to establish culture conditions for *R. eutropha* growth on palm oil for PHA production and the monitoring of the palm oil consumption. Malaysia is one of the biggest global producers of palm oil, which therefore is available in large quantities at a very competitive price compared to other plant oils. Palm oil has a high carbon content, which makes it an attractive carbon feedstock for PHA production with *R. eutropha*. In order to utilize palm oil as carbon source, *R. eutropha* secretes a lipase to emulsify the hydrophobic oil in the aqueous culture media. In terms of lipid content, palm oil contains mostly TAGs, which are continuously degraded during the emulsification process to DAGs, MAGs and finally to FFA, which are than consumed as carbon source for cell growth and PHA production of *R. eutropha*. The natural emulsification process leads to an extended culture lag phase, where it is not possible to take homogenous samples of the cultures until the oil has been emulsified. Chapter II described a plant oil emulsification method with gum arabic as an emulsification agent, which allowed shortening of the lag phase in culture, since the palm oil was bioavailable starting from the inoculation of the culture. Gum arabic was also not used as carbon or nitrogen source for cell growth. Through the pre-emulsification of the palm oil, it was possible to take homogenous samples throughout the entire duration of the cultures. During Chapter II, a lipid extraction method was developed, which allowed the full extraction of TAGs, DAGs MAGs and FFA from the culture media. Commonly used hexane extraction is only suitable for TAG recovery (Kahar et al. 2004). With the developed chloroform/methanol extraction, it was possible to quantify the palm oil that was used during the cultivation and to create a lipid profile. In six fold pre-emulsified palm oil fermentations, the function of the established methods were shown to be reproducible. During the cultivation, TAG molecules were broken, whereas the concentration of DAGs, MAGs and FFA increased. PHB was synthesized at a rate of 0.61 g PHB/ g PO over the time of fermentation, whereas the rate was 0.84 g PHB/ g PO during PHB production phase when nitrogen was limited and the residual biomass stagnated. The PHB yields are similar to other reported values in the literature where plant oil was used as the carbon source (Kahar et al. 2004; Ng et al. 2010)

One principal aim of this thesis was to develop a high-yield PHA production process with recombinant *R. eutropha* strains, which can produce the novel copolymer P(HB-*co*->10mol%HHx) using palm oil as the main carbon feedstock.

In Chapter III, metabolic engineering of *R. eutropha* H16 (wild type) is described, in order to generate recombinant strains which are capable of producing the desired copolymer. The engineered strains were screened in shaking flask cultivation experiments to determine both P(HB-*co*-HHx) accumulation and molar HHx content of PHA when palm oil was the sole carbon source. Two promising PHA production strains were identified: Re2058/pCB113 and Re2160/pCB113. The native class I PHA-Synthase (*phaC*) was deleted from both strains, since it cannot synthesize the desired *scl*-co-*mcl* polymer, P(HB-*co*-HHx). Additionally, the three *phaB* gene homologs where knocked out in Re2160/pCB113, which prevents the classic HB precursor synthesis, starting from acetyl-CoA (conversion of acetoacetyl-CoA to HB-CoA is blocked). The plasmid pCB113 carries a modified class I *phaC* gene from *R. aetherivorans*, which enabled *R. eutropha* to produce P(HB-*co*-HHx). In order to boost the molar HHx concentration in the polymer, the *phaJ* gene from *P. aeruginosa* was also inserted into pCB113. PhaJ catalyzes the formation of HHx-CoA molecules from enoyl-CoA, a β-oxidation intermediate.

Re2058/pCB113 accumulated 73% PHA/CDW with an HHx concentration of 13 mol% in shaking flask experiments, with 1% palm oil and 0.05% NH_4Cl in minimal media after 72 h, Re2061/pCB113, which could not use the released acetyl-CoA during β-oxidation of FFA for PHA production, accumulated 64% PHA/CDW with a high HHx concentration of 24 mol%.

To increase the total amount of PHA produced, first fermentation batch conditions with 4% palm oil, 0.4% NH_4Cl and no use of an emulsifying agent, were developed to increase the biomass. Whereas the described emulsification strategy with gum arabic in Chapter II is convenient to analyze bacterial growth with plant oils, or presumably other hydrophobic carbon sources, the use of an emulsifying agent is not desirable for an industrial process. It increases production costs through the amount used and through the additional energy consuming emulsification step. The use of gum arabic in industrial fermentations could also negatively influence the downstream processing.

Re2058/pCB113 produced under the described batch conditions 71% PHA/CDW with 21 mol% HHx and reached a total PHA production of 18 g/L. Re2160/pCB113 produced under the same conditions 66% PHA/CDW with 30 mol% HHx and reached a total PHA production of 12 g/L. It is notable that the HHx concentration in both strains was initially at

much higher levels (>40 mol%) and decreased over the course of experiment. This phenomenon is probably caused through decreasing competitions of specific molecule pools between metabolic pathways after the onset of nutrient limitation. For example, after nitrogen limitation, acetyl-CoA is no longer required for cell growth and can be turned more readily into HB-CoA molecules. This results in a change in the ratio of HB and HHx precursor molecule pool sizes. HHx precursor molecules are, through the overexpression of the heterologous *phaJ* gene, present consistently at higher levels upon the beginning of the cultures, which explains the very high HHx concentrations at these time points.

Re2058/pCB113 was chosen to further increase the total production of PHA, since it accumulated more PHA/CDW as Re2160/pCB113 and met the criteria of high HHx concentration in the polymer. In Chapter IV two fed-batch fermentation strategies are described with feeding both, palm oil and the nitrogen source (urea or NH_4Cl/NH_4OH). The strategy of using urea as the nitrogen source resulted in among the highest reported yields of a production a *scl*-co-*mcl* PHA copolymer. At the end of the fermentation, a total CDW of 140 g/L with a PHA content of 74% PHA/CDW and 19 mol% HHx where reached. The total productivity was >1 g PHA/L/h, which indicated that the process is scalable at an industrial level. The high biomass concentrations reached here also demonstrate the strong plasmid stability in Re2058/pCB113. No antibiotic pressure was needed to keep the plasmid in the cells, since the strain is proline autotrophic because of the deletion of a gene encoding an enzyme involved in proline synthesis (*proC*). The strain is “addicted” to the plasmid and as a result keeps it, since it carries the missing *proC* gene. The molecular weight of the polymer decreased over time from ~500,000 Da to 330,000 Da. This effect could be the result of polymer degradation during cultivation, or because of the formation of shorter polymer chains by the end of cultivation. Also, the developed methods from Chapter II were used to monitor the oil consumption of the cells during the fed-batch fermentation with urea. Over the entire fermentation, 0.63 g PHA/g PO was synthesized, whereas the rate was 0.78 g PHA/g PO, during PHA production phase, when nitrogen was limited. The production rate is comparable with the results in Chapter II. The concentration of TAGs continuously decreased over the course of the experiments, which indicates a balanced palm oil feeding, whereas the concentration of FFA increased until the end of the fermentation. The concentration of DAGs, MAGs were changing throughout the fermentation, which indicates that *R. eutropha,* respective its lipase, has no preferences for one or the other to cleave the FA from the glycerol backbone. However, a preference in the consumption of unsaturated FA was observed by analyzing the FA profile of the recovered lipids. Whereas the concentration of all FA stayed

almost constant during the feeding period, the concentration of the saturated FA stearic acid decreased five-fold in the media and the concentration of the unsaturated linoleic acid decreased four-fold in that same time frame.

In Chapter V, alternative carbon feedstocks to palm oil were evaluated for the production of PHA. Waste animal fats came into focus, since they are available in large quantities and also present at local markets which will most likely limit the need to import carbon feedstocks from other areas. They also have a high carbon content, but they do not compete with the food production industry. However, there is significant competition with the biodiesel industry concerning waste animal fats. Therefore, the main focus was on waste animal fats with low qualities and a high amount of FFA. Biodiesel generally cannot be effectively converted during basic catalysis from fat containing high amounts of FFA, since there occurs a recovery issue of the fatty acid methyl esters from the raw glycerol. The main chosen waste animal fat (ANiFAT_R2) had a FFA content of >50% and a high T_m of >45°C. These two parameters made it also very difficult to use this fat as carbon feedstock for fermentations. *R. eutropha* was not able to emulsify this fat under standard conditions, in shaking flask or batch fermentation experiments, as described in Chapter III. Therefore, the emulsification method described in Chapter II was adapted for initial experiments. For shaking flask experiments, also a careful pre-warming of the equipment during culture preparation enabled *R. eutropha* to emulsify, with extended lag phase, the waste animal fats on its own. This did not work for cultivation in fermenters, due to the different mixing and aeration techniques compared to shaking flasks. However, it was discovered that non-emulsified waste animal fat could be fed into an existing waste animal fat emulsion that was initially created using gum arabic. Based on this result, an emulsification strategy for waste animal fats was developed that does not need any chemical or mechanically pre-treatment. *R. eutropha* was first grown with 1% of a waste animal fat with a low T_m (<35°C), respectively with waste plant oil, which was liquid at RT. After the initial emulsion was built, liquefied waste animal fat was fed continuously to the fermentation broth. At the end of the best fed-batch fermentation with waste plant oil/ANiFAT_R2 (1% + 5%) and 0.45% urea, Re2058/pCB113 accumulated 58% PHA/CDW with 19 mol% HHx. The final CDW was 45 g/L, which resulted in a total PHA production of 26 g/L. The produced CDW and requisite HHx concentration, with the amount of nitrogen used, are comparable to the extended-batch fermentation shown in Chapter IV. FA determination of the animal waste fats showed a similarity to the FA profile of palm oil, which explains the comparable HHx contents of the polymer produced from waste animal fat and palm oil. The high T_m of ANiFAT_R2 caused several operational issues through clogging

of the feeding lines. Even when the fat was heated at 80°C, fed continually, and the feeding lines were heated with water/infrared light, this could not prevent clogging of the feed lines at all times. Also foaming, through the high content of FFA, was a much bigger issue than during the palm oil fermentations (Chapter IV). These described issues prevented at this time a continuation of the fed-batch fermentation process in order to yield comparable cell densities, as shown in Chapter IV. However, the PHA yield reached from directly using low quality waste animal fats, a very cheap biogenic waste material, is unique in the literature at the time of writing. Moreover, it is a very promising approach to accelerate PHA commercialization through providing the possibility to use an inexpensive carbon feedstock (US$50–200) that is not dependent on the biodiesel or food industries.

R. eutropha stores PHA in the form of intracellular granules. Solvent recovery with halogenated solvents like chloroform and using methanol as precipitant is still the standard method for recovery of high-purity PHA. In order to recover the PHA produced in Chapter IV and Chapter V from the surrounding biomass, a recovery method using non-halogenated solvents from wet and dry biomass was developed in Chapter VI. The copolymer with high HHx content (>20 mol%) was soluble up to 10% in MIBK, EA, MEK and BA. However, through increasing viscosity and impurities, 2% PHA solutions were used for PHA recovery. MIBK and EA were used as the main solvents, since they had the lowest solubility in water, which enhances the separation of waste water during the process. The low density of the non-halogenated solvents also simplified the processing, since the organic phase was the top phase and therefore better separable form waste water and cell debris. Hexane and heptane where used as precipitants, because of the big differences in the boiling points as compared to the chosen solvents. Recovery of spent solvent through distillation is possible and is required to decrease production costs. Purity levels from >99% were reached during PHA recovery. The recovery process was scaled up to 3 L starting from wet cells with a total recovery yield of up to 93%. During the recovery with MIBK, a unique separation technique was observed, which allowed the separation of P(HB-*co*-HHx), depending on its HHx content, in two groups. A PHA/MIBK-solution was observed with polymer containing >15 mol% HHx, and a PHA/MIBK-gel was observed with polymer containing <15 mol% HHx. The separation is based on the different solubilities of PHA based on the HHx content and occurs by cooling down the sample after the extraction to RT. P(HB-*co*-~10mol%HHx), could not be recovered with MIBK and remained in the residual cells. The separation of P(HB-*co*-HHx) within one batch and the decreasing HHx concentration during PHA synthesis (see Chapter III–V) shows that the different monomers are randomly distributed throughout an individual polymer chain.

The findings detailed in Chapters III–VI show that the HHx content of the polymer can be regulated through the carbon source, the bacterial strain used, the total fermentation time and the recovery technique.This thesis showed the development of a closed cycle for the accumulation, production and recovery of novel PHA biopolymers from plant oils and biogenic waste.

Unique P(HB-*co*-HHx) polymers with varying HHx concentrations were produced with feedstocks which are: inexpensive, renewable, and available in large quantities and, in terms of waste animal fats, have very low competition to other industries. High PHA production with >100 g/L PHA was demonstrated. Finally, an effective recovery process using non-halogenated solvents is shown together with a unique PHA separation technique during PHA recovery.

Opportunities for future work

Strain engineering

R. eutropha secretes a lipase, which allows for the emulsification of oil/fat in the aqueous media. The initial emulsification step is critical for growth on lipid based carbon sources. With increasing T_m of the oil/fat, the lag phases also increased or even prevented an emulsification of the supplied oil/fat. Besides the presented emulsification strategies (Chapter II; Chapter V) an overexpression of the lipase potentially can enhance the emulsification of oil/fat. Lu et al. showed a significant shortening of the lag phase, respective with initial emulsification time, of palm oil cultures during shaking flask experiments with a recombinant *R. eutropha* strain that overexpressed the native lipase gene *lipA* (Lu et al. 2013). However, using the same strain as the biocatalyst for palm oil fermentation led to massive over-foaming at the beginning of the fermentation and therefore led to termination of the experiment (data not shown). A possible solution would be the development of an inducible, reversible lipase overexpression system, which only overexpresses *lipA* until the initial emulsion is built. Light-mediated control of gene expression in bacteria and yeast has been reviewed previously (Drepper et al. 2011). It is imaginable to use light in the headspace of the fermenter vessel to control lipase activity in an engineered *R. eutropha* strain.

The molecular weight of PHA produced from Re2058/pCB113 decreased from ~500,000 Da to 330,000 Da over the course of the experiment. This has also been seen previously in other *R. eutropha* cultivations (Taidi et al. 1995). It has been reported that a molecular weight between 500,000 to 700,000 is preferred for many PHA processing applications (Noda et al. 2005). To increase the molecular weight for polymer produced with

Re2058/pCB113, the gene for the PHA synthase, *phaC,* could be removed from the PHA production plasmid pCB113 and inserted to the chromosome of Re2058. This would lead to a decreased expression of *phaC*, which would lead to a lower concentration of PhaC in the cell. The decreased concentration of PHA synthase would potentially lead to a reduction in initiation of new polymer chains, but at the same time enhance the propagation of longer polymer chains. It has been proposed previously that a greater expression of PHA synthase leads to shorter PHA chains (Sim et al. 1997). Another approach to increase the molecular weight of the PHA could be the deletion of the five depolymerase genes $phaZ_{1\text{-}5}$ of *R. eutropha*, to prevent depolymerisation during cultivation time. Since, it has been shown that some PHA depolymerases are active during the PHA production phase (Lawrence et al. 2005).

Waste lipids as carbon feedstock for PHA production

This chapter part was modified from a previously published article in Applied Microbiology and Biotechnology, 2014. 98: 1469-1483 'Lipid and fatty acid metabolism in *Ralstonia eutropha:* relevance for the biotechnological production of value-added products' Sebastian L. Riedel, Jingnan Lu, Ulf Stahl and Christopher J. Brigham © Springer-Verlag Berlin Heidelberg.

Outlook for PHA production using VFA, plant oils and waste fats as the sole C-source.

For a successful PHA production using *R. eutropha* as a biocatalyst, the interplay of several factors is needed. First, a strain is needed that accumulates the desired PHA polymer in high levels per CDW (>70%) without the addition of any antibiotics in the culture. Second, a widely available, inexpensive carbon feedstock is needed. Third, feeding strategies that allow for the accumulation of both high cell densities and high levels of PHA per CDW in the shortest possible time window are necessary for a maximum space time yield during fed-batch fermentation.

Efficient laboratory production of *scl* and *mcl* PHA copolymers during fed-batch fermentations have been shown with wild type and recombinant strains of *R. eutropha*, respectively, using (waste) plant oils, as sole carbon source, which were liquid at RT (Chapter I; Table 1.2). The next step would be to increase copolymer production to industrial scale. Furthermore, the adaptation of feeding strategies to other TAG-based inexpensive waste streams, such as industrial waste animal fats, is desirable. These fats are more challenging to use in a fed-batch fermentation scenario than the oils described above, since most animal fats are solid at RT (Chapter V). Using mixtures of VFA, experimentally proven models have been shown to diversify the *scl* monomer composition of P(HB-*co*-HV) (Yu et al. 2002; Yang et al. 2010). The idea is to alternate the composition of the VFA mixture to create tailor-made

polymers with desired properties. However, these studies have only been performed in low-density shake flask cultures, which are not suitable for production. These encouraging models need to be expanded and adapted to fed-batch fermentations to realize high productivity (*e.g.*, how fixed ratios over the course of the fermentation, changing ratios during different stages of growth and PHA production, and/or alternating concentrations of the feed stream have an influence over polymer composition).

Also, there is still potential to be reached in total PHA production with the discussed fed-batch fermentations (Chapter I; Table 1.2). The type of PHA monomer and the particular concentration of each monomer have great influence on the polymer properties. Recent metabolome studies by Fukui et al., (Fukui et al. 2013) using *R. eutropha* H16 have indicated the presence of greater intracellular 3HB-CoA and 3HHx-CoA pools during PHB production phase when octanoate (representing fatty acid β-oxidation) was used, instead of fructose as the sole carbon source. Varying pool concentrations of PHA precursor molecules could have an influence of the PHA monomer composition (Lindenkamp et al. 2012).

PHA recovery

The PHA recovery is one of the major costs during the production process. For the characterization of new polymers and some applications as medical products, high purity polymers are necessary. To meet these criteria, a solvent based recovery is still the first choice. PHA copolymers with high *mcl*-monomer composition can be recovered with non-halogenated solvents (Chapter VI), which makes the process “greener” for the environment and less harmful for the operator. For the next step, the descried PHA recovery process needs to be up-scaled to industrial level in an ex-proved facility. Besides upscale, several optimizations are possible. Washing of the precipitated polymer could be done with ethanol instead of hexane or heptane to reduce production cost. To save energy, the extraction process could be extended, but done at lower temperature. Instead of centrifugation a sedimentation process should be evaluated. Koller et al. and Madkour et al. reviewed recently the ongoing process development of PHA recovery (Koller et al. 2013; Madkour et al. 2013). However, working with base as sodium hydroxide solution degrades the molecular weight of the polymer. Detergent based recovery often leads to large amount of waste waters. A potentially favorable option is suggested to recover the polymer first mechanically followed by a solvent extraction using non-halogenated solvents. Therefore, fewer solvents would be needed for the extraction process.

PHA characteristics and application

The fed-batch fermentation described in Chapter IV and Chapter V, followed by using the recovery strategy developed in Chapter VI leads to the accumualation of, for a lab-scale PHA production, large amounts of PHA polymer. Also, through use of different recombinant strains, carbon sources and recovery techniques, P(HB-*co*-HHx) with a broad range of HHx concentrations is available. As a next step, more characteristics, besides purity, molecular weight and HHx content need to be determined to find a suitable and best possible application for the produced polymers. First, thermal analyses (data not shown) via differential scanning calorimetry provided compelling evidence to the connection between the HHx content of the copolymer and its thermal properties, *e.g.* T_m and glass transition temperature. The injection moulding parameter of the polymers should be developed and optimized to brighten the potential applications, followed by more mechanical testing. Static parameters should be investigated by (micro-) hardness, Young's modulus, and tensile strength and elongation tests. Dynamic parameters, such as notch impact strength should be investigated as well. The influence of "material aging" caused by UV rays, temperature change or air moisture should be investigated. While indeed PHA is a biodegradable material, it may be necessary to determine optimal recycling conditions for these copolymers, which would potentially decrease production costs and strengthen PHA's image as the overall environmentally friendly material.

Final thoughts

PHA Bioplastic is expensive. Current prices are ranging from 6–15 € per kg (Endres and Siebert-Raths 2011), depending on the production process. The main price factors are the carbon feedstock and the recovery process. The use of cheaper carbon sources is mandatory. A price range for PHA, using waste animal fats as feedstock, of 1–3 €/kg is conceivably. However, even by a large scale industrial production, the product price will not be able to compete with petroleum based plastic (0.8 €/kg PET, Alibaba 2015). Therefore PHA products have to target high valuable consumables, as medical products. Bulk markets will probably stay closed, until the price for crude oil increases. This thesis began when the buzzword "peak oil" was very actual and the world started making great efforts to look to alternative fuels. However, within one year the price for one barrel oil dropped by 50% (US$100 at 09/15/14 to US$48.14 at 09/13/15, Bloomberg 2015). The Organization of the Petroleum Exporting Countries (OPEC) continues to produce large amounts of oil, and with Iran a (new) potential world supplier waiting to begin to export more oil, depending on its changing geopolitical (trade sanction) situations (Petherick 2015). Moreover, with new (optimized) techniques, such

as hydrofracturing (fracking) (Howart et al. 2011), oil sand recovery (Tollefson 2012), using of biopolymers to enhance the properties of the conveying liquid during oil pumping (Wintershall holding GmbH 2015) or even exploitation of new grounds in deep sea (Ramirez-Llodra et al. 2011) or Antarctica (estimated storage of 200 billion barrels, Teller 2014); oil will be plentiful and plastic made from it will be cheap. Only a massive reduction in plastic consumption (buzzwords: plastic bag, packing), a stepwise substitution of petroleum based plastics with biodegradable polymers, together with a recycling system, could reduce the environmental disaster we are facing in this and the next century.

Nevertheless, there is a trend to produce convential plastics from renewable carbon sources, as *e.g.* bio-PET30, since MAG, one of the PET precursor molecules can be chemically synthesizes starting from bioethanol, which is available at industrial scale (30% MAG + 70% PTA → PET). Corn and sugar cane are major substrates for bioethanol production (Pimentel and Patzek 2005; de Souza Dias et al. 2015). With the trend of bio-PET30 or even a "bio-PET100", which was recently announced to be available at industrial scale from Coca Cola in 2020 (Guzman 2012), the competition of food or land to biofuel and bioplastic will increase dramatically in the future (based on an increasing bioethanol production, Pimentel and Patzek 2005). What is worse is that the public do not have the understanding, enhanced due to commercials (buzzword: plantbottle™), that bio-PET is not biodegradable and pollutes the environment when entering the nature, the same way as normal PET.

Hopefully this thesis can help to accelerate the industrial commercialization of PHA, a totally bio-based and biodegradable polymer in common habitats, by giving the opportunity to use a mostly competition free (*e.g.* to the biofuel or food industry) biogenic waste carbon feedstock for its production.

PHA for a better world!

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List of Abbreviations

Abbreviation	
AA	acetic acid
AN	acid number
ATPS	aqueous two-phase systems
BA	butyric acid or butyl acetate
CA	cellulose acetate or category
CDW	cell dry weight
C/N ratio	carbon/nitrogen ratio
C-source	carbon source
DAG	diacylglycerol
DIN	German Institute for Standardization
EA	ethyl acetate
FA	fatty acid
FAE	fatty acid esters
FDA	US Food and Drug Administration
FFA	free fatty acids
Gly	Glycerol
GPC	gel permeation chromatography
HA	3-hydroxyalkanoate
HB	3-hydroxybutyrate
HD	3-hydroxydecanoate
HHx	3-hydroxyhexanoate
HO	3-hydroxyoctanoate
HPLC	high performance liquid chromatography
HV	3-hydroxyvalerate
IA	isoamyl alcohol
LA	lactic acid
LAS	linear alkyl benzene sulfonic acid
LDPE	low-density polyethylene
MAG	monoglycerol or monoethylene glycol
mcl	medium chain length
MEK	methyl ethyl ketone

MIT	Massachusetts Institute of Technology
MIBK	methyl isobutyl ketone
M_n	number average molecular weight
Mt	mega ton
M_w	weight average molecular weight
OD_{600}	optical densities at 600 nm
PA	propionic acid
PBS	polybutylene succinate
PCL	Polycaprolactone
PDI	polydispersity index
PE	Polyethylene
PEG	polyethylene glycol
PEL	permissible exposure limit
PET	polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
P(HB-*co*-HHx)	poly(hydroxybutyrate-*co*-hydroxyhexanoate)
P(HB-*co*-HO)	poly(hydroxybutyrate-*co*-hydroxyoctanoate)
P(HB-*co*-HV)	poly(hydroxybutyrate-*co*-hydroxyvalerate)
PLA	poly lactic acid
PP	Polypropylene
PrOH	Propanol
PTA	purified terephthalic acid
PTFE	Polytetrafluoroethylene
PTT	polytrimethylene terephthalate
RCDW, rCDW	residual cell dry weight
Rcells	residual cell mass
RM	residual moisture
rpm	revolutions per minute
RT	room temperature
RY	recovery yield
scl	short chain length
SDS	sodium dodecyl sulfate
sFA	saturated fatty acid
STY	space time yield

TAG	Triacylglycerol
TLC	thin layer chromatography
T_m	melting temperature
Triton X-100	4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol
TSB	tryptic soy broth
Tween 80	polyoxyethylene (20) sorbitan monooleate
VFA	volatile fatty acids
Vvm	volume per volume per minute
Wgly	waste glycerol
WFO	waste frying oil
WRO	waste rapeseed oil

Resume

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