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**Tailored Medium-Chain-Length
Poly[(*R*)-3-hydroxyalkanoates]:
Biosynthetic and Chemical Approaches**

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SUMMARY

Polyhydroxyalkanoates (PHAs) are polyesters accumulated in a wide variety of bacteria as reserve material, when bacteria are supplied with an excess of carbon substrates, whereas at the same time the supply of nitrogen limits growth. PHAs are stored in form of granules and serve as carbon and energy source. The so called medium-chain-length (mcl) PHAs, a group of PHAs with a monomer length of 6 to 12 carbon atoms, have not only interesting polymer characteristics but are in addition biodegradable and can be produced from renewable resources. Furthermore, mclPHAs exhibit a high biocompatibility which allows an application of these polyesters in the medical sector. Although mclPHAs are considered as alternative to commercial petrochemical plastics since a while, so far, they were not commercially successful. The main reason for this may be due by the high production costs for PHAs compared with synthetic plastics. Present research therefore focuses on applications of this material in the medical sector, where only few petrochemical competing materials exist. For that purpose, tailor-made PHAs are required that fulfill clearly defined properties. Although to date, more than 100 different mclPHA monomers were identified, only a few mclPHAs have been made in quantities sufficient for a detailed analysis and characterization.

This doctoral thesis deals in a first part with the biosynthetic production and characterization of functionalized, tailored mclPHAs. Thereby the synthesis of tailored mclPHAs containing unsaturated side chains was the primary goal. First of all, the production of olefinic mclPHAs from mixtures of octanoic acid and undecenoic acid in batch and dual (carbon, nitrogen) nutrient limited chemostat cultures of *Pseudomonas putida* GPo1 was investigated. It could be shown that the monomeric composition of the accumulated PHAs depend in both production systems to a certain extent on the growth rate. Since the growth rate can be set exactly in chemostat cultures, this production method was evaluated to be the best for the production of tailored, olefinic mclPHAs (chapter 2). In a next step it was investigated to improve the thermal properties of olefinic mclPHAs. Therefore carbon substrate mixtures of phenylvaleric acid, octanoic acid and undecenoic acid were fed to chemostat cultures of *Pseudomonas putida* GPo1. The carbon to nitrogen (C_0/N_0) ratios in the cultivation media were chosen in such a way that dual (carbon, nitrogen)

limited growth resulted in each case. Five new, tailored PHAs could be produced that all contained an olefinic monomer ratio of 10 mol% but differed in the ratio of aromatic and aliphatic monomers. Thermal analysis of these PHAs showed a linear correlation between the glass transition temperatures of these polymers and the amounts of aromatic monomers (chapter 3). Furthermore, a two-stage continuous culture system was evaluated for the production of block polymers (chapter 4). The PHAs produced from mixtures of octanoic acid and undecenoic acid were characterized by gel permeation chromatography, differential scanning calorimetry and by crosslinking experiments. It turned out that the accumulated PHAs were mainly blends composed of an aliphatic and an olefinic polymer.

In the second part of this doctoral thesis, olefinic mclPHAs produced from octanoic acid and undecenoic acid, were chemically modified in order to obtain novel mclPHAs with improved properties. Firstly comb polymers were produced by modifying the olefinic side chains by a two-step synthesis (chapter 5). In contrast to the starting polymers, the modified polymers were crystalline materials with a filamentary texture. Furthermore silica nanoparticles were coupled to the terminal side chain double bonds of different olefinic PHAs (chapter 6). This resulted in glass-like mclPHAs with clearly higher melting points than the unmodified starting polymers.

The results obtained in this doctoral thesis show that tailored mclPHAs can be produced by biosynthetic procedures as well as by chemical modifications efficiently and in sufficient amounts for detailed material studies. This know-how of production provides a basis for the identification of new applications of mclPHAs in medicine or industry.

ZUSAMMENFASSUNG

Polyhydroxyalkanoate (PHAs) sind Polyester, die in einer Vielzahl von Bakterien als Reservestoff akkumuliert werden, wenn den Bakterien Kohlenstoffsubstrate im Überschuss angeboten werden, gleichzeitig aber das Stickstoffangebot das Wachstum limitiert. Die in Form von Granula eingelagerten PHAs dienen dabei den Mikroorganismen als Kohlenstoff- und Energiespeicher. Die sogenannten medium-chain-length (mcl) PHAs, eine Polyestergruppe deren Monomerlänge zwischen 6 bis 12 Kohlenstoffatomen liegt, haben nicht nur interessante Polymereigenschaften, sondern zeichnen sich auch dadurch aus, dass sie biologisch abbaubar sind und mit Hilfe von nachwachsenden Rohstoffen produziert werden können. Darüber hinaus weisen mclPHAs eine hohe Bioverträglichkeit auf, was eine Anwendung dieser Polyester im medizinischen Bereich ermöglicht. Obwohl mclPHAs seit einiger Zeit als Alternative zu kommerziellen, petrochemisch hergestellten Kunststoffen gehandelt werden, haben sie sich bis heute kommerziell nicht durchgesetzt. Der Hauptgrund dafür dürfte wohl darin liegen, dass PHAs gegenüber synthetischen Kunststoffen einen höheren Preis aufweisen. Die heutige Forschung fokussiert sich denn auch auf einen Einsatz dieses Werkstoffs im Medizinalsektor, wo es nur wenige petrochemische Konkurrenzmaterialien gibt. Gefragt sind hier massgeschneiderte PHAs, die klar definierte Anforderungen erfüllen. Obwohl bis heute weit über 100 verschiedene mclPHA Monomere gefunden wurden, sind nur ein paar Wenige mclPHAs in Mengen produziert worden, die eine detaillierte Analyse und Charakterisierung erlaubten.

Die hier vorliegende Doktorarbeit beschäftigt sich im ersten Teil mit der biosynthetischen Herstellung und Charakterisierung von funktionalisierten, massgeschneiderten mclPHAs. Im Fokus stand dabei die Synthese von massgeschneiderten mclPHAs mit ungesättigten Seitenketten. Als erstes wurde die Produktion von olefinischen mclPHAs aus Oktansäure und Undecensäure in Batch und doppelt (Kohlenstoff, Stickstoff) limitierten kontinuierlichen Kulturen von *Pseudomonas putida* GPO1 untersucht. Dabei konnte gezeigt werden, dass in beiden Produktionssystemen die Monomerzusammensetzung des gebildeten PHAs bis zu einem gewissen Masse von der Wachstumsrate abhängt. Da sich in kontinuierlichen Kulturen die Wachstumsrate genau einstellen lässt, wurde diese Produktions-

methode als geeignetes Verfahren zur Synthese von massgeschneiderten olefinischen mclPHAs evaluiert (Kapitel 2). In einem weiteren Schritt wurde dann versucht die thermischen Eigenschaften von olefinischen mclPHAs zu verbessern. Dazu wurden Kohlenstoffsubstratgemische aus Phenylvalerat, Oktansäure und Undecensäure kontinuierlichen Kulturen von *P. putida* GPo1 zugeführt. Die Kohlenstoff zu Stickstoff- (C_0/N_0) Verhältnisse im Kultivationsmedium wurde dabei so gewählt, dass jeweils doppelt (Kohlenstoff, Stickstoff) limitiertes Wachstum resultierte. Fünf neue massgeschneiderte PHAs konnten so produziert werden, die alle einen olefinischen Monomeranteil von 10 mol% aufwiesen, sich jedoch im Anteil an aromatischen und aliphatischen Monomeren unterschieden. Thermische Analysen dieser PHAs zeigten einen linearen Zusammenhang zwischen dem Glasspunkt der Polymere und deren Gehalt an aromatischen Monomeren (Kapitel 3). Im Weiteren wurde ein kontinuierliches Zweistufen-Kultivationsystem zur Produktion von Blockpolymere evaluiert (Kapitel 4). Die aus Oktansäure und Undecensäure gebildeten PHAs wurden mittels Gel-Permeations-Chromatographie, dynamischer Differenzkalorimetrie sowie durch Quervernetzungsexperimente charakterisiert. Es zeigte sich, dass die PHAs überwiegend Mischungen aus einem aliphatischen und einem olefinischen Polymer darstellten.

Im zweiten Teil dieser Doktorarbeit wurden olefinische mclPHAs aus Oktansäure und Undecensäure chemisch modifiziert, um neuartige mclPHAs mit verbesserten Eigenschaften zu entwickeln. Es wurden zunächst Kammpolymere hergestellt, indem die olefinischen Seitenketten über eine Zweistufensynthese modifiziert wurden. Die modifizierten Polymere waren im Gegensatz zum Ausgangspolymer kristallin und zeigten eine faserartige Textur (Kapitel 5). Des Weiteren wurden Siliziumnanoteilchen an die endständigen Doppelbindungen verschiedener olefinischer PHAs gekoppelt (Kapitel 6). Daraus resultierten glasartige mclPHAs mit deutlich höheren Schmelzpunkten als die Ausgangspolymere.

Die Resultate dieser Doktorarbeit zeigen, dass massgeschneiderte mclPHAs sowohl durch biosynthetische Verfahren als auch durch chemische Modifikationen effizient und in genügenden Mengen für detaillierte Materialstudien hergestellt werden können. Dieses Know-how bildet die Basis für die Identifizierung neuer Anwendungen von mclPHAs in der Medizin oder Industrie.

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CHAPTER 1

General Introduction

INTRODUCTION

Today, at the beginning of the twenty-first century, petrochemical plastics are omnipresent materials. The widespread use of plastics is the result of their extraordinary versatility and relatively low price. Much of the plastic that is produced is used for packaging but also for building materials, consumer products, automobiles, furniture, electrical components, and many other miscellaneous end uses. In 2003 the global plastic production was 202 million tons and the percentage increase by the year 2010 is expected to be 5% each year (Kunststoffe, 2004).

With the increased use of plastics, people have become concerned about their impact on the environment. One concern refers to the crude oil from which they are produced. Crude oil will probably run out at the end of the twenty-first century and therefore the use of this limited and nonrenewable resource for the large-scale manufacture of plastics clearly cannot be designated as a long-term and trendsetting technology. A second major concern is plastic waste. Moore et al. (Moore et al., 2001) reported that in surface waters between the coasts of California and Hawaii, the mass of plastic was approximately six times that of plankton.

Thus, the development of biodegradable plastics obtained from renewable resources has become an important topic of research and several biopolymers have been checked as alternatives to petrochemical plastics: cellulose, starch, chitin, polylactic acid, and poly(3-hydroxyalkanoates) (PHAs). PHAs are thermoplastic, biodegradable polyesters that are synthesized by many bacteria as carbon and energy storage compounds. They have been drawing much attention because of their similar material properties to conventional plastics (Lee, 1996). However, the commercialization of these materials has been prevented mainly by their high production cost. In 1996, the price of BIOPOL™ (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)) was at 16 US\$ kg⁻¹ (Lee, 1996) and therefore 20 to 30 times more expensive than the widely spread polyethylene, polystyrene or polypropylene. However, in the long run, it is possible that advances in fermentation and purification technology as well as the development of superior bacterial strains by recombinant DNA techniques could make the industrial scale production of these biopolymers competitive with oil-based synthetic polymers. Furthermore there are also approaches to reduce the production cost via agricultural production of PHAs after effective transfer of the essential genes into plants. The feasibility of this route has

been demonstrated in small plants such as *Arabidopsis thaliana* (Porier et al., 1995) but the transfer of this technology into crop plants like tobacco or potato with acceptable production levels is still at the research stage (Bohmert et al., 2002; Arai et al., 2004).

History of PHA

Polyhydroxyalkanoates (PHAs) are polyesters synthesized and stored in the form of intracellular granules by numerous bacteria. Poly(3-hydroxybutyrate) (PHB) was the first PHA discovered by the French scientist Lemoigne (Lemoigne, 1926). The polymer consists of 3-hydroxybutyric acid monomers that are linked through an ester bond between the 3-hydroxyl group and the carboxylic group of the next monomer (Figure 1.1). PHB was long thought to be the only type of polyester produced by microorganisms until polyesters containing hydroxyalkanoates units longer than hydroxybutyrate were isolated from microorganisms in sewage sludge in 1974 (Wallen and Rohwedder, 1974). That polymer was a copolymer containing 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as major components. Although the potential usefulness of PHB had already been recognized in 1962 (Baptist, 1962), the interest in the biopolymer remained directed almost exclusively at their physiological role (Douderoff and Stanier, 1959; Schlegel et al., 1961). In the mid seventies, when the crude oil price increased due to a predicted end of the oil reserves, the search for alternative plastic material led to industrial interest in PHAs. In 1982 Imperial Chemical Industries (ICI) began producing "BIOPOL", a copolyester containing randomly arranged units of 3HB and 3HV (Lenz and Marchessault, 2005). In 1993 Zeneca Bioproducts took along the polyester fermentation activities initiated by ICI and in 1996 Monsanto bought the production business from Zeneca. In 1998 Monsanto terminated its activity in this area and closed the PHB/HV production plant. However, several companies remain active in the PHB, PHB/HV production field such as Procter & Gamble and Metabolix in the USA and Biomer in Germany.

Beside the so called short-chain-length poly(3-hydroxyalkanoates) (scIPHAs), which consist only of the two monomers 3-hydroxybutyrate and 3-hydroxyvalerate, de Smet et al. (1983) observed in 1983 inclusion bodies consisting of a copolymer of 3-hydroxyoctanoate and 3-hydroxyhexanoate, when *Pseudomonas putida* GPo1

(ATCC 29347) (formerly *Pseudomonas oleovorans*) was grown in a two-liquid-phase batch culture with octane under ammonium limiting growth conditions. Some years later, Lageveen et al. (Lageveen et al. 1988) demonstrated that when *P. putida* GPo1 was grown on C₆ to C₁₂ n-alkanes and n-alkenes under ammonium limiting growth conditions, PHAs were formed which contain C₆ to C₁₂ 3-hydroxy fatty acids. These PHAs were classified as medium-chain-length (mcl) PHAs and were subsequently found in other fluorescent pseudomonads such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, or *Pseudomonas fluorescens* (Haywood et al., 1989; Huisman et al., 1989).

Although mclPHAs exhibit enormous variety in their monomeric composition, to date they have not been produced on a commercial scale. This is due in part because the final mclPHA concentration and cellular content obtained have been relatively low compared with those same parameters for sclPHAs. Initial studies showed that in a fed-batch culture of *P. putida* GPo1, mclPHA concentration and mclPHA cellular content obtained in 38 h were 12.1 g L⁻¹ and 33 wt%, resulting in a mclPHA productivity of 0.32 g L⁻¹ h⁻¹ (Preusting et al., 1993). In contrast, in a fed-batch culture of *Alcaligenes eutrophus*, sclPHA concentration and sclPHA content obtained in 50 h were 121 g L⁻¹ and 76 wt%, resulting in a sclPHA productivity of 2.42 g L⁻¹ h⁻¹ (Kim et al., 1994).

However, production of mclPHAs by *P. putida* in fed batch cultures has been improved significantly (Lee et al., 2000b). By using phosphorous limitation and an optimized feeding strategy, the mclPHA concentration and mclPHA content obtained in 38 h were increased to 72.6 g L⁻¹ and 51.4 wt%, respectively, resulting in a high mclPHA productivity of 1.91 g L⁻¹ h⁻¹. Jung et al. (Jung et al. 2001) demonstrated that reasonably efficient production of mclPHAs can also be achieved in continuous culture systems. By using a two-stage continuous process a mclPHA productivity of 1.06 g L⁻¹ h⁻¹ was determined.

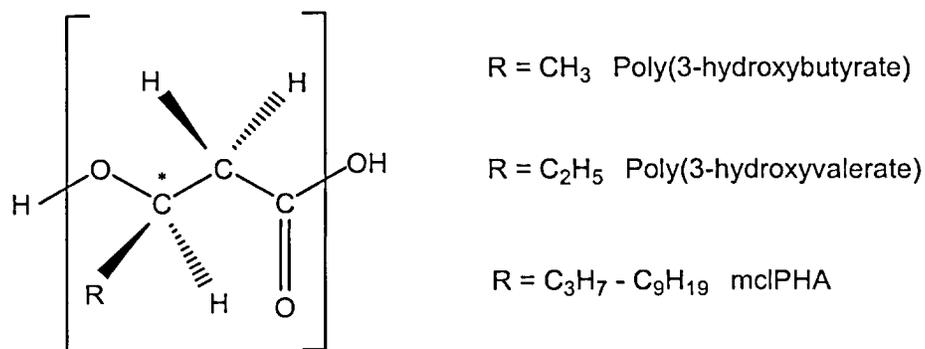


Figure 1.1: Chemical structure of different poly(3-hydroxyalkanoates) (PHAs). All monomers have one chiral center (*) in the R position.

Chemical structure and diversity of mclPHA

MclPHAs exhibit a wide variety in their monomeric composition and more than 100 different monomers have been found in biosynthetic mclPHAs (de Rijk et al., 2002). To date, *P. putida* GPO1 is the best investigated mclPHA producer. Besides a large variety of linear (de Smet et al., 1983; Brandl et al., 1988; Lageveen et al., 1988; Gross et al., 1989) or branched side chains (Fritzsche et al., 1990b; Lenz et al., 1992), various mclPHAs with side chains containing functional groups such as carbon-carbon double (Lageveen et al., 1988) and triple bonds (Kim et al., 1998), acetoxy and ketone (Jung et al., 2000), or aromatic groups (Fritzsche et al., 1990a; Curley et al., 1996; Kim et al., 1999) have been produced in *P. putida* GPO1 (Figure 1.2).

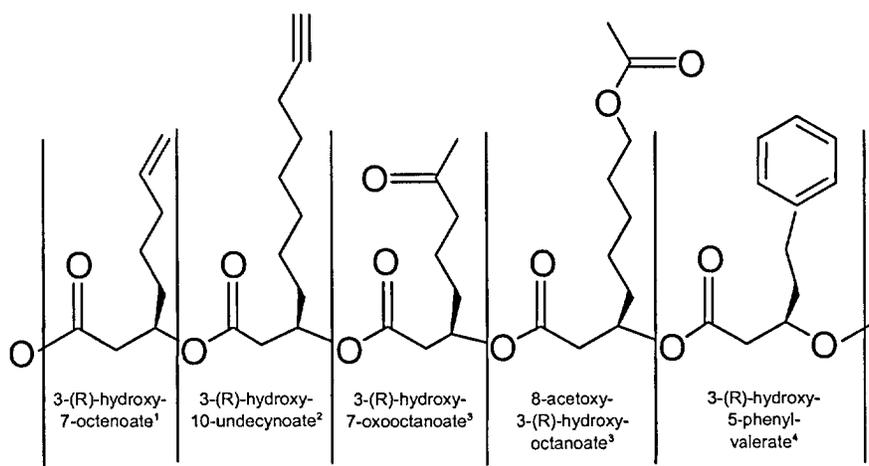


Figure 1.2: Examples of mclPHAs containing functional groups. (¹Lageveen et al., 1988; ²Kim et al., 1998; ³Jung et al., 2000; ⁴Fritzsche et al., 1990a).

Furthermore, side chains with chlorinated (Doi and Abe, 1990), brominated (Kim et al., 1992), fluorinated (Abe et al., 1990; Hori et al., 1994) as well as cyanoalkyl (Lenz et al., 1992) or epoxy (Bear et al., 1997) groups have been reported in mclPHAs accumulated by *P. putida* GPo1. Thus, *P. putida* GPo1 exhibits an unusual versatility in its ability to incorporate a multiplicity of mcl-monomers into PHAs. This feature, as well as the fact that *P. putida* GPo1 accumulates mclPHAs up to a cellular content of 63 wt% (Jung et al., 2001), makes this strain an excellent candidate for the production of tailored mclPHAs.

Pathways in the synthesis of mclPHAs

Depending on the bacterial host, different pathways are involved in the synthesis of mclPHAs. When *P. putida* GPo1 was grown on fatty acids, alkanes or alkanols consisting of 6 to 12 carbon atoms, mclPHAs were found composed of monomers that were structurally closely related to the feed carbon source (Lageveen et al., 1988). The number of carbon atoms in the monomers were identical to the numbers of carbon atoms in the substrate supplied or have been shortened by 2, 4 or 6 carbon atoms. However, no mclPHA formation was observed when *P. putida* GPo1 was grown on small organic molecules such as glucose or citrate (de Smet et al., 1983; Haywood et al., 1989). Therefore it was suggested that intermediates of the fatty acid β -oxidation cycle such as 2-*trans*-enoyl-CoA, S-3-OH-acyl-CoA, 3-keto-acyl-CoA are channeled to mclPHA synthesis (Lageveen et al., 1988). However, since mclPHA monomers are in the *R*-form, an additional biosynthetic step is required between the intermediates of the fatty acid β -oxidation cycle and the mclPHA synthesis. Whether this is the product of a reaction catalyzed by a *R*-specific enoyl-CoA hydratase, by a 3-OH-acyl-CoA epimerase or by a 3-keto-acyl-CoA reductase is unknown (Figure 1.3).

In contrast to *P. putida* GPo1, most pseudomonads belonging to rRNA homology group I also synthesize mclPHAs from acetyl-CoA via *de novo* fatty acid synthesis (Haywood et al., 1990; Timm and Steinbüchel, 1990; Huijberts et al., 1992). Rehm et al. (Rehm et al. 1998) identified a *R*-3-OH-acyl-ACP-transferase that catalyzed the conversion of *R*-3-OH-acyl-ACP to *R*-3-OH-acyl-CoA and therefore links *de novo* fatty acid synthesis to mclPHA synthesis (not indicated in Figure 1.3).

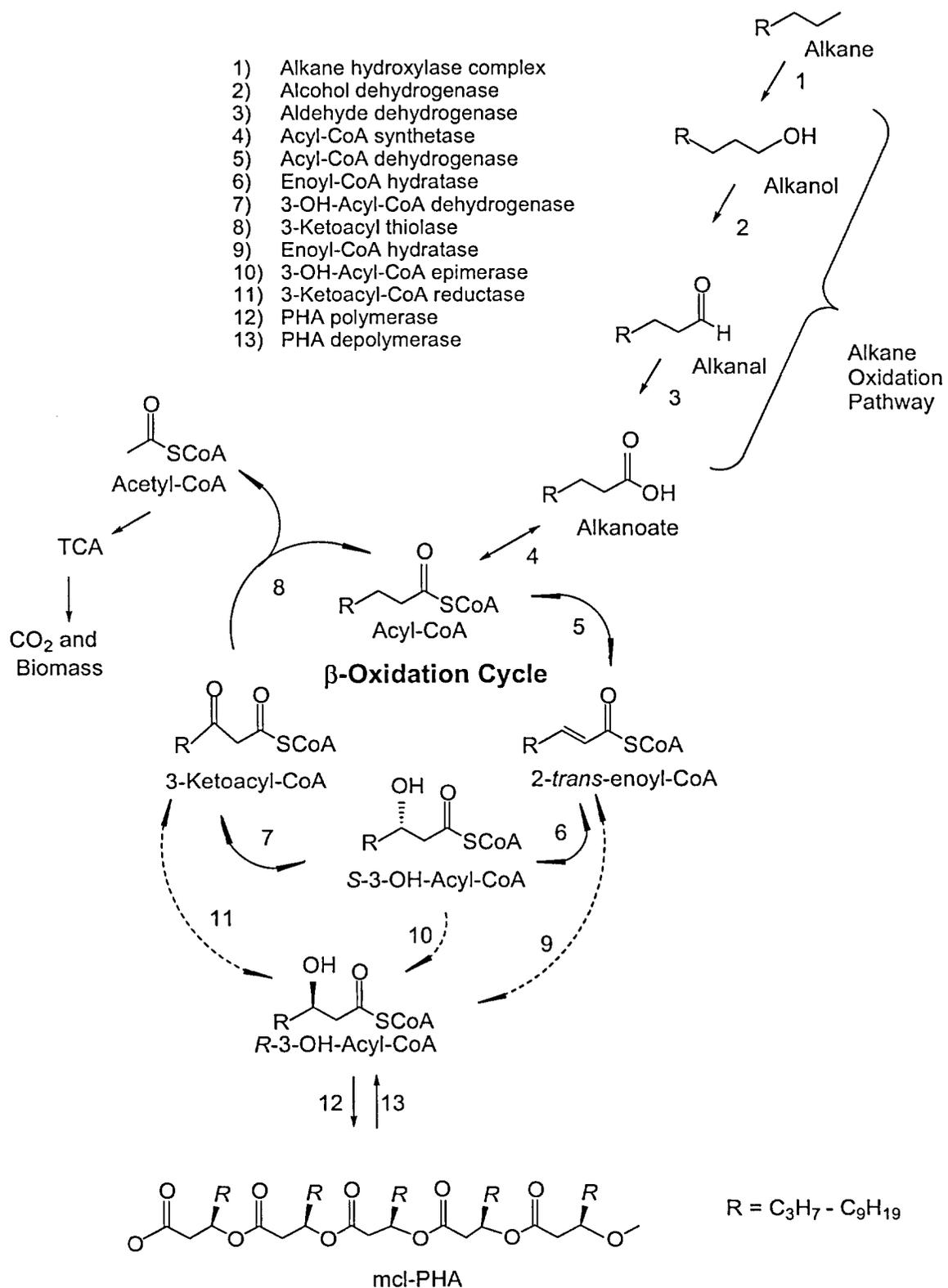


Figure 1.3: Pathways in the synthesis of mclPHAs in *P. putida* GPO1. The OCT plasmid enables *P. putida* GPO1 to degrade alkanes via the alkane oxidation pathway (van Beilen et al., 1994). Dashed arrows represent hypothetical steps.

Biotechnological production systems of PHAs

Besides the *in vitro* biosynthesis of PHAs from synthetic 3-hydroxyacyl-CoA precursors (Gerngross and Martin, 1995; Kamachi et al., 2001) and the synthesis in transgenic plants (Poirier et al., 1992; Poirier et al., 1995; Bohmert et al., 2002; Arai et al., 2004), the method of choice is the fermentative production using bacteria. Various culture methods such as batch, fed-batch or continuous culture can be used for this. Batch fermentations are technically simple and a common method to produce small amounts of PHAs. In its simplest form, a batch experiment can be performed in a shake flask and no expensive equipment is required. Generally, PHA accumulation is triggered after the onset of a nutrient limitation such as limitation of nitrogen, phosphorus, magnesium or oxygen while a suitable carbon source is in excess (Lee, 1996). However, a major disadvantage of this cultivation technique is that substrate toxicity may impair cell growth during the initial growth phase (long lag phase), or even inhibit cell growth. Furthermore, insufficient aeration may prevent exponential cell growth (Preusting et al., 1993). Batch cultivation may also be problematic regarding the homogeneity of the accumulated PHA because the monomeric composition or the molecular mass of the polymer can be a function of time (Kim et al., 1991).

An artificially prolonged batch culture, called fed-batch culture, is often used for the production of large amounts of PHA. This cultivation technique is made up of a batch culture that is continuously supplemented with nutrients after it enters the late exponential phase (Pirt, 1975). The advantage of fed-batch cultures in general is the high cell densities that can be obtained (Preusting et al., 1993; Wang and Lee, 1997; Lee et al., 1999). A problem of this process is that the cells grow at a decreasing growth rate. This is because nutrients supplied at a constant rate are consumed by an ever increasing biomass concentration. This can lead to unexpected losses in PHA production (Suzuki et al., 1986) or in a shift in the polymer composition.

The continuous production of PHA in a chemostat is the most controlled cultivation method. A chemostat is essentially a perfusion reactor that is continuously supplied with sterile medium. The volume in the reactor is kept constant by the continuous removal of culture broth. Once such a system is in equilibrium (steady-state), cell

number, nutrition, and productivity remain constant over time (Herbert et al., 1956). Only recently it has been shown that PHA production can be triggered in a chemostat under simultaneous limitation by carbon (C) and nitrogen (N) substrates (Durner et al., 2000; Durner et al., 2001; Zinn et al., 2003; Hartmann et al., 2004). Durner et al. (Durner et al. 2000) defined a so called dual nutrient (C,N) limited growth regime (DNLGR) and showed it to be a function of the dilution rate and the carbon to nitrogen ratio (C_0/N_0) of the medium feed (see Figure 1.4).

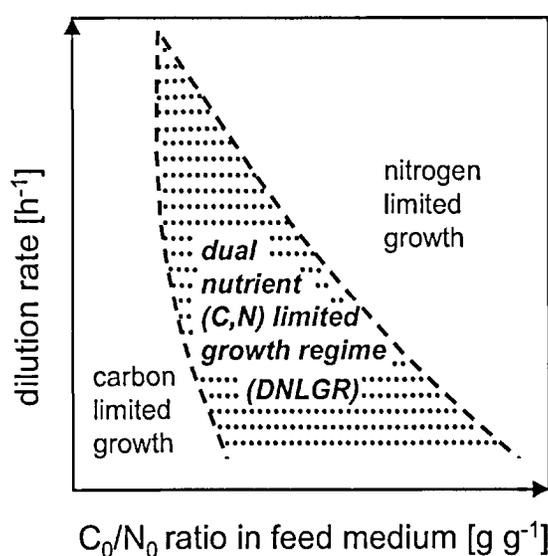


Figure 1.4: The dual nutrient (C,N) limited growth regime (DNLGR) is a function of the dilution rate and the carbon to nitrogen ratio (C_0/N_0) of the medium feed. The DNLGR is broader at low growth rates and becomes narrower at high growth rates. The zone is a function of the growth rate, the redox state of the carbon source and the microorganism (Egli, 1991).

The shape of this DNLGR can be determined experimentally or calculated from the elemental yield coefficients ($Y_{X/C}$ and $Y_{X/N}$) obtained under single-nutrient-limited growth conditions according to the method of Egli and Quayle (Egli and Quayle 1986). Within the DNLGR all fed C- and N-substrates are completely metabolized and it was proposed that this dual nutrient (C,N) limited growth is a suitable method to produce PHA even from a toxic substrate. Further, it has been demonstrated that this DNLGR offers an excellent tool to tailor PHA composition during biosynthesis. Zinn et al. (Zinn et al. 2003) found that when a culture of *Ralstonia eutropha* was grown in a chemostat under simultaneous limitation by carbon (butyric and/or valeric

acid) and nitrogen (ammonia), the composition of the isolated poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/HV) was a function of the carbon substrate mixture. They demonstrated that the fraction of 3-hydroxyvalerate in PHB/HV could be reproducibly controlled between 0 and 62 mol% and the melting temperature was significantly influenced by the polymer composition. The authors demonstrated that the melting temperature could be tuned between 178 °C and 80 °C via the controlled incorporation of 3-hydroxyvalerate in PHB/HV.

Chemical modifications of biosynthetic PHAs

Although chemical modifications offer an interesting tool to modulate the basic polymer properties or to create functionalities which are impossible to introduce by biosynthesis, detailed studies on the chemical modification of biosynthetic PHAs are still rare.

In general, two kinds of chemical modification of biosynthetic PHAs can be distinguished. The first one involves chemistry on the polymer backbone, for example the complete hydrolysis of the PHA polymer to chiral hydroxyalkanoate monomers (de Roo et al., 2002). Also the degradation to PHA oligomers followed by the synthesis of block-copolymers was described (Hirt et al., 1996a; Hirt et al., 1996b; Saad et al., 1999; Andrade et al., 2002a; Andrade et al., 2002b). The second kind of modification targets the polymer side chain. In particular, reactions analogous to those used with petro based polymers have been carried out with mclPHAs containing unsaturated side chains: cross-linking (de Koning et al., 1994; Dufresne et al., 2001; Hazer et al., 2001), epoxidation (Bear et al., 1997), conversion of double bonds to diols (Lee et al., 2000a) and conversion of double bonds to carboxylic groups (Kurth et al., 2002; Stigers and Tew, 2003) have thus far all been reported.

Applications of PHAs

PHAs are thermoplastic, biodegradable polyesters that exhibit material properties similar to those seen for conventional plastics (Lee, 1996). Hence the majority of the PHA applications which were initially explored were aimed at replacing petrochemical polymers currently in use for packaging and coating applications. PHAs were initially used mainly in the manufacture of bottles and fibres for biodegradable packaging

materials (Holmes 1985). However, applications of PHAs are not restricted to these areas. Due to their biocompatibility, PHAs represent a class of polymers with an immense potential for medical applications. PHAs have been investigated as drug carriers (Pouton and Akhtar, 1996; Sendil et al., 1999) or as scaffold material in tissue engineering (Sodian et al., 2000). Moreover, the use of PHAs as skin substitutes, cardiovascular fabrics, bone graft substitutes or internal fixation devices (e.g. screws) has also been considered (Zinn et al., 2001). Finally, PHAs are also considered as source for the synthesis of enantiomerically pure chemicals since all of the PHA monomers are in (*R*)-configuration (Witholt and Lageveen, 1986; Haywood et al., 1988; Madison and Huisman, 1999). Such *R*-3-hydroxyalkanoic acids are valuable synthons and can be widely used as starting materials for synthesis of antibiotics, pharmaceuticals, vitamins, flavours and pheromones (Qun et al., 2005).

Aim and scope of the thesis

As previously mentioned, PHAs are natural thermoplastic polyesters with great potential in industrial and medical applications. For that purpose, tailor-made PHAs are required that fulfill clearly defined properties. The aim of the work presented in this thesis was the production and characterization of tailored mclPHAs, obtained directly via biosynthesis (Chapters 2-4) or by chemical modifications of biosynthetic mclPHAs (Chapters 5 and 6).

Chapter 2 describes the production of olefinic mclPHAs from mixtures of octanoic acid and 10-undecenoic acid in batch and dual nutrient (C,N) limited chemostat cultures of *Pseudomonas putida* GPo1. In a batch culture, where *P. putida* GPo1 was grown on a mixture of octanoic acid (58 mol%) and 10-undecenoic acid (42 mol%), it was found that the fraction of aliphatic monomers was slightly lower in mclPHA produced during exponential growth than during late stationary phase. Thus the monomeric composition changed over time indicating different kinetics for the two carbon substrates. Chemostat experiments showed that the dual nutrient (C,N) limited growth regime (DNLGR) for 10-undecenoic acid coincided with the one for octanoic acid. Five different chemostats on equimolar mixtures of octanoic acid and 10-undecenoic acid within the DNLGR revealed that the monomeric composition of mclPHA was not a function of the carbon to nitrogen (C_0/N_0) ratio in the feed medium but rather of the dilution rate. **Chapter 3** documents the production of mclPHAs from

different mixtures of octanoic, 10-undecenoic, and 5-phenylvaleric acid in a chemostat under dual nutrient (C,N) limited growth conditions. Five new, tailor-made copolymers were produced and consisted of poly(3-hydroxy-5-phenylvalerate-co-3-hydroxyalkanoates-co-3-hydroxy- ω -alkenoates), poly(HP-co-HA-co-HE), with increasing amounts of aromatic side chains (A: 0%, B: 3%, C: 19%, D: 42% and E: 59%), approximately 10 mol% unsaturated side chains and decreasing amounts of saturated side chains. This concept allowed the tailored synthesis of novel, olefinic PHAs with increased glass transition temperatures due to the integration of phenyl groups into the polymer. **Chapter 4** describes the cultivation of *P. putida* GPo1 in a sequence of two dual nutrient (C,N) limited chemostat cultures at different dilution rates. The cells were supplied with different amounts of octanoic acid in the first fermentor and different amounts of 10-undecenoic acid in the second fermentor in order to see whether it is possible to produce block copolymers in such a two-stage continuous culture system. The polymers isolated from the second-stage fermentor contained different amounts of aliphatic and olefinic PHA monomers. Their characterization by gel permeation chromatography, differential scanning calorimetry and their crosslinking ability indicated that the obtained mclPHAs were not block copolymers but mainly a physical mixture of polymers produced from octanoic acid (PHO) and 10-undecenoic acid (PHU). The crosslinking-extraction experiments indicated that these polymers might contain small amounts of repeating units from PHO and PHU.

The next two chapters deal with chemical modifications of biosynthetic mclPHAs. **Chapter 5** presents the synthesis of comb polymers by a two-step synthesis from a bacterial poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate] containing 25 mol% terminal side chain double bonds. The radical addition reaction of 11-mercaptoundecanoic acid to the side chain alkenes produced a derivative containing thioether bonds with terminal carboxyl functionalities, which was subsequently transformed into the amide or ester derivative using tridecylamine or octadecanol, respectively. This modification led to comb polymers which were crystalline materials. **Chapter 6** documents the synthesis of inorganic-organic hybrid polymers. Mercaptopropyl-isobutyl-POSS (POSS-SH) was linked via a free radical addition reaction to the side-chain double bonds of bacterial poly(3-hydroxyalkanoate-co-3-hydroxyalkenoate), (PHAE). PHAE with 11.5, 55, 78, and 97 mol% of double bonds were used to produce a series of POSS-PHAE inorganic-organic hybrid materials

with increasing amounts of POSS. The reactions proceeded with high yields and minor side reactions, which was confirmed with NMR and GPC experiments. The appearance of these POSS-based biopolyesters varied from non-sticky and elastic, to brittle and glass-like with increasing POSS content. The chemical attachment of POSS to PHAE increased the glass transitions and melting points. As a result, melting points could effectively be tuned between 48 °C and 120 °C. **Chapter 7** presents the conclusions of this thesis and discusses the importance of PHAs with tailor-made properties.

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CHAPTER 2

Tailor-made Olefinic Medium-Chain-
Length Poly[(*R*)-3-hydroxyalkanoates]
by *Pseudomonas putida* GPo1: Batch
versus Chemostat Production

Abstract: Functionalized medium-chain-length polyhydroxyalkanoates (mclPHAs) have gained much interest in research on biopolymers because of their ease of chemical modification. Tailored olefinic mclPHA production from mixtures of octanoic acid and 10-undecenoic acid was investigated in batch and dual nutrient (C,N) limited chemostat cultures of *Pseudomonas putida* GPo1 (ATCC 29347). In a batch culture, where *P. putida* GPo1 was grown on a mixture of octanoic acid (58 mol%) and 10-undecenoic acid (42 mol%), it was found that the fraction of aliphatic monomers was slightly lower in mclPHA produced during exponential growth than during late stationary phase. Thus the total monomeric composition changed over time indicating different kinetics for the two carbon substrates. Chemostat experiments showed that the dual nutrient (C,N) limited growth regime (DNLGR) for 10-undecenoic acid coincided with the one for octanoic acid. Five different chemostats on equimolar mixtures of octanoic acid and 10-undecenoic acid within the DNLGR revealed that the monomeric composition of mclPHA was not a function of the carbon to nitrogen (C_0/N_0) ratio in the feed medium but rather of the dilution rate. The fraction of aliphatic monomers in the accumulated mclPHA was slightly lower at high dilution rates and increased towards low dilution rates, again indicating different kinetics for the two carbon substrates in *P. putida* GPo1.

INTRODUCTION

Pseudomonas putida GPo1 (ATCC 29347) (commonly known as *Pseudomonas oleovorans*, van Beilen et al., 2001) is a member of the rRNA homology group I of fluorescent pseudomonads, which is able to accumulate poly[(R)-3-hydroxyalkanoates] of medium-chain-length side-chains (mclPHAs) as intracellular carbon and energy storage compounds (Huisman et al., 1989; Timm and Steinbüchel, 1990). These mclPHAs, which contain C6-C12 3-hydroxyalkanoate monomers, are natural polyesters with glass transition temperatures (T_g) between -44 and -30 °C and melting temperatures (T_m) between 39 and 61 °C (Gross et al., 1989; Preusting et al., 1990; Witholt and Kessler, 1999). Generally the material has low crystallinity and is rather flexible and soft. Today, more than 100 different monomers have been shown to be incorporated into mclPHAs (de Rijk et al., 2002). PHA accumulation in *P. putida* GPo1 has been reported to be enhanced, when the bacteria are exposed to a surplus of carbon, while cell growth is restricted due to the

absence of an essential nutrient such as nitrogen (Lageveen et al., 1988). Such conditions can be achieved in batch or fed-batch cultures (Brandl et al., 1988; Gross et al., 1989; Preusting et al., 1993). Recently, it has also been shown in chemostat cultures of *P. putida* GPo1 that PHA production can take place when nitrogen and carbon are limiting growth simultaneously (dual nutrient (C,N) limited growth) (Durner et al., 2000; Zinn et al., 2003). Various mclPHAs with side chains containing functional groups, for example carbon-carbon double (Lageveen et al., 1988) and triple bonds (Kim et al., 1998), acetoxy and ketone (Jung et al., 2000), or aromatic groups (Curley et al., 1996; Fritzsche et al., 1990a; Kim et al., 1999) have been produced. The presence of unsaturated side chains provides sites for chemical modifications like crosslinking (de Koning et al., 1994; Dufresne et al., 2001; Hazer et al., 2001), epoxidation (Bear et al., 1997), conversion to carboxylic (Kurth et al., 2002; Stigers and Tew, 2003) and diol groups (Lee et al., 2000a), or the synthesis of crystalline comb polymers (Hany et al., 2004a). In this work, we investigated the production of olefinic mclPHAs from mixtures of octanoic acid and 10-undecenoic acid in batch and dual nutrient (C,N) limited chemostat cultures. In particular we were interested which system is more suitable to produce olefinic mclPHAs with a defined monomeric unit composition.

MATERIALS AND METHODS

P. putida GPo1 (ATCC 29347) was kept as frozen stock at -80 °C in 15 % glycerol and used for all experiments. For the preparation of inocula, 1 mL frozen stock culture was added to 100 mL of minimal medium in 300 mL shake flasks and grown at 30 °C until the optical density had reached an OD (450 nm) of 1.5. The minimal medium contained per liter: 3.5 g $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 7.5 g K_2HPO_4 , 3.7 g KH_2PO_4 , and 2.9 g $\text{Na}_3\text{citrate} \cdot 2\text{H}_2\text{O}$ (Vogel and Bonner, 1956). The pH was adjusted to 7.1 with 10 M NaOH. This medium was autoclaved and subsequently supplemented with filter sterilized $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 mL L⁻¹, 1M) and 1 mL L⁻¹ of MT (mineral trace element) stock solution which contained per liter: 2.78 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.98 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.81 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 M HCl (Lageveen et al., 1988). Fifty mL of shake-flask culture were used as inocula for shake flasks and for chemostat culture experiments. Inocula

for batch experiments in the fermentor were prepared in the same way, except that the cells were precultivated with the same carbon source that was later used.

The minimal medium for all batch experiments (shake flasks / fermentor) contained per liter: 1.67 g $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 7.5 g K_2HPO_4 , 3.7 g KH_2PO_4 , and 10-undecenoate or mixtures of 10-undecenoate and octanoate, respectively. The pH was adjusted to 7.1 and the media were supplemented with filter sterilized MgSO_4 and MT as above. For chemostat cultivation the following medium was used (per liter): 1 g KH_2PO_4 , 0.71 g $(\text{NH}_4)_2\text{SO}_4$, and 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Further, 1 L of the medium was supplemented with 1 mL of 10 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (in 1 M HCl) and 1 mL of chemostat culture mineral trace element (CCMT) stock solution containing per liter: 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.98 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.81 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 g EDTA at pH 4 (Durner et al., 2001). Forty liter of this medium were then filter sterilized into gamma sterilized 50 L medium bags (Flexboy, Stedim S.A., Aubagne Cedex, France). Particular mixtures of octanoic and 10-undecenoic acid were pumped directly into the culture vessel by using a dosimat (Metrohm, Herisau, Switzerland). The carbon to nitrogen ratio of the feed (C_0/N_0) was set by the pump rates of the ammonium containing minimal medium and the carbon sources.

Cultivation conditions: Batch experiments in shake flasks were performed in 1 L Erlenmeyer flasks containing 500 mL minimal medium. Cells were cultivated for 24 h under aerobic conditions in a temperature-controlled shaker at 30 °C and 140 rpm (Lab-Therm LT-W, Kühner AG, Birsfeld, Switzerland). All batch experiments in the fermentor, as well as all chemostat culture experiments were performed in a 3.7 L laboratory bioreactor (KLF 2000, Bioengineering, Wald, Switzerland) with a working volume of 2.8 L. The cultures were run at 30 °C and the pH was maintained at 7.0 ± 0.05 by automated addition of either 2 M NaOH or 2 M H_2SO_4 . The dissolved oxygen tension was monitored continuously with an oxygen probe (Mettler Toledo, Greifensee, Switzerland) and care was taken that it remained above 35 % air saturation. Chemostat culture experiments were performed at different dilution rates ($0.1 - 0.4 \text{ h}^{-1}$). For this, the bioreactor was placed on a balance which controlled a harvest pump to keep the mass of the culture broth constant. The culture was collected in a 10 L harvest tank which was cooled with ice in order to avoid intracellular degradation of PHA.

Sample preparation: Cells were spun down at 4500 x g for 15 min at 4 °C. The pellet was washed with nano-pure water, lyophilized for 48 hours, and stored in a desiccator. Samples of 50 mL of culture supernatant were stored at – 20 °C before further analyses of residual nutrients. During batch experiments, samples of 50 mL of culture broth were taken and treated in the same way.

Cell dry weight (CDW): Cells were collected on preweighed polycarbonate filters (pore-size 0.2 µm, Nuclepore, Sterico AG, Dietikon, Switzerland). The filters were first washed with 10 mM MgCl₂, dried overnight at 110 °C, cooled down in a desiccator over silicagel and weighed. An appropriate volume of cell suspension (5 to 10 mL) was then filtered through the preweighed filter. The filters were dried again at 110 °C overnight and the weight difference was used to calculate the concentration of the biomass in the culture.

Analyses of the substrate concentrations in the culture supernatant: Ammonium was measured by using a photometric ammonium test (Spectroquant, Merck, Darmstadt, Germany). The detection limit of this method was 0.01 mg L⁻¹ NH₄-N. The method was linear up to concentrations of 3.0 mg L⁻¹ NH₄-N. If necessary, samples were diluted with nano-pure water. Octanoate and 10-undecenoate were measured by reversed-phase liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS), which was performed using an esquire high capacity trap (Bruker Daltonics, Bremen, Germany) equipped with an Agilent HP1100 binary pump. The fatty acids were separated on a C18 Nucleosil 2 x 250 mm column (3 µm, 100 Å, Macherey-Nagel Inc., Easton, PA, USA). Mobile phases consisted of 0.1 % acetic acid in nano-pure water (A) and 0.1 % acetic acid in acetonitrile (B). Separation was achieved using a linear gradient from 100 % A to 100% B in 10 minutes. The flow rate was 0.2 mL min⁻¹ with injection volumes of 7.5 µL. The mass spectrometer was operated in negative ion mode with a voltage of + 4.6 keV and a desolvation gas flow of 8 L min⁻¹ at 350 °C. The fatty acids were recorded from their extracted ion chromatograms (EIC) at m/z 143 for octanoic acid and m/z 183 for 10-undecenoic acid, respectively. Quantification occurred in the range of 2-8 ppm after dilution of the samples in acetonitrile/double distilled water (1/1 v/v). For the calibration of the system 2, 5 and 10 ppm standards of the corresponding fatty acids were used.

PHA extraction: PHA was extracted directly from lyophilized cells. Cells were pulverized and transferred into pure methylene chloride (60 g CDW in 1 L methylene

chloride). After stirring the suspension overnight, the solution was filtered and concentrated by distillation at 60 °C at 0.35 bar until the solution became viscous. The polymer was then precipitated in ice-cold methanol (final ratio (v/v) of CH₂Cl₂/MeOH = 1/6). After removal of the liquids by filtration, the PHA was vacuum-dried (30 °C, 30 mbar) for at least one day.

PHA analysis: PHA monomers were obtained after acid catalyzed hydrolysis of PHA. The hydrolytic step was adapted from the procedure for poly(3-hydroxybutyrate) (Riis and Mai, 1988). A known amount of about 10 mg lyophilized cells were weighed into a 10 mL pyrex tube. Then, 1 mL of methylene chloride containing 1.5 mg 3-hydroxyisovaleric acid as internal standard and 1 mL of a mixture of *n*-propanol / hydrochloric acid (80/20 v/v) were added. The tube was capped and heated for 3h at 100 °C. After cooling, 2 mL of nano-pure water were added and the tube shaken on a laboratory mixer. The organic layer was then dried with anhydrous sodium sulfate. The derivatized samples were analyzed in methylene chloride solution on a GC (Hewlett Packard 5890/II, Urdorf, Switzerland) equipped with a flame ionization detector (FID). The separation was made on a Supelcowax 10 column, 30 m x 0.25 mm, 0.5 μm (Supelco, Buchs, Switzerland). The GC parameters were as follows: temperature of the injector 250 °C, temperature of the FID detector 285 °C, He gas flow 3 mL min⁻¹, split ratio 1:10, 3 μL of injection, and the oven temperature program was: 120 °C, 1 min isotherm, 120-280 °C with 10 °C min⁻¹, 1 min isotherm. The propylesters of 3-hydroxyacid monomers were identified by comparing the sample retention times with the commercially available 3-hydroxy standards of butanoic, hexanoic, octanoic, decanoic, and dodecanoic acid after propanolysis. Quantification was done via a calibration function generated from a mixture of 3-hydroxyisovaleric acid and the 5 standards after propanolysis as described above, and interpolating the response factors for monomers not available commercially.

For the determination of T_g and T_m, differential scanning calorimetry (DSC) was used. Samples of 8 – 14 mg PHA were weighed into aluminum pans and analyzed with a DSC 30 (Mettler Toledo, Greifensee, Switzerland). The samples were cooled down to -80 °C within 10 minutes. After temperature equilibration, the sample was heated to 100 °C at a heating rate of 10 °C min⁻¹. Molecular weights (number average (M_n) and weight average (M_w)) were determined by gel permeation chromatography (GPC, Waters 150, Milford MA, USA) equipped with a RI detector.

The system was calibrated by using 10 polystyrene standards with known Mw (2×10^3 to 2.13×10^6 g mol⁻¹) and low polydispersity ($M_w/M_n \leq 1.09$). Forty mg of every sample were dissolved in 10 mL THF for 2 hours. Aliquots of 100 μ L of the polymer solution were chromatographed with pure THF as the solvent phase through 2 GPC-columns (Mixed-Bed, Viscothek, Houston, USA) at a flow rate of 1 mL min⁻¹.

RESULTS AND DISCUSSION

McIPHA accumulation by *P. putida* GPo1 in batch culture (shake flask experiments). To study the tailored production of olefinic mcIPHAs in batch cultures, *P. putida* GPo1 was grown in 500 mL minimal medium on different mixtures of octanoic acid and 10-undecenoic acid. A relatively high initial carbon to nitrogen ratio ($C_0/N_0 = 20$ g g⁻¹) was set, to assure that exponential growth was terminated by nitrogen exhaustion, while carbon remained in excess. The cell dry weight, the PHA content and compositions of the resulting polymers obtained after 24 h cultivation time are summarized in Table 2.1.

Table 2.1: Production of PHA by *P. putida* GPo1 under different growth conditions in batch cultures.

<i>Experimental conditions</i> ^{a)}	A	B	C	D
<i>C-source feed [mol%]</i>				
octanoate	90	75	50	25
10-undecenoate	10	25	50	75
<i>Biomass and PHA production</i> ^{b)}				
Cell dry weight [g L ⁻¹]	1.43	1.37	1.51	1.39
PHA content during steady state [% CDW]	29	30	38	32
<i>PHA composition (mol%)</i>				
Σ 3-hydroxyalkanoates (HA)	91.7	75.2	49.7	20.4
3-hydroxyoctanoate	83.0	69.1	46.0	18.8
3-hydroxyhexanoate	8.7	6.1	3.7	1.6
Σ 3-hydroxyalkenoates (HE)	8.3	24.8	50.3	79.6
3-hydroxy-10-undecenoate	1.7	6.2	15.7	22.3
3-hydroxy-8-nonenoate	5.9	17.1	32.0	53.1
3-hydroxy-6-heptenoate	0.7	1.5	2.6	4.2

a) $N_0 = 0.112$ g L⁻¹; $C_0/N_0 = 20$ g g⁻¹.

b) cells were collected after cultivation for 24 h.

Cell and PHA production were different for experiment C and almost identical for experiments A, B and D. The monomer compositions of the accumulated mclPHAs reflected almost the corresponding carbon feed mixtures for all experiments.

MclPHA accumulation by *P. putida* GPo1 in batch culture (fermentor experiments). Cell growth and PHA accumulation were followed as a function of time. In the first experiment, *P. putida* GPo1 was grown on a mixture of octanoic acid (15 mM = 58 mol%) and 10-undecenoic acid (11 mM = 42 mol%). A high initial carbon to nitrogen ratio ($C_0/N_0 = 26 \text{ g g}^{-1}$) was set, so that exponential growth was terminated by nitrogen exhaustion. Three different phases could clearly be identified. During phase 1 (Figure 2.1) *P. putida* GPo1 grew exponentially at a maximum specific growth rate (μ_{max}) of 0.38 h^{-1} . The cellular PHA content towards the end of phase 1 was 9 wt%, indicating that *P. putida* GPo1 began to accumulate mclPHA at significant rates during the exponential growth phase. Phase 2 began after all ammonia was utilized. In contrast to previous reports (Durner et al., 2001) the PHA-free biomass (calculated as the difference between the total cell dry weight and the PHA concentration in the culture) did not remain constant after the onset of nitrogen limitation, but showed a clear increase from 0.89 g L^{-1} to 1.15 g L^{-1} instead. Pedrós-Alio et al., (Pedrós-Alio et al., 1985) reported a threefold increase in the cell volume of *A. eutrophus* during PHA accumulation. Steinbüchel and coworkers (Steinbüchel et al., 1995) concluded that such increase in cell volume necessarily enlarges the volume of the cytoplasmic membrane and since phospholipids constitute to a significant fraction of the total cellular dry matter of bacteria (e.g. 9.1 wt% in *Escherichia coli* (Neidhart and Umbarger, 1996)), PHA accumulation in *A. eutrophus* and possibly also in other bacteria must be accompanied by a considerable net biosynthesis of phospholipids. In addition, PHA accumulates in granules which are formed in the cytoplasm of bacteria (de Smet et al., 1983). These PHA granules are covered by a phospholipid monolayer with proteins (Steinbüchel et al., 1995). Therefore, the formation of PHA granules and the associated development of the phospholipid monolayer with proteins contribute also to an increase of the PHA-free biomass per cell. Consequently, the total PHA-free biomass in the culture has to increase.

The cellular PHA content increased during phase 2 from 9 wt% to 34 wt% and the averaged specific PHA accumulation rate (calculated as g PHA accumulated per g of

PHA-free biomass per h) was $0.08 \text{ g (g}^*\text{h)}^{-1}$. Immediately succeeding phase 2, a third phase could be identified where the PHA-free biomass remained constant but the total biomass increased further. This increase was exclusively due to the intracellular accumulation of mclPHA and the cellular PHA content was 40.2 wt% at the end of phase 3, indicating that the cellular adaptation to nitrogen starvation was terminated. The specific PHA accumulation rate remained constant in this phase at $0.08 \text{ g (g}^*\text{h)}^{-1}$.

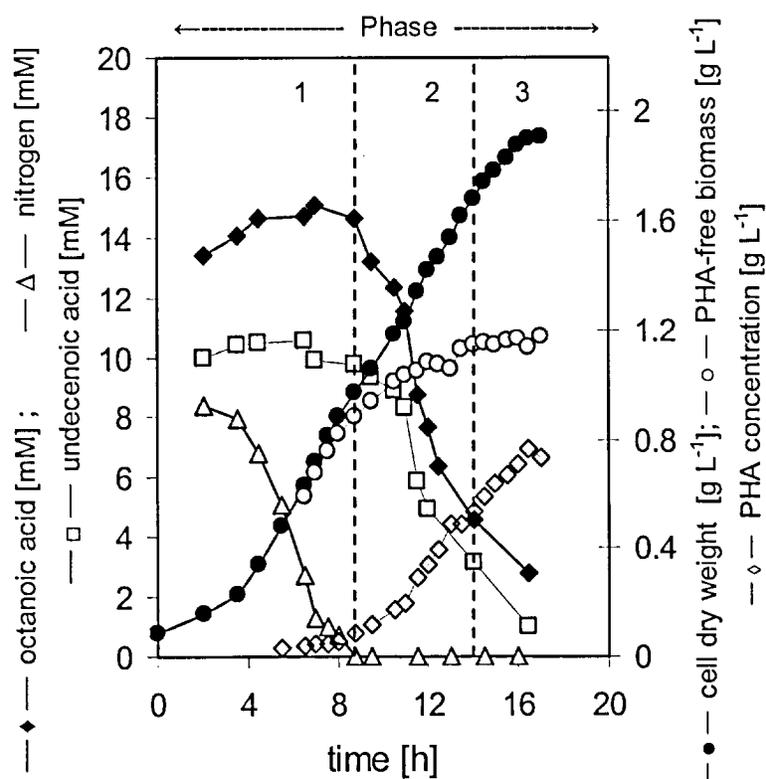


Figure 2.1: Accumulation of olefinic mclPHAs by *P. putida* GPo1 during batch growth with a mixture of octanoate (15 mM) and 10-undecenoate (11 mM).

The monomeric composition of the accumulated PHA is shown in Figure 2.2. The accumulated polymer consisted not only of 3-hydroxyoctanoate and 3-hydroxy-10-undecenoate, but also of monomers that were two carbon units shorter (3-hydroxyhexanoate, 3-hydroxy-8-nonenoate and 3-hydroxy-6-heptenoate) because of the β -oxidation of the carbon sources. The data show that the monomeric composition of the accumulated PHA changed slightly from phase 1 to phase 3. The oxidation of octanoate seems to be enhanced in the first two phases and diminished in phase 3. The averaged specific carbon consumption rates during phase 2 (calculated as g carbon used per g of averaged PHA-free biomass per h) was

0.18 g C (g of PHA-free biomass \cdot h) $^{-1}$ for octanoate and 0.16 g C (g of PHA-free biomass \cdot h) $^{-1}$ for 10-undecenoate. In phase 3, specific carbon consumption rates were calculated for octanoate of 0.06 g C (g of PHA-free biomass \cdot h) $^{-1}$ and 0.1 g C (g of PHA-free biomass \cdot h) $^{-1}$ for 10-undecenoate. The PHA composition in phase 3 was constant around 51 mol% 3-hydroxyoctanoate, 5 mol% 3-hydroxyhexanoate, 11 mol% 3-hydroxy-10-undecenoate, 30 mol% 3-hydroxy-8-nonenoate, 3 mol% 3-hydroxy-6-heptenoate and reflected the carbon composition of the feed medium (58 mol% octanoate, 42 mol% 10-undecenoate).

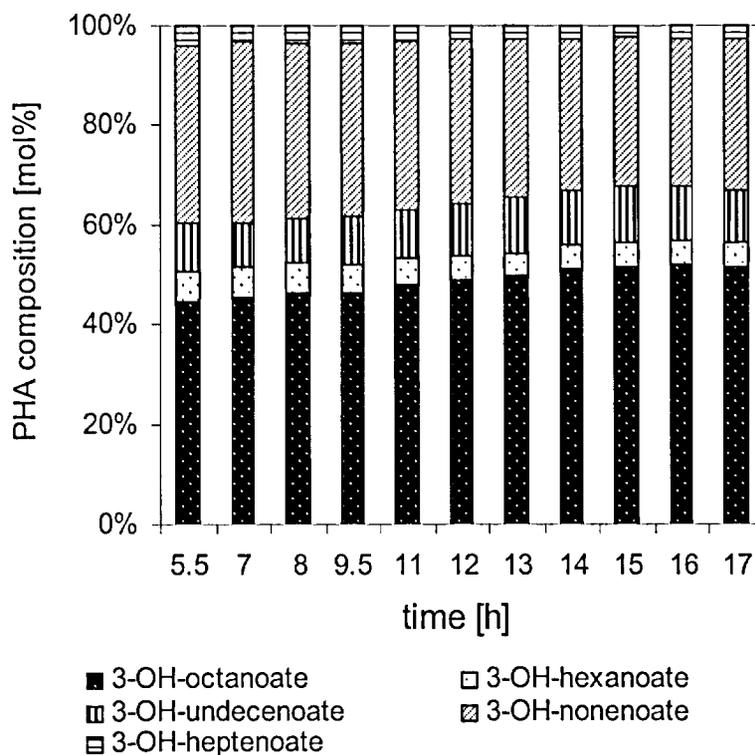


Figure 2.2: Monomeric composition of the olefinic mclPHAs accumulated during batch growth with a mixture of octanoate (15 mM) and 10-undecenoate (11 mM).

In the second batch experiment, which was performed in the fermentor, *P. putida* GPo1 was grown with 10-undecenoate as sole source of carbon (Figure 2.3). The relative concentrations of the nitrogen (NH₄-N) and of carbon (10-undecenoate) were adjusted such that ammonia was consumed to completion, thereby terminating the exponential growth phase, whereas the carbon source remained in excess.

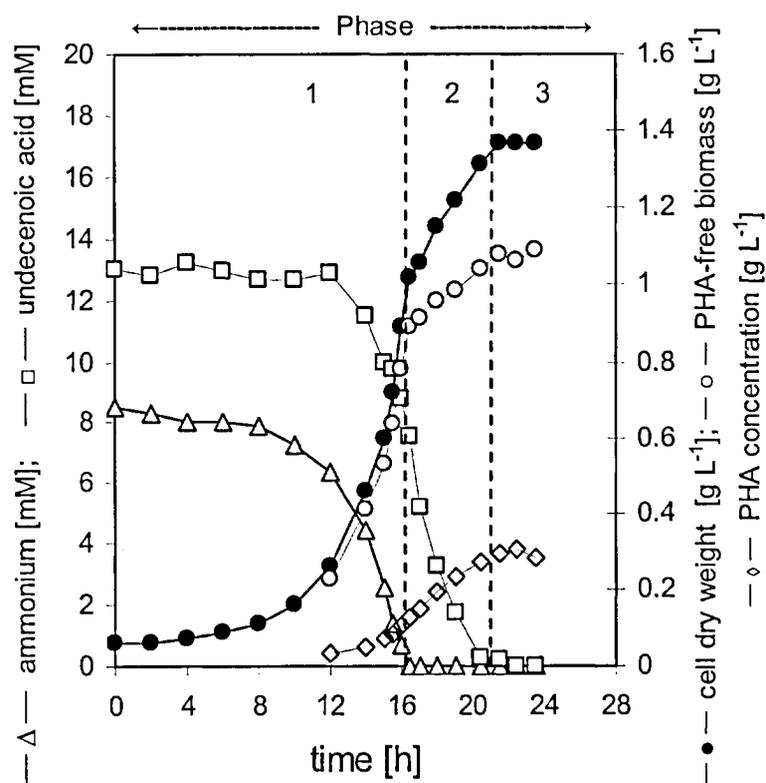


Figure 2.3: Accumulation of olefinic mclPHAs by *P. putida* GPo1 during batch growth on 10-undecenoate as sole carbon source (13 mM).

In analogy to the first batch experiment (Figure 2.1), three different phases could be identified. In phase 1 the cells grew exponentially with a maximum specific growth rate (μ_{max}) of 0.42 h^{-1} . MclPHA accumulation was observed also during the exponential growth phase and the cellular PHA content was 12 wt% at the end of phase 1. The carbon and nitrogen growth yields during exponential growth were $Y_{X/C} = 1.24 \text{ g g}^{-1}$ and $Y_{X/N} = 7.32 \text{ g g}^{-1}$, respectively. During phase 2, the PHA-free biomass increased from 0.89 g L^{-1} to 1.08 g L^{-1} and the cellular PHA content increased from 12 wt% to 21 wt%. In contrast to the first batch experiment, the PHA-free biomass and the cellular PHA content were lower at the end of phase 2 and no increase of the total biomass due to pure PHA accumulation in phase 3 was observed. We propose that the difference between this experiment and the first batch experiment was caused by the early onset of a carbon limitation during phase 2.

Figure 2.4 shows the monomeric composition of the accumulated PHA. The results suggest that oxidation of 10-undecenoate to 8-nonenolate and 6-heptenolate did not change throughout the whole batch experiment. The molar distribution of the PHA-

monomers was around 22 mol% 3-hydroxy-10-undecenoate, 71 mol% 3-hydroxy-8-nonenote and 7 mol% 3-hydroxy-6-heptenoate.

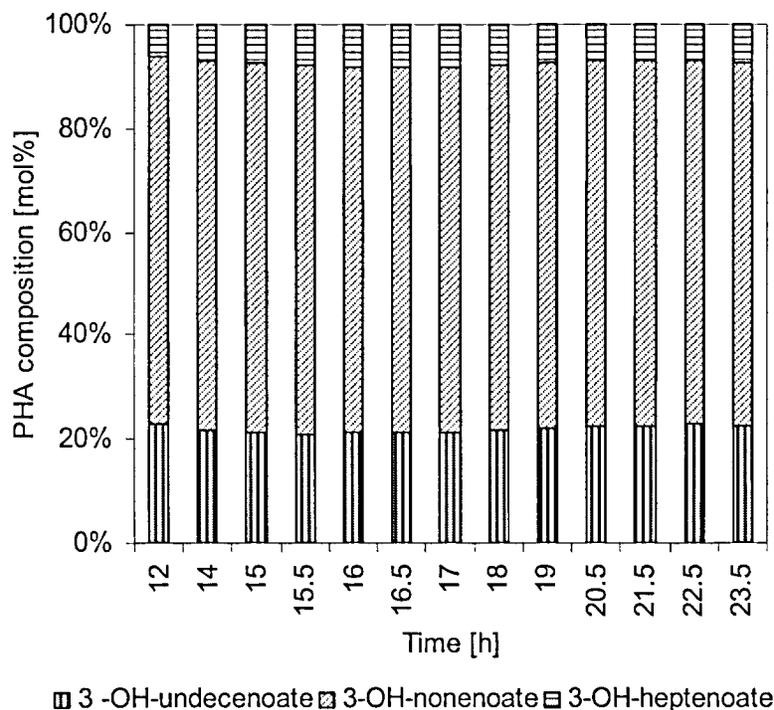


Figure 2.4: Monomeric composition of the olefinic mclPHAs accumulated during batch growth on 10-undecenoate as sole carbon source (13 mM).

Determination of the dual nutrient (C,N) limited growth regime (DNLGR) in chemostat cultures on 10-undecenoate. MclPHA can be accumulated by *P. putida* GPo1 in chemostat cultures during dual nutrient (C,N) limited growth, i.e., during simultaneous nitrogen (N) and carbon (C) limitation (Durner et al., 2000; Hartmann et al., 2004; Zinn et al., 2003). Durner et al., (Durner et al., 2000) defined a so called dual nutrient (C,N) limited growth regime (DNLGR) and showed it to be a function of the dilution rate and the carbon to nitrogen ratio (C_0/N_0) of the medium feed (see Figure 2.5). The shape of this DNLGR can be determined experimentally or calculated from the elemental yield coefficients ($Y_{X/N}$ and $Y_{X/C}$) obtained under single-nutrient-limited growth conditions according to the method of Egli and Quayle (Egli and Quayle 1986). *P. putida* GPo1 was therefore cultivated in chemostat cultures with 10-undecenoate under carbon as well as nitrogen limited growth conditions at

different dilution rates. The obtained elemental yield coefficients (Table 2.2) were used to calculate the boundaries of the DNLGR.

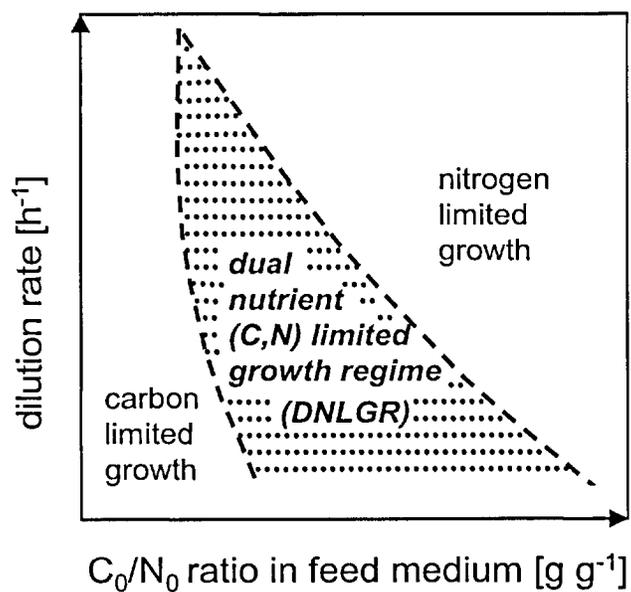


Figure 2.5: The dual nutrient (C,N) limited growth regime (DNLGR) is a function of the dilution rate and the carbon to nitrogen ratio (C_0/N_0) of the medium feed. The zone is a function of the growth rate, the redox state of the carbon source and the microorganism (Egli, 1991).

Table 2.2: Culture parameters, cell dry weights and PHA contents, residual substrate concentrations, elemental growth yield and dual nutrient (C,N) limited growth regime (DNLGR) characteristics for chemostat cultures of *P. putida* GPo1.

<i>Growth conditions</i>								
Dilution rate [h ⁻¹]	0.1		0.2		0.3		0.4	
Carbon to nitrogen ratio [g g ⁻¹]	4 ^{a)}	23 ^{b)}	4 ^{a)}	18 ^{b)}	4 ^{a)}	14 ^{b)}	4 ^{a)}	10 ^{b)}
Calculated boundaries of the DNLGR ^{c)}	7.79	19.11	5.71	14.32	5.61	11.65	5.42	8.85
<i>Culture and cellular characteristics</i>								
Cell dry weight (CDW) [g L ⁻¹]	0.48	1.75	0.63	1.59	0.69	1.45	0.74	1.30
<i>Nitrogen utilization</i>								
Residual nitrogen in supernatant [g L ⁻¹]	0.073	0	0.045	0	0.043	0	0.039	0
Nitrogen consumption [g L ⁻¹]	0.077	0.15	0.105	0.15	0.107	0.15	0.111	0.15
Growth Yield coefficient for nitrogen Y _{XN}	6.23	11.66	6.00	10.60	6.45	9.67	6.67	8.67
<i>Carbon utilization</i>								
Residual 10-undecenoate in supernatant [g L ⁻¹]	0	0.83	0	0.79	0	0.50	0	0.25
Carbon consumption [g L ⁻¹]	0.6	2.86	0.6	2.14	0.6	1.74	0.6	1.32
Growth Yield coefficient for carbon Y _{XC}	0.8	0.61	1.05	0.74	1.15	0.83	1.23	0.98
<i>PHA characteristics</i>								
PHA content during steady state [% CDW]	5	46	5	34	4	28	6	17
Specific PHA accumulation rate [g (g*h) ⁻¹] ^{d)}	0.015	0.239	0.015	0.144	0.012	0.109	0.018	0.057
Volumetric PHA production rate [g (L*h) ⁻¹]	0.002	0.081	0.006	0.108	0.008	0.122	0.018	0.088

a) Carbon limited growth condition.

b) Nitrogen limited growth condition.

c) C₀/N₀ ratio in the feed medium [g g⁻¹] = (Y_{XN} / Y_{XC}).

d) Grams of PHA accumulated per gram of PHA-free biomass per hour.

Figure 2.6 depicts the DNLGR for chemostat cultures of *P. putida* GPo1 with respect to 10-undecenoate and ammonium. The known DNLGR for chemostat cultures of *P. putida* GPo1 with respect to octanoate and ammonium (Durner et al., 2000) is also shown in this figure.

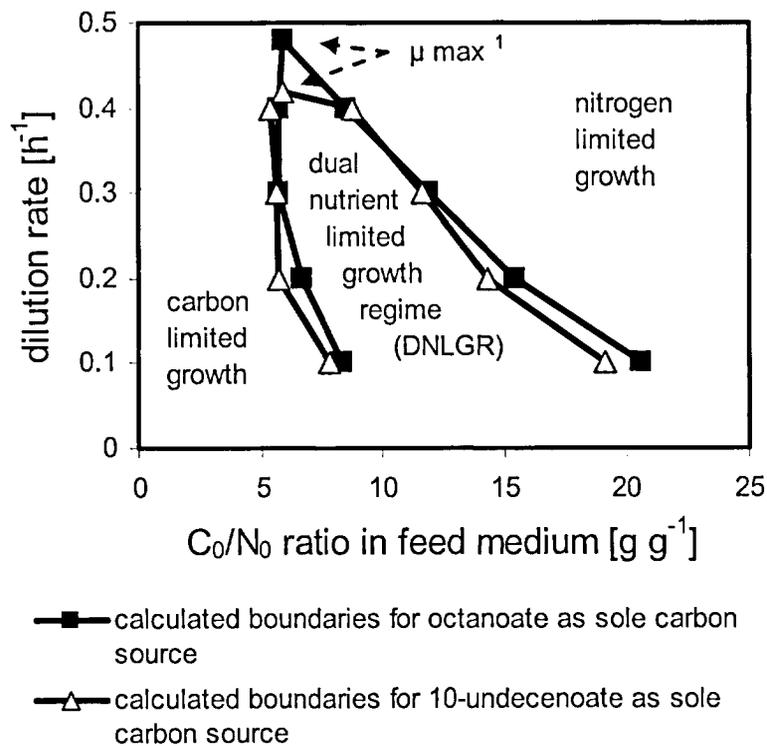


Figure 2.6: Extension of the dual nutrient (carbon,nitrogen) limited growth zone for the cultivation of *P. putida* GPo1 with 10-undecenoate and ammonia as function of the dilution rate and the C_0/N_0 ratio of the feed medium. The boundaries were calculated from carbon and nitrogen yield coefficients obtained under single nutrient limited growth conditions according to Egli and Quayle 1986. The dual nutrient (C,N) limited growth zone during cultivation of *P. putida* GPo1 with octanoate and ammonium is also shown in this figure (Data taken from Durner et al., 2000). ¹⁾ The growth yield coefficients for μ_{max} with 10-undecenoate as sole carbon source were determined from the batch experiment (see Figure 3) and refer to the rest biomass produced during exponential phase (data for octanoate were also taken from Durner et al., 2000).

The DNLGR is similar for growth on octanoate and 10-undecenoate (see Figure 2.6). The DNLGR was narrower at higher growth rates, became wider and was shifted towards higher C_0/N_0 ratios with decreasing growth rates. Egli (Egli 1991) postulated that the redox state of the carbon substrate determines the shape and position of the

DNLGR. Since octanoate and 10-undecenoate have an almost identical redox state, it had indeed to be expected that both substrates result in almost identical DNLGR. The boundaries of the DNLGR (calculated from the elemental yield coefficients $Y_{X/N}$ and $Y_{X/C}$ obtained under single nutrient limited growth conditions) are shown in Table 2.2. Under carbon limitation, $Y_{X/N}$ was constant for all dilution rates. In contrast $Y_{X/C}$, which is the sum of a growth associated term and the maintenance energy coefficient (Pirt 1975), decreased towards lower dilution rates, as a result of the increasing contribution of maintenance energy to the total carbon source consumption. Therefore, the location of the lower boundary of the DNLGR depends on this variable influence of maintenance energy to the total carbon source consumption, which is again a function of the growth rate.

Both $Y_{X/C}$ and $Y_{X/N}$ changed under nitrogen limited growth conditions. The change in $Y_{X/C}$ was due to the same reasons discussed above, that is, the increasing influence of maintenance energy coefficient with decreasing growth rate. In contrast to $Y_{X/C}$, $Y_{X/N}$ for total biomass increases under nitrogen limited growth conditions with decreasing growth rates. The cellular PHA content increased for cells cultivated at lower dilution rates (Table 2.2). Therefore $Y_{X/N}$ calculated for the total biomass (PHA-free biomass and PHA) has to increase towards lower dilution rates. As a consequence $Y_{X/N}$ was affected by the PHA content of the cells, which in turn influenced the location of the upper boundary of DNLGR.

Production of tailored olefinic mclPHAs using dual nutrient (C,N) limited growth conditions in chemostat cultures. Table 2.2 shows that PHA production increased with decreasing growth rates under nitrogen limited growth conditions, with specific PHA accumulation rates increasing from $0.057 \text{ [g (g}\cdot\text{h)}^{-1}]$ at a dilution rate of 0.4 h^{-1} , to $0.239 \text{ [g (g}\cdot\text{h)}^{-1}]$ at a dilution rate of 0.1 h^{-1} . The volumetric PHA production rate under nitrogen limited conditions was highest ($0.122 \text{ [g (L}\cdot\text{h)}^{-1}]$) at a dilution rate of 0.3 h^{-1} . However, the extension of the DNLGR was widest at a dilution rate of $D = 0.1 \text{ h}^{-1}$ and the maximum cellular PHA content was highest under nitrogen limited growth conditions at this dilution rate (see Table 2.2). Therefore, olefinic mclPHAs were produced from different mixtures of octanoic acid and 10-undecenoic acid in chemostat cultures of *P. putida* GPo1 at a dilution rate of $D = 0.1 \text{ h}^{-1}$ (Table 2.3, experiments E-K). The carbon to nitrogen ratio (C_0/N_0) of the medium was kept constant at 15 g g^{-1} for all experiments, which is well within DNLGR (see Figure 2.6).

The cell dry weights did not differ significantly from experiments E to K. The cellular PHA content of the cells varied between 27 wt% for experiments F and I and 37 wt% for E and H. These differences may result from errors in the experimental settings (e.g. when setting the C_0/N_0 ratio of the medium) or from errors in the CDW or GC measurements.

Table 2.3: PHAs produced in chemostat cultures grown on different carbon mixtures ($D = 0.1 \text{ h}^{-1}$; $C_0/N_0 = 15 \text{ g g}^{-1}$).

<i>Experimental conditions</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>	<i>I</i>	<i>K</i>
<i>C-source feed [mol%]</i>						
octanoate	100	90	75	50	25	0
10-undecenoate	0	10	25	50	75	100
<i>Biomass and PHA production</i>						
Cell dry weight [g L^{-1}]	1.35	1.27	1.34	1.28	1.36	1.31
PHA content during steady state [% CDW]	37	27	29	37	27	30
<i>PHA composition (mol%)</i>						
Σ 3-hydroxyalkanoates (HA)	100	89.8	73.0	46.7	23.3	0
3-hydroxyoctanoate	86.2	76.1	61.1	39.6	19.3	0
3-hydroxyhexanoate	13.8	13.7	11.9	7.1	4.0	0
Σ 3-hydroxyalkenoates (HE)	0	10.2	27	53.3	76.7	100
3-hydroxy-10-undecenoate	0	1.8	4.6	11.0	13.7	15.6
3-hydroxy-8-nonenoate	0	8.4	18.8	36.8	52.6	69.5
3-hydroxy-6-heptenoate	0	0	3.6	5.5	10.4	14.9

The monomeric compositions of the polymers from experiments E-K clearly reflected the corresponding carbon mixtures of octanoic acid and 10-undecenoic acid fed to the cells. For example, in experiment H the cells were supplied with an equimolar mixture of octanoic and 10-undecenoic acids, and the resulting polymer contained 46.7 mol% aliphatic and 53.3 mol% olefinic monomers.

Table 2.4 shows molecular weights (number (Mn) and weight (Mw) averages), polydispersity (Mw/Mn), and thermal properties (glass transition (Tg) and melting (Tm) temperatures) of polymers E-K. Mw and Mn values were typical for molecular weights reported for mclPHAs (Witholt and Kessler, 1999). The thermal properties depended on the olefinic monomer content of the PHAs. Tg decreased from – 33.1 °C in polymer E to – 49.3 °C in polymer K, while Tm decreased from 58.1 °C for polymer E to 39.9 °C for polymer H. Polymers I and K did not display any crystalline melting endotherms, indicating that these polymers were completely amorphous.

Table 2.4: GPC data and thermal properties of the five polymers (E-K) produced in chemostat cultures.

Polymer	Mw	Mn	Mw/Mn	Tg	Tm
E	286'000	118'000	2.4	-33.1	58.1
F	251'000	132'000	1.9	-35.9	50.8
G	253'000	113'000	2.2	-39.5	44.5
H	290'000	156'000	1.9	-44.6	39.9
I	278'000	118'000	2.4	-47.4	-
K	290'000	122'000	2.4	-49.3	-

Production of tailored olefinic mclPHAs in chemostat cultures at different dilution rates and different carbon to nitrogen feed ratios. Table 2.5 summarizes the effect of different dilution rates and different carbon to nitrogen feed ratios on cell and PHA production as well as on the composition of the resulting PHAs during steady-state. The composition of the carbon source (50 mol% octanoic acid and 50 mol% 10-undecenoic acid) was kept constant in all experiments.

Table 2.5: Production of PHA by *P. putida* GPo1 under different growth conditions in chemostat cultures.

<i>Experimental conditions</i> ^{a)}	<i>L</i>	<i>M</i> ^{b)}	<i>N</i>	<i>O</i>	<i>P</i>
Dilution rate [h ⁻¹]	0.1	0.1	0.2	0.2	0.3
Carbon to nitrogen ratio in feed medium [g g ⁻¹]	12	15	10	12	8
<i>Biomass and PHA production</i>					
Cell dry weight [g L ⁻¹]	1.17	1.28	1.21	1.44	1.11
PHA content during steady state [% CDW]	23	37	17	24	12
<i>PHA composition (mol%)</i>					
Σ 3-hydroxyalkanoates (HA)	46.1	46.7	43.1	42.5	41.7
3-hydroxyoctanoate	38.7	39.6	36.6	36.6	35.4
3-hydroxyhexanoate	7.4	7.1	6.5	5.9	6.3
Σ 3-hydroxyalkenoates (HE)	53.9	53.3	56.9	57.5	58.3
3-hydroxy-10-undecenoate	10.6	11.0	11.2	11.9	11.3
3-hydroxy-8-nonenoate	37.3	36.8	40.4	40.7	41.6
3-hydroxy-6-heptenoate	6.0	5.5	5.3	4.9	5.4

a) A constant mixture of C source (50 mol% octanoate and 50 mol% 10-undecanoate) was used.

b) Data for M taken from experiment H (Table 2.3).

The cell dry weight increased with increasing dilution rate (constant C_0/N_0 ratio; experiments L and O) due to the reduced influence of maintenance energy as discussed before. Further, the cell dry weight increased with increasing C_0/N_0 ratios (experiments L and M; or N and O) caused by the PHA content of the cells. The monomeric composition of the resulting mclPHAs was not affected by the C_0/N_0 ratio of the feed medium. The two polyesters that were produced at a dilution rate of 0.1 h⁻¹ (experiments L and M) had almost identical monomeric compositions. The same is true for the two polymers produced at a dilution rate of 0.2 h⁻¹ (experiments N and O). The monomer composition varied slightly at different dilution rates. For example the molar content of 3-hydroxyoctanoate decreased with increasing growth rate, indicating that the oxidation rate of octanoate was enhanced. This finding agrees with the results obtained in the first batch experiment where *P. putida* GPo1 was grown

on a mixture of octanoic acid and 10-undecenoic acid (58 mol% / 42 mol%). Also there the oxidation of octanoate was enhanced during exponential growth and in the early PHA accumulation phase (see Figures 2.1 and 2.2). Maximum growth rate for *P. putida* GPo1, grown on octanoic acid as sole carbon source is reported in literature (Durner et al., 2001) to be 0.48 h^{-1} . For 10-undecenoic acid as sole carbon source, we determined in this work μ_{max} to be 0.42 h^{-1} (see Figure 2.3). We propose that the observed changes in the monomeric composition is probably caused by different kinetics of the two carbon substrates e.g. a different efficiency in the carbon uptake rate. Such an effect is probably less pronounced in slow growing cells than in fast growing cells, leading to the observed changes in the monomeric composition in batch as well as in chemostat culture experiments.

Table 2.6: GPC data of the five polymers (L-P) produced in chemostat cultures at different carbon to nitrogen ratios in the feed medium and dilution rates.

Polymer	Mw	Mn	Mw/Mn
L	308'000	170'000	1.8
M ^{a)}	290'000	156'000	1.9
N	309'000	177'000	1.8
O	288'000	136'000	2.1
P	282'000	132'000	2.1

a) Data for M taken from experiment H (Table 2.3 and 2.4).

The molecular weights and the polydispersity (Mw/Mn) of the polymers L-P are shown in Table 2.6. According to experiments E-K, molecular weights were also here in the typical range reported for mclPHAs (Witholt and Kessler, 1999) and are independent of the growth rate as well as on C_0/N_0 ratio.

CONCLUSIONS

The presence of functional groups in mclPHAs provides sites for chemical modifications, which may be useful to modify physical properties or to create chemical groups which cannot be introduced directly by biosynthesis.

In this work, we investigated the tailored production of olefinic mclPHAs from mixtures of octanoic acid and 10-undecenoic acid in batch and dual nutrient (C,N) limited chemostat cultures. We could demonstrate that in batch as well as in

chemostat cultures, the monomeric composition depended to a certain degree on the growth rate. In a batch culture, where *P. putida* GPo1 was grown on a mixture of octanoic acid (58 mol%) and 10-undecenoic acid (42 mol%), it was found that the fraction of aliphatic monomers was slightly lower in mclPHA produced during exponential growth than during late stationary phase. Thus, the monomeric composition changed over time. Such a time variable monomeric composition of mclPHAs produced in batch cultures has been observed also in previous studies for the production of mclPHAs obtained from mixtures of 5-phenylvaleric acid and nonanoic acid (Kim et al., 1991). In contrast, in chemostat cultures the fraction of aliphatic monomers in the accumulated polymer was constant during steady-state. We conclude that chemostat culture is the most suitable method to produce PHAs with an exact defined monomeric unit composition.

ACKNOWLEDGEMENTS

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CHAPTER 3

Tailored Biosynthesis of Olefinic
Medium-Chain-Length Poly[(*R*)-3-
hydroxyalkanoates] in *Pseudomonas*
putida GPo1 with Improved Thermal
Properties

René Hartmann, Roland Hany, Thomas Geiger, Thomas Egli, Bernard Witholt and
Manfred Zinn. 2004. *Macromolecules* 37, 6780-6785.

Abstract: Mixtures of 5-phenylvalerate, octanoate, and 10-undecenoate were fed to a chemostat culture (dilution rate = 0.1 h^{-1}) of *Pseudomonas putida* GPo1 under well-defined dual nutrient (C,N) limited growth conditions. Five new, tailor-made copolymers were produced and consisted of poly(3-hydroxy-5-phenylvalerate-co-3-hydroxyalkanoates-co-3-hydroxy- ω -alkenoates), poly(HP-co-HA-co-HE), with increasing amounts of aromatic side chains (A: 0%, B: 3%, C: 19%, D: 42% and E: 59%), approximately 10 mol% unsaturated side chains and decreasing amounts of saturated side chains. Based on NMR analysis of polymer E, it was concluded that the incorporation of the substrates occurred randomly. The HP-content determined the glass transition temperature, which increased linearly from $-38.7 \text{ }^{\circ}\text{C}$ for poly(0%HP-co-90%HA-co-10%HE) to $-6.0 \text{ }^{\circ}\text{C}$ for poly(59%HP-co-31%HA-co-10%HE).

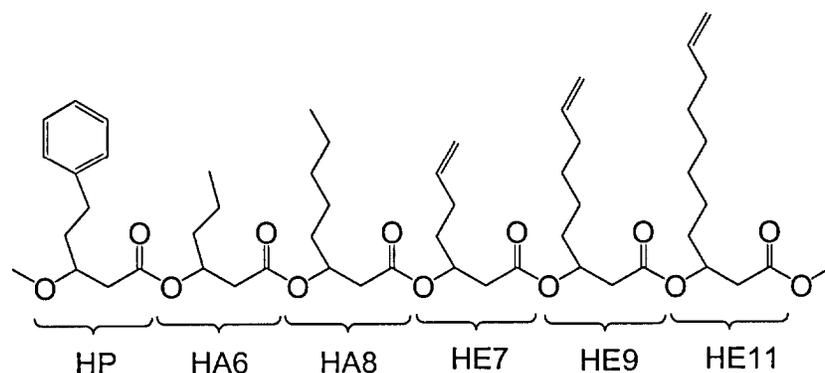
INTRODUCTION

Poly-[(*R*)-3-hydroxyalkanoates] (PHAs) are biodegradable and biocompatible polymers of high molecular weight synthesized by a wide variety of microorganisms (Doi, 1990; Steinbüchel and Valentin, 1995). PHAs have received increased attention due to their potential applications in coatings, as medical implants, as controlled drug release systems or as a source of chiral monomers (Gursel and Hasirci, 1995; Atkins and Peacock, 1996; Zinn et al., 2001; de Roo et al., 2002). MclPHAs (which contain medium-chain-length C6 – C12 alkanate monomers) are mostly amorphous and soft-sticky with glass transition temperatures (T_g) between -44 and $-30 \text{ }^{\circ}\text{C}$ and melting temperatures (T_m) between 39 and $61 \text{ }^{\circ}\text{C}$ (Gross et al., 1989; Preusting et al., 1990; Witholt and Kessler, 1999). Various mclPHAs with side chains containing functional groups, for example carbon-carbon double (Lageveen et al., 1988) and triple bonds (Kim, et al., 1998), acetoxy and ketone (Jung et al., 2000), or aromatic groups (Fritzsche et al., 1990a; Curley et al., 1996; Kim et al., 1999) have been produced. Among the latter ones, mclPHAs containing phenyl groups, such as poly[(*R*)-3-hydroxy-5-phenylvalerate] ($T_g = 13 \text{ }^{\circ}\text{C}$) (Fritzsche et al., 1990a), or (co)-polymers from 6-phenylhexanoic acid, 7-phenylheptanoic acid, and 8-phenyloctanoic acid (T_g between -14.8 and $-1.3 \text{ }^{\circ}\text{C}$) (Abraham et al., 2001) were completely amorphous, but showed an increase in the glass transition temperature.

The presence of functional groups in mclPHAs provides sites for chemical modifications, which may be useful to modify physical properties or to create chemical groups which cannot be directly introduced by biosynthesis. Polymer-analogous reactions on unsaturated mclPHAs have been studied in detail, and crosslinking (de Koning et al., 1994; Dufresne et al., 2001; Hazer et al., 2001), epoxidation (Bear et al., 1997), or conversion to carboxylic (Kurth et al., 2002; Stigers and Tew, 2003) and diol groups (Lee et al., 2000a) have been reported so far.

In this work, chemostatic culture conditions (also called continuous culture) were applied to produce tailored mclPHAs. A chemostat is essentially a perfusion reactor that is continuously supplied with sterile medium. The (culture) volume in the reactor is kept constant by the continuous removal of culture broth. Once such a system is in equilibrium (steady-state), cell number, nutrition, and productivity remain constant over time (Herbert et al., 1956). As we have shown previously (Durner et al., 2000; Durner et al., 2001), *Pseudomonas putida* GPo1 can be grown under simultaneous limitation by carbon (C) and nitrogen (N) substrates in a chemostat. Under such growth conditions all fed C- and N-substrates are completely metabolized and therefore no toxicity of C-substrates is detected (Egli and Zinn, 2003).

In this study, different mixtures of octanoic, 10-undecenoic, and 5-phenylvaleric acid were used to produce mclPHAs (Scheme 3.1) in a chemostat under dual nutrient (C,N) limited growth conditions. This concept allowed the tailored synthesis of novel, olefinic PHAs with increased glass transition temperatures due to the integration of phenyl groups.



Scheme 3.1: Structural formula of poly(HP-co-HA-co-HE). HP: 3-hydroxy-5-phenylvalerate; HA6: 3-hydroxyhexanoate; HA8: 3-hydroxyoctanoate; HE7: 3-hydroxy-6-heptenoate; HE9: 3-hydroxy-8-nonenoate; HE11: 3-hydroxy-10-undecenoate.

MATERIALS AND METHODS

Pseudomonas putida GPo1 (ATCC 29347) (formerly *P. oleovorans*) was kept as frozen stock at -80 °C in 15% glycerol and was used for all experiments. For the preparation of inocula for continuous cultures, 1 mL frozen stock culture was added to 100 mL of minimal medium in 300 mL shake flasks and grown overnight at 30 °C. One liter of minimal medium contained 3.5 g $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 7.5 g K_2HPO_4 , 3.7 g KH_2PO_4 , and 2.9 g $\text{Na}_3\text{Citrate} \cdot 2\text{H}_2\text{O}$ (Durner et al., 2001). The pH was adjusted to 7.1 with 10 M NaOH. This medium was autoclaved and subsequently supplemented with filter sterilized $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 mL L^{-1} , 1M) and 1 mL L^{-1} of MT (mineral trace element) stock solution which contained per liter: 2.78 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.98 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.81 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 M HCl (Durner et al., 2001). Fifty mL of shake-flask culture were used to inoculate the bioreactor.

For continuous cultivation the following medium was used (per liter): 1 g KH_2PO_4 , 0.71 g $(\text{NH}_4)_2\text{SO}_4$, and 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Further, 1 L of the medium was supplemented with 1 mL of 10 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (in 1 M HCl) and 1 mL continuous culture mineral trace element (CCMT) stock solution containing per liter: 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.98 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.81 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 g EDTA at pH 4 (Durner et al., 2001). Forty liter of this medium were then filter sterilized into gamma sterilized 50 L medium bags (Flexboy, Stedim S.A., Aubagne Cedex, France). Particular mixtures of octanoic, 10-undecenoic, and 5-phenylvaleric acid were pumped directly into the culture vessel by using a dosimat (Metrohm, Herisau, Switzerland).

Cultivation conditions: All continuous culture experiments were performed in a 3 L laboratory bioreactor (KLF 2000, Bioengineering, Wald, Switzerland) with a working volume of 2.5 L. The dilution rate (D) was set to 0.1 h^{-1} , which means that each hour 10 % of the working volume is removed and continuously replaced with sterile minimal medium. The nitrogen content of the minimal medium was 150 mg nitrogen L^{-1} . In contrast, the carbon feed rates of the continuous cultures were altered for each experiment in order to obtain particular carbon to nitrogen ratios (C_0/N_0) in the feed medium. The cultures were run at 30 °C, and the pH was maintained at 7.0 ± 0.05 by automated addition of either 2 M NaOH or 2 M H_2SO_4 . The dissolved oxygen tension was monitored continuously with an oxygen probe (Mettler Toledo, Greifensee,

Switzerland) and care was taken that it remained above 35 % air saturation. The culture volume was kept constant with an overflow tube that was connected to a continuously running harvest pump. The culture was collected in a 10 L harvest tank which was placed in a refrigerator (4 °C).

Sample preparation: Cells were spun down at 4500 x g for 15 min at 4 °C. The pellet was washed with nano-pure water, lyophilized for 48 hours, and stored in a desiccator. Samples of 20 mL of culture supernatant were stored at – 20 °C before further analyses of residual nutrient concentrations.

Cell dry weight (CDW): Cells were collected on preweighed polycarbonate filters (pore-size 0.2 µm, Nuclepore, Sterico AG, Dietikon, Switzerland). The filters were first washed with 10 mM MgCl₂, dried overnight at 110 °C, cooled down in a desiccator over silicagel and weighed. An appropriate volume of cell suspension (5 to 10 mL) was then filtered through the preweighed filter. The filters were dried again at 110 °C overnight, and the weight difference was used to calculate the concentration of the biomass in the culture.

Analyses of the substrate concentrations in the culture supernatant: Ammonium was measured by using a photometric ammonium test (Spectroquant, Merck, Darmstadt, Germany). The detection limit of this method was 0.01 mg L⁻¹ NH₄-N. The method was linear up to concentrations of 3.0 mg L⁻¹ NH₄-N. If necessary, samples were diluted in nano-pure water. Octanoate, 10-undecenoate, and 5-phenylvalerate were measured by a modified gaschromatographic method (de Roo et al., 2002; Lageveen et al., 1988). Samples were acidified by mixing an equal volume of 15 % v/v sulphuric acid in ethanol after adding 2 g L⁻¹ benzoic acid methylester as internal standard. They were directly injected (column: DB5, 30 m x 0.32 mm, MSP Friedli & Co, Koeniz, Switzerland) and measured with a flame-ionization detector.

PHA extraction: PHA was extracted directly from the lyophilized cells. Cells were pulverized and transferred into pure methylene chloride (60 g CDW in 1 L methylene chloride). After stirring the suspension overnight, the solution was filtered and concentrated by distillation at 60 °C until the solution became viscous. The polymer was then precipitated into ice-cold methanol (final ratio (v/v) of CH₂Cl₂/MeOH = 1:6). After removal of the solvents by filtration, the PHA was vacuum-dried (30 °C, 30 mbar) for at least one day.

PHA analysis: The cellular content and the monomeric composition were determined by gaschromatography as well as by NMR spectroscopy. For gaschromatography a known amount of 4-9 mg lyophilized cells were transferred into a 10 mL pyrex tube with a teflon cap. Two mL of chloroform containing 0.1 mg mL^{-1} methylbenzoate as internal standard and 2 mL of 15 % H_2SO_4 v/v in methanol were added for the methanolysis at $100 \text{ }^\circ\text{C}$ for 150 min. Subsequently, the sample was cooled on ice and 1 mL of nanopure water was added. The aqueous phase was removed and the resulting organic phase was dried by adding Na_2SO_4 . This sample was used for injection in the GC (GC 8575 MEGA 2, Fisons Instruments, Rodano, Italy; column: DB 5, 30 m x 0.32 mm; MSP Friedli & Co., Koeniz, Switzerland; split ratio 1:10, initial temperature $80 \text{ }^\circ\text{C}$, $8 \text{ }^\circ\text{C}/\text{min}$ to $300 \text{ }^\circ\text{C}$). The efficiency of the methanolysis was tested by using 1 mg extracted PHA from octanoate which was treated in the same way and was used for calibration.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ASX-400 NMR spectrometer at 300 K. For proton (^1H) NMR spectra 10 mg of polymer were dissolved in 0.7 mL chloroform-d, and 20 – 30 mg of polymer were used for carbon (^{13}C) spectra. Chemical shifts are given in parts per million (ppm) relative to the signal of chloroform as internal reference (^1H NMR: 7.26 ppm; ^{13}C NMR: 77.7 ppm). The ^1H (^{13}C) NMR spectra were recorded at 400.13 MHz (100.61 MHz) with the following parameters: 5 μs (3 μs) 45° pulse lengths, 32 (10K) transients, 3800 Hz (22400 Hz) spectral widths, 32K (64K) data points, and 10 s (5 s) relaxation delays. The ^{13}C NMR spectra were recorded in the inverse gated mode.

For the determination of T_g and T_m differential scanning calorimetry (DSC) was used. Samples of 8 – 14 mg PHA were weighed into aluminum pans and analyzed with a DSC 30 (Mettler Toledo, Greifensee, Switzerland). The samples were cooled down to $-80 \text{ }^\circ\text{C}$ within 10 minutes. After temperature equilibration, the sample was heated to $100 \text{ }^\circ\text{C}$ at a heating rate of $10 \text{ }^\circ\text{C min}^{-1}$. Molecular weights (number average (M_n) and weight average (M_w)) were determined by gel permeation chromatography (GPC, Waters 150, Milford MA, U.S.A.) equipped with a RI detector. The system was calibrated by using 10 polystyrene standards with known M_w (2×10^3 to $2.13 \times 10^6 \text{ g mol}^{-1}$) and low molecular weight distributions ($M_w/M_n \leq 1.09$). Forty mg of every sample were dissolved in 10 mL THF for 2 hours. Aliquots of 100 μL of the polymer solution were chromatographed with pure THF as the solvent

phase through 2 GPC-columns (Mixed-Bed, Viscothek, Houston, U.S.A.) at a flow rate of 1 mL min^{-1} .

RESULTS AND DISCUSSION

For the determination of the dual nutrient limited growth regime, *Pseudomonas putida* GPo1 was grown in a chemostat at a dilution rate of 0.1 h^{-1} . The C_0/N_0 ratio in the feed medium was changed by variation of the carbon concentration in the feed medium (a constant mixture of 55 mol% octanoate, 10 mol% 10-undecenoate and 35 mol% 5-phenylvalerate) only; the nitrogen supply was kept constant at $N_0 = 150 \text{ mg nitrogen L}^{-1}$. The medium was designed in such a way that only nitrogen and carbon could limit growth, all other nutrients being in excess.

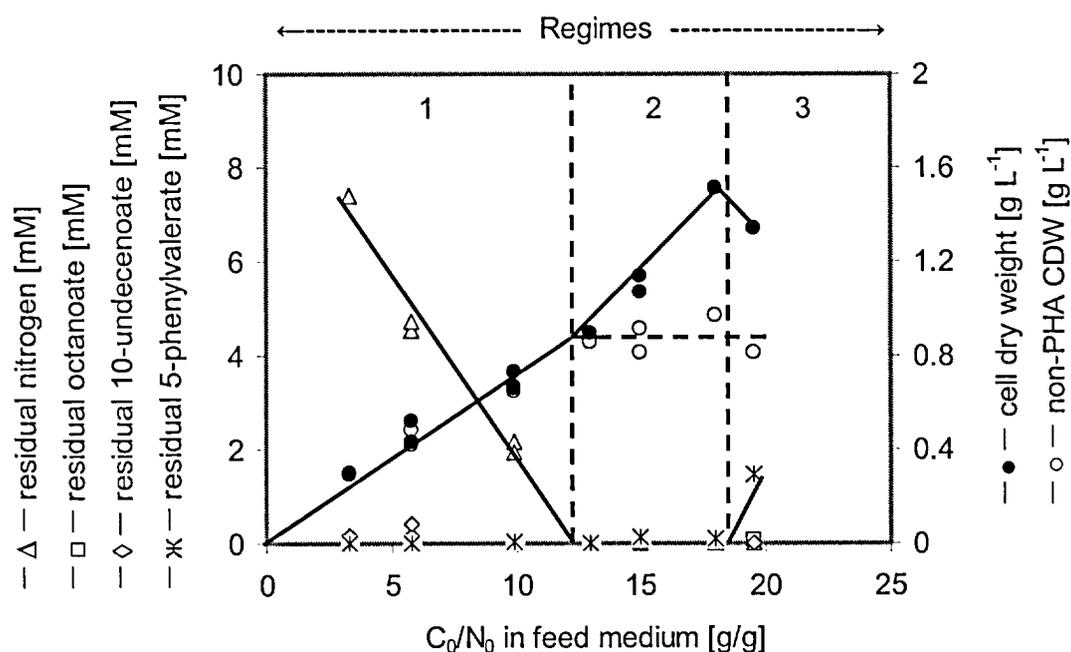


Figure 3.1: Growth and PHA content of *Pseudomonas putida* GPo1 in a chemostat ($D = 0.1 \text{ h}^{-1}$) at different C_0/N_0 ratios in the feed medium. A constant mixture of C source (55 mol% octanoate, 10 mol% 10-undecenoate and 35 mol% 5-phenylvalerate) was used.

Growth regimes

Three different growth regimes were found by increasing the carbon concentration in the feed medium, in agreement with earlier findings by Durner et al., 2001. The first growth regime (Figure 3.1) went from a C_0/N_0 ratio of 0 to 12.1 g g^{-1} . In this regime, the cells grew under carbon limitation. A linear increase of the cell dry weight (CDW) was observed by increasing the carbon concentration in the feed medium. Thus, the concentration of bacteria in the culture broth increased with increasing C_0/N_0 ratios. A concomitant decrease of the residual nitrogen concentration in the culture supernatant occurred, since a higher concentration of microorganisms consumed more nitrogen. In the second regime, which began at $C_0/N_0 = 12.1 \text{ g g}^{-1}$ and went as far as $C_0/N_0 = 18.3 \text{ g g}^{-1}$, no residual nitrogen was detected in the culture supernatant, indicating that growth was limited by carbon and nitrogen simultaneously. Thus, neither residual nitrogen nor residual carbon (octanoate, 10-undecenoate or 5-phenylvalerate) was found in the culture supernatant in this dual nutrient (C,N) limited regime. In fact, the cell dry weight continued to increase and even showed a higher yield in this second regime than under single carbon limitation: the biomass yield $Y_{X/C}$ (which is the yield coefficient of g biomass formed in the culture per g carbon substrate used) (Herbert et al., 1956) was calculated and increased from $Y_{X/C} = 0.47 \text{ g g}^{-1}$ in regime 1 to $Y_{X/C} = 0.58 \text{ g g}^{-1}$ at a C_0/N_0 ratio of 18.0 g g^{-1} . This can be explained by the fact that there was very little PHA formation in regime 1. In this growth regime, 5-phenylvalerate was completely metabolized to 3-phenylpropionate, which has been reported not to be a growth substrate for *P. putida* GPo1 (Fritzsche et al., 1990a). Therefore, under C limited growth conditions, only two carbon atoms from 5-phenylvalerate were used for biomass production. In regime 2, on the other hand, 5-phenylvalerate served not only as growth substrate, but was also incorporated into PHA as 3-hydroxy-5-phenylvalerate. Consequently, a higher fraction of the carbon atoms of this compound was used and the carbon yield was higher in the dual nutrient (C,N) limited growth zone.

In regime 3, where the carbon input increased above a C_0/N_0 ratio of 18.3 g g^{-1} , unutilized 5-phenylvalerate was detected in the culture supernatant and the biomass concentration decreased slightly, which was also observed in a previous study (Durner et al., 2000). This can be explained by a decreased cell viability due to a toxic effect of 5-phenylvalerate (Pirt, 1975).

The PHA-free biomass remained approximately constant in the dual nutrient limited (regime 2) and the nitrogen limited growth regime 3. The cells accumulated significant amounts of mclPHA in growth regimes 2 and 3, increasing from about 4 wt% at a C_0/N_0 ratio of 13 g g^{-1} , to 36-40 wt% at the boundary of regimes 2 and 3.

Biosynthesis of tailored, olefinic mclPHAs

Pseudomonas putida GPo1 was then grown on five different substrate mixtures of octanoate, 10-undecenoate and 5-phenylvalerate in the feed medium at a constant C_0/N_0 ratio of 15 g g^{-1} (Table 3.1, experiments A – E), which is well within the dual nutrient limited growth zone (see Figure 3.1). The content of 10-undecenoate in the carbon feed was kept constant at 10 mol%, whereas the feed content of 5-phenylvalerate was gradually increased from 0 mol% to 35 mol%, and octanoate was decreased from 90 mol% to 55 mol%.

Table 3.1: Compositions of the carbon mixtures fed to the continuous cultures ($D = 0.1 \text{ h}^{-1}$; $C_0/N_0 = 15 \text{ g g}^{-1}$) and of the resulting polymers. a) From ^1H NMR spectra, b) See Scheme 3.1, c) Polymer D, from ^{13}C NMR spectra.

Experimental conditions		A	B	C	D	E
<u>C-source feed</u> [mol%]	5-Phenylvalerate	-	5	15	25	35
	Octanoate	90	85	75	65	55
	10-Undecenoate	10	10	10	10	10
<u>Cell and PHA production</u>						
Cell dry weight [g L^{-1}]		1.05	0.98	1.02	1.05	1.06
PHA content during steady state [% CDW]		29	27	18	16	20
<u>PHA composition</u>						
Poly(HP-co-HA-co-HE) [mol%] ^{a)}	HP ^{b)}	-	3	19	42	59
	HA = (HA6 + HA8) ^{b)}	90	81	65	47	HA6 = 6.3 ^{c)}
						HA8 = 40.7 ^{c)}
	HE = (HE7 + HE9 + HE11) ^{b)}	10	16	16	11	HE7 = 2.5 ^{c)}
HE9 = 6.8 ^{c)}						
					HE11 = 1.7 ^{c)}	

Because of the β -oxidation of the carbon sources to PHA precursors with one or more C_2 -units less, PHA copolymers consisting of 3-hydroxyphenylvalerate (HP, Scheme 3.1), 3-hydroxy-alkanoates (HA6, HA8) and 3-hydroxyalkenoates (HE7, HE9, HE11) were produced. No 3-hydroxy-3-phenylpropionate was found in the accumulated polymers. This agrees with the findings of Fritzsche et al (Fritzsche et al., 1990a), who showed that neither 3-phenylpropionate nor 3-hydroxy-3-phenylpropionate were substrates for growth or PHA formation.

The cell dry weights during steady-states did not significantly differ from experiments A to E, whereas the PHA content of the cells decreased slightly with increasing 5-phenylvalerate content in the feed medium. We propose that this PHA decrease was caused by a shift of the dual nutrient (C,N) limited growth regime, because 5-phenylvalerate cannot be metabolized completely under carbon limitation. This is in contrast with the results obtained for pure n-alkanoic acids (Brandl et al., 1988). As a result, more carbon equivalents are required in order to yield the same biomass. In fact, Durner and co-workers (Durner et al., 2000) observed in a chemostat culture ($D = 0.1 \text{ h}^{-1}$) of *P. putida* GPo1 grown on octanoic acid only that the lower boundary of the dual nutrient (C,N) limited growth regime was at $10.6 \text{ mol mol}^{-1}$ (9.1 g g^{-1}) and the yield of biomass ($Y_{X/C}$) was 0.83 g g^{-1} . In the present work, we determined for the same growth conditions but a different carbon feed mixture (55 mol% octanoate, 10 mol% 10-undecenoate, and 35 mol% 5-phenylvalerate) a lower boundary at $C_0/N_0 = 12.1 \text{ g g}^{-1}$, and for $Y_{X/C}$ a value of 0.47 g g^{-1} . Therefore, with increasing content of 5-phenylvalerate, the dual nutrient (C,N) limited growth regime shifted towards higher C_0/N_0 values. In practice, this means that for identical C_0/N_0 ratios but increasing 5-phenylvalerate contents in the feed medium, the PHA content has to decrease. The limited data set of our experiments supports this explanation (compare Table 3.1).

PHA composition

The monomeric composition of poly(HP-co-HA-co-HE) was determined from ^1H and ^{13}C NMR spectra. The aromatic [HP], saturated [HA = (HA6 + HA8)] and unsaturated [HE = (HE7 + HE9 + HE11)] fractions were readily obtained from ^1H NMR spectra by integration of the aromatic signals resonating at $\delta = 7.15 \text{ ppm}$, the methyl groups at 0.87 ppm , and the olefinic protons at 5.76 ppm (Table 3.1). The signals of the different saturated and unsaturated units overlapped in the ^1H NMR spectrum, but the resonances of HA6 and HA8, and these of HE7, HE9 and HE11 were separated

in the ^{13}C NMR spectra (for example $\delta(-\text{CH}_3)$ of HA6 = 14.4 ppm, of HA8 = 14.6 ppm; $\delta(=\text{CH}_2)$ of HE7 = 115.9 ppm, of HE9 = 115.2 ppm, of HE11 = 114.9 ppm). Integration of these (small) resonances yielded the ratios HA6 : HA8 and HE7 : HE9 : HE11, and with the data from ^1H NMR, the complete composition could be estimated. As an example, the composition of poly(HP-co-HA-co-HE) from experiment D is shown in Table 3.1.

The fraction of HE units (10 – 16 mol%) in the polymers from experiments A – E approximately reflected the constant 10 mol% 10-undecenoate of the feed media. However, the content of HP was larger compared to the content of 5-phenylvalerate in the feed. For example, cells fed with 35 mol% 5-phenylvalerate, 55 mol% octanoate, and 10 mol% 10-undecenoate produced a polymer with 59 mol% HP units, 31 mol% HA units, and 10 mol% HE units. The increase of the HP content was accompanied by a strong decrease of the HA units. Figure 3.2 illustrates these changes in polymer composition.

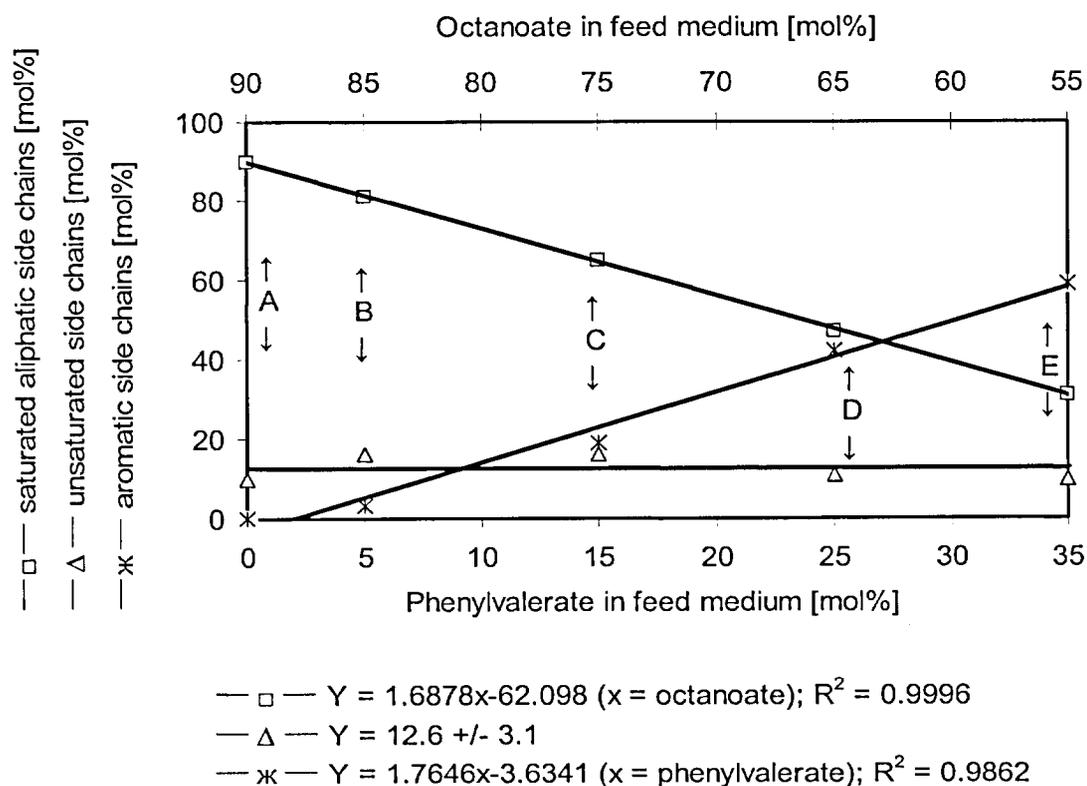


Figure 3.2: Linear regressions of the copolymer contents of poly(HP-co-HA-co-HE) produced in continuous cultures ($D = 0.1 \text{ h}^{-1}$; $C_0/N_0 = 15 \text{ g g}^{-1}$) with 10-undecenoate (10 mol%), 5-phenylvalerate (0 – 35 mol%), and octanoate (90 – 55 mol%) respectively.

The increase of the HP unit, the decrease of the HA units and the almost constant amounts of HE units may be explained by the different substrate affinity to the carbon sources used in these experiments. It is known that octanoic acid is a good growth substrate for *Pseudomonas putida* GPo1, whereas this strain grows only slowly on 5-phenylvaleric acid (Fritzsche et al., 1990a; Durner et al., 2001). Thus, the consumption of octanoic acid during steady-state conditions is faster than the consumption of 5-phenylvaleric acid, leading to the observed changes of polymer composition from experiments A-E.

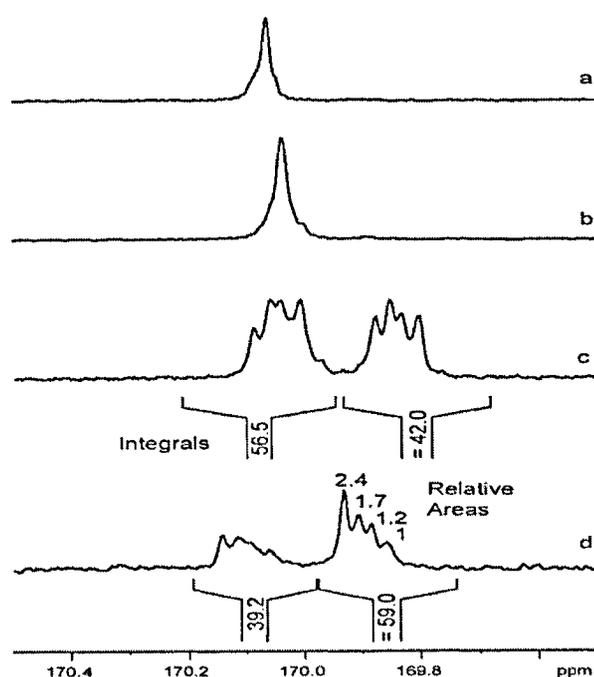


Figure 3.3: ^{13}C NMR carbonyl chemical shift region of mclPHAs from a) feed 100 mol% octanoate, b) experiment A, c) experiment D, and d) experiment E.

^{13}C NMR spectra of the carbonyl region of various mclPHAs are shown in Figure 3.3. Figure 3.3a shows a single carbonyl resonance at 170.07 ppm for poly(HA6-co-HA8) (a polymer that was produced from octanoate as the only carbon source). The same is true for the carbonyl carbons (170.04 ppm) of poly(90%HA-co-10%HE) from experiment A, as shown in Figure 3.3b. For polymers containing 5-phenylvalerate units, such as poly(42%HP-co-47%HA-co-11%HE) and poly(59%HP-co-31%HA-co-10%HE) from experiments D and E, the carbonyl region was split into two groups of

four peaks (Figures 3.3c and 3.3d). The splitting can be related to the sensitivity of the carbonyl carbons to their chemical environment and reflects the sequence distribution of the monomeric units along the polymer chain. By comparison with the chemical shifts of poly(HA6-co-HA8) and poly(90%HA-co-10%HE), the group of four lines at 170.05 ppm (Figure 3.3c) and 170.10 ppm (Figure 3.3d) were assigned to the HA and HE units, while the four lines at 169.84 ppm (Figure 3.3c) and 169.9 ppm (Figure 3.3d) came from HP monomeric unit. This assignment was confirmed by the area of the NMR signals; in Figure 3.3c, the intensity of the HP unit was set to 42.0 (mol%, Table 3.1), with a resulting intensity of 56.5 (mol%) for HA and HE, close to the expected value of 58 (mol%) for the sum of the fractions of HA (47 mol%) and HE (11 mol%). The same was true for poly(59%HP-co-31%HA-co-10%HE) in Figure 3.3d. The splitting into four lines was attributed to triads HP(HA/HE)*HP, HP(HA/HE)*(HA/HE), (HA/HE)(HA/HE)*HP, and (HA/HE)(HA/HE)*(HA/HE) at high frequency, and to HPHP*HP, HPHP*(HA/HE), (HA/HE)HP*HP and (HA/HE)HP*(HA/HE) at low frequency. The resolution of the (HA/HE) centered triads was poor, but the HP unit of poly(59%HP-co-31%HA-co-10%HE) could be fit to a sum of four Lorentzian lines with relative intensities of approximately 2.4 : 1.7 : 1.2 : 1. The calculated intensities for a statistically random copolymer are 2.1 : 1.4 : 1.4 : 1. The experimental intensities deviate only slightly from the values expected for a random copolymer, and the differences may be the result of the poor spectral resolution and the rather low NMR signal-to-noise ratio. However, the observed line splitting in the NMR spectra let us conclude that *Pseudomonas putida* GPo1 incorporated 5-phenylvalerate, octanoic acid and 10-undecenoic acid without any preferential sequence, producing a random copolymer.

Table 3.2: GPC data of the five polymers (A-E) produced in continuous cultures ($D = 0.1 \text{ h}^{-1}$; $C_0/N_0 = 15 \text{ g g}^{-1}$).

Polymer	Mw	Mn	Mw/Mn
A	191'000	86'000	2.2
B	246'000	120'000	2.1
C	227'000	104'000	2.2
D	214'000	83'000	2.6
E	358'000	124'000	2.9

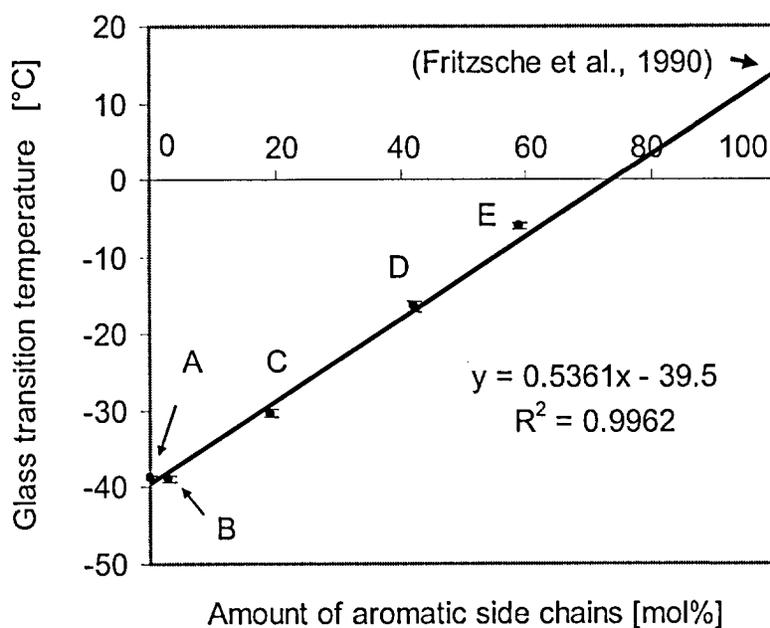


Figure 3.4: Glass transition temperature (T_g) as a function of the monomeric composition.

Physical properties

Molecular weights (number average (M_n) and weight average (M_w)) as well as the molecular weight distribution (M_w/M_n) of the different polymers A-E are shown in Table 3.2. M_w and M_n values were typical for molecular weights reported for mclPHAs (Witholt and Kessler, 1999), with the weight average value for sample E, containing the largest fractions of HP units, close to $M_w = 350'000$ for the homopolymer poly(3-hydroxy-5-phenylvalerate) (Fritzsche et al., 1990a).

Depending on the amount of 3-hydroxy-5-phenylvalerate, the polymers showed different thermal properties (Figure 3.4). T_g increased linearly from $-38.7\text{ }^\circ\text{C}$ in polymer A to $-6.0\text{ }^\circ\text{C}$ in polymer E. An extrapolation to 100% aromatic side chains would lead to a glass transition temperature of about $14.1\text{ }^\circ\text{C}$ for poly(3-hydroxy-5-phenylvalerate). This is in good agreement with the literature value of $T_g = 13\text{ }^\circ\text{C}$ for this homopolymer (Fritzsche et al., 1990a). This means that the glass transition temperatures of mclPHAs with 10 mol% olefinic side chains can be predicted also for HP fractions larger than 59 mol% (as reported in this work) over a total range of approximately $50\text{ }^\circ\text{C}$. The dependence of T_g on the fraction of phenyl groups parallels the findings for several copolymers of poly(3-hydroxybutyrate-co-X) with X = 3-hydroxypropionate, 4-hydroxybutyrate, 3-hydroxyvalerate, and 3-hydroxyhexanoate, where the values of T_g of all copolymers decreased linearly with the increase of the second monomer unit content (Feng et al., 2002).

Polymer A showed a melting point at $40.1\text{ }^\circ\text{C}$. In contrast, polymers of experiments B to E containing phenyl groups did not display any crystalline melting endotherm. It is known that the homopolymer poly(3-hydroxy-5-phenylvalerate) as well as phenyl group bearing (co-)polymers from 6-phenylhexanoate, 7-phenylheptanoate, and 8-phenyloctanoate are amorphous (Curley et al., 1996; Abraham et al., 2001). We found here that the incorporation of as little as 5 mol% phenyl groups leads to completely amorphous olefinic mclPHAs. On the other hand, crystalline mclPHAs have been produced from 5-(4-tolyl)valeric acid and 8-(*p*-methylphenoxy)octanoic acid (Curley et al., 1996; Kim et al., 1999). As a natural extension of this work, it would be interesting to use such aromatic containing substrates for the production of polymers of type poly(3-hydroxy-5-(4-tolyl)valerate-co-HA-co-HE). This would enable the production of crystalline mclPHAs with olefinic side chains, where not only T_g , but also T_m can be adjusted in a predictable way over a wide temperature range.

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CHAPTER 4

Conceptual Study for the Biosynthesis
of Block Copolymers in a Two-Stage
Continuous Culture System of
Pseudomonas putida GPo1

Abstract: *P. putida* GPo1 was cultivated in a sequence of two dual nutrient (C,N) limited chemostat cultures. The cells were supplied with different amounts of octanoic acid in the first fermentor and different amounts of 10-undecenoic acid in the second fermentor. The polymers isolated from the second-stage fermentor contained different amounts of aliphatic and olefinic PHA monomers. Their characterization by gel permeation chromatography, differential scanning calorimetry and their crosslinking ability indicated that the obtained mclPHAs were mainly blends of polymers produced from octanoic acid (PHO) and 10-undecenoic (PHU). The crosslinking-extraction experiments indicated that these polymers might contain small amounts of repeating units from PHO and PHU.

INTRODUCTION

Poly[(*R*)-3-hydroxyalkanoates] (PHAs) are natural polyesters accumulated by numerous bacteria as intracellular carbon and energy storage compounds. Generally, mclPHA accumulation is triggered under growth conditions such as limitation of nitrogen, phosphorus, magnesium or oxygen while a suitable carbon source is in excess (Lee, 1996). Recently, it has been shown in chemostat cultures of *P. putida* GPo1 that mclPHA accumulation occurs when nitrogen and carbon are limiting growth simultaneously (Durner et al., 2000; Zinn et al., 2003; Hartmann et al., 2004).

Medium-chain-length poly[(*R*)-3-hydroxyalkanoates] (mclPHAs), which contain C6 to C12 3-hydroxy alkanoate monomers exhibit a wide variety in their monomeric composition and more than 100 different monomers were found in biosynthetic mclPHAs (de Rijk et al., 2002). To date, *Pseudomonas putida* GPo1 (formerly *Pseudomonas oleovorans*) is the best investigated mclPHA producer. Besides a large variety of linear (de Smet et al., 1983; Brandl et al., 1988; Lageveen et al., 1988; Gross et al., 1989) or branched side chains (Fritzsche et al., 1990b; Lenz et al., 1992), various mclPHAs with side chains containing functional groups such as carbon-carbon double (Lageveen et al., 1988) and triple bonds (Kim et al., 1998), acetoxyl and ketone (Jung et al., 2000), or aromatic groups (Fritzsche et al., 1990a; Curley et al., 1996; Kim et al., 1999) have been produced in *P. putida* GPo1. Several reports in the literature describe a chemical modification of PHAs containing unsaturated side chains (de Koning et al., 1994; Bear et al., 1997; Lee et al., 2000a;

Dufresne et al., 2001; Hazer et al., 2001; Kurth et al., 2002; Stigers and Tew 2003). Thus, olefinic PHAs represent an interesting group of mclPHAs. Ballistreri and co-workers (Ballistreri et al., 1992) reported that PHAs obtained from *P. putida* GPo1 were random copolymers, when grown on mixtures of 10-undecenoic acid with either octanoic acid or nonanoic acid. We recently demonstrated that mixtures of 5-phenylvaleric acid, octanoic acid, and 10-undecenoic acid fed to a chemostat culture of *P. putida* GPo1 resulted in random copolymers, too (Hartmann et al., 2004).

To date only a few reports can be found in the literature where the biosynthetic production of block copolymers was attempted. Such biodegradable block copolymers are seen as promising biomaterials by virtue of the possibility to manipulate their amphiphilic, mechanical and physical properties by adjusting the ratio of the constituting blocks (Kumar et al., 2001). Kim and co-workers (Kim et al., 1997) investigated whether it is possible to produce block copolymers when *P. putida* GPo1 was grown in a batch culture and sequentially fed with nonanoic acid (NA) and 10-undecenoic acid (UA). They concluded that the obtained polymers were mainly blends of PHA(NA) and PHA(UA), although a fractionation study indicated that small amounts of the mclPHA contained repeating units from both substrates.

In this study, we examined the possibility of producing block copolymers in a sequence of two dual nutrient (C,N) limited chemostat cultures where the cells were supplied with octanoic acid in the first and 10-undecenoic acid in the second fermentor.

MATERIALS AND METHODS

Cultivation conditions: *P. putida* GPo1 (ATCC 29347) was used for all experiments. For the preparation of inocula, 1 mL frozen stock culture was added to 100 mL of minimal medium in 300 mL shake flasks and cells were grown overnight at 30 °C. The minimal medium contained per liter: 3.5 g $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, 7.5 g K_2HPO_4 , 3.7 g KH_2PO_4 , and 2.9 g $\text{Na}_3\text{citrate} \cdot 2\text{H}_2\text{O}$ (Vogel and Bonner 1956). The pH was adjusted to 7.1 with 10 M NaOH. This medium was autoclaved and subsequently supplemented with filter sterilized $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 mL L^{-1} , 1M) and 1 mL L^{-1} of mineral trace element stock solution which contained per liter: 2.78 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.47 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.98 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.81 g $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.17 g

CuCl₂*2H₂O, and 0.29 g ZnSO₄*7H₂O in 1 M HCl (Lageveen et al., 1988). Fifty mL of shake-flask culture were used to inoculate the first fermentor.

For continuous cultivation the following medium was used (per liter): 1 g KH₂PO₄, 0.71 g (NH₄)₂SO₄, and 0.25 g MgSO₄*7H₂O. Further, 1 L of the medium was supplemented with 1 mL of 10 mM FeSO₄*7H₂O (in 1 M HCl) and 1 mL of mineral trace element stock solution containing per liter: 1.47 g CaCl₂*2H₂O, 1.98 g MnCl₂*4H₂O, 2.81 g CoSO₄*7H₂O, 0.17 g CuCl₂*2H₂O, 0.29 g ZnSO₄*7H₂O, and 10 g EDTA at pH 4 (Durner et al., 2001). Forty liter of this medium were then filter sterilized into gamma sterilized 50 L medium bags (Flexboy, Stedim S.A., Aubagne Cedex, France). Octanoic and 10-undecenoic acid were pumped directly into the culture vessels by using a dosimat (Metrohm, Herisau, Switzerland).

Two-stage continuous culture system: The two-stage continuous culture system consisted of two 3.7 L laboratory bioreactor in series (KLF 2000, Bioengineering, Wald, Switzerland), both with a working volume of 2.8 L (Figure 4.1). All cultures were run at 30 °C and the pH was maintained at 7.0 ± 0.05 by automated addition of either 2 M NaOH or 2 M H₂SO₄. The dissolved oxygen tension was monitored continuously with an oxygen probe (Mettler Toledo, Greifensee, Switzerland) and care was taken that it remained above 35 % air saturation. In the first-stage fermentor, cells were first grown on the same minimal medium used for the preparation of inocula. When the culture entered the stationary phase, the continuous culture system was started. Generally dilution rates of both fermentors were controlled by the medium supply of the first-stage fermentor (F1). The culture volume in F1 was kept constant by an overflow tube that was connected to a peristaltic pump continuously transferring broth to the second-stage fermentor (F2). The second-stage fermentor was previously sterilized with 2.8 L of a phosphate buffer (7.5 g L⁻¹ K₂HPO₄ and 3.7 g L⁻¹ KH₂PO₄). Cells from the F2 were harvested by using again an overflow tube connected to a continuously running peristaltic pump and finally collected in a 10 L harvest tank which was cooled on ice in order to avoid intracellular degradation of PHA. At the end of each fermentation experiment, the culture broth from F1 was also collected and treated in the same way as described for F2.

The carbon to nitrogen ratio (C₀/N₀) of the feed medium was set by the pump rates of the ammonium containing minimal medium and the carbon source. The use of two syringe pumps allowed the setting of different C₀/N₀ ratios in F1 and F2 as well as their supply with different carbon substrates (octanoic acid and 10-undecenoic acid).

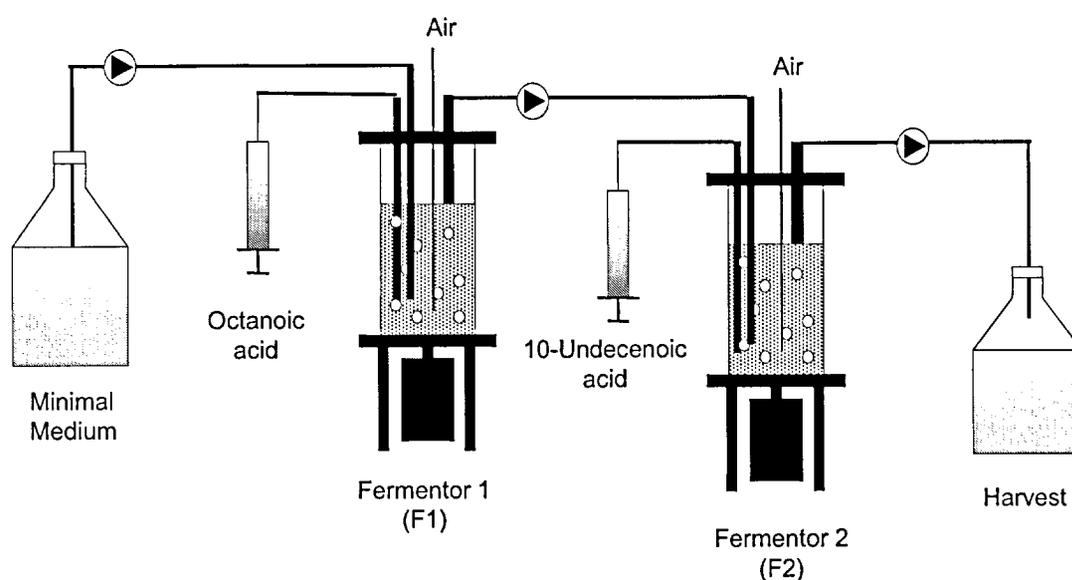


Figure 4.1: Experimental set-up of two-stage continuous culture system.

Sample preparation: Cells were spun down at 4500 x g for 15 min at 4 °C. The pellet was washed with deionised water, lyophilized for 48 hours, and stored in a desiccator.

Cell dry weight (CDW): Cells were collected on preweighed polycarbonate filters (pore-size 0.2 μm , Nuclepore, Sterico AG, Dietikon, Switzerland). The filters were first washed with 10 mM MgCl_2 , dried overnight at 110 °C, cooled down in a desiccator over silicagel and weighed. An appropriate volume of cell suspension (5 to 10 mL) was then filtered through the preweighed filter. The filters were dried again at 110 °C overnight and the weight difference was used to calculate the concentration of the biomass in the culture.

Analyses of the substrate concentrations in the culture supernatant: Ammonium was measured by using a photometric ammonium test (Spectroquant, Merck, Darmstadt, Germany). The detection limit of this method was 0.01 mg L^{-1} $\text{NH}_4\text{-N}$. The method was linear up to concentrations of 3.0 mg L^{-1} $\text{NH}_4\text{-N}$. If necessary, samples were diluted with nano-pure water. Octanoate and 10-undecenoate were measured by reversed-phase liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS), which was performed using an esquire high capacity trap (Bruker Daltonics, Bremen, Germany) equipped with an Agilent HP1100 binary pump. The fatty acids were separated on a C18 Nucleosil 2 x 250 mm column (3 μm , 100 Å, Macherey-Nagel Inc., Easton, PA, USA). Mobile

phases consisted of 0.1 % acetic acid in nano-pure water (A) and 0.1 % acetic acid in acetonitrile (B). Separation was achieved using a linear gradient from 100 % A to 100% B in 10 minutes. The flow rate was 0.2 mL min^{-1} with injection volumes of $7.5 \text{ }\mu\text{L}$. The mass spectrometer was operated in negative ion mode with a voltage of + 4.6 keV and a desolvation gas flow of 8 L min^{-1} at $350 \text{ }^\circ\text{C}$. The fatty acids were recorded from their extracted ion chromatograms (EIC) at m/z 143 for octanoic acid and m/z 183 for 10-undecenoic acid, respectively. Quantification occurred in the range of 2-8 ppm after dilution of the samples in acetonitrile/double distilled water (1/1 v/v). For the calibration of the system 2, 5 and 10 ppm standards of the corresponding fatty acids were used.

PHA extraction: mclPHA was extracted directly from lyophilized cells. Cells were pulverized and transferred into pure methylene chloride (60 g CDW in 1 L methylene chloride). After stirring the suspension overnight, the solution was filtered and concentrated by distillation. The polymer was then precipitated in ice-cold methanol (final ratio (v/v) of $\text{CH}_2\text{Cl}_2/\text{MeOH} = 1/6$). After removal of the liquids, the mclPHA was vacuum-dried ($30 \text{ }^\circ\text{C}$, 30 mbar) for at least one day.

PHA analysis: The cellular mclPHA content and the monomeric composition were determined using a modified gas chromatography procedure described by Riis and Mai (Riis and Mai, 1988) as well as by NMR spectroscopy. For gas chromatography a known amount of about 10 mg lyophilized cells were weighed into a 10 mL pyrex tube. Then, 1 mL of methylene chloride containing 1.5 mg 3-hydroxyisovaleric acid as internal standard and 1 mL of a mixture of *n*-propanol / hydrochloric acid (80/20 v/v) were added. The tube was capped and heated for 3h at $100 \text{ }^\circ\text{C}$. After cooling, 2 mL of nano-pure water were added and the tube shaken on a laboratory mixer. The organic layer was then dried with anhydrous sodium sulfate. The derivatized samples were analyzed in methylene chloride solution on a GC (Hewlett Packard 5890/II, Urdorf, Switzerland) equipped with a flame ionization detector (FID). The separation was made on a Supelcowax 10 column, $30 \text{ m} \times 0.25 \text{ mm}$, $0.5 \text{ }\mu\text{m}$ (Supelco, Buchs, Switzerland). The GC parameters were: temperature of the injector $250 \text{ }^\circ\text{C}$, temperature of the FID detector $285 \text{ }^\circ\text{C}$, He gas flow 3 mL min^{-1} , split ratio 1:10, $3 \text{ }\mu\text{L}$ of injection. The oven temperature program was: $120 \text{ }^\circ\text{C}$, 1 min isotherm, $120\text{-}280 \text{ }^\circ\text{C}$ with $10 \text{ }^\circ\text{C min}^{-1}$, 1 min isotherm. The 3-hydroxyalkanoate propylesters were identified by comparing the sample retention times with the commercially available 3-hydroxy standards of butanoic, hexanoic, octanoic, decanoic, and dodecanoic acid

after propanolysis. Quantification was done via a calibration function generated from a mixture of 3-hydroxyisovaleric acid and these 5 standards after propanolysis as described above, and interpolating the response factors for monomers not available commercially.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ASX-400 NMR spectrometer at 300 K. For proton (^1H) NMR spectra 10 mg of polymer were dissolved in 0.7 mL chloroform-d, and 20 – 30 mg of polymer were used for carbon (^{13}C) spectra. Chemical shifts are given in parts per million (ppm) relative to the signal of chloroform as internal reference (^1H NMR: 7.26 ppm; ^{13}C NMR: 77.7 ppm). The ^1H (^{13}C) NMR spectra were recorded at 400.13 MHz (100.61 MHz) with the following parameters: 5 μs (3 μs) 45° pulse lengths, 32 (10K) transients, 3800 Hz (22400 Hz) spectral widths, 32K (64K) data points, and 10 s (5 s) relaxation delays. The ^{13}C NMR spectra were recorded in the inverse gated mode.

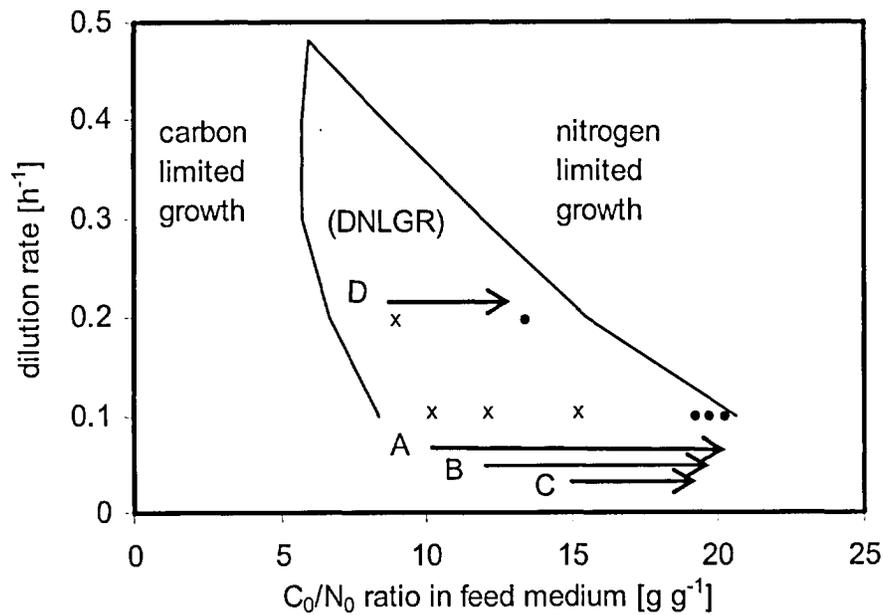
Melting (T_m) and glass transition temperatures (T_g) were determined by differential scanning calorimetry (DSC). Samples of 8 – 14 mg mclPHA were weighed into aluminum pans and analyzed with a DSC 30 (Mettler Toledo, Greifensee, Switzerland). The samples were cooled down to -80 °C within 10 minutes. After temperature equilibration, the sample was heated to 100 °C at a heating rate of 10 °C min^{-1} . Molecular weights (number average (M_n) and weight average (M_w)) were determined by gel permeation chromatography (GPC, Waters 150, Milford MA, USA) equipped with a RI detector. The system was calibrated by using 10 polystyrene standards with known M_w (2×10^3 to 2.13×10^6 g mol^{-1}) and low molecular weight distributions ($M_w/M_n \leq 1.09$). Forty mg of every sample were dissolved in 10 mL THF for 2 hours. Aliquots of 100 μL of the polymer solution were chromatographed with pure THF as the solvent phase through 2 GPC-columns (Mixed-Bed, Viscotek, Houston, USA) at a flow rate of 1 mL min^{-1} .

Crosslinking of PHAs: The mclPHA crosslinking was adapted from the procedure described by Kim et al. (Kim et al., 1997). Mixtures of known amounts of PHAs produced from octanoic acid and 10-undecenoic acid alone, as well as olefinic PHAs obtained from the two-stage continuous culture system were crosslinked using a borane tetrahydrofuran complex solution ($\text{BH}_3\text{-THF}$). 400 mg of polymers were dissolved in 8 mL of a mixture of methylene chloride / tetrahydrofuran (1/1 v/v) and 2 mL of $\text{BH}_3\text{-THF}$ solution (1M) were added. Gel was produced immediately after the

addition of the BH_3 -THF solution. Thirty minutes after gel formation, a few drops of nano-pure water were added, followed by the addition of 50 mL of methylene chloride. The mixture was shaken on a laboratory mixer and let stand for additional 30 minutes at room temperature. The soluble fraction was then filtered through a glass tube clogged with fibreglass and the dissolved polymer was recovered by evaporating the solvents. This fraction was weighed and analyzed for olefinic monomers by ^1H NMR.

RESULTS AND DISCUSSION

MclPHA can be accumulated by *P. putida* GPo1 in continuous cultures during dual nutrient (C,N) limited growth, i.e., during simultaneous nitrogen (N) and carbon (C) limitation (Durner et al., 2000; Zinn et al., 2003; Hartmann et al., 2004). Durner et al., 2000 (Durner et al., 2000) defined a so called dual nutrient (C,N) limited growth regime (DNLGR) for the carbon source octanoic acid and the nitrogen source ammonium and showed it to be a function of the dilution rate and the carbon to nitrogen ratio (C_0/N_0) of the medium feed. Based on this DNLGR, four different experimental settings (A-D) were set (Figure 4.2). The cells were first supplied with minimal medium and octanoic acid in F1. Here, the carbon to nitrogen ratios (C_0/N_0) were chosen such that dual nutrient (C,N) limited growth occurred in F1 (A = 10.9, B = 13.4, C = 15.9 and D = 9.7). Culture broth transferred to F2 was further supplied with only 10-undecenoic acid in F2. The supply of 10-undecenoic acid in F2 corresponds to shifts of A = 9.8, B = 6.4, C = 3.2 and D = 5 C_0/N_0 units, keeping the total C_0/N_0 ratio in F1 and F2 within the DNLGR. Analyses of the culture supernatant showed that dual nutrient (C,N) limited growth did indeed take place in F1 and F2 for all experimental settings (A-D). This corroborates the findings of chapter 2 that the DNLGR for 10-undecenoic acid coincided with that for octanoic acid. The results obtained for the four different experimental settings are summarized in Table 4.1.



— DNLGR for octanoate and ammonium ($N_0 = 150 \text{ mg L}^{-1}$)
as a function of the dilution rate

Figure 4.2: Dual nutrient (C,N) limited growth regime (DNLGR) for the carbon sources octanoic acid and the nitrogen source ammonium ($N_0 = 150 \text{ mg L}^{-1}$) as a function of the dilution rate (Durner et al., 2000). Four different experimental settings (A-D) were set. The cells were first supplied with minimal medium and octanoic acid in F1. The carbon to nitrogen ratios (C_0/N_0) were chosen such that dual nutrient (C,N) limited growth occurred in F1. After transfer of the culture broth to F2, cells were supplied with only 10-undecenoic acid. Thus, the supply of 10-undecenoic acid in F2 occurred such that the total C_0/N_0 ratios of F1 and F2 were still within the DNLGR.

Table 4.1: Growth conditions in a two-stage continuous culture system of *P. putida* GPo1, cell dry weight, PHA content, and compositions of the resulting polymers.

<i>Experimental conditions</i>								
Fermentation	A		B		C		D	
Fermentor	1	2	1	2	1	2	1	2
Dilution rate (h ⁻¹)	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2
Carbon to nitrogen units (g g ⁻¹)	10.9 ^{a)}	9.8 ^{b)}	13.4 ^{a)}	6.4 ^{b)}	15.9 ^{a)}	3.2 ^{b)}	9.7 ^{a)}	5 ^{b)}
<i>Biomass and PHA production</i>								
Cell dry weight (CDW) (g L ⁻¹)	1.13	1.53	1.26	1.48	1.35	0.78	1.22	1.32
PHA content during steady state (% CDW)	24.2	52.4	32.6	50.3	31.4	53.8	22.7	38.8
<i>PHA composition (mol%)</i>								
Σ 3-hydroxyalkanoates	100	19.4	100	35.4	100	59.8	100	46.2
3-hydroxyoctanoate	85.6	16.8	86.8	30.5	84.7	51.2	83.7	38.6
3-hydroxyhexanoate	14.4	2.6	13.2	4.9	15.3	8.6	16.3	7.6
Σ 3-hydroxyalkenoates	0	80.6	0	64.6	0	40.2	0	53.8
3-hydroxy-10-undecenoate	-	24.1	-	19.1	-	7.5	-	19.1
3-hydroxy-8-nonenoate	-	48.0	-	38.1	-	27.8	-	31.5
3-hydroxy-6-heptenoate	-	8.5	-	7.4	-	4.9	-	3.2

a) Cells were supplied with minimal medium ($N_0 = 150$ mg) and octanoic acid in F1.

b) Culture broth transferred from F1 supplied with only 10-undecenoate (The supply of 10-undecenoic acid correspond to a shift of 9.8, 6.4, 3.2 and 5 C_0/N_0 units (calculated on $N_0 = 150$ mg; see also Figure 4.2).

The dilution rates in fermentations A, B and C were 0.1 h⁻¹ in both fermentors, whereas the supply of carbon substrates to single fermentors differed. In fermentation A, a cell dry weight of 1.13 g L⁻¹ with a cellular mclPHA content of 24.2 % (wt/wt) was determined in F1. After transfer of the culture broth to F2, a further increase of the total biomass to 1.53 g L⁻¹ was observed, due to the ongoing

intracellular accumulation of mclPHA. Here, the cellular mclPHA content increased to 52.4 % (wt/wt). The resulting polymer obtained from F2 was composed of 19.4 mol% aliphatic mclPHA monomers and 80.6 mol% olefinic mclPHA monomers. For fermentation B, the cell dry weight (1.26 g L^{-1}) as well as the cellular mclPHA content (32.6 % (wt/wt)) was slightly higher in F1. This was to be expected since the C_0/N_0 ratio was higher than in fermentation A. Similar to fermentation A, the cell dry weight and the mclPHA content of the cells increased in the F2. The resulting polymer obtained from F2 in fermentation B exhibited a smaller fraction of olefinic mclPHA monomers (64.6 mol%) due to the increased supply of octanoic acid to F1 and the reduced supply of 10-undecenoic acid to F2. The results from fermentation C differed for the pattern observed from fermentations A and B. Here, the cell dry weight (1.35 g L^{-1}) was higher in F1, but no increase in the cellular mclPHA content (31.4 % (wt/wt)) was detected. More important the cell dry weight in F2 decreased to 0.78 g L^{-1} whereas the cellular mclPHA content increased to 53.8 % (wt/wt). Fermentation experiment D was performed at a dilution rate of 0.2 h^{-1} in both fermentors. Although the C_0/N_0 ratio ($C_0/N_0 = 9.7 \text{ g g}^{-1}$) in F1 was lower than in fermentation A, the observed cell dry weight of 1.22 g L^{-1} was higher than in fermentation A. As discussed in previous reports (Durner et al., 2000; Zinn et al., 2004) the carbon growth yield $Y_{X/C}$ increases towards higher dilution rates. Therefore the cell dry weight in F1 in fermentation D was higher, although the C_0/N_0 ratio was slightly lower than in fermentation A. According to fermentations A and B, a further increase of the total biomass in F2 to 1.32 g L^{-1} as well as an increase in cellular mclPHA content to 38.8 % (wt/wt) was observed. The resulting polymer obtained from F2 was here composed of 46.2 mol% aliphatic mclPHA monomers and 53.8 mol% olefinic mclPHA monomers.

Determination of Pirt parameters (m , $Y_{X/C}^{EG}$) for growth of *P. putida* GPo1 on 10-undecenoate: This significant decrease in the cell dry weight in F2 during fermentation experiment C let us presume that the supply of 10-undecenoic acid to F2 was not sufficient to meet the demand for cell energy maintenance. To determine the maintenance energy coefficient, *P. putida* GPo1 was grown in series of continuous cultures under carbon limitation (10-undecenoate, $C_0/N_0 = 4.0 \text{ g g}^{-1}$; $N_0 = 150 \text{ mg}$) at different dilution rates. According to the Pirt theory (Pirt 1965; Pirt 1975),

the experimentally determined carbon growth yield $Y_{X/C}^{C\ lim}$, is a function of the specific growth rate (μ). This fact is considered in the equation of Pirt:

$$\frac{1}{Y_{X/C}^{C\ lim}} = \frac{1}{Y_{X/C}^{EG}} + \frac{m}{\mu} \quad [1.1]$$

where $Y_{X/C}^{C\ lim}$ is the observed biomass yield on used carbon, $Y_{X/C}^{EG}$ is the true biomass yield on carbon without the influence of maintenance carbon consumption, m is the maintenance coefficient, and μ the specific growth rate (in steady-state cultures $\mu =$ dilution rate). A double reciprocal plot of $Y_{X/C}^{C\ lim}$ against μ gives a linear correlation with an intercept of $Y_{X/C}^{EG}$ and a slope m (Figure 4.3).

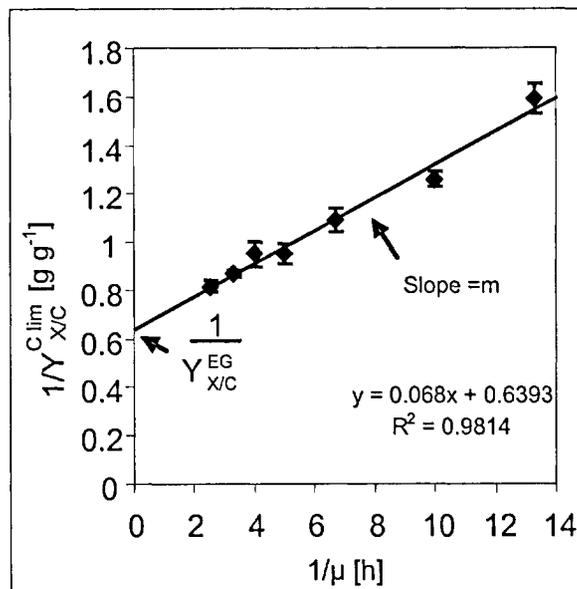


Figure 4.3: Determination of Pirt parameters for growth of *P. putida* GPO1 on 10-undecenoate. The experimental carbon yield was measured in 7 independent steady-state experiments with limiting carbon concentrations ($C_0/N_0 = 4.0 \text{ g g}^{-1}$) in the feed medium.

Figure 4.3 shows that the maintenance energy coefficient m for 10-undecenoate was $0.068 \text{ g g}^{-1} \text{ h}^{-1}$ which means that for growth of *P. putida* GPO1 on 10-undecenoate, one gram of cell dry weight needs a supply of 68 mg carbon during one hour just to meet the demand for cell energy maintenance. The true biomass yield $Y_{X/C}^{EG}$ for growth of *P. putida* GPO1 on 10-undecenoate was determined to be 1.56 g g^{-1} which means that without the influence of maintenance carbon consumption, 1 g of carbon (from 10-undecenoate) leads to a total biomass of 1.56 g.

From Table 4.1 it can be seen that for fermentation C the cell dry weight was 1.35 g L⁻¹ in F1. Considering the working volume of 2.8 L, a total biomass of 3.78 g needs a carbon supply of 0.257 g during one hour just to meet the demand for cell energy maintenance. Indeed, the carbon supply of F2 was 0.134 g C h⁻¹. As a consequence, a significant part of the cells may have died off. This assumption was also confirmed by determining the number of colony forming units (cfu) on TSA plates (results not shown). Here we observed a reduction of cfu's of about 50 % in F2. Finally, it has to be pointed out that the cellular mclPHA content increased to 53.8 % (wt/wt) in the second fermentor for fermentation C. Here we propose that the reduction in cell number was such that the supply of 10-undecenoate was not only sufficient to meet the demand for cell energy maintenance of surviving cells, but also allowed them to produce mclPHA.

Physical properties of the polymers produced in the two-stage continuous culture systems: Molecular weights (number average (Mn) and weight average (Mw)) as well as the molecular weight distribution (Mw/Mn) of the different polymers produced in fermentations A, B, C and D are shown in Table 4.2.

Table 4.2: GPC data of the different polymers produced in fermentations A, B, C and D.

Fermentation	A		B		C		D	
	1	2	1	2	1	2	1	2
Mw ^{a)}	219	255	302	282	218	255	232	220
Mn ^{a)}	121	132	152	135	121	132	119	114
Mw/Mn	1.8	1.9	2.0	2.1	1.8	1.9	1.9	1.9

a) [$\times 10^3$ g mol⁻¹]

From Table 4.2 it can be seen that the molecular weights (Mw and Mn) as well as the molecular weight distribution were approximately constant. Assuming that the polymers isolated from F2 produced in fermentations A-D were block copolymers, the molecular weights would be expected to increase. However, our results did not show an increase in the molecular weights of the mclPHAs isolated from F2. This indicates that the polymers obtained from the F2 were not block copolymers of repeating units

from aliphatic and olefinic mclPHA monomers. The DSC thermograms for mclPHAs that were isolated from F2 produced in fermentations A-D are shown in Figure 4.2. In addition thermograms for polymers produced from octanoic acid (PHO) and 10-undecenoic (PHU) alone are also shown.

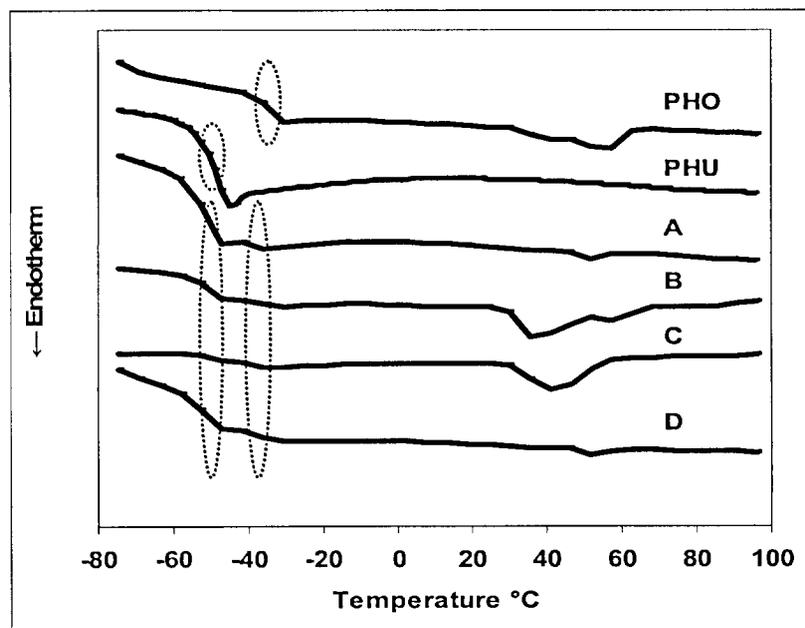


Figure 4.4: DSC thermograms of mclPHAs isolated from F2 in fermentations A-D. In addition thermograms for polymers produced from octanoic acid (PHO) and 10-undecenoic (PHU) alone are also shown.

The DSC thermogram for PHO showed a glass transition temperature (T_g) at -34 °C and two melting temperatures (T_m) at 40 °C and 56 °C. The presence of two T_m 's is a known phenomenon and can be explained by the presence of crystalline domains with different sizes and/or morphologies (Ehrenstein et al., 2003). In contrast, PHU has a very low degree of crystallinity and crystallizes very slowly (Kim et al. 1995). Therefore only a T_g at -49 °C and no T_m was detected for this polymer. The DSC thermograms for mclPHAs that were isolated from F2 produced in fermentations A-D, all revealed two T_g 's at approximately -47 °C and -36 °C. These observed T_g 's corresponded to those of the mclPHAs produced from octanoic acid (PHO) and 10-undecenoic acid (PHU) alone. Regarding the T_m 's of the four mclPHAs that were isolated from F2 produced in fermentations A-D, the polymers exhibit one or two melting temperatures. They were observed approximately around 35 - 40 °C (B and C) and at 52 - 57 °C (A, B and D) and can be attributed to the two T_m 's observed for

PHO, indicating that crystalline domains with different sizes and/or morphologies also occur in these polymers. These results suggest that the mclPHAs isolated from F2 produced in fermentations A-D are either block copolymers (PHO-*block*-PHU-*block*) or blends of PHO and PHU. However, the presence of random copolymers can be excluded here, since random copolymers exhibit only one glass transition temperature between those of PHO and PHU (Kim et al., 1995).

Crosslinking experiments of the polymers produced in the two-stage continuous culture systems: MclPHAs isolated from F2 produced in fermentations A-D, were crosslinked using a borane tetrahydrofurane complex solution (BH₃-THF). BH₃-THF crosslinks only PHA monomers that contain carbon-carbon double bonds (Kim et al., 1997). The produced gel was washed with an excess of methylene chloride and the soluble fraction was analyzed. Assuming that the mclPHAs isolated from F2 were block copolymers (PHO-*block*-PHU-*block*), the weight of the soluble fraction would be expected to be very small. Table 4.3 summarizes the results obtained from crosslinking-extraction experiments.

Table 4.3: Results of the crosslinking-extraction experiments.

Polymer isolated from F2 produced in fermentation	Weight of polymer (mg) ^{d)}	Weight of saturated repeating units in the polymer (mg)	Weight of extracted polymer (mg)	Mol% of unsaturated repeating units in extracted polymer
I ^{a)}	402.5	100.5	98.7	0
II ^{b)}	409.8	201.1	196.6	0
III ^{c)}	403.4	302.6	296.5	0
A	403.4 ± 1.2	68.5 ± 0.2	60.4 ± 1.2	0.3 ± 0.6
B	402.2 ± 3.4	130.3 ± 1.1	109.5 ± 1.6	0.0 ± 0.0
C	401.8 ± 2.4	224.4 ± 1.3	212.9 ± 2.5	0.0 ± 0.0
D	402.0 ± 1.3	170.8 ± 0.6	143.8 ± 2.8	2.8 ± 4.9

a) Control prepared by mixing PHO and PHU (weight ratio 1:3)

b) Control prepared by mixing PHO and PHU (weight ratio 1:1)

c) Control prepared by mixing PHO and PHU (weight ratio 3:1)

d) Weight of polymer used for crosslinking experiment

Mixtures of known amount of PHO and PHU (I, II, III) were used as control for crosslinking-extraction experiments. Independent of the weight ratio of PHO and PHU, the weight of the extracted polymer was in close agreement to the weight of saturated repeating units in the polymer. Therefore, the used amount of $\text{BH}_3\text{-THF}$ seemed to be sufficient to crosslink all carbon-carbon double bonds, even when the polymer contained a high amount of olefinic mclPHA monomers (control III). This postulation was confirmed by analyzing the soluble fraction using ^1H NMR. In all three control experiments no unsaturated monomeric units were detected.

The weight of saturated repeating units for the mclPHAs isolated from F2 produced in fermentations A-D were calculated from the monomeric composition obtained from gas chromatography or NMR spectroscopy, respectively. Crosslinking-extraction experiments for these polymers were carried out as triplicates. Table 4.3 shows that the weights of the extracted polymers were slightly below the theoretical calculated weights of saturated repeating units. The mole percent of unsaturated units in the extracted polymers were below 3 mol%. For mclPHAs produced in fermentations A, B and D, the weights of the extracted polymers were around 85 % of the theoretical calculated weights. For the polymer produced in fermentation B a value of 95 % was calculated. Here we assume that this higher value for the polymer produced in fermentation B must be related to the observed decrease in cell dry weight in F2. It might be that growing polymer chains got lost due to cell lysis and therefore, these growing polymer chains were no longer available for an elongation with mclPHA precursors derived from 10-undecenoic acid in F2.

CONCLUSIONS

PHAs are biocompatible and biodegradable polyesters and have gained considerable significance in medical applications, which include release systems, implant materials and scaffolds in tissue engineering (Zinn et al., 2001). In this study here, we were interested whether it was possible to produce block copolymers in a sequence of two dual nutrient (C,N) limited chemostat cultures. Such biodegradable block copolymers have been found as promising biomaterials by virtue of their ability to manipulate their amphiphilic behaviour, mechanical and physical properties by adjusting the ratio of the constituting blocks (Kumar et al., 2001). The results obtained in this study indicate that independently of the dilution rate or the carbon supply to the fermentors,

the obtained mclPHAs were not pure block copolymers (PHO-*block*-PHU-*block*) but mainly blends of PHO and PHU. Nevertheless, the crosslinking-extraction experiments indicated that these polymers might contain small amounts of repeating units from PHO and PHU. This finding might be explained by the existence of different polymer chain populations. Only polymer chains that are in the elongation process during the transfer from F1 to F2 can develop repeating units from PHO and PHU. In contrast, polymer chains where the elongation process is already terminated in F1, or where the elongation process only starts in F2, will not contain repeating units from PHO and PHU. This conceptual study revealed that the biosynthetic production of biodegradable block copolymers in a two-stage continuous culture system of *P. putida* GPo1 clearly has some limits. We therefore conclude that it might be easier to produce pure block copolymers by linking single block segments via chemical synthesis as shown before (Hirt et al., 1996b; Andrade et al., 2002a).

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CHAPTER 5

Chemical Synthesis of Crystalline Comb Polymers from Olefinic Medium- Chain-Length Poly[3- hydroxyalkanoates]

Roland Hany, Christine Böhlen, Thomas Geiger, René Hartmann, Jumpei Kawada,
Manfred Schmid, Manfred Zinn and Robert H. Marchessault. 2004.

Macromolecules 37, 385-389.

Abstract: Comb polymers were produced in a two-step synthesis from a bacterial poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate] (PHOU, **1**) containing 25 mol% terminal side chain double bonds. The radical addition reaction of 11-mercapto-undecanoic acid to the side chain alkenes of **1** produced derivative **2** containing thioether bonds with terminal carboxyl functionalities, which were subsequently transformed into the amide (**3**) or ester (**4**) derivatives using tridecylamine or octadecanol, respectively. The reactions proceeded to completion with little side reactions, which was confirmed with NMR and GPC experiments. The resulting comb polymers **3** and **4** were white crystalline materials. ^{13}C CP/MAS NMR spectra and X-ray diffraction results suggested a crystalline textural two-phase organization into polyethylene-like domains and regions characteristic of poly[3-hydroxyalkanoates] (PHAs). The breadth of the decomposition steps in thermal gravimetric analysis and the diffuse melting endotherms confirmed the solid-state organization as composed of nanosize crystallites of both polyethylene and PHAs.

INTRODUCTION

Poly[3-hydroxyalkanoates] (PHAs) are high molecular weight biodegradable and biocompatible polymers synthesized by a wide variety of microorganisms (Doi, 1900; Steinbüchel and Valentin, 1995). The best known PHAs are poly[3-hydroxybutyrate] (PHB) and its copolymers with 3-hydroxyvalerate, poly[3-hydroxybutyrate-co-3-hydroxyvalerate] (PHB-co-HV). PHB and PHB-co-HV are highly crystalline, thermoplastic materials with a degree of crystallinity of 60 % or more at all compositions. On the other hand, medium-chain-length PHAs (mclPHAs), which contain C6 – C12 alkanate monomers, are elastomeric thermoplastics of lower crystallinity, melting points (T_m) in the 45 – 60 °C range and glass transition temperatures (T_g) down to – 40 °C. X-ray diffraction patterns have suggested that PHAs with aliphatic side chains of average lengths between C5 and C7 crystallize with participation of both the main and side chains (Marchessault et al., 1990). The main chain crystallizes as a 2_1 helix in an orthorhombic chain lattice with two molecules per unit cell, whilst the side chains form ordered sheets with trans zigzag conformations. The degree of crystallinity for poly[3-hydroxyoctanoate] is approximately 25 – 33 % (Marchessault et al., 1990), and its crystalline state can be understood as a case where the helical backbone acts as a slow-moving fiber with

relatively mobile hydrocarbon side chains acting as a continuous matrix (Morin and Marchessault, 1992).

Depending on the carbon source, mclPHAs can contain side chains with functional groups and, among many others, polymers with terminal carbon-carbon double (Lageveen et al., 1988) and triple bonds (Kim et al., 1998), acetoxy and ketone (Jung et al., 2000), or aromatic groups (Fritzsche et al., 1990a; Curley et al., 1996; Kim et al., 1999; Abraham et al., 2001) have been produced. Among the latter, mclPHAs containing phenyl groups such as poly[3-hydroxy-5-phenylvalerate] ($T_g = 13\text{ }^\circ\text{C}$ (Fritzsche et al., 1990a)), or (co-)polymers from 6-phenylhexanoic acid, 7-phenylheptanoic acid, and 8-phenyloctanoic acid (T_g between -14.8 and $-1.3\text{ }^\circ\text{C}$ (Abraham et al., 2001)) have shown an increase in the glass transition temperature, but were completely amorphous. In contrast, poly[3-hydroxy-5-(*p*-tolyl)valerate] with $T_g = 18\text{ }^\circ\text{C}$, $T_m = 95\text{ }^\circ\text{C}$ (Curley et al., 1996) and a mclPHAs from 8-(*p*-methylphenoxy)octanoic acid with $T_g = 14\text{ }^\circ\text{C}$ and $T_m = 97\text{ }^\circ\text{C}$ (Kim et al., 1999) have been reported to crystallize. A crystallinity of 20 % has been determined for a copolymer of poly[3-hydroxyoctanoate] (PHO) containing 33 mol% undecenoate moieties (Dufresne et al., 2001).

The presence of functional groups in mclPHAs also provides sites for chemical modification, again directed to affect physical polymer properties or creating chemical groups which cannot be directly introduced by bioproduction. In particular, polymer-analogous reactions on mclPHAs containing unsaturated side chains have been studied in detail, and crosslinking (de Koning et al., 1994; Dufresne et al., 2001; Hazer et al., 2001), epoxidation (Baer et al., 1997), and conversion of double bonds to diol (Lee et al., 2000a) and carboxylic groups (Kurth et al., 2002; Stigers and Tew, 2003) have been reported so far. As an additional method, we report here on the functional conversion of PHA side chain alkenes into carboxylic groups via the free-radical coupling of a substituted thiol, and on their subsequent esterification or transformation into corresponding carboxamide derivatives.

EXPERIMENTAL SECTION

Biosynthesis of PHOU: PHOU was produced in a chemostat culture of *Pseudomonas putida* Gpo1 (ATTC 29347) at a dilution rate of 0.1 h^{-1} under multiple (C,N) nutrient limitations (Durner et al., 2001). Cells were fed with octanoic acid (75 mol%), 10-

undecenoic acid (25 mol%) and a mineral medium. The medium was designed in such a way that only nitrogen and carbon limited growth, whereas all other nutrients were in excess. Nitrogen concentration was kept constant at $N_0 = 10.7$ mM, and a C/N ratio of 17.5 mol/mol resulted in simultaneous growth limitation by carbon and nitrogen as well as PHA accumulation (Durner et al., 2001).

Synthesis of PHOU derivatives **2**, **3**, and **4**: All reagents were used as purchased from Fluka or Aldrich. For the synthesis of **2**, the starting polyester **1**, 11-mercaptoundecanoic acid (4 equivalents of PHOU double bonds) and AIBN (2,2'-azobisisobutyronitrile, 0.2 equivalents) were dissolved in toluene (1:10 w1/v) under argon. The solution was heated to 75 °C for 16 – 20 hours, cooled to r.t. (room temperature) and dropped into ice-cold methanol (1:10 v/v). The raw product was dissolved twice in a minimal amount of CH_2Cl_2 and precipitated in a tenfold excess of methanol for further purification, then **2** was dried under high vacuum. For the synthesis of **3**, **2** (1 equivalent of $-COOH$), tridecylamine (1.2 equivalents) and HBTU (O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate, 1.5 equivalents) were dissolved in a 2:1 mixture of dry DMF/ CH_2Cl_2 (1:10 w2/v) under argon at r.t., then 2.5 equivalents of triethylamine were slowly added. The solution was stirred for 5 hours at r.t., precipitated twice in methanol and dried. For the synthesis of **4**, a solution of DCC (dicyclohexylcarbodiimide, 1.1 equivalents of $-COOH$ of **2**) and 4-pyrrolidinopyridine (0.2 equivalents) in dry CH_2Cl_2 was added under argon to a 0 °C cold solution of 1-octadecanol (1.1 equivalents) and **2** in dry CH_2Cl_2 . The mixture was stirred for 2 hours at 0 °C and 20 hours at r.t., filtered, precipitated twice in methanol and dried. Yields of **2**, **3**, and **4** were 74, 57 and 69 %.

Polymer Characterization: NMR experiments in solution were performed on a Bruker ASX-400 spectrometer. The measurements were carried out at 300 K with samples of typically 10 – 20 mg of polymer dissolved in 0.7 ml $CDCl_3$. Chemical shifts are given in ppm relative to the remaining signals of chloroform as internal reference (1H NMR: 7.26 ppm; ^{13}C NMR: 77.7 ppm) or relative to neat liquid nitromethane ($\delta(^{15}N) \equiv 0$ ppm) as external reference. The ^{15}N chemical shift of derivative **3** was obtained from a gradient-selected $^1H,^{15}N$ HSQC spectra (Kay et al., 1992).

Molecular weights were determined by gel permeation chromatography (GPC, Waters 150, Milford MA, U.S.A.) equipped with a RI-detector. The system was calibrated by using 10 polystyrene standards with known Mw (2×10^3 to 2.13×10^6 g mol $^{-1}$) and low molecular weight distributions ($M_w/M_n \leq 1.09$). 40 mg of every sample

was dissolved in 10 ml THF for 2 hours. Aliquots of 100 μl of the polymer solution were chromatographed at 35 °C with pure THF as solvent phase through 2 GPC-columns (Mixed-Bed, Viscotek, Houston, U.S.A.) at a flow rate of 1 ml min⁻¹.

X-ray diffraction data were collected by using a Nicolet XRD at 45 kV and 25 mA at room atmosphere. A copper X-ray anode was used to provide CuK α radiation. A flat film camera was also used to study small angle X-ray diffraction under room condition with a Philips X-ray generator employing Ni-filtered CuK α radiation generated at 40 kV and 20 mA.

¹³C cross-polarization/magic angle spinning (CP/MAS) solid-state NMR spectra were recorded by a Chemagnetics CMX-300 instrument operating at 75.4 MHz. Samples were packed in 7.5 mm PENCIL rotors and spun at 4000 Hz.

Thermal analysis (DTA and DSC): Differential thermal analysis, DTA, was performed using a SEIKO TG/DTA 220 instrument over the temperature range from room temperature to 500 °C by ramping at 10 °C / min. For differential scanning calorimetry (DSC) a DSC Q1000 calorimeter from TA Instrument was used. Pretreatment of samples was as follows: firstly, sample endotherms were recorded from room temperature to 70 °C by 10 °C / min ramping; secondly, they were cooled to -70 °C by 40 °C / min ramping and kept at that temperature for 10 min. Afterwards, the 2nd DSC run, with ramping at 10 °C / min, provided the melting temperature (T_m) from the observed endotherm.

RESULTS AND DISCUSSION

PHOU (1) production was carried out using *Pseudomonas putida* GPo1 (ATCC 29347) in a chemostat culture as described earlier (Durner et al., 2001). PHOU was extracted directly from the lyophilized cells (Zinn et al., 2003). Cells were pulverized and transferred into pure methylene chloride. After stirring the suspension overnight, the solution was filtered and concentrated by distillation at 60 °C until the solution became viscous. The polymer was then precipitated into ice-cooled methanol. After removal of the solvents by filtration, PHOU was vacuum-dried. The steady-state conditions in a continuous culture are ideally suited to produce PHAs with tailored copolymer composition by taking advantage of the adjustable effect of feed mixture on polymer composition (Zinn et al., 2003). The carbon feed consisted of a mixture of 75 mol% octanoate and 25 mol% undecenoate, which indeed resulted in PHOU with

25 mol% monomer units containing unsaturated side chains. However, because of the conversion of the carbon source by fatty acid degradation (β -oxidation) to monomers units which had two carbons less, 3-hydroxyhexanoate, 3-hydroxynonenoate, and 3-hydroxyheptenoate units were also incorporated into the resulting polymer. The copolymer composition of PHOU was determined from ^{13}C NMR spectra and is indicated in Figure 1.

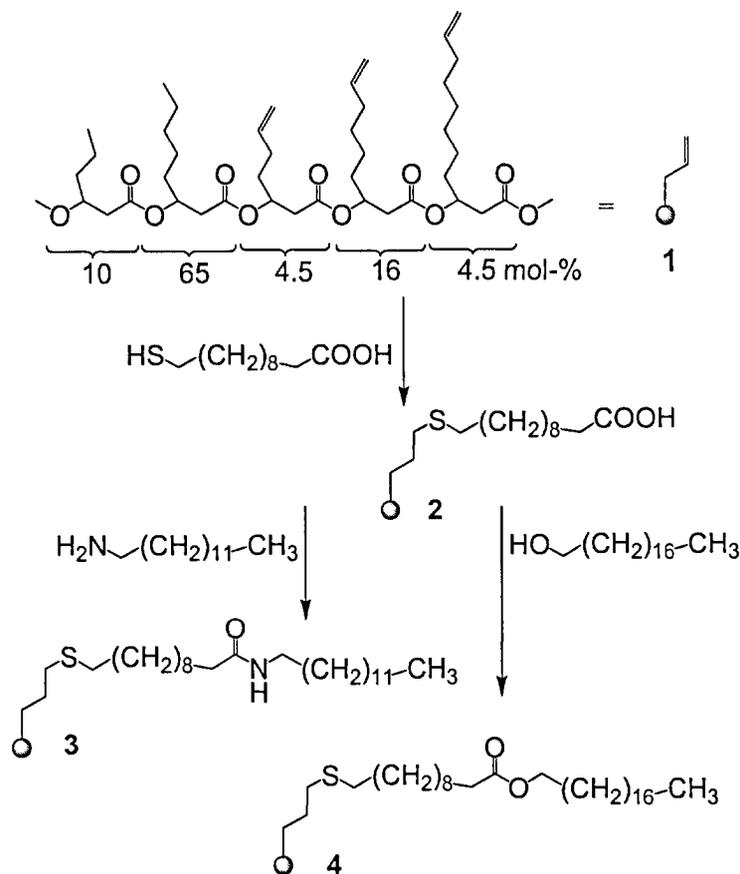


Figure 5.1: Synthetic route to comb polymers 3 and 4.

Figure 1 also shows the reaction scheme investigated to convert PHOU in a two-step synthesis into comblike PHA derivatives 3 and 4. We extended here the existing library for the chemical transformations of unsaturated side chain double bonds (de Koning et al., 1994; Bear et al., 1997; Lee et al., 2000a; Dufresne et al., 2001; Hazer et al., 2001; Kurth et al., 2002; Stigers and Tew, 2003) with the free-radical addition of 11-mercaptoundecanoic acid. Radical addition reactions of HS-R compounds

occur with anti-Markovnikov regioselectivity on terminal alkenes, usually under mild conditions and in good yields (Stranix et al., 1997). The reaction was carried out in toluene at 75 °C with AIBN as radical initiator. PHA derivative **2** was isolated from the reaction mixture and purified by precipitation into cooled methanol. **2** was then coupled with tridecylamine and octadecanol to synthesise derivatives **3** and **4** using common activation agents (HBTU (Knorr et al., 1989) and DCC (Neises and Steglich, 1978), respectively) for the carboxylic function. Again, the products were isolated from the reaction mixtures and purified by precipitation into cooled methanol, a common non-solvent for all polymers **1** – **4**.

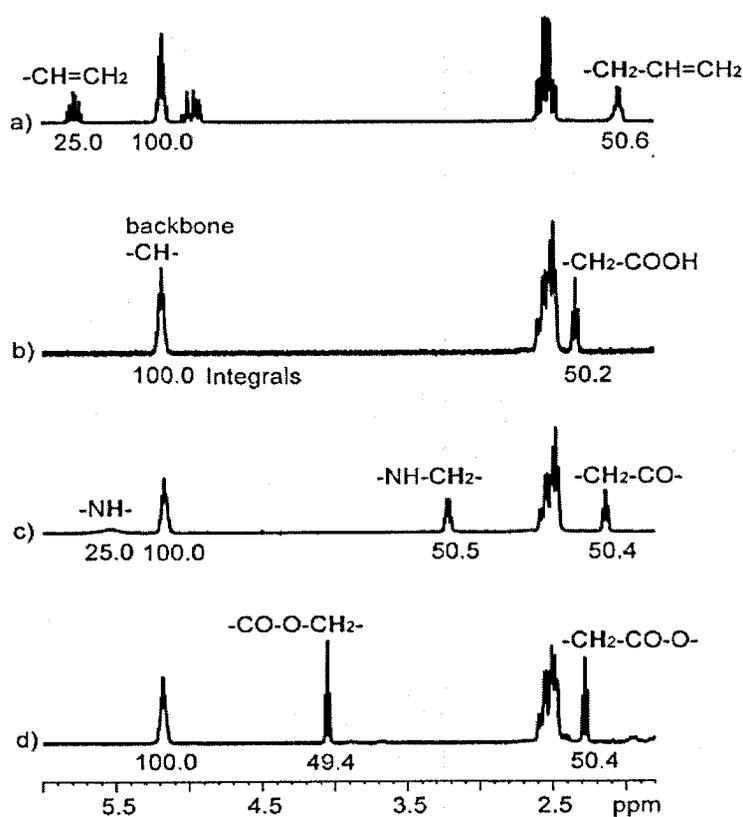


Figure 5.2: Parts of ^1H NMR spectra of (a) PHOU, **1**, (b) derivative **2**, (c) derivative **3**, and (d), derivative **4**. The numbers **1** - **4** above refer to the chemical structures shown in Figure 1 while the numbers under certain resonances are relative intensity values.

Reaction conversions were monitored with solution ^1H NMR spectroscopy. Figure 2 shows parts of the corresponding spectra with the crucial resonances assigned. In each spectrum the intensity of the methine backbone protons at 5.18 ppm was set to 100 (mol%). The intensity value of 25.0 for $-\text{CH}=\text{CH}_2$ in Figure 2a then means, that 25 mol% of monomer units in PHOU contained a double bond. These signals are completely absent in the spectrum of derivative **2** (Figure 2b), suggesting quantitative conversion of the alkene functions. In addition, the expected signals of the methylene protons adjacent to the carboxyl group appear at 2.34 ppm. The signal intensity of 50.2 for these (2 equivalent) protons suggests the complete conversion of the alkene groups to thioethers in derivative **2**. We mention that the analogous reaction with 4-methyl-7-thiocoumarin (with the aim to synthesise a fluorescence-labelled PHA) did not succeed. This can be explained by the stability of the arylthiyl radical, providing for more alternate reaction opportunities.

By similar arguments it can be derived from the ^1H NMR spectra shown in Figure 2c and Figure 2d that also the conversions of the carboxyl groups in derivative **2** to the amide (derivative **3**) and the ester (derivative **4**) were quantitative and free from significant side reactions. The formation of the amide bond was further corroborated from its ^{15}N chemical shift at -263.9 ppm, close to the reported value $\delta(^{15}\text{N}) = -257.5$ ppm for nylon-6 (Berger et al., 1997). DCC has been recently used for the preparation of graft copolymers of PHA carboxyl groups and poly(ethylene glycol) or poly(lactic acid) (Renard et al., 2003), too. However, the condensation of the acid groups did not lead to complete conversion and the coupling reactions were accompanied by side reactions and formation of insoluble products. This was not the case here and the conversions of the functional groups were quantitative. Reaction products were only gathered as these materials sticking to a rapidly stirring mixer, and no attempts were made to obtain the remaining parts by centrifugation, which were visible as a finely dispersed PHA clouding in the methanol solutions. This explains the lower yields which were between 57 and 74 % (as calculated from the product weights).

Table 5.1: PHAs molecular weights measured by GPC.

PHA	Mn	Mw/Mn
PHOU (1)	107500	2.3
derivative 2	111500	2.6
derivative 3	128100	1.7
derivative 4	141300	3.2

The molecular weight remained essentially constant upon addition of 11-mercaptoundecanoic acid to the PHOU double bonds (Table 5.1) and increased when the carboxyl groups of **2** were transformed into the carboxamide (**3**) and ester (**4**) derivatives. This course of molecular weights is consistent with the chemistry and confirms the smooth conversion of the carboxyl groups. For derivative **2**, the average unit weight M_0 is 196.7 g mol^{-1} , and the average degree of polymerization is $P_n(\mathbf{2}) = M_n/M_0 = 111500/196.7 = 567$. Average theoretical molecular weights of $137000 \text{ g mol}^{-1}$ for **3** and $147000 \text{ g mol}^{-1}$ for **4** can be calculated from this. These values are slightly higher than the experimental results (Table 5.1), but the differences may be explained in terms of differences in the hydrodynamic volumes of the products in THF due to the presence of new side chains, and considerable side reactions with by-products not detectable by ^1H NMR spectroscopy can be excluded.

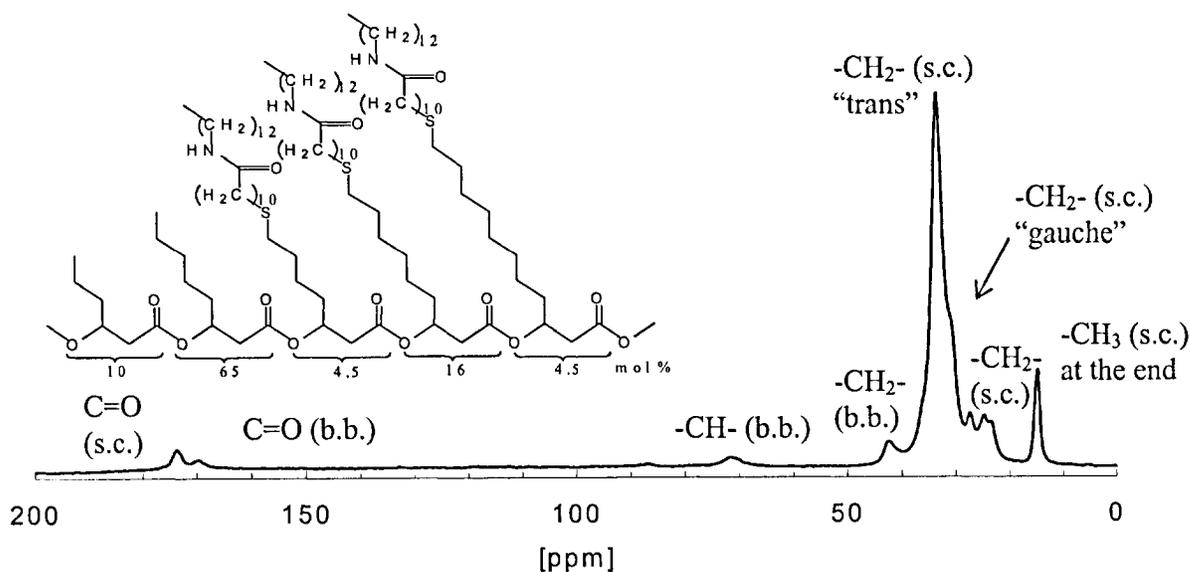


Figure 5.3: ^{13}C CP/MAS solid-state NMR spectrum of PHA derivative **3**. b.b.: backbone, and s.c.: side chain.

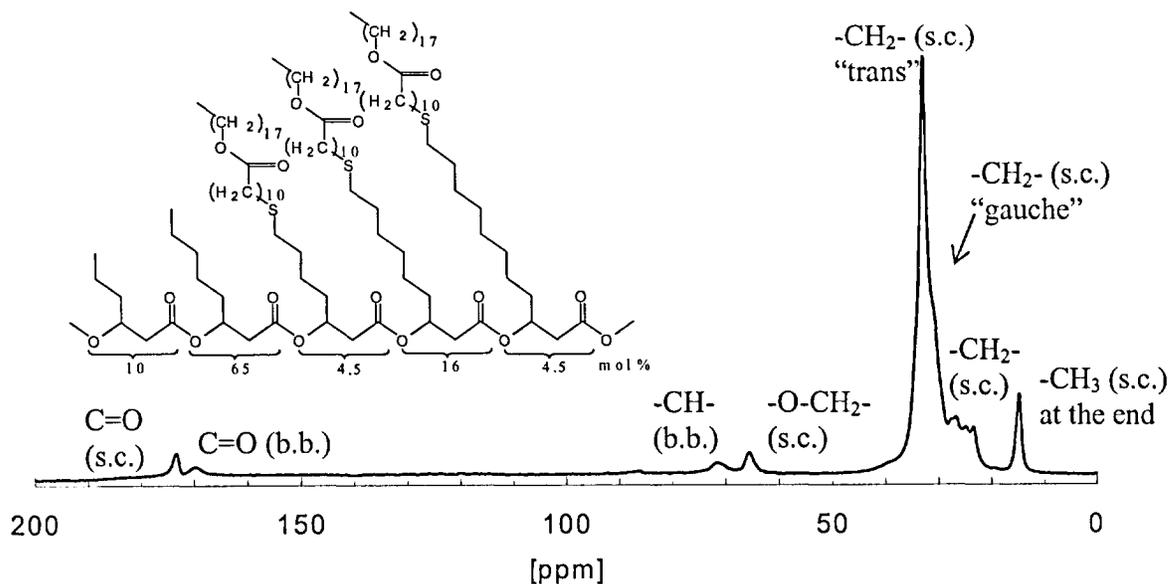


Figure 5.4: ^{13}C CP/MAS solid-state NMR spectrum of PHA derivative 4. b.b.: backbone, and s.c.: side chain.

PHA derivatives 3 and 4 showed well defined ^{13}C CP/MAS solid-state NMR spectra in Figures 3 and 4. Both spectra have two kinds of resonances from carbonyl carbons at 170 and 174 ppm; the former value is typical of PHAs backbone chain, the latter is from the derivatives side chains. PHA derivative 3 has two other resonances from the backbone chain at 42 ppm for $-\text{CH}_2-$ and at 71 ppm for $-\text{CH}-$, similar to PHB and PHV. The resonance from $-\text{CH}_2-$ groups at 33.6 ppm in Figure 3 suggests that the methylene groups in the derivative side chains are in the trans conformation. Crystalline polyethylene (PE) has the same chemical shift when all methylene groups are "trans" (Koenig, 1992). However, a shoulder peak at 30 ppm indicates that some methylene groups are "gauche", which is also observed in melt-crystallized PE (Koenig, 1992). The resonances from 23 to 27 ppm are from methylene groups in the PHA side chains as well as the derivative side chains. The resonance at 15 ppm is due to the methyl groups at the end of side chains. There are no resonances from 100 to 140 ppm, suggesting that derivative 3 did not have terminal double bonds, thus the $\text{HS}-(\text{CH}_2)_{10}-\text{COOH}$ reagent reacted with all double bonds. This confirms the results from ^1H NMR spectra in solution (Figure 2).

Similar conclusions apply to the PHA derivative 4 which also has resonances from the backbone chain at 72 and 170 ppm (Figure 4). In this case, the $-\text{CH}_2-$ resonance

from the backbone overlapped with the large $-\text{CH}_2-$ resonance of the derivative side chains. The resonance at 66 ppm in derivative 4 is from the $-\text{CH}_2-$ group next to the ester oxygen in the derivative side chain, differing from the derivative 3.

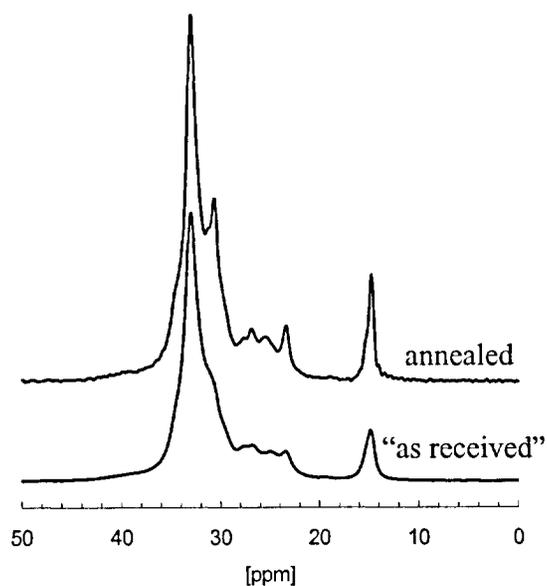


Figure 5.5: ^{13}C CP/MAS solid-state NMR spectra of PHA derivative 4 before and after annealing. The spectrum of annealed derivative 3 was almost identical.

NMR spectra of derivatives 3 and 4 were recorded again after annealing. Figure 5 is the ^{13}C CP/MAS NMR spectra of derivative 4 and shows that the resonance at 30 ppm is more intense than that for the “as received” sample, indicating that the polyethylene phase has improved its degree of order. This was also observed in the NMR spectra of annealed derivative 3.

Table 5.2: Bragg d -spacings and 2θ values for PHA derivatives **3** and **4**.

derivative 3		derivative 4	
spacing [nm]	2θ [deg]	spacing [nm]	2θ [deg]
1.5 ~ 2.0	4.4 ~ 5.9	1.3 ~ 2.0	4.4 ~ 6.8
0.783	11.31	0.773	11.45
0.454	19.54	0.459	19.29
		0.415 *	21.43 *
		0.378 *	23.51 *

* Corresponding to PE powder diffraction.

Samples of PHA derivatives **3** and **4** showed better resolved X-ray powder diffraction traces after annealing, when the samples were melted and annealed 10 °C below the melting temperatures. The X-ray powder diffraction traces do not show diffraction peaks smaller than 5 degree (2θ) and the diffractometer traces are not well-resolved. Therefore, an X-ray flat plate camera was also used to complement the diffractometer data. Observed d -spacings and 2θ of both samples are listed in Table 5.2. The d -spacings for derivatives **3** and **4** were not all the same as those for PHO (Marchessault et al., 1990), even though both derivatives had 65 mol% PHO monomer units. Both derivatives have "the long d -spacing" ($d = 1.5$ - 2.0 nm) which is also observed in PHO fiber diagram (Marchessault et al., 1990). This long spacing was detected by recording an X-ray film pattern using a 12 cm "film to sample" distance. It was of broader width in derivatives **3** and **4** because the PHO crystallites are small and disordered by the crystallization of the side chains. It can not be decided whether the reflections: $d = 0.454$ and 0.459 nm in derivatives **3** and **4** are characteristic of PHO or PE. However, the PHA derivative **4** has two main diffraction peaks of PE, 0.415 and 0.378 nm, which suggests that the long $-\text{CH}_2-$ side chains crystallized with PE-like structure. PHA derivative **4** has better crystallization properties than derivative **3**. Thermal gravimetric traces of both derivatives with PHB as a reference were recorded as shown in Figure 6. Both derivatives show two-step decomposition curves different from PHB. The thermal gravimetric trace for PHO is similar to PHB and does not display a two-step degradation (Marchessault and Yu, 2002). Therefore, the two-step degradation suggests that the chain backbone

degrades first and the side chains degrade next because PE is more heat stable than PHAs. Although PHB and PHO decompose completely to H₂O and CO₂ before 300 °C, the derivatives persisted beyond 400 °C.

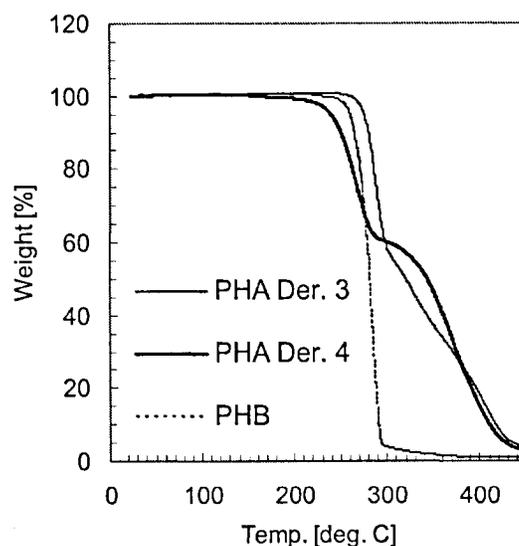


Figure 5.6: Thermal gravimetric traces of PHA derivatives 3, 4 and of PHB. The trace of PHO is almost identical to PHB.

Table 5.3: Melting points for PHAs.

PHA	T _m [°C]
PHB ²⁸	170
PHO ²⁸	46-61
derivative 3	46*, 66
derivative 4	46

* very small endotherm.

DSC data showed broad melting endotherms at 66 and 46 °C for derivative 3, and derivative 4 had a single endotherm at 46 °C (Table 5.3). The 46 °C endotherm can be associated with PHA and the 66 °C endotherm probably reflects the influence of amide linkages in the derivative side chains. Derivative 4 had a higher enthalpy in its single endotherm compared to derivative 3, which supports that derivative 4 was a more crystalline material. Glass transition temperatures were observed at -43.6 °C for PHOU and at -38.2 °C for 2, but no T_g transition was observable for derivatives 3

and **4**, even not after rapid quenching from the melt. This is in keeping with the high degree of crystallinity for derivatives **3** and **4**, associated with a fast rate of crystallization which is characteristic of polyethylene.

CONCLUSIONS

The starting PHOU material provides options for producing a variety of derivatives through reaction at the double bonds. In this work, we investigated the conversion of double bonds to thioethers via the free-radical addition of 11-mercaptoundecanoic acid. Preliminary results showed that the reaction works equally well with 11-mercapto-1-undecanol; in this way, it will be possible to generate PHAs containing side chains with hydroxyl functional groups as reactive intermediates. Also the activation of the carboxyl groups by DCC or HBTU are well established procedures, and further esterification and amidification reactions will therefore not be limited to the reagents 1-octadecanol and tridecylamine used in this work.

The crystalline derivatives **3** and **4** showed ^{13}C solid-state NMR spectra similar to PHO, since the 3-hydroxyhexanoate and 3-hydroxyoctanoate monomer units also contributed 75 mol% in PHOU. However, the intensity of the resonance at 33.6 ppm was much larger, being derived from methylene groups of the derivative side chains. After annealing the samples, both derivatives showed evidence in the NMR spectra that PE chains folded to crystallize since the well-defined shoulder at 30 ppm ("gauche" conformation) is evidence of chain folding. Due to the relatively long polymethylene side chains affecting the PHO crystal organization, the X-ray diffraction patterns of derivatives **3** and **4** did not match exactly with PHO reflections. The results also suggest that the long $-\text{CH}_2-$ chains in derivative **4** crystallized in a polyethylene-like phase. This was not so for **3** and the reason might be the presence of amide linkages and hydrogen bond effects derived therefrom.

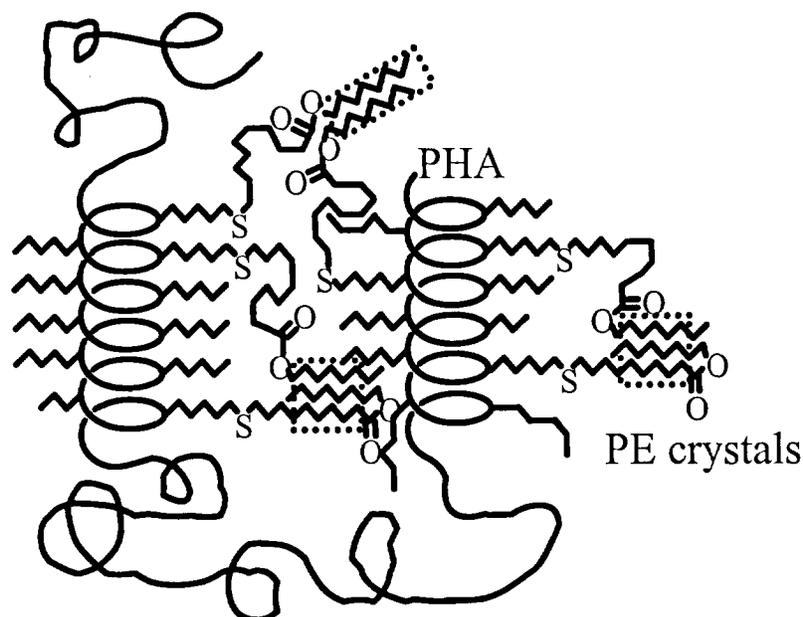


Figure 5.7: Proposed model of PHA derivative 4. The PE crystals are surrounded by a dotted line.

Both derivatives showed a two-step degradation in the thermal gravimetric traces, thus differing from PHAs. The greater thermal resistance is due to decomposition of carbon-carbon bonds in PE from the derivative side chains. This supports the X-ray data conclusions and suggests that the derivatives crystallized in two phases, one of which is predominantly PE-like. Based on these results, we propose a PHA derivative model as shown in Figure 7 for derivative 4. This schematic is meant to show the solid texture of the polyethylene-like phase which could be a useful property for stabilizing the crystallization of the PHOU and eliminating its well known stickiness in film form.

ACKNOWLEDGEMENTS

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CHAPTER 6

Chemical Synthesis and Characterization of POSS- Functionalized Poly[3- hydroxyalkanoates]

Roland Hany, René Hartmann, Christine Böhlen, Sandro Brandenberger, Jumpei Kawada, Christiane Löwe, Manfred Zinn, Bernard Witholt and Robert H. Marchessault. 2005. *Polymer* 46, 5025-5031.

Abstract: A polyhedral oligomeric silsesquioxane containing seven isobutyl groups and one mercaptopropyl group (POSS-SH) was linked via a free radical addition reaction to the side-chain double bonds of bacterial poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate], (PHAЕ). PHAE with 11.5, 55, 78, and 97 mol% of double bonds were used to produce a series of inorganic-organic hybrid materials, PHAE-POSS, with increasing amounts of POSS. The reactions proceeded with high yields and limited side reactions, confirmed with NMR and GPC experiments. X-ray diffraction and ^{13}C solid-state NMR analysis of these nanocomposite materials suggested the favoured crystallization of POSS, with PHAE functioning as a non-crystalline combining matrix. This is in contrast to blends of POSS-SH and PHAE, where incompatibility occurred and the two components crystallized independently. The covalent links between PHAE and POSS imposed spatial constraint which resulted in disordered crystalline POSS domains and limited crystal sizes. With increasing POSS content, the appearance of these POSS-based biopolyesters changed from non-sticky and elastic to brittle and glass-like. The covalent linking of POSS-SH to PHAE increased the heat stability such as glass transitions, and melting points could be tailored between 48 °C and 120 °C.

INTRODUCTION

In the field of inorganic-organic hybrid polymers, there is growing interest in polyhedral oligomeric silsesquioxane-based systems (Lichtenhan et al., 1995; Li et al., 2001; Pittman et al., 2003; Pyun et al., 2003; Phillips et al., 2004). Silsesquioxanes with the general $\text{RSiO}_{1.5}$ -stoichiometry include random structures, ladder structures, and cage or partial cage structures. Polyhedral oligomeric silsesquioxanes with specific cage structures have been designated by the abbreviation POSS (Li et al., 2001). The most representative members of this POSS family consist of Si_8O_{12} arranged in an inner eight-cornered cage, while the corners of this nanosized cluster are substituted with organic groups (R). Depending on the number of organic groups bearing reactive functionalities, POSS can be classified as nonfunctional, monofunctional, or polyfunctional.

POSS chemicals with reactive functionalities are suitable for polymerization, grafting or surface bonding. The incorporation of these nanoparticle reinforcing agents (Schmidt et al., 2002) into polymeric materials can lead to property improvements,

such as temperature and oxidation resistance (Gonzalez et al., 2000), surface hardening, and reduced flammability (Devaux et al., 2002). A variety of POSS-containing copolymers have been synthesized (Lichtenhan et al., 1995; Li et al., 2001; Pittman et al., 2003; Pyun et al., 2003; Phillips et al., 2004), and these hybrid materials have potential in areas such as stabilized semiconducting polymers in optoelectronic devices (Xiao et al., 2003; Chou et al., 2005) or as low-dielectric-constant materials in the microelectronics industry (Leu et al., 2003).

Here, we report on the POSS-functionalization of poly[3-hydroxyalkanoates], PHAs, which are a family of high molecular weight polyesters synthesized by a wide variety of microorganisms (Steinbüchel and Valentin, 1995). The best-known PHA is poly[3-hydroxybutyrate], a highly crystalline biodegradable thermoplastic, also available as a random copolymer with valerate. On the other hand, medium-chain-length mclPHAs (Lageveen et al., 1988), which contain C6 – C12 alkanolate monomers, are soft and/or sticky thermoplastic elastomers. MclPHAs can contain side chains with functional groups (Steinbüchel and Valentin, 1995; Hartmann et al., 2004), which provide sites for further chemical modification, directed to modulate the basic polymer properties or to create functionalities which are impossible to introduce by biosynthesis (Kurth et al., 2002).

Because they are biodegradable and biocompatible polyesters, PHAs are attractive for medical applications including release systems, implants and scaffolds in tissue engineering (Zinn et al., 2001). However, the lack of structural and functional stability prevents currently available PHAs from having wide-spread commercial impact. To take advantage of the property enhancements of polymers reinforced with nanofillers (Schmidt et al., 2002; Phillips et al., 2004), we covalently attached a monofunctional POSS molecule containing seven isobutyl and one mercaptopropyl group, POSS-SH, via a free radical addition reaction to the side-chain alkenes of poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate], PHAE, and report on the characterization of these novel POSS-based biopolyesters.

EXPERIMENTAL

Biosynthesis of Poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate], PHAE. PHAE was produced in a chemostat culture of *Pseudomonas putida* GPo1 (ATTC 29347) at a dilution rate of 0.1 h^{-1} under multiple (C,N) nutrient limited growth conditions

(Durner et al., 2001). Cells were fed with different mixtures of octanoate, 10-undecenoate, and a mineral medium. The medium was designed in such a way that only nitrogen and carbon limited growth, whereas all other nutrients were in excess. The ratio of the feeds of octanoate and 10-undecenoate was adjusted to control the fraction m of side-chain double bonds in PHAE- m . Thus, PHAE-11.5 with 11.5 mol% alkenes, as well as PHAE-55, PHAE-78, and PHAE-97 were produced.

Synthesis of PHAE- m -POSS. All reagents were purchased from Fluka or Aldrich. Mercaptopropyl-isobutyl-POSS (POSS-SH) was further purified by sublimation under vacuum (approximately 3 mbar) at 210 – 230 °C. POSS-SH is highly soluble in hexane, CHCl_3 or THF at room temperature, but only in boiling acetone. The solubility of POSS-SH in acetone at room temperature (4.4 g L^{-1}) was determined as follows: 250 mg POSS-SH in 25 mL acetone was stirred for 16 h. 10 mL of the suspension were then centrifuged, 5 mL of the solution collected in a weighed flask, the solvent evaporated and the flask dried at 10^{-6} bar. The amount of residue was determined by weight. For the synthesis of PHAE-11.5-POSS, 10 g of the starting polyester (8.2 mmol PHAE double bonds), 14.6 g of POSS-SH (16.4 mmol), and 269 mg of AIBN (1.64 mmol, 2,2'-azoisobutyronitrile) were dissolved in 100 mL toluene under argon. The solution was heated to 75 °C for 18 h, cooled to room temperature, dropped into 1 L of ice-cold methanol, and the precipitate (21.3 g of PHAE-11.5-POSS and POSS-SH) was then dried under high vacuum. POSS-SH was removed from PHAE-11.5-POSS by slowly stirring the raw product in acetone (1 : 10 w/v) at room temperature, the suspension was centrifuged, the supernatant collected, the solvent removed and PHAE-11.5-POSS was then dried under high vacuum. The synthesis of PHAE- m -POSS, with $m = 55, 78,$ and $97,$ was carried out as described above. However, in contrast to PHAE-11.5-POSS, these products were not soluble in acetone. Therefore, POSS-SH could be removed by repeated boiling in refluxing acetone and collecting the sediment after centrifugation.

Polymer characterization. Proton NMR experiments in solution were performed on a Bruker AV-400 spectrometer. The measurements were carried out at 300 K with typically 10 mg of sample dissolved in 0.7 mL solvent. Chemical shifts are given in ppm relative to the remaining signal of chloroform at 7.26 ppm. Cross polarization, magic angle spinning (CP/MAS) ^{13}C NMR spectra were recorded on a Chemagnetics CMX-300 instrument operating at 75.4 MHz. Samples were packed in 7.5 mm rotors and spun at 4 kHz. A contact time of 3 ms and a recycle delay of 3 s were used.

Single pulse Bloch decay spectra were obtained using a 60 ° pulse and a recycle delay of 3 s. Molecular weights were determined by gel permeation chromatography (GPC, Viscotek, Houston, TX) equipped with a RI detector. The system was calibrated by using 10 polystyrene standards with known Mw (2×10^3 to 2.13×10^6 g mol⁻¹) and low polydispersity (Mw/Mn \leq 1.09). 40 mg of every sample was dissolved in 10 mL THF overnight. Aliquots of 100 μ L of the polymer solution were chromatographed at 35 °C with pure THF as solvent phase through 2 GPC-columns (Mixed-Bed, Viscotek, Houston, TX) at a flow rate of 1 mL min⁻¹. X-ray diffraction data were recorded using a Bruker AXS D8 Discover instrument with a GADDS powder diffractometer and CCD camera detector. Cu K α radiation was generated at 40 kV and 40 mA. Thermal gravimetric analysis was performed with 4 – 10 mg of sample from room temperature to 650 °C on a 2950 thermogravimetric analyzer from TA Instruments. The gas flow (oxygen or nitrogen) was 20 mL min⁻¹, and the heating rate was 5 °C min⁻¹. To prevent the loss of the powdered POSS-SH (and decomposition products) in the gas stream, the aluminium pot was covered with a perforated top. Differential scanning calorimetry measurements in a nitrogen atmosphere were carried out on a Mettler Toledo Star^e DSC System. 9 – 15 mg of sample were heated from room temperature to 200 °C, subsequently removed, and quenched in liquid nitrogen. DSC data were then recorded for the temperature program –80 °C to 200 °C, cooling to –80 °C, followed by heating to 350 °C, all at a rate of 10 °C min⁻¹.

RESULTS AND DISCUSSION

Different feed mixtures of octanoate and 10-undecenoate in a chemostat culture of *Pseudomonas putida* GPo1 were used for the biosynthesis of poly[3-hydroxyalkanoates-co-3-hydroxyalkenoates] with varying fractions *m* of side chains with terminal double bonds, PHAE-*m*. The conversion of the carbon sources by fatty acid degradation (β -oxidation) lead to the incorporation of shorter monomer units as well, and we adopted the experimentally (Hany et al., 2004a) determined ratios 3-hydroxyhexanoate : 3-hydroxyoctanoate = 1 : 6.5, and 3-hydroxyheptenoate : 3-hydroxynonenoate : 3-hydroxyundecenoate = 1 : 3.6 : 1, for all PHAE copolymers of this work. The reaction scheme for the free-radical addition of POSS-SH to the side-chain double bonds of PHAE is shown in Figure 6.1. The reactions were carried out in toluene and AIBN was the radical initiator, as described recently for the similar

transformations using 11-mercapto-1-undecanol (Hany et al., 2004b) or 11-mercaptoundecanoic acid (Hany et al., 2004a).

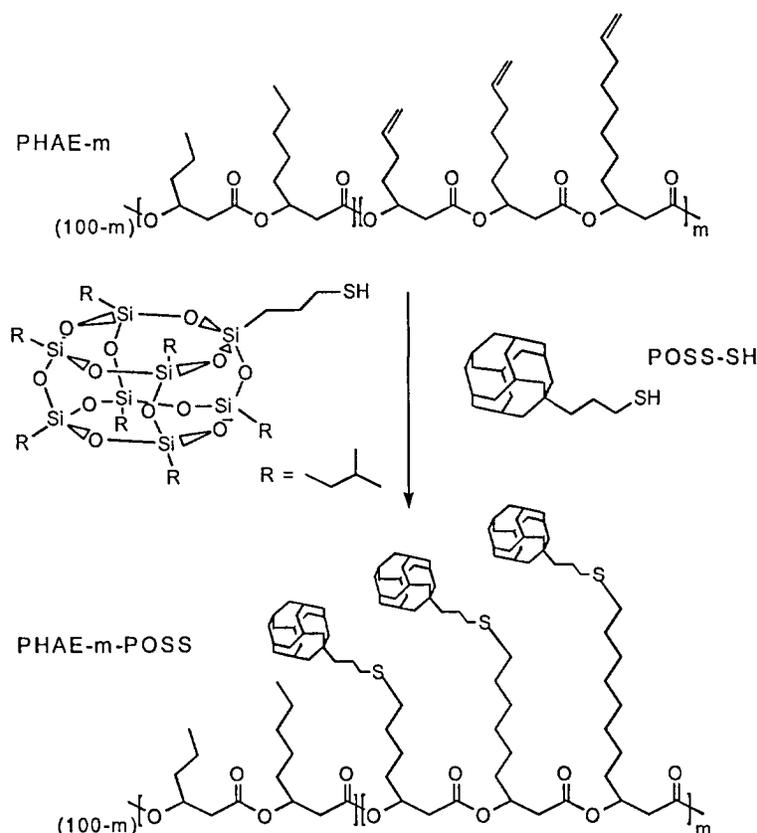


Figure 6.1: Free radical addition reaction of mercaptopropyl-isobutyl-POSS (POSS-SH) to the side-chain double bonds of poly[3-hydroxyalkanoate-co-3-hydroxyalkenoate], PHAE.

Reaction conversions were obtained from ^1H NMR spectra. Figure 6.2a shows the spectrum of POSS-SH with the assignment of the isobutyl and mercaptopropyl resonances. In the NMR spectra of the starting polyesters, as displayed in Figure 6.2b for PHAE-11.5, the intensity of the methine backbone protons was set to 100 (mol%). The fraction of unsaturated side-chain double bonds (11.5 %) was then directly obtained from the intensity of the alkene resonances at 5.77 ppm, and was cross-checked via the intensity of the three equivalent methyl protons of 3-hydroxyoctanoate and 3-hydroxyhexanoate at 0.88 ppm ($266/3 = 88.7$), representing the rest of 88.5 mol% of saturated monomer units. The remaining intensity of 1.1 for the alkene resonances in the NMR spectrum of PHAE-11.5-POSS shown in Figure 6.2c points to a conversion of 10.4 mol% alkene groups during the reaction. From the

POSS NMR resonances b (one proton) and a (two protons) of the seven isobutyl groups, a POSS content of 10.5 mol% can be calculated. The good agreement between the decrease of the olefinic resonances (conversion of 90.4 % of all double bonds) and the appearance of POSS (suggesting the conversion of 91.2 % of the alkenes into thioether groups) in the NMR spectrum of PHAE-11.5-POSS agrees with the smooth free-radical addition of thiols to PHAE observed recently (Hany et al., 2004a; Hany et al., 2004b) and excludes significant side reactions. We note, that due to the purification process and the limited solubility of POSS-SH at room temperature (see Experimental Section), PHAE-11.5-POSS was still contaminated with approximately 4 weight % of unreacted POSS-SH. The other PHAE-m-POSS samples could be cleaned completely from excess POSS-SH. Also for these reactions, the degree of conversion of double bonds as determined from the decrease of alkene resonances or the appearance of POSS agreed within 8 %. The results and the weight % of POSS in the PHAE-m-POSS copolymers are summarized in Table 6.1.

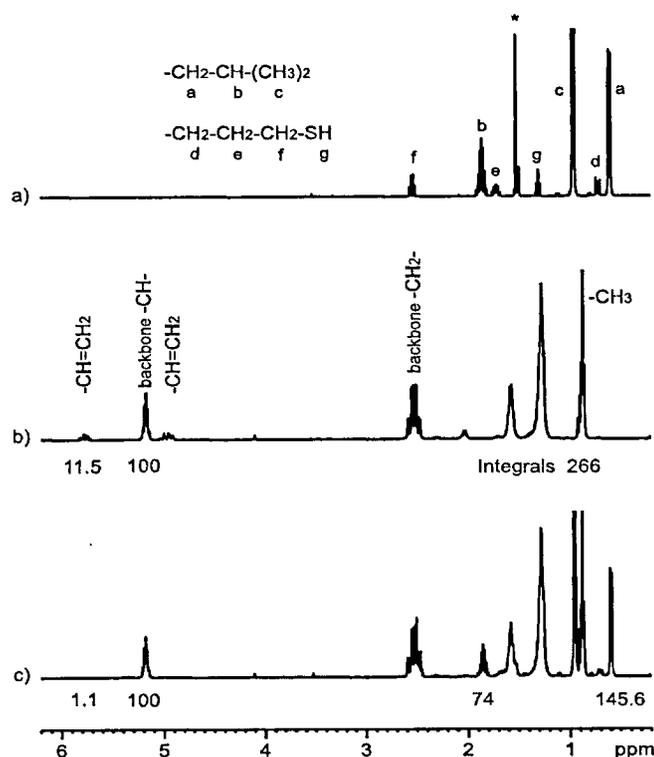


Figure 6.2: ^1H NMR spectra of (a) POSS-SH, (b) PHAE-11.5, and (c), PHAE-11.5-POSS. The chemical structures are shown in Figure 6.1. The numbers under certain resonances are relative intensity values and residual solvent signal is marked with * at 1.50 ppm in (a).

Table 6.1: Compositions and Characteristics of Poly[3-hydroxyalkanoates-co-3-hydroxyalkenoates], PHAE-m, and PHAE-m-POSS Derivatives^a

sample	conversion of PHAE double bonds [%] ^b	POSS in copolymers [wt %]	Mn exp ^c	Mw/Mn exp	Mn exp / Mn calc	Tg [°C]	Tm [°C]
POSS-SH			0.56	1.1	0.63		257 ^d
PHAE-11.5			106	2.1		-36	53
PHAE-55			90	2.2		-44	41
PHAE-78			94	2.1		-47	
PHAE-97			107	2.2		-48	
PHAE-11.5-POSS	91.2	40.1	107	4.5	0.60	-35	48
PHAE-55-POSS	72.7	70.8	161	2.6	0.52	-34	90
PHAE-78-POSS	67.9	75.8	177	2.4	0.44	-38	105
PHAE-97-POSS	74.5	80.8	215	4.2	0.39	-36	120

a) m denotes the fraction [%] of side-chain double bonds in PHAE, see Figure 6.1.

b) From ¹H NMR data.

c) [$\times 10^3 \text{ g mol}^{-1}$].

d) An additional small endotherm was measured at 23.5 °C, see Figure 6.6.

Molecular weights were measured to monitor the polymer-analogous reactions (see Table 6.1). Mn values of typically $100000 \text{ g mol}^{-1}$ and Mw/Mn = 2 were found for the starting polyesters (Preusting et al., 1990), and the molecular weights increased for the PHAE-m-POSS reaction products. Mn values were also calculated for PHAE-m-POSS, based on the known starting copolymer compositions, the experimental Mn values for PHAE-m and the reaction conversions. These calculated molecular weights were considerably higher than the experimental results, and decreasing ratios Mn exp/Mn calc from 0.6 to 0.39 with increasing POSS content were obtained (see Table 6.1). The same was true for POSS-SH alone, where Mn exp/Mn calc was 0.63. Therefore, these differences must be explained with differences in terms of the hydrodynamic volumes of the products in THF due to the presence of POSS in the side chains, and are not indicative of considerable side reactions with byproducts not

detectable by ^1H NMR spectroscopy. Low molecular weight data of POSS copolymers based on GPC with polystyrene calibration have been mentioned in the literature recently (Pyun and Matyjaszewski, 2000).

Considering the size of the POSS molecule with a diameter of approximately 1.5 nm, the high conversion yields are surprising. For PHAE-97-POSS, the conversion of 74.5 % of double bonds means that 72 % of all PHAE side chains had covalently linked to a bulky POSS molecule. It must be noted that the reactions were not optimized for high conversions, since we added, for economic reasons, only a two molar excess of POSS-SH, instead of the four molar excess of thiols we used for the quantitative transformations of PHAE alkene groups earlier (Hany et al., 2004a; Hany et al., 2004b). Qualitatively, the incorporation of POSS had a strong influence on the appearance of thin films cast from chloroform solutions on microscope slides. Films of PHAE-11.5 could be removed and were highly elastic, whereas PHAE-97 was a material of honey-like consistency too sticky to be peeled off from the glass surface. For the POSS copolymers, the trend was reversed: PHAE-11.5-POSS appeared as a non-sticky, but still elastic film material, and PHAE-97-POSS formed a highly transparent and very brittle, glass-like film. Finally, for blends of PHAE-11.5 or PHAE-97 with POSS-SH, demixing was observed and the material looked like POSS powder stuck together with PHAE. Obviously, the covalent attachment of POSS-SH to PHAE imposed spatial constraint and hindered the phase separation process of the copolymers (Phillips et al., 2004).

The X-ray powder pattern of POSS-SH (see Figure 6.3) showed four main diffraction peaks at 8.3° , 11.1° , 12.1° and 19.3° (2θ) in the diffraction diagram which correspond to 1.07, 0.80, 0.73 and 0.46 nm, respectively. These values are typical for POSS (Waddon et al., 2002; Waddon and Coughlin, 2003). Five diffraction peaks at 4.9° , 11.7° , 18.3° , 19.5° and 21.3° (2θ) were observed in the X-ray powder diffractogram of PHAE-11.5, which clearly differed from the values obtained for POSS-SH. The d spacing value at 4.9° corresponds to 1.8 nm and is a characteristic of crystalline medium-chain-length PHA, such as poly(3-hydroxyoctanoate) (Marchessault et al., 1990). This observation is reasonable because PHAE-11.5 contains 88.5 % of 3-hydroxyhexanoate and 3-hydroxyoctanoate monomer units as well. PHAE-11.5-POSS did not have the diffraction peaks derived from PHAE, especially the characteristic diffraction at 4.9° (2θ) was missing, but it showed all the typical diffractions of POSS in the X-ray pattern, suggesting that independent

crystallization of POSS was favoured over that of PHAE-11.5. A blend of PHAE-11.5 and POSS-SH displayed an X-ray pattern as the superposition of both components and the diffractions from PHAE-11.5 were evident. The X-ray powder pattern of PHAE-97-POSS was similar to that for PHAE-11.5-POSS, meaning that the crystalline component of PHAE-97-POSS was only POSS in this case, too. The POSS diffraction at 8.3° is of broader width for both PHAE-POSS derivatives shown in Figure 6.3, which points to disordered crystalline domains and limited crystallite sizes.

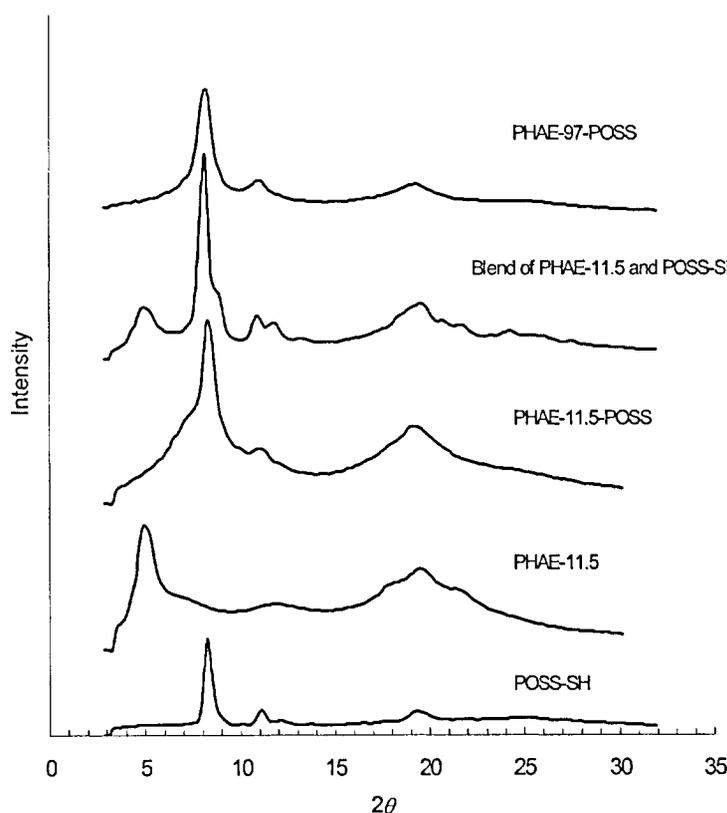


Figure 6.3: X-ray powder diffraction diagrams for POSS-SH, PHAE-11.5, and PHAE-m-POSS derivatives.

The model for crystalline medium-chain-length PHA reported previously (Dufresne et al., 2001) consists of two principal spacings in a sheet-like structure: one spacing corresponding to 1.8 nm is due to the periodicity between helical main chains with twofold symmetry in the sheets (Marchessault et al., 1990), the other is interplanar spacing with 0.46 nm between the sheets. This organization was disrupted when POSS-SH was covalently attached to PHAE, and PHAE-11.5-POSS did not show a 1.8 nm maximum indicating that PHAE became a non-crystalline matrix for the

crystalline POSS. The same was true for PHAE-97-POSS. When a blend was examined, the crystalline structures of both POSS-SH and PHAE were maintained, which supports the qualitative observation of demixing and phase separation for cast films described above.

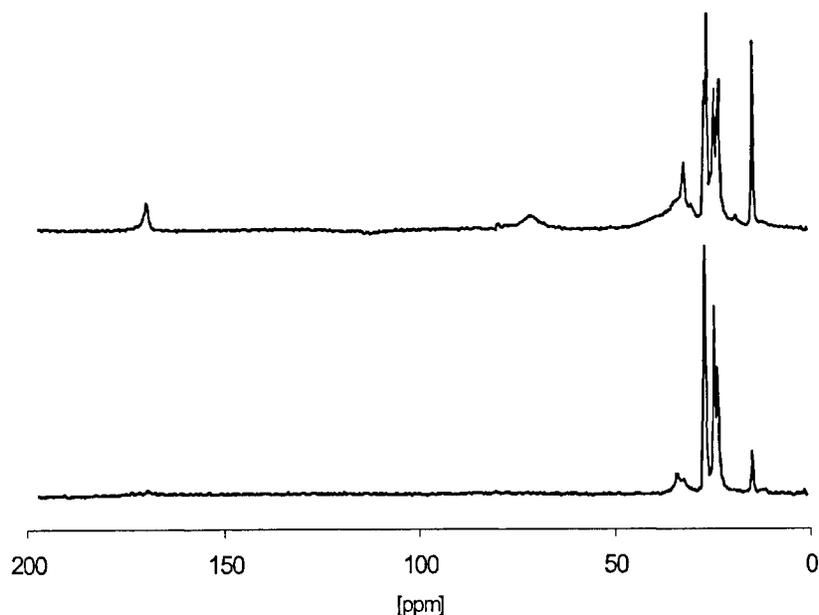


Figure 6.4: Single pulse Bloch decay MAS (a) and CP/MAS (b) ^{13}C NMR spectra of PHAE-11.5-POSS.

Solid-state ^{13}C NMR spectra of PHAE-11.5-POSS are shown in Figure 6.4. The single-pulse Bloch decay MAS experiment was used to detect both the mobile and more amorphous, as well as the more crystalline and thus rigid components. In Figure 6.4a, POSS resonances are visible at 24, 25 and 29 ppm. For the PHAE component, the resonances for the backbone carbonyl carbons, -CH- and -CH₂- groups, appear at 172, 72 and 39 ppm, and for the side-chain -CH₂- and -CH₃ groups they appear at 24, 25, 31, 34, and 15 ppm (Marchessault et al., 1990; Morin and Marchessault, 1992). As expected, no resonances are visible from the PHAE alkene groups at 115 and 139 ppm, which proves that most double bonds have reacted with POSS-SH. Compared to pure PHAE-11.5 (data not shown), the NMR signals of the backbone carbons are broadened in Figure 6.4a, implying that PHAE was in a more disordered environment after reaction with POSS-SH. The cross-polarization (CP) spectra of PHAE-11.5-POSS in Figure 6.4b shows mainly POSS resonances, suggesting that the PHAE component was mobile and non-crystalline. This is in

keeping with the X-ray results displayed in Figure 6.3. Also for a blend of PHAE-11.5 and POSS-SH, the CP spectrum was equivalent to the superposition of both individual components, confirming that the crystallization of POSS only was favoured in the derivatives. The spectrum of PHAE-97-POSS was similar to that of PHAE-11.5-POSS, and, because of the high weight fraction and dominant signal intensities of POSS in the PHAE-97-POSS sample, no relevant differences were detectable between the single-pulse and CP NMR spectra.

Selected thermal gravimetric traces are shown in Figure 6.5. The weight loss of PHAE-11.5 in an oxygen atmosphere started at approximately 270 °C, and the weight loss at 300 °C was greater than 80 %. PHAE-11.5 decomposed almost completely for temperatures greater than 500 °C. In nitrogen, the decomposition behaviour was similar, and the gravimetric trace was shifted to lower temperatures by approximately 10 °C. All PHAE samples decomposed identically. The decomposition of POSS-SH in oxygen started at 300 °C, the 20 % weight loss occurred at 340 °C, and the weight residue was 52.8 % at 650 °C. This can be explained by the oxidative decomposition of POSS into SiO₂, since the calculated weight residue for this transformation is 53.9 %, close to the experimental value. When POSS-SH was heated under nitrogen, the weight loss started at 290 °C, the 90 % weight loss occurred at 400 °C, and no residue remained above 600 °C. This points to a complete transformation into volatile products or sublimation of POSS-SH in a nitrogen atmosphere. A steady increase of the thermal stability with increasing POSS amounts was observed for the PHAE-POSS derivatives in oxygen, and the 20 % weight loss increased from 290 °C for PHAE-11.5-POSS to 320 °C for PHAE-97-POSS. Again, the weight residues at 650 °C can be explained with remaining SiO₂. This was confirmed by comparison with the calculated SiO₂ residues from the known weight fractions of POSS in PHAE-m-POSS (Table 6.1). These values are 21.6 % for PHAE-11.5-POSS and 44.2 % for PHAE-97-POSS, close to the experimental weight residues shown in Figure 6.5.

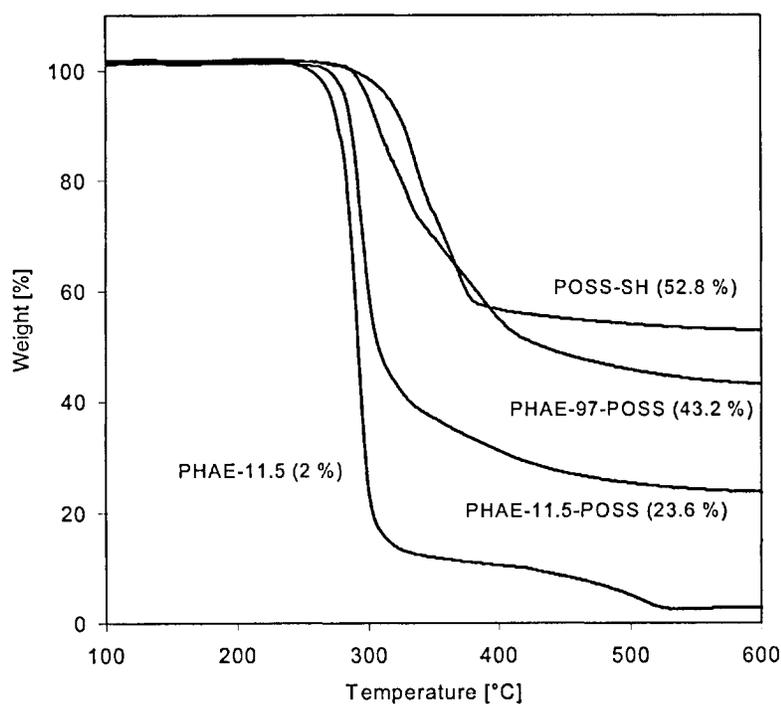


Figure 6.5: Thermal gravimetric traces in O₂ atmosphere of POSS-SH, PHAE with 11.5 mol% side-chain double bonds, and selected PHAE-POSS derivatives. The numbers in brackets denote the experimental weight residues at 650 °C.

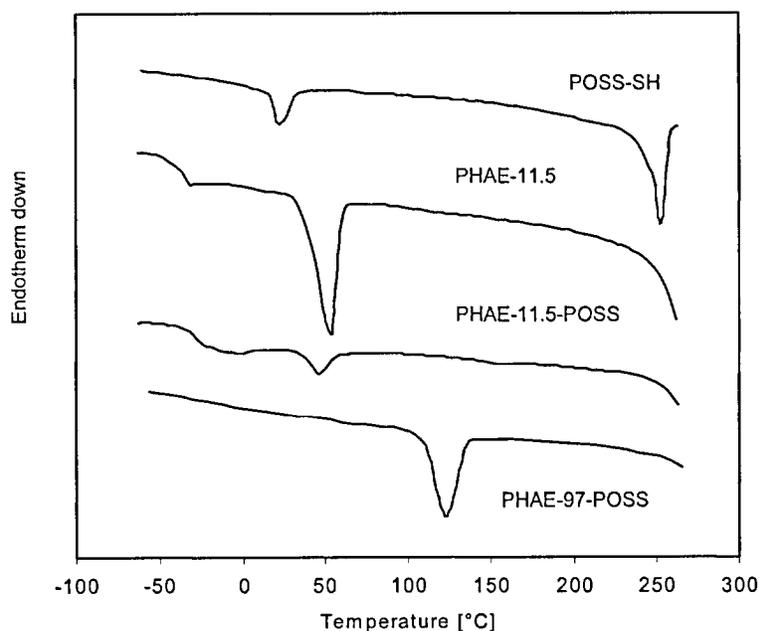


Figure 6.6: DSC traces of POSS-SH, PHAE-11.5, and PHAE-m-POSS derivatives. The presented traces were taken after slowly cooling down the samples to -80 °C. For PHAE-97-POSS, the glass transition was observable only after quenching in liquid nitrogen (see Table 6.1).

Figure 6.6 shows DSC traces for selected samples. The melting endotherm, T_m , of POSS-SH is at 257 °C (Table 6.1), which is close to the TGA weight loss onset temperature of approximately 290 °C. A further small endotherm of unknown origin is visible at 23.5 °C. In preliminary measurements we observed a similar solid-state transformation at 50 °C for the structurally related chloropropyl-isobutyl-POSS molecule ($T_m = 265$ °C). Melting points at 53 °C and 41 °C were observed for PHAE-11.5 and PHAE-55, whereas PHAE with higher contents of unsaturated side groups are known to be amorphous (Preusting et al., 1990). The POSS content in the copolymers had a drastic effect on the melting points. For PHAE-11.5-POSS, T_m was 48 °C, slightly lower than T_m of PHAE-11.5, but increased up to 120 °C for PHAE-97-POSS. As shown by XRD diffraction, these melting endotherms are due to the crystalline phase of POSS, and the lower melting points suggest small and imperfect crystals. It is interesting to note that T_m of PHAE-97-POSS is considerably higher than the highest melting point achieved for PHA variants obtained by incorporation of modified fatty acids. There, a maximum $T_m = 100$ °C has been obtained for a PHA containing fluorinated phenoxy side groups (Takagi et al., 2004). The glass transition temperatures for the PHAE-POSS copolymers varied only slightly between $T_g = -34$ °C and -38 °C. A comparison of the starting polyesters with their products shows that T_g remained constant for PHAE-11.5-POSS, and increased by approximately 10 °C for the other POSS derivatives. This has been observed in many other copolymers when sufficient POSS was incorporated, and has been explained by the POSS-POSS and POSS-PHAE interactions, resulting in retarded polymer chain mobility and T_g enhancement (Li et al., 2001). However, an initial T_g decrease at low POSS contents due to the inert diluent role of POSS was not observed here (Xu et al., 2002).

CONCLUSIONS

The POSS-based biopolyesters of this work form a new family of organic-inorganic hybrid materials. The incorporation of POSS significantly changed the physical properties of mclPHAs and could eliminate their notorious stickiness. These findings agree well with the results of our previous study on the functionalization of mclPHA double bonds with long polyethylene-like chains (Hany et al., 2004a). Clearly, the potential application of these POSS-based biopolyesters has yet to be demonstrated.

Recent results indicate that such systems might well be classified as nontoxic and suitable for food contact and not harmful if swallowed (McCusker et al., 2005)

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CHAPTER 7

General Conclusions

A bioplastic is needed for the future

Nowadays, it seems that we are living in an age of plastic. There is a multiplicity of products made of plastic such as transparencies, bottles, cups, furniture or miscellaneous consumer products. Further plastic is used in the building industry or for means of transportation like cars or airplanes. The term plastic includes various polymers such as polyethylene, poly(vinyl chloride), polypropylene, polystyrene or poly(ethylene terephthalate), which are all petroleum based and produced in the range of millions of tons per year (Kunststoffe, 2004). Besides the low price of these materials and the possibility to set them in nearly any desired shape, one common feature of these materials is that they are stable in almost every environment. This characteristic makes them suitable for many applications, but on the other hand, this characteristic also causes serious environmental problems. It is known that petroleum based plastics accumulate in the environment. Visually they are disintegrated when plasticizers have leached out or UV photooxidation causes oxidation of the polymers. However this process is very slow and plastics are therefore effectively recalcitrant to mineralization. For example a discarded plastic sandwich bag may be around long after the sandwich is gone and its consumer is deceased.

Plastic waste is not only generated on land but also on sea. Millions of pounds of plastics are produced at sea each year. Moore et al. (Moore et al., 2001) reported that in surface waters between the coasts of California and Hawaii, the mass of plastic was approximately six times that of plankton. The analysis of preserved seawater samples from 1960 to present day revealed that the fiber content correlated with the world plastic fiber production (Thomson et al., 2004). Government regulations now ban the dumping of plastic at sea but to control compliance with regulations is costly and expensive.

A second major disadvantage of common plastics is that they are all based on crude oil. Worldwide reserves have been estimated to be in the range of 200 billion tons, which is enough for only fifty years or so at the current rate of consumption (Stevens, 2002). Probably crude oil will never become totally exhausted but the use of our limited and nonrenewable supply of fossil resources for the large scale manufacture of plastics is a legitimate environmental concern. The first time that people became

concerned that global crude oil reserves might be depleted was in the mid-seventies. At that time crude oil prices increased due to a predicted end of the oil reserves and an artificially induced shortage by the oil producing countries. From that moment on, the development of plastics based on renewable resources became an important research topic and several biopolymers have been checked as alternatives to petrochemical plastics.

The use of biopolymers is a reasonable approach to tackle both problems. They can be produced from renewable resources and they are biodegradable. A few biopolymers have already been successfully commercialized, such as Mater-Bi (Novamont), Ecofoam (National Starch), poly(lactic acid) (Cargill Dow LCC), Bionolle (Showa Denko), and Biopol (Metabolix and Biomer). The world-wide consumption of biodegradable polymers has increased from 14'000 tons in 1996 to an estimated 68'000 tons in 2001. Clearly, the fraction of biopolymers is still marginal in comparison to the global production of synthetic polymers, which amount to about 202 million tons in 2003 (Kunststoffe, 2004). But the market for biopolymers is expected to grow quickly. For example, on the basis of an estimate, the volume of poly(lactic acid) production alone in 2020 will be 3.6 million tons per year (Gross and Kalra, 2002).

Polyhydroxyalkanoates: The plastic of the future?

Poly(3-hydroxyalkanoates) (PHAs) are thermoplastic, biodegradable polyesters that are synthesized by many bacteria as carbon and energy storage compounds. They have been drawing much attention because their material properties are similar to those of conventional plastics (Lee, 1996) and were designated "the plastic of the future" (Hänggi, 1995; Page, 1995). However, the commercialization of these materials has been prevented mainly by their high production cost. In 1996, the price of BIOPOL™ (poly(hydroxybutyrate-co-3-hydroxyvalerate)) was 16 US\$ kg⁻¹ (Lee, 1996) and therefore 20 to 30 times more expensive than the widely used polyethylene, polystyrene or polypropylene.

Today, costs of about 2 US\$ kg⁻¹ are discussed for PHAs produced through microbial fermentation. At this writing, Procter & Gamble Chemicals and Kaneka Corporation of Osaka, Japan announced that they have finalized a joint development agreement for

the completion of research and development leading to the commercialization of NODAX™ H, chemically known as poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). This ambitious development program plans the scale up of the fermentation and extraction process to a commercial scale by the year 2006 and targets a price of less than 2.3 US\$ per kilogram.

Although most research on biopolymers has been driven by the desire to develop alternatives for synthetic polymers, biopolymers have additional merits such as biocompatibility. Therefore, biopolymers are excellent candidates for applications where biocompatibility is desired. This feature is predominantly demanded for materials used for medical applications. The market for biomaterials is expected to reach a volume of 10 billion US\$ by the year 2010 (Zinn and Hany, 2005) and there is no doubt that PHAs will find usage as a material in the medical sector, where there are only a few competing petrochemical materials (Steinbüchel and Fächtenbusch 1998; Zinn et al., 2001). Whether PHAs will play an important role in the future, no one knows. Present research in our laboratories focuses on applications of this material in the medical sector but clearly, PHAs hold the potential to replace petrochemical plastics in many areas of applications. It will probably take another decade before large scale applications are realized - but when this happens, it will be because PHAs offer interesting, and sustainable polymeric materials from renewable resources.

Tailored PHAs by biosynthesis and chemical modifications

The aim of the work presented in this thesis was the production and characterization of tailored mclPHAs, obtained directly via biosynthesis (Chapters 2-4) or by chemical modifications of biosynthetic mclPHAs (Chapters 5 and 6). The results obtained in chapters 2 and 3 clearly demonstrate that the biosynthetic production in chemostat cultures under dual nutrient (C,N) limited growth conditions allows adjusting the degree of functionality as well as the thermal properties of olefinic mclPHAs. Certainly, the biosynthetic production, as it is described in this thesis, is expensive. The daily production of tailored olefinic mclPHAs (chapter 2) is around 2.5 grams and a rough estimation would lead to a production price of about 100 sFr. per gram. However, current experiments in our laboratories show that the volumetric production

rate can be increased by increasing the initial nitrogen concentration in the feed medium. This leads to a percentile reduction of the capital investment (personnel costs, laboratory maintenance etc.) per produced mass of PHA and will therefore decrease the production price.

The conceptual study of the biosynthetic production of biodegradable block copolymers in a two-stage continuous culture system of *P. putida* GPO1 (chapter 4) revealed that this approach is rather limited. It might be easier to produce pure block copolymers by linking single block segments via chemical synthesis as shown before (Hirt et al., 1996b; Andrade et al., 2002a). Generally, chemical modifications offer an interesting tool to modulate the basic polymer properties or to create functionalities which are impossible to be introduced by biosynthesis. In this work, we extended the existing library for polymer-analogous reactions on olefinic mclPHAs. In chapter 5 comb polymers were produced by a two-step synthesis. For example, derivative 4 was produced by a radical addition reaction of 11-mercaptoundecanoic acid to the side chain alkenes followed by an esterification of the terminal carboxyl group using octadecanol. This chemical modification led to a crystalline material and the well known stickiness of the starting polymer could be eliminated. This makes the polymer interesting for medical applications where crystalline materials are required (e.g. osteogenesis) although a heat sterilization of this material is not possible. Finally, in chapter 6 a series of POSS-PHAE inorganic-organic hybrid materials were produced. With increasing POSS content, the appearance of these POSS-based biopolyesters varied from non-sticky and elastic, to brittle and glass-like.

Today, detailed studies on the chemical modification of biosynthetic PHAs are still rare although chemical modification opens a wide field to produce new types of biopolymers which cannot be produced by biosynthesis. For instance, by the covalent linkage of peptides, proteins or drugs to olefinic (mcl)PHAs, scaffolds for tissue engineering could be developed which support the attachment and growth of a desired cell type. Further, Hany et al. (Hany et al, 2004b) demonstrated that olefinic mclPHAs can be functionalized in such a way that they gain the potential to protect surfaces from biofouling.

The results obtained in this doctoral thesis show that tailored mclPHAs can be produced by biosynthetic procedures as well as by chemical modifications efficiently

and in sufficient amounts for detailed material studies. This know-how of production provides a basis for the identification of new applications of medium-chain-length polyhydroxyalkanoates in medicine or industry.

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